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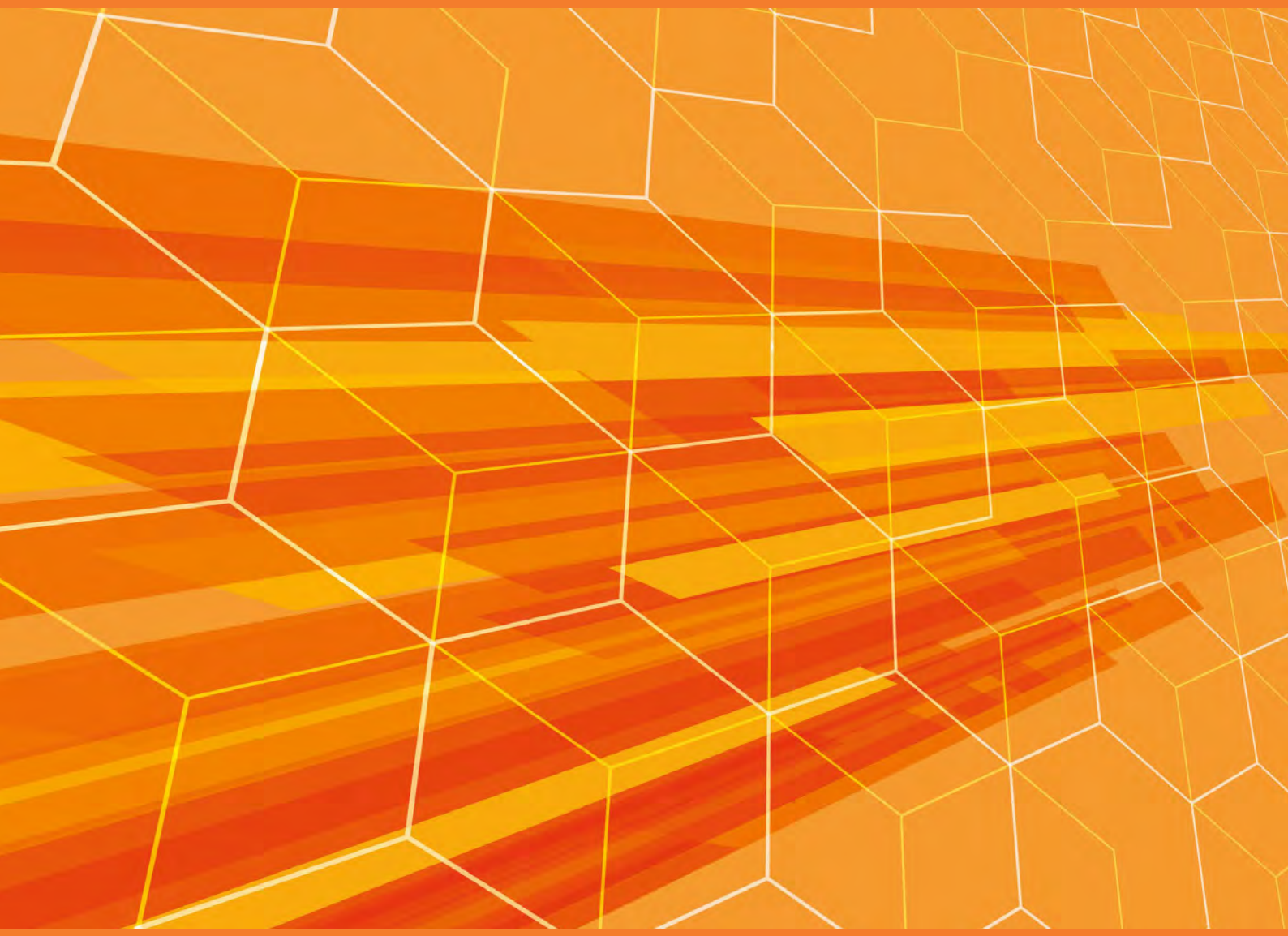
DTI | Drug Target Insights



ABOUTSCIENCE

DTI | GENERAL INFORMATION

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Drug Target Insights covers current developments in all areas of the field of clinical therapeutics and focusing on molecular drug targets which include disease-specific proteins, receptors, enzymes, and genes. The journal seeks to elucidate the impact of new therapeutic agents on patient acceptability, preference, satisfaction and quality of life.

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EDITORIAL

Natural Products & Phytotherapeutics: why a new section?

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According to one of the most authoritative reports focusing on natural products as sources of new drugs, the use of natural products and their synthetic derivatives is still pivotal in the discovery of new drugs (1). Indeed, among the new drugs approved (N = 1881) in the last four decades, about 25% are natural products (Fig. 1A). This scenario is particularly relevant for antibacterial and anticancer agents (Fig. 1B, C).

This should not be surprising. Since ancient times, humanity has made use of medicinal plants to heal itself, and even today, traditional medicine represents the dominant health care system in many parts of the world and for billions of people (2). This is the case of herbal medicines, the cornerstone of phytotherapy, which include, according to the World Health Organization (WHO), 'herbs, herbal materials, herbal preparations and finished herbal products that contain, as active ingredients, parts of plants, other plant materials or combinations thereof' (3). Several famous examples could be cited, from aspirin to many anticancer drugs (Tab. I).

However, natural product research still suffers from some important limitations. First, the validation of traditional uses. Despite hundreds (or even thousands) of preclinical (in vitro/in vivo) studies, evidence in humans is still scanty, due to the paucity of clinical trials evaluating the real efficacy of natural products. Second, the poor oral bioavailability of natural products. Phytochemicals are xenobiotics metabolized, detoxified and eliminated by phase I and II metabolizing enzymes and phase III transporters involved in efflux mechanisms. This drawback can be bypassed by proper (nano) formulation. Third, natural does not always mean safe. The safety of natural products is rarely investigated and the available information is scanty, as are the phytochemical-drug interactions with possible changes in therapeutic efficacy for some drugs with a narrow therapeutic index (4). These issues call for an evidence-based approach to be followed even for phytotherapeutics, where randomized controlled trials are at the top of the evidence-based pyramid (5).

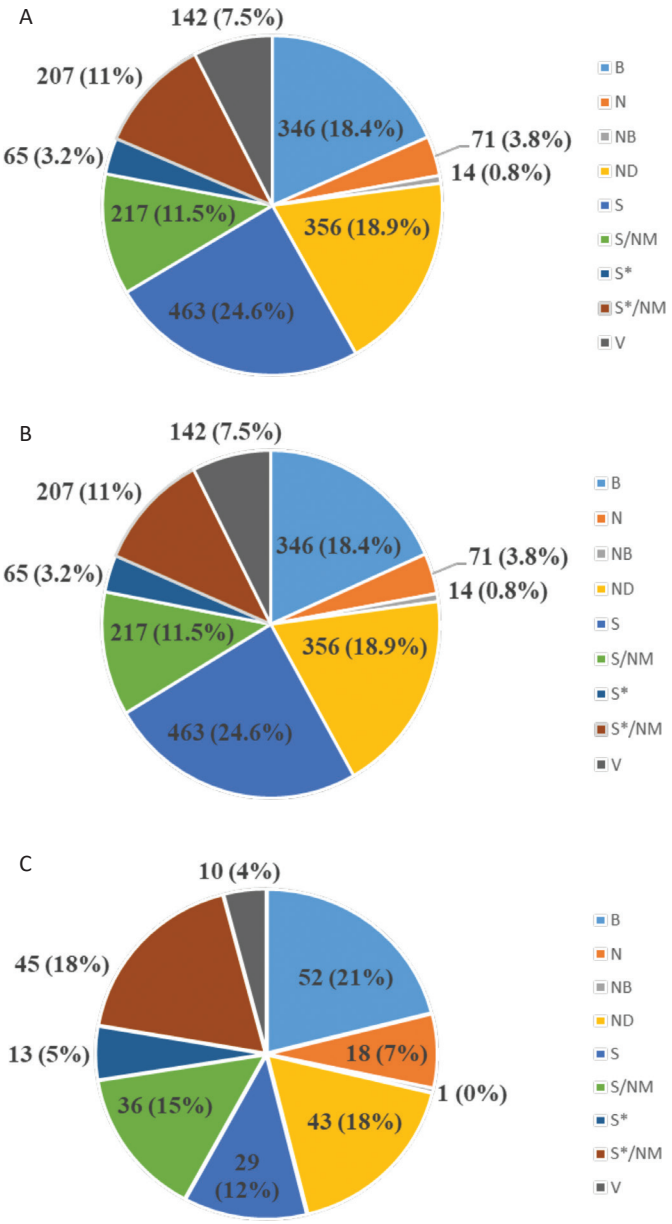

Fig. 1 - A) All new approved drugs by source from 1981 to 2019 (N = 1881). B) All antibacterial drugs by source from 1981 to 2019 (N = 162). C) All anticancer drugs by source from 1981 to 2019 (N = 247). *Categories of sources: B = biological; N = natural product; NB = natural product – botanical; ND = natural product derivative; S = synthetic; S* = synthetic (with pharmacophore from a natural product); V = vaccine. Subcategory: NM = natural product mimic. Adapted from Newman and Cragg (1).*

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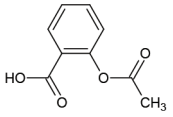
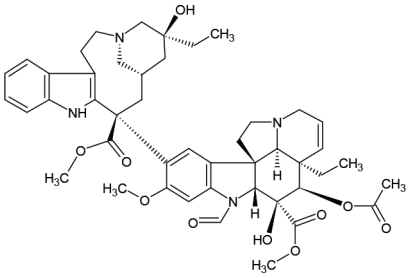
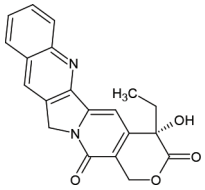
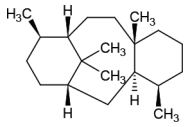
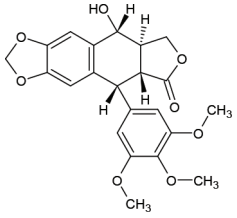
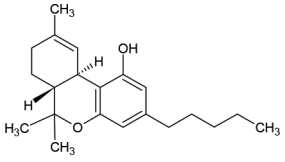
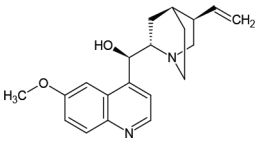
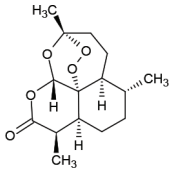
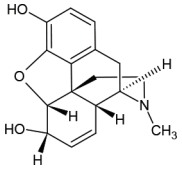
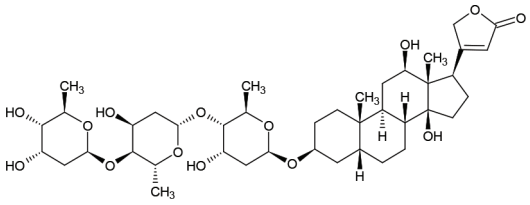
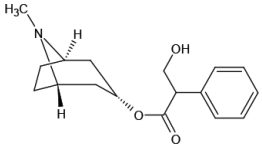
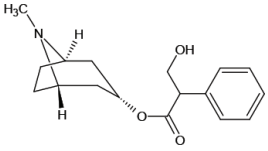
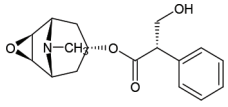
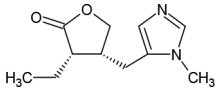
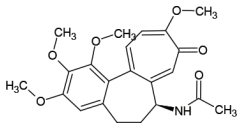
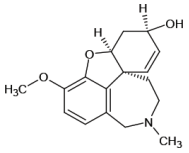
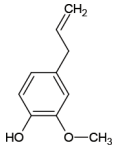
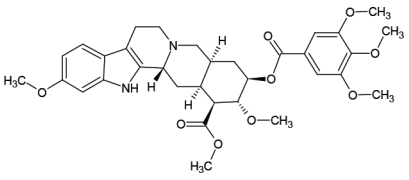
TABLE I - Selected examples of drugs developed from medicinal plants

Medicinal plant	Drugs	Indications	Chemical structure
Salix spp.	Acetylsalicylic acid	Anti-inflammatory, antiaggregant	
Catharanthus roseus	Vinca alkaloids (vincristine, vinblastine, vinorelbine)	Anticancer	
Camptotheca acuminata	Camptothecin derivatives (topotecan, irinotecan)	Anticancer	
Taxus brevifolia	Taxane derivatives (paclitaxel, docetaxel, cabazitaxel)	Anticancer	
Podophyllum peltatum	Podophyllotoxin derivatives (etoposide, teniposide)	Anticancer	
Cannabis sativa	Cannabinoids (tetrahydrocannabinol, cannabidiol)	Psychotropic	
Cinchona spp.	Quinine	Antimalarial	
Artemisia annua	Artemisinin	Antimalarial	

Medicinal plant	Drugs	Indications	Chemical structure
Papaver somniferum	Morphine, codeine	Analgesic	
Digitalis spp.	Glicosidi digitalici (digoxin, digitoxin)	Cardiotonic	
Atropa belladonna	Atropine	Anticholinergic	
Hyoscyamus niger	Hyoscyamine	Anticholinergic	
Datura stramonium	Scopolamine	Anticholinergic	
Pilocarpus jaborandi	Pilocarpine	Cholinergic	
Colchicum autumnale	Colchicine	Antigout	
Galanthus spp.	Galantamine	Cholinesterase inhibitor	
Syzygium aromaticum	Eugenol	Antiseptic, anesthetic	
Rauwolfia serpentina	Reserpine	Antihypertensive	

Not least, the combination of natural products with conventional drugs offers another area of application that should be pursued extensively. This has previously been investigated with natural products used in combination with anticancer drugs and antimicrobials. This therapeutic approach was able to (chemo)sensitize chemoresistant cancer cells, fungi and bacterial strains by inhibiting the cellular active efflux system, a conserved drug resistance mechanism that pumps xenobiotics out of the cell. The rationale for the use of natural products is based on their multitarget action mechanism of particular interest in the treatment of disorders with multistage pathogenesis. In this complex scenario, natural products still offer the best options for finding new active agents/templates and provide the unlimited potential for discovering new structures that can lead to effective drugs in a variety of communicable and non-communicable diseases.

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REVIEW

A systematic review of mucoadhesive vaginal tablet testing

Ismin Zainol Abidin[1], Emma J. Murphy[1–3], Gustavo Waltzer Fehrenbach[4], Emanuele Rezoagli[5,6], Noel Gately[1], Ian Major[1]

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ABSTRACT

Drug administration through the vaginal tract is one of the oldest modalities of pharmacotherapy, and it is also one of the most explored. Since the vaginal cavity has a wide surface area, a plentiful blood supply, and a complex network of blood arteries, it can evade hepatic first-pass metabolism and obtain high local drug concentrations. Vaginal pills look to be a good dose form since they are simple to use, portable, and can easily deliver the required amount of medicine. Vaginal formulations, on the other hand, are vulnerable to rapid expulsion due to the vaginal tract's self-cleaning action, which reduces the formulation's efficiency. Currently, there is an increasing amount of focus on mucoadhesive vaginal formulation research and development to fix the formulation at the place where the medicine can be released and/or absorbed. This article examines all of the strategies used by researchers to develop a mucoadhesive vaginal tablet that is safe, effective, and comfortable for the user.

Keywords: Dissolution, Mucoadhesion, Physicochemical, Swelling

Introduction

Over the past three decades, the vaginal route has gained relevance in modern medicine as a route for drug delivery and is now considered an option for several therapeutic strategies, specifically for female-related conditions. Several advantages have been claimed for vaginal drug delivery in managing local conditions and achieving systemic effects. In the case of managing local conditions, vaginal administration means that lower doses can be effective (compared to the oral route), which frequently leads to reduced systemic exposure and can prevent side effects (1,2). Owing to its large surface area, rich blood supply, and the presence of a dense network of blood vessels, the vagina serves as a promising site for systemic drug delivery (3). Its relatively high permeability to many drug compounds (including several with high molecular weight) also allows for drug transport across the vaginal mucosa and access into the blood circulation, presenting in many cases higher flux levels than those observed via intestinal tissues (2,4). The vaginal route may prove to be of particular importance in the case of drugs undergoing extensive hepatic metabolism, since it avoids the hepatic first-pass effect (1). Furthermore, it permits the elimination of possible degradation in the gastrointestinal tract and the effect of the drug directly at the site of application (5).

Traditionally, the vaginal route has been used for delivery of locally acting drugs such as antibacterial, antifungal, antiprotozoal, antiviral, labor-inducing, and spermicidal agents, prostaglandins, and steroids (6). Recently, there has been increased interest and effort in the development of vaginal formulations such as microbicides that provide effective contraception and protection against transmission of various sexually transmitted diseases (STDs), including acquired immunodeficiency syndrome (AIDS) (3). There are many different vaginal products in the market to treat different vaginal conditions, for example, Canesten® for antifungal and Nuvaring® as contraceptive (7,8). One of the leading vaginal

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TABLE I - List of some vaginal tablet patent applications for different treatments

Patent ID	Author/inventor	Title	Treatment/ condition	Date of application	Current status
CN1307986C	Chen Z.	Vaginal effervescent tablets for inflammation and their preparation	Gynecological inflammation	2004-06-08	Granted (2007-04-04) Anticipated expiration (2024-06-08)
AU2012210296B2	Mogna G., Mogna L., Strozzi G.P.	Effervescent composition in solid form for use in vaginal application for the treatment of vaginal infections	Vaginal infections	2011-01-28	Granted (2017-01-05) Anticipated expiration (2032-01-24)
CN106420726A	Jiang Dingyu, Zhang Yongwei, Zhu Ming	Clotrimazole vaginal tablets	Vulvovaginal candidiasis	2016-06-30	Pending
WO2020201515A1	Ellervik U., Manner S., Sterner O., Strevens H., Lindberg N., Säfholm A.	Vaginal tablet formulation	Vaginal microbial infections	2019-04-05	Publication (2020-10-08)
US20200155621A1	Thoral C., Tchoreloff P., Mazel V., Busignies V., Nivoliez A.	Mucoadhesive sustained-release vaginal tablet	Probiotic strain: *Lactobacillus*	2014-03-10	Pending
W02022218487A1	Crouzier T., Schimpf U.	A vaginal contraceptive composition for reinforcement of the cervical mucus barrier properties	Contraceptive	2021-04-12	Publication (2022-10-20)

These patents have been sourced from Google Patent and accessed on November 28, 2022.

tablets available in the market is Vagifem® by Novo Nordisk, which was first introduced in 1988. It is an option for local estrogen therapy that continuously releases steroid in the vagina for a consistent dose of hormone (9). There are many other vaginal tablets that are currently in the process of patent application and are listed in Table I. These applications are in various stages of the patenting process, some has been granted and anticipating expiry, some are still pending, and some have just published a research article under the patent ID.

Vaginal products do not need to be sterile; they are usually cheap and relatively easy to manufacture (2). It also allows easy and comfortable self-administration and rarely requires the intervention of a health-care provider (1). Marketed vaginal dosage forms include solutions (douches), semisolids (creams, ointments, and gels), and solid formulations (tampons, capsules, pessaries, suppositories, films, sponges, powders, and special controlled release devices like the intravaginal ring) as well as other types of formulations such as aerosols and particulate systems integrated in adequate drug delivery systems (3,6,10). However, the use of vaginal formulations can be limited due to poor drug retention in the vaginal tract, as they are removed in a short time by the tract's self-cleansing action (3). The low residence time often leads to disappointing experiences such as leakages and messiness, which cause loss of formulation from the application site, giving rise to inadequate formulation and hence lack of effectiveness (11). Therefore, frequent daily doses are often required to maintain an effective drug concentration, which further complicates application and contributes to low patient acceptability and, thus, poor compliance (3,4,6,12,13).

Extensive research and innovative attempts have been made to develop vaginal formulations to meet clinical and user requirements. To overcome these limitations, researchers have focused their attention on the development of new delivery systems that can prolong the drug residence time in the vaginal cavity, basically by using mucoadhesive formulations (3,12,14,15). The general principles of the mucoadhesive vaginal drug delivery system will be discussed further in a later section of this review.

Mucoadhesive vaginal drug delivery systems are superior to conventional ones due to their ability to prolong drug residence time at the application site, leading to improved bioavailability and efficacy (12). The number of products based on new vaginal drug delivery systems has significantly increased, and this growth is expected to continue in the near future (3). With various types of formulations available, the popularity of vaginal products can be different among women from different backgrounds and countries (6). Nonetheless, tablets and gels (films) are among the most popular vaginal formulations (3,6,12). Even with major advancement in the gel (hydrogel) and film formulations, moderate vaginal leakage was still observed and daily administration was required (16). Therefore, vaginal tablets still often represent the typically acceptable dosage form with stability-related advantages and an economical choice for both manufacturers and users (17,18), thus advocating its role and relevance in the vaginal drug delivery system. So, it won't be obsolete just yet.

This review aims to assemble and discuss the key parameters and unique methodologies that should be considered when evaluating vaginal tablet formulations. This review will not go into any specific medicinal substances or polymers; instead, it will concentrate on the tactics and evaluation

methods that can aid in the development of an effective vaginal tablet formulation. To the author's knowledge, this is the first systematic review that compiles numerous assessment methodologies required to develop a new mucoadhesive vaginal tablet formulation.

Methods

Eligibility criteria

Inclusion criteria

i. All original studies designing and formulating mucoadhesive vaginal tablets, regardless of its therapeutic treatment
ii. Publication years between 2000 and November 2021
iii. Articles in English language
iv. Articles published in scholarly peer-reviewed journals

Exclusion criteria

i. Articles of studies on other mucoadhesive vaginal dosage forms (e.g., semisolids and liquids)
ii. Articles of studies on vaginal pessaries, suppositories, and pellets because it has been identified that they are different from tablet dosage forms
iii. Articles of studies of mucoadhesive vaginal tablet for veterinary use
iv. Articles of studies that compare between two different dosage forms (e.g., tablets and films)

Information sources

A search of relevant papers between years 2000 and 2021 was made via chosen electronic databases available in the Technology University of Shannon: Midlands Midwest (TUS) Library online search engine. The databases included PubMed, Science Direct, Multidisciplinary Digital Publishing Institute (MDPI) and Academic Search Complete (Ebsco). The search was done between August 19 and November 11, 2021, for any new relevant publications.

Search strategy

The search in the databases was carried out using the keyword "mucoadhesive vaginal tablets" or "vaginal tablets + mucoadhesive" or "vaginal tablets + evaluations" or "vaginal tablet testing + mucoadhesion."

Study selection and data collection process

The initial stage involves screening the titles. Titles that specify a dosage form other than vaginal tablets (e.g., films, gels) and for veterinary purposes are essentially excluded. In the case of vague titles, a quick scan through the abstract is conducted to identify the words, vaginal tablet or tablet/s. Titles that do not use the term "vaginal tablet" are considered as vague titles. Following that, the abstracts were screened to ensure that they meet the inclusion criteria. The full text of eligible abstracts is then accessed and reviewed.

Two reviewers were involved in the selection process. Articles were collected individually; the other reviewer replicated the search strategy and listed the articles deemed eligible for the review. Excluded articles from each reviewer were confirmed with each other and eligible articles are compiled. Studies that were deemed eligible were compiled in an Excel spreadsheet (Microsoft 365 application, version 2201). Data were collected systematically and analyzed using Excel spreadsheet.

Data items

Data extracted from each study include: (i) information of the tablets (e.g., use, physical appearance, etc.), (ii) all the methods used to measure the physicochemical properties of the tablets, (iii) all the methods used to assess the mucoadhesion property of the tablet, (iv) all the methods used to evaluate drug release profile of the tablet, and (v) other relevant methods used to evaluate the technical working ability of the tablet. For each outcome, effect measures were determined via difference in means and standard deviation. All graphs to presenting the results were prepared using Excel software (Microsoft 365 application, version 2201).

Study risk of bias assessment

Each article was assessed for risk of bias using the Cochrane Collaborations tool (19). The following biases were examined: (i) bias in selection/sampling, (ii) performance bias associated with the allocation of interventions during the study, (iii) attrition bias associated with the handling of incomplete outcome data, (iv) reporting bias associated with selective outcome reporting, (v) measurement bias associated with the use of non-validated data collection criteria, and (vi) analysis bias associated with the omission of necessary statistical coefficients associated with the study.

Synthesis methods

The full text of each article was scrutinized in sequence and grouped according to the data items mentioned in Section 2.5. Data were collected systematically and tabulated and analyzed (means and standard deviation) using Excel software (Microsoft 365 application, version 2201). All graphs prepared to present the results were prepared using the same software.

Results

Study selection

There were 772 research articles that were identified as a result of the keyword search of mucoadhesive vaginal tablets across five electronic databases. The breakdown is as follows: (i) PubMed, 65 articles; (ii) Science Direct, 624 articles; (iii) MDPI, 5 articles; (iv) Scopus, 77 articles, and (v) Ebsco, 1 article. From the initial screening of the titles, a total of 63 research articles were deemed eligible to proceed for abstract screening. At this stage, the research articles

were sub-divided into two categories according to the study characteristic: (i) research articles with a single formulation of vaginal tablet (study of inter-batches variation) and (ii) vaginal tablets formulation with polymer intervention to improving the mucoadhesive properties of the formulation (comparison study between modified and non-modified formulations). The subcategories were to facilitate the reviewers in understanding the study design and characteristic of the articles. Following the abstract screening, 43 research articles were deemed eligible to proceed for full-text review and data collection. For most excluded research articles, the study focuses more on a specify drug compound or polymer rather than the technology of tablet manufacturing and performance. In addition, four other research articles were then excluded during the full-text review, due to one being outdated (20) and three research articles having non-vaginal tablet-related outcomes (21-23). In these research articles, most formulations were simply made into KBr disks to test the mucoadhesive property exclusively, no further tablet manufacturing parameters were considered. Thus, it is considered non-vaginal tablet-related outcomes. To avoid further limitations, unaccessible research articles were agreed to not be included in this review. Figure 1 shows the flow diagram of the selection process and that at the end of this process, 39 research articles were included in this review.

Study characteristics

Table II summarizes the study characteristics of the research articles reviewed. All of the vaginal tablets were prepared by direct compression, with most having flat-faced and round or cylindrical shape. More of the study characteristics are discussed in Section 4.2.

The studies were divided into two subcategories depending on the study design: either (a) inter-batch comparison or (b) polymer intervention. The study background of the articles reviewed is summarized in Table III. Inter-batch comparison studies involved vaginal tablet formulations that were prepared in different batches varying in different types and/or ratios, and/or combinations of mucoadhesive polymers in each batch. Some are natural polymers, some in combination

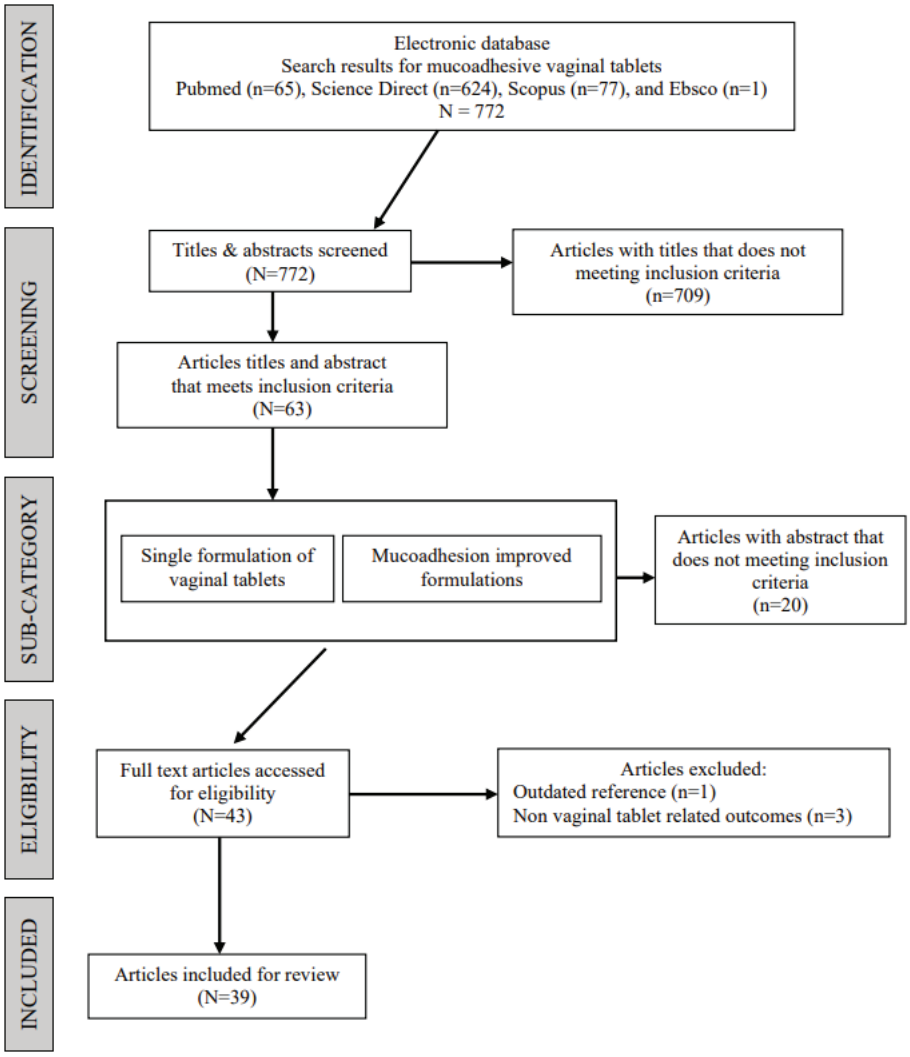

Fig. 1 - PRISMA flow diagram of the study selection process.

TABLE II - Demographic of the research articles reviewed in alphabetical order

Research article	Author	Study design	Vaginal tablet design	Treatment application	Study title
1	Abidin et al (2020)	Inter-batch comparison (n* = 6)	Bilayer tablet, flat faced, round shaped	Anticancer	A bilayer vaginal tablet for the localized delivery of disulfiram and 5-fluorouracil to the cervix
2	Abu El-Enin et al (2020)	Inter-batch comparison (n* = 6)	Core-in-cup tablet flat faced, round shaped	Preterm labor	Formulation, development, in vivo pharmacokinetics and pharmacological efficacy evaluation of novel vaginal bioadhesive sustained core-in-cup salbutamol sulphate tablets for preterm labor
3	Baki et al (2009)	Inter-batch comparison (n* = 6)	Flat faced, round shaped	Vaginal health management	Formulation of a solid intravaginal matrix system to prolong the pH-decreasing effect of lactic acid
4	Baloglu et al (2011)	Polymer intervention	Flat faced, round shaped	Antifungal	In vitro evaluation of mucoadhesive vaginal tablets of antifungal drugs prepared with thiolated polymer and development of a new dissolution technique for vaginal formulations
5	Bartkowiak et al (2018)	Inter-batch comparison (n* = 6)	Flat faced, round shaped	Antifungal	Surface and swelling properties of mucoadhesive blends and their ability to release fluconazole in a mucin environment
6	Bhat et al (2010)	Inter-batch comparison (n* = 6)	Flat faced, round shaped	Vaginal infection	Bioadhesive controlled release clotrimazole vaginal tablets
7	Cazorla-Luna et al (2019)	Inter-batch comparison (n* = 15)	Flat faced, round shaped	HIV prevention	Chitosan-based mucoadhesive vaginal tablets for controlled release of the anti-HIV drug tenofovir
8	Cevher et al (2014)	Inter-batch comparison (n* = 4)	Flat faced, round shaped	Antifungal	Bioadhesive tablets containing cyclodextrin complex of itraconazole for the treatment of vaginal candidiasis
9	Cevher et al (2008)	Inter-batch comparison (n* = 3)	Flat faced, round shaped	Antifungal	Preparation and characterisation of natamycin: g-cyclodextrin inclusion complex and its evaluation in vaginal mucoadhesive formulations
10	El-Kamel et al (2002)	Inter-batch comparison (n* = 4)	Flat faced, round shaped	Antibacterial	Chitosan and sodium alginate–based bioadhesive vaginal tablets
11	Fitaihi et al (2017)	Inter-batch comparison (n* = 17)	Flat faced, round shaped	Antifungal	Role of chitosan on controlling the characteristics and antifungal activity of bioadhesive fluconazole vaginal tablets
12	Gupta et al (2013)	Inter-batch comparison (n* = 6)	Flat faced, round shaped	Antimycotic	Bioadhesive vaginal tablets containing spray dried microspheres loaded with clotrimazole for treatment of vaginal candidiasis
13	Gök et al (2017)	Polymer intervention	Flat faced, round shaped	Did not specify	The effects of the thiolation with thioglycolic acid and l-cysteine on the mucoadhesion properties of the starch-graft-poly(acrylic acid)
14	Hani et al (2016)	Inter-batch comparison (n* = 10)	Flat faced, round shaped	Antifungal	Development of a curcumin bioadhesive monolithic tablet for treatment of vaginal candidiasis
15	Hassan et al (2017)	Inter-batch comparison (n* = 12)	Flat faced, round shaped	Hormone therapy	Mucoadhesive tablets for the vaginal delivery of progesterone: in vitro evaluation and pharmacokinetics/ pharmacodynamics in female rabbits
16	Hombach et al (2009)	Polymer intervention	Flat faced, round shaped	Vaginal infection	Development and in vitro evaluation of a mucoadhesive vaginal delivery system for nystatin
17	Kailasam et al (2010)	Inter-batch comparison (n* = 5)	Flat faced, round shaped	Antibacterial	Formulation and evaluation of once daily mucoadhesive vaginal tablet of metronidazole

(*Continued*)

TABLE II - (*Continued*)

Research article	Author	Study design	Vaginal tablet design	Treatment application	Study title
18	Kast et al (2002)	Polymer intervention	Flat faced, round shaped	Vaginal infection	Design and in vitro evaluation of a novel bioadhesive vaginal drug delivery system for clotrimazole
19	Khan et al (2017)	Inter-batch comparison (n* = 8)	Did not specify	HIV prevention	Formulation and evaluation of once daily mucoahdesive vaginal tablet of metronidazole
20	Khan et al (2014)	Inter-batch comparison (n* = 8)	Did not specify	HIV prevention	Formulation and evaluation of mucoadhesive vaginal tablets of tenofovir disoproxil fumarate
21	Lupo et al (2017)	Polymer intervention	Flat faced, round shaped	Antibacterial	Entirely S-protected chitosan: a promising mucoadhesive excipient for metronidazole vaginal tablets
22	Notario-Pérez et al (2019)	Inter-batch comparison (n* = 5)	Flat faced, round shaped	HIV prevention	Tenofovir hot-melt granulation using Gelucire® to develop sustained-release vaginal systems for weekly protection against sexual transmission of HIV
23	[a]Notario-Pérez et al (2017)	Inter-batch comparison (n* = 12)	Flat faced, round shaped	HIV prevention	Optimization of Tenofovir release from mucoadhesive vaginal tablets by polymer combination to prevent sexual transmission of HIV
24	[b]Notario-Pérez et al (2017)	Inter-batch comparison (n* = 12)	Flat faced, round shaped	HIV prevention	Influence of chitosan swelling behaviour on controlled release of tenofovir from mucoadhesive vaginal systems for prevention of sexual transmission of HIV
25	Nowak et al (2015)	Polymer intervention	Flat faced, round shaped	Did not specify	Preactivated hyaluronic acid: a potential mucoadhesive polymer for vaginal delivery
26	Pacheco-Quito et al (2020)	Inter-batch comparison (n* = 8)	Flat faced, round shaped	Vaginal infection	Carrageenan-based acyclovir mucoadhesive vaginal tablets for prevention of genital herpes
27	Paczkowska et al (2020)	Inter-batch comparison (n* = 6)	Did not specify	Vaginal infection	Mucoadhesive chitosan delivery system with ***Chelidonii herba*** lyophilized extract as a promising strategy for vaginitis treatment
28	Palade et al (2013)	Inter-batch comparison (n* = 12)	Did not specify	Did not specify	***In vitro*** evaluation of 5-fluorouracil dissolution profiles from vaginal bioadhesive tablets
29	Patel A. et al (2012)	Inter-batch comparison (n* = 9)	Did not specify	Antifungal	Design, development and in vitro evaluation of sertaconazole mucoadhesive vaginal tablet
30	Patel A. et al (2011)	Inter-batch comparison (n* = 10)	Did not specify	Antifungal	Development and evaluation of mucoadhesive vaginal tablet of sertaconazole for vaginal candidiasis
31	Patel G.M. et al (2010)	Inter-batch comparison (n* = 9)	Did not specify	Antifungal	A novel effervescent bioadhesive vaginal tablet of ketoconazole: formulation and invitro evaluation
32	Pendekal et al (2013)	Inter-batch comparison (n* = 9)	Flat faced, round shaped	Anticancer	Hybrid drug delivery system for oropharyngeal, cervical and colorectal cancer—in vitro and in vivo evaluation
33	Pendekal et al (2012)	Inter-batch comparison (n* = 9)	Flat faced, round shaped	Anticancer	Development and characterization of chitosan-polycarbophil interpolyelectrolyte complex-based 5-fluorouracil formulations for buccal, vaginal and rectal application
34	Perioli et al (2009)	Inter-batch comparison (n* = 6)	Flat faced, round shaped	Antibacterial	FG90 chitosan as a new polymer for metronidazole mucoadhesive tablets for vaginal administration
35	Perioli et al (2011)	Inter-batch comparison (n* = 5)	Flat faced, round shaped	Vaginal infection	New solid mucoadhesive systems for benzydamine vaginal administration

Research article	Author	Study design	Vaginal tablet design	Treatment application	Study title
36	Sánchez et al (2017)	Inter-batch comparison (n* = 4)	Double layer, flat faced, round shaped	Vaginal infection	A novel double-layer mucoadhesive tablet containing probiotic strain for vaginal administration: design, development and technological evaluation
37	Szymańska et al (2014)	Inter-batch comparison (n* = 4)	Flat faced, round shaped	Vaginal infection	Vaginal chitosan tablets with clotrimazole—design and evaluation of mucoadhesive properties using porcine vaginal mucosa, mucin and gelatine
38	Tunpanich et al (2019)	Inter-batch comparison (n* = 5)	Capsule shaped	Hormone therapy	Mucoadhesive sustained-release tablets for vaginal delivery of ***Curcuma comosa*** extracts: preparation and characterization
39	Valenta et al (2001)	Polymer intervention	Flat faced, round shaped	Hormone therapy	Development and in vitro evaluation of a mucoadhesive vaginal delivery system for progesterone

HIV = human immunodeficiency virus.
*n = number of batches formulated and compared to.

TABLE III - Background characteristic of research articles reviewed

Author	Study background	Polymers used
Abidin et al (2020)	Designed a bilayer vaginal tablet. A comparison study between batches formulated using different ratios of polymers in individual layers.	Chitosan, poly(acrylic acid)
Abu El-Enin et al (2020)	Designed a core-in-cup vaginal tablet. A comparison study between batches formulated using different combinations of mucoadhesive polymers.	Carbopol, hydroxypropyl methylcellulose, hydroxyethylcellulose, polyethylene glycol
Baki et al (2009)	Studied the applicability of solid matrix system for tablet formulations. A comparison study between batches formulated with different concentrations of API.	Hydroxypropyl methylcellulose, microcrystalline cellulose
Baloglu et al (2011)	A comparison study between batches formulated using thiolated and non-thiolated mucoadhesive polymers. Article includes polymer modification steps and structure characterization methods.	Poly(acrylic acid), poly(acrylic acid)-cysteine (thiolated)
Bartkowiak et al (2018)	A comparison study between batches formulated using different blends of mucoadhesive polymers.	Carbopol, polycarbophil, chitosan, hydroxyethylcellulose, hydroxypropyl methylcellulose
Bhat et al (2010)	A comparison study between batches formulated using mixtures of natural mucoadhesive polymer with HPMC in different ratios.	Hydroxypropyl methylcellulose, sodium carboxymethylcellulose, guar gum
Cazorla-Luna et al (2019)	A comparison study between batches formulated using CHN alone and in combination with natural mucoadhesive polymers. The different blends of polymers were used to generate spontaneous polyelectrolyte complexes. Article includes structure characterization methods of the complexes formed.	Chitosan, pectin, locust bean gum
Cevher et al (2014)	This study focuses on forming inclusion complex of cyclodextrin with the chosen API to improve their aqueous solubility. The complex was then formulated into sustained-release vaginal tablet.	Hydroxypropyl methylcellulose, carbopol, xanthan gum
Cevher et al (2008)	A comparison study between batches formulated using different polymer ratios. This study focuses on forming inclusion complex of cyclodextrin with the chosen API to improve their aqueous solubility. The complex was then formulated with a polymer at different ratios until high mucoadhesion to the vaginal mucosa is achieved.	Hydroxypropyl methylcellulose, carbopol, xanthan gum
El-Kamel et al (2002)	A comparison study between batches formulated using mixtures of anionic polymers with CHN. The polymer combinations were used to generate spontaneous polyelectrolyte complexes.	Chitosan, microcrystalline cellulose, sodium carboxymethylcellulose, sodium alginate
Fitaihi et al (2017)	A comparison study between batches formulated using different types of polymers physically blended with CHN at different ratios.	Chitosan, hydroxypropyl methylcellulose, guar gum, sodium carboxymethylcellulose, polyvinyl pyrrolidone

(*Continued*)

TABLE III - (*Continued*)

Author	Study background	Polymers used
Gupta et al (2013)	Incorporated microspheres of the API into a vaginal tablet formulation. A comparison study between batches formulated using the API in the pure form and the API microspheres.	Eudragit RS®, Eudragit RL, carbopol, hydroxypropyl methylcellulose, sodium carboxymethylcellulose
Gök et al (2017)	This study investigated the effect of thiolation reagents on the thiolation process. Article includes polymer modification and confirmation methods. A comparison study between batches formulated using thiolated and non-thiolated mucoadhesive polymers.	Poly(acrylic acid), poly(acrylic acid)-cysteine (thiolated), poly(acrylic acid)-thioglycolic acid (thiolated)
Hani et al (2016)	A comparison study between batches formulated using different types of polymers and at different ratios.	Hydroxypropyl methylcellulose, xantham gum, guar gum
Hassan et al (2017)	A comparison study between batches formulated using different types of polymers.	Carbopol, hydroxypropyl methylcellulose, chitosan, sodium alginate
Hombach et al (2009)	A comparison study between batches formulated using two different thiolated polymers (thiomers); the conjugates of poly(acrylic acid).	Poly(acrylic acid), poly(acrylic acid)-cysteine, poly(acrylic acid)-cysteamine
Kailasam et al (2010)	A comparison study between batches formulated using wo different types of polymer in different ratios.	Carbopol, hydroxypropyl methylcellulose
Kast et al (2002)	Investigated the effect of thiolation by using various amounts of thiolating agent. A comparison study between batches formulated using thiolated and non-thiolated polymers.	Chitosan, chitosan-thioglycolic acid (thiolated)
Khan et al (2017)	Investigated the application of PCP as the matrix forming polymer with other types of polymers. A comparison study between batches formulated with different polymer combinations.	Carbopol, chitosan, sodium carboxymethylcellulose
Khan et al (2014)	Formulation and evaluation of mucoadhesive vaginal tablets of tenofovir disoproxil fumarate.	Hydroxypropyl methylcellulose, carbopol, chitosan, sodium carboxymethylcellulose
Lupo et al (2017)	Investigated the effect of enhanced modification by protecting the thiol groups on thiolated polymers to avoid oxidation and increase the stability of the polymer. A comparison study between batches formulated using S-protected thiomer and non-thiolated polymer.	Chitosan, S-protected chitosan
Notario-Pérez et al (2019)	Evaluated and compared batches of vaginal tablets formulated using granules prepared by hot-melt granulation method.	Chitosan, hydroxypropyl methylcellulose, polyvinyl pyrrolidone
Notario-Pérez et al (a) (2017)	A comparison study between batches formulated using different polymer combinations.	Hydroxypropyl methylcellulose, chitosan, Eudragit RS®, guar gum
Notario-Pérez et al (b) (2017)	A comparison study between batches formulated using natural, semisynthetic, and synthetic polymers.	Chitosan, hydroxypropyl methylcellulose, Eudragit RS®, guar gum
Nowak et al (2015)	Investigated the effect of enhanced modification by using preactivated thiomer on polymer stability.	Hyaluronic acid-cysteine-6-mercaptonicotinamide
Pacheco-Quito et al (2020)	A comparison study between batches formulated using different types of polymers and at different amount.	Iota-carrageenan, hydroxypropyl methylcellulose
Paczkowska et al (2020)	Developed vaginal drug delivery system using lyophilized extract with CHN carrier.	Chitosan, hydroxypropyl methylcellulose, microcrystalline cellulose
Palade et al (2013)	A comparison study between batches formulated using acrylic acid derivatives and cellulose derivatives.	Carbopol, metolose
Patel A. et al (2012)	A comparison study between batches formulated with different combination of polymers. In addition, this study included an effervescent feature in the formulation to enhance the swelling of the tablets.	Carbopol, chitosan, sodium carboxymethylcellulose, methyl cellulose, hydroxypropyl methylcellulose, hydroxyethylcellulose, sodium alginate
Patel A. et al (2011)	A comparison study between batches formulated with different combination of polymers. In addition, this study included an effervescent feature in the formulation to enhance the swelling of the tablets.	Carbopol, chitosan, sodium carboxymethylcellulose, methyl cellulose, hydroxypropyl methylcellulose, hydroxyethylcellulose, sodium alginate
Patel G.M. et al (2010)	A comparison study between batches formulated with different combination of polymers. In addition, this study included an effervescent feature in the formulation to enhance the swelling of the tablets.	Hydroxypropyl methylcellulose, carbopol, sodium carboxymethylcellulose, chitosan, methyl cellulose, sodium alginate

Author	Study background	Polymers used
Pendekal et al (2013)	This study investigated spontaneous interpolymer complexes between CHN and alginate. It is thought to be a newer and efficient form of polymeric carriers and will make the tablet more versatile in its application.	Chitosan, alginate
Pendekal et al (2012)	This study investigated spontaneous interpolymer complexes between CHN and CP. It is thought to be a newer and efficient form of polymeric carriers and will make the tablet more versatile in its application.	Chitosan, carbopol
Perioli et al (2009)	A comparison study between batches that includes different type of polymers blended in different ratios.	Chitosan, polyvinyl pyrrolidone, polycarbophil
Perioli et al (2011)	A comparison study between batches using mucoadhesive polymers alone and in combinations.	Hydroxypropyl methylcellulose, carbopol
Sánchez et al (2017)	Designed a two-layered vaginal tablet. A comparison study between batches formulated using mixtures with different polymeric ratios.	Sodium carboxymethylcellulose, carbopol, chitosan
Szymańska et al (2014)	This study formulated vaginal tablets using CHN as a matrix and investigated two different mucosa surrogate as simple adhesive models.	Chitosan, silicified microcrystalline cellulose, sodium stearyl fumarate
Tunpanich et al (2019)	A comparison study between batches formulated using different types of polymers and their blends in different ratios.	Polycarbophil, hydroxypropyl methylcellulose
Valenta et al (2001)	A comparison study between batches formulated using non-thiolated and thiolated CHN. The effect of varied amount of TGA on the thiomer was investigated. The study included modification steps and structure characterization methods.	Chitosan, chitosan-thioglycolic acid (thiolated)

with synthetics ones. In addition, there were some articles that compared different batches that were made using solutions with different pH (13), some with different modification of the drug compound itself instead of the polymer (24). On the other hand, studies with polymer intervention involved modification to a selected mucoadhesive polymer into thiomers. Thiomers (thiolated polymers) are currently thought to be a new generation of mucoadhesive polymers, as they have thiol group side chains that can form inter- and/or intrachain disulfide bonds and improve the cohesive property of a formulation (25). Therefore, these studies include tablet evaluations comparing between batches formulated using non-thiolated and thiolated polymers.

Risk of bias in studies

The risk of bias from the reviewed articles is shown in Table IV. The risk of bias is indicated by symbols; positive "+" indicates that there was risk of bias and negative "–" indicates that there were no/less risk of bias. There was no risk of bias in selection and sampling as this was not applicable to the study design of all the reviewed articles. Performance bias refers to the improved results when a control goes through intervention. This was seen in articles with polymer intervention as formulation using manipulated polymers performed better compared to formulations with non-manipulated polymer. Therefore, there was some risk of bias mostly in articles that included polymer interventions. All reviewed articles were thought to have low risk of both attrition and reporting bias as there were no incomplete outcome data and there were reports on both good and bad batches in the articles, respectively. Some performance assessments were done using self-constructed or modified apparatus setup, thus increasing the risk of measurement bias, as compared to if they were to use a standardized and calibrated apparatus/equipment. Results in all the articles were expressed as mean values ± standard deviation and are indicated in the text. However, some studies conducted further statistical analysis to establish significant differences between groups (formulated batches) compared. These studies have reduced their risk of analysis bias.

Discussion

Mucoadhesive drug delivery systems

There has been extensive research done to circumvent the limitations of discomfort (i.e., messiness, leakage), short stay, and frequent dosing due to insufficient therapeutic dosage of vaginal formulations (3,14,15). Innovative and novel attempts have been made to develop vaginal formulations that consider both the clinical as well as the user's requirements. Mucoadhesive drug delivery systems exploit the useful property of mucoadhesion of certain biopolymers on interaction with mucus that is present at the targeted physiological sites, for example, the vaginal mucosa (24), though in the absence of goblet cells and the lack of direct release of mucin, the vaginal epithelium is still considered as a mucosal surface (24). Mucin is a glycoprotein that makes up the most part of mucus on mucosal surfaces. Mucin exhibits electrostatic, hydrophobic, and coupling effect, which makes it possible for good adherence of a number of substances to the vaginal mucosa (5). Mucoadhesive polymers that bind to mucin or epithelial surfaces increase the residence time of the dosage form at the action or absorption site, and thus could be useful in solving bioavailability problems resulting from a too short stay of the pharmaceutical dosage form at the absorption site (26).

TABLE IV - Bias assessment for each research article reviewed

Author	Sampling Bias	Performance Bias	Attrition Bias	Reporting Bias	Measurement Bias	Bias in Analysis
Abidin et al (2020)	–	–	–	–	–	–
Abu El-Enin et al (2020)	–	–	–	–	–	–
Baki et al (2009)	–	–	–	–	–	+
Baloglu et al (2011)	–	+	–	–	+	–
Bartkowiak et al (2018)	–	–	–	–	–	+
Bhat et al (2010)	–	–	–	–	+	–
Cazorla-Luna et al (2019)	–	–	–	–	–	–
Cevher et al (2014)	–	–	–	–	–	–
Cevher et al (2008)	–	–	–	–	–	–
El-Kamel et al (2002)	–	–	–	–	+	+
Fitaihi et al (2017)	–	–	–	–	–	–
Gupta et al (2013)	–	–	–	–	+	–
Gök et al (2017)	–	+	–	–	–	+
Hani et al (2016)	–	–	–	–	+	+
Hassan et al (2017)	–	–	–	–	+	–
Hombach et al (2009)	–	+	–	–	–	–
Kailasam et al (2010)	–	–	–	–	+	+
Kast et al (2002)	–	+	–	–	–	–
Khan et al (2017)	–	–	–	–	+	+
Khan et al (2014)	–	–	–	–	+	+
Lupo et al (2017)	–	+	–	–	–	–
Notario-Pérez et al (2019)	–	–	–	–	–	–
[a]Notario-Pérez et al (2017)	–	–	–	–	–	–
[b]Notario-Pérez et al (2017)	–	–	–	–	–	+
Nowak et al (2015)	–	+	–	–	+	+
Pacheco-Quito et al (2020)	–	–	–	–	–	–
Paczkowska et al (2020)	–	–	–	–	–	+
Palade et al (2013)	–	–	–	–	–	–
Patel A. et al (2012)	–	+	–	–	+	–
Patel A. et al (2011)	–	+	–	–	+	+
Patel G.M. et al (2010)	–	+	–	–	+	–
Pendekal et al (2013)	–	–	–	–	+	–
Pendekal et al (2012)	–	–	–	–	+	+
Perioli et al (2009)	–	–	–	–	+	+
Perioli et al (2011)	–	–	–	–	+	+
Sánchez et al (2017)	–	–	–	–	+	+
Szymańska et al (2014)	–	–	–	–	–	–
Tunpanich et al (2019)	–	–	–	–	–	–
Valenta et al (2001)	–	+	–	–	+	+

The inclusion of mucoadhesive polymers into vaginal formulations intensifies the contact between formulation and the vaginal mucosa (27,28). Mucoadhesion happens in two stages. Suitable parameters such as wettability, swelling, and hydration of the polymer can ensure close contact between polymer and mucosal layer. This establishment of contact is the first stage of mucoadhesion. Any material can adhere to the mucosa thanks to its viscous nature, but there can be no real adhesion without an interrelation between some specific chemical groups in the polymers and biological tissues, or without establishing an interpenetration of chains (29). Therefore, the second stage of mucoadhesion involves the activation of the polymer in the presence of moisture (hydration), including wetting and swelling of the formulation. The moisture plasticizes the system, which allows the release of mucoadhesive particles and their connection with the mucin by forming van der Waals or hydrogen bonds (5). This then facilitates intimate contact between the formulation and the underlying absorptive surface (14). The main purpose of mucoadhesive drug delivery system is to remain fixed (localization) at the point where the drug's release and/or absorption can occur (29). This is the great advantage as it prolongs the residence time at the targeted site of application (3,27,30). Hence, the drug's uptake and bioavailability may be increased, frequency of dosing reduced, and patient compliance improved (24,31). Apart from prolongation of drug release at the site of absorption, drug targeting to the affected site can also be realized (24).

Therefore, the polymers used in these mucoadhesive formulations must be able to adhere to the vaginal mucosa and modulate the drug release from the dosage form. Mucoadhesive polymers should ideally be biocompatible, biodegradable, and non-toxic (11). Commonly employed mucoadhesive polymers can be derived either from synthetic or natural sources (24). Additionally, after hydration, these polymers form hydrophilic matrices that can often be used to produce controlled/sustained-release formulations through their hydration, swelling, and/or erosion (28). Properly designed mucoadhesive vaginal tablets should be able to hydrate and gel very slowly, leading to a prolonged release of the drug to provide a long-term therapeutic effect with improved efficacy, reduced frequency of administration, and minimized drug side effect (32,33).

Vaginal tablets

As mentioned, vaginal dosage forms have been developed and used clinically for many years to deliver gynecological drugs and/or for local therapy of female-related conditions. As shown in Figure 2, the vaginal tablets formulated in all the research articles reviewed is a form of local therapy for female-related conditions. It has been reported that vaginal infections affect nearly 75% of adult women at least once in their lifetime (13). Therefore, it reflects on the research (Tab. II) that has been done with 56% formulated vaginal tablets to treat vaginal infections, including formulations that are antibacterial, antifungal, and antimycotic. There are increasing attention and more recent developments to tackle other female-related conditions such as HIV

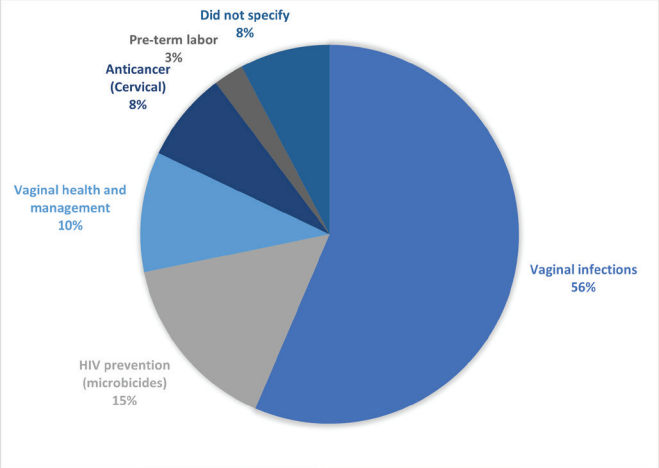

Fig. 2 - Overview of the application for the mucoadhesive vaginal tablets formulated in the 39 research articles reviewed.

preventions (15%), vaginal health and management such as hormone therapy (10%), cervical cancer (8%), and preterm labor (3%). About 8% of the research articles reviewed did not specify the application for the vaginal tablet formulated because their research focuses on developing the roles of the carrier in their mucoadhesive vaginal tablets regardless of its therapeutic purpose.

Compared to semi-solid systems, solid formulations have the advantage of high dose accuracy and long-term stability (29). Tablets are one of the best means of drug delivery because they are uncomplicated to formulate, and have the feasibility for mass industrial production at a low cost (11,34). Tablets are usually prepared by direct compression, which is an easy, rapid, and cheap method (35). Similar to tablets intended for other administrative routes, vaginal tablets also have advantages including portability, avoidance or antimicrobial agents for preservation, and ease of storage and handling (11,18). Furthermore, the application is convenient, with no applicators or supervision needed, giving the user an added advantage of discretion (18,34).

Tablets are a recurring trend because they permit controlling effects on the drug dissolution. Recently, considerable attention has been paid on novel and controlled release systems to provide a long-term therapeutic concentration of drug following a single administration (36). There has been major advancement in the development of vaginal tablets that increase vaginal residence time and are capable of delivering the active agent for an extended period at a predictable rate using mucoadhesive polymers (3,27,32). It can be an appropriate therapeutic strategy as the required quantity of the drug can be readily administered; with the prolonged residence time, high drug levels at the target site are achievable, simultaneously minimizing unnecessary drug exposure and side effect in other parts of the body (26,32). Mucoadhesive vaginal tablets are relatively easy to insert and do not cause leakages (37). A controlled drug release can be achieved over several hours, if the delivery system does not disintegrate too early (38). In general, the tablet softens and adheres to the vaginal mucosa and is retained in position until dissolution

and/or release is complete. They are designed to melt in the vaginal cavity and release the drug for several hours (3). A controlled drug release can be achieved over several hours if the formulation does not disintegrate too early (38). After a short time, the presence of tablet is reported to be no longer noticeable to the patient (3).

Referring to the tablet shapes and designs from the reviewed studies, 80% (31 research articles) are flat-faced cylindrical-shaped tablets, compared to a few (3 research articles) that used an ovoid, concave, and capsule shape and 5 research articles that did not specify the shape of the tablet (Tab. II). However, in these studies the research articles did mention using a disc when testing for the mucoadhesive property of the formulations. The diameters of the tablets range from 15 to 5 mm; 13 mm is the most popular diameter (11 research articles). However, recent research has started using smaller sized tablets. Tablets kept at a smaller size offer a more efficient drug release ability, reduce the risk of an inhomogeneous blend or non-uniform drug content in finished tablets, and can also improve patient comfort and thus compliance (18).

Strategies in formulating mucoadhesive vaginal tablets

The strategies in designing a mucoadhesive vaginal tablet adopted by the reviewed articles are divided into six main categories (Fig. 3). The strategies used are listed as follows: (i) polymer blends (46%), (ii) thiomers (18%), (iii) intermolecular complexes (18%), (iv) physical design (10%), (v) micro-particulate technology (5%), and (vi) formulation technique (3%). The percentages are the number of research articles that used the design strategy respectively. The categories were decided by the reviewers based on the theoretical application/theme used in designing the tablets. As the advancement of mucoadhesive drug system continues, there is a vast number of novel strategies that are being invented and it can get quite competitive. Therefore, this review focuses greatly on the assessment methods that are conducted by the research articles reviewed, to evaluate the degree of mucoadhesion for vaginal tablet formulation.

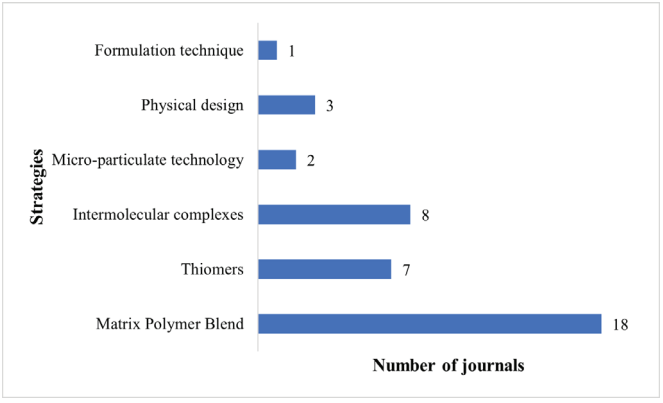

Fig. 3 - The main categories of the strategies adopted in the research articles reviewed in designing their mucoadhesive vaginal tablets.

Physical evaluations of tablets/physicochemical properties

Tablet formulations are evaluated to comply to specific pharmacopoeia requirements, including precompression (e.g. tabletability, flowability), tablet uniformity, friability, disintegration time, tablet ejection force, and dissolution performance and any other relevant evaluations (18). If any of these requirements are not met, the formulation should be excluded and new formulation is designed and re-evaluated to address identified deficiencies (18).

Precompression evaluation investigates the powdered mixtures' compressibility profile or matrix granules before compression. The flowability determines the mechanical behavior of the powders, which can affect the tablets' weight, hardness, and content uniformity (39). For example, powder flowability can determine the ease with which the powder can be fed into the die and non-flowing powders can also cause the non-uniformity incorporation of drug compounds in the powders (40). Therefore, flow properties of pharmaceutical powders are critical to manufacturing. There are various methods that can be used as an indication of powder flowability, including measurement of angle of repose, bulk density, tapped density, and Carr's compressibility index (CI) or Hausner ratio (HR) (39). Angle of repose (°) is the steepest slope of the unconfined material, measured from a horizontal plane (41). Repose angles are reported within the range of 30°–55°. It is described that less than 30° are powders that has high flowability and more than 55° are non-flowing powders (41). Bulk and tapped densities are commonly reported together, as it measures the volume occupied by the powder (of known mass), g/mL, in a graduated cylinder mounted onto a tapping platform. The volume occupied before tapping is reported as the bulk density and after a range of 250–1,000 taps, the volume obtained is reported as the tapped density (18). Both these densities are used to calculate CI and HR to characterize flowability. CI uses a percentage scale of 0–100, where 0–10% indicates excellent flowability. HR has a range of 1.00–1.50, where 1.00–1.11 indicates excellent flowability (18,40). Poor flowability of powders are indicated with >31 CI and >1.60 HR. Any value in between can describe the powder's flowability as good, fair, and passable. These evaluations are to some extent interrelated to one another and help to describe the particles' flow and how readily the material undergoes a change in volume when compressed (18,32).

Good compressibility, on the other hand, describes a material capable of achieving desired tablet hardness at low compression pressure. It is important to remember that any unnecessary increase in compression pressure can induce physical changes upon the compressed material (37). Therefore, careful selection of optimal compression pressure to be employed in manufacturing the tablets is important. A pressure will not physically damage the material and can produce tablets with good tensile strength.

Figure 4 shows the list of the types of tablet evaluations that have been conducted by the research articles reviewed (Tab. V). About 54% and 44% of the research articles conducted the hardness and friability test, respectively. These are considered fundamental evaluations as it ensures that the formulations have satisfactory tablet strength to withstand

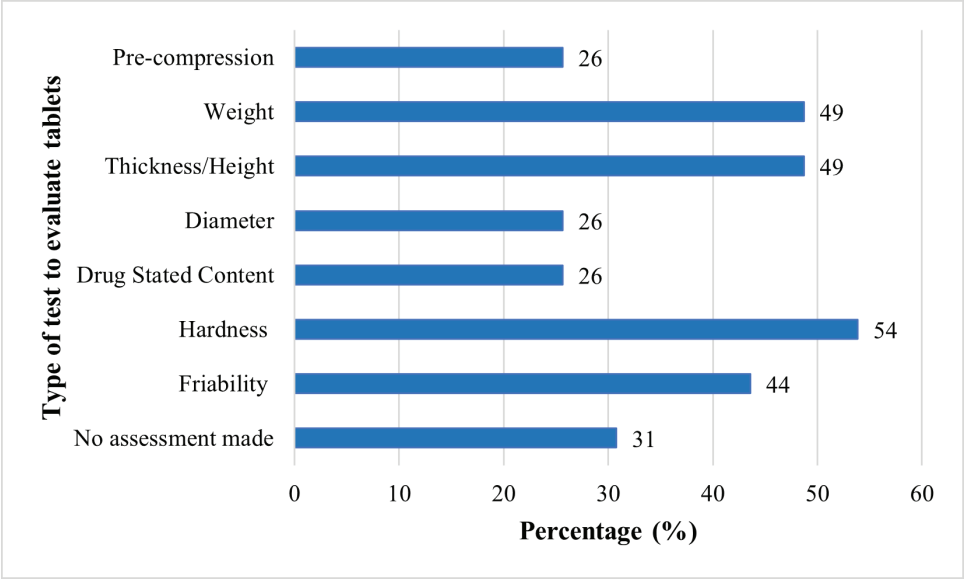

Fig. 4 - This bar chart shows the type and frequency of the tablet evaluations conducted within the research articles reviewed.

TABLE V - Physicochemical evaluations conducted by each research article reviewed

Author	Precompression	Uniformity measurement				Durability measurement	
		Weight	Height/ thickness	Diameter	Drug content	Hardness	Friability
Abidin et al (2020)	√	√	√	√	√	√	√
Abu El-Enin et al (2020)	–	√	–	–	√	√	√
Baki et al (2009)	√	–	√	√	–	–	√
Baloglu et al (2011)	–	√	√	√	–	√	√
Bartkowiak et al (2018)	–	–	–	–	–	–	–
Bhat et al (2010)	–	√	–	–	√	√	√
Cazorla-Luna et al (2019)	–	√	√	√	–	√	–
Cevher et al (2014)	–	√	–	–	–	√	√
Cevher et al (2008)	–	–	–	–	–	–	–
El-Kamel et al (2002)	–	–	–	–	√	–	–
Fitaihi et al (2017)	√	√	√	–	√	√	√
Gupta et al (2013)	–	√	√	–	√	√	√
Gök et al (2017)	–	–	–	–	–	–	–
Hani et al (2016)	–	√	√	–	√	√	√
Hassan et al (2017)	–	√	√	–	√	√	√
Hombach et al (2009)	–	–	–	–	–	–	–
Kailasam et al (2010)	–	–	–	–	–	–	–
Kast et al (2002)	–	–	–	–	–	–	–
Khan et al (2017)	√	√	√	√	–	√	√
Khan et al (2014)	√	√	√	√	–	√	√
Lupo et al (2017)	–	–	–	–	–	–	–
Notario-Pérez et al (2019)	–	–	–	–	–	–	–
[a]Notario-Pérez et al (2017)	–	–	–	–	–	–	–
[b]Notario-Pérez et al (2017)	–	–	–	–	–	–	–

(*Continued*)

TABLE V - (*Continued*)

Author	Precompression	Uniformity measurement				Durability measurement	
		Weight	Height/ thickness	Diameter	Drug content	Hardness	Friability
Nowak et al (2015)	–	–	–	–	–	–	–
Pacheco-Quito et al (2020)	–	√	√	√	–	–	–
Paczkowska et al (2020)	–	√	√	√	–	√	–
Palade et al (2013)	–	–	–	–	–	–	–
Patel A. et al (2012)	–	√	√	–	–	√	–
Patel A. et al (2011)	–	–	–	–	–	√	√
Patel G.M. et al (2010)	–	√	√	–	–	√	–
Pendekal et al (2013)	–	–	–	–	–	–	–
Pendekal et al (2012)	–	–	–	–	–	–	–
Perioli et al (2009)	–	–	√	–	–	√	√
Perioli et al (2011)	–	–	√	–	–	√	√
Sánchez et al (2017)	–	√	√	√	–	√	–
Szymańska et al (2014)	–	√	√	√	√	√	√
Tunpanich et al (2019)	√	√	√	–	√	√	√
Valenta et al (2001)	–	–	–	–	–	–	–

abrasion or chipping during packaging, handling, and shipping (18,26). Evaluations of tablet's hardness typically involve crushing actions of individual tablets and the crushing force used can be reported in Newtons (N) and/or kilograms (kg). The crushing force reported serves as an information to avoid crushing the tablets during manufacturing and packaging processes. Friability evaluation involves subjecting tablets in circular motions and the result is reported as the weight difference of the tablets before and after the test, in percentage. Weight loss of below 1% is ideal. The friability test can help indicate the brittleness of the tablets, which helps to prevent chipping and abrasion of the tablets (18).

Weight, thickness/height, diameter, and drug content measurements are evaluations of uniformity in the tablets manufactured. The evaluations of tablet uniformity are typically reported as an average and standard deviation. Weight, thickness/height, and diameter of any individual tablets should not deviate more than 5% from the average measurement. Drug content within 95–105% from the expected content is considered acceptable (18). The tablet uniformity is important as it is an indication of a consistent tablet formulation and manufacturing process (26). Furthermore, uniformed tablets can also contribute to consistent tablet performance.

Swelling assessment

Mucoadhesive drug delivery systems require mucoadhesive polymers that can adhere to the vaginal mucosa and swell rapidly in aqueous environmental conditions (36). Some studies have suggested that tablet swelling is an important parameter to be studied before considering mucoadhesion (35). The swelling characteristic of a polymer contributes to their adhesive capacity and in order to manifest maximum adhesive strength, an optimum water uptake (hydration) is needed for the polymer particles (26). However, if the hydration is too high, the adhesion property is expected to be reduced due to the competition between water molecules and the active groups in the mucin chains of the vaginal mucosa to bind to the functional groups of the polymer (18). Therefore, the swelling property (degree of hydration) should be investigated and considered when designing the mucoadhesive vaginal tablet formulations. About 90% of the vaginal tablets in the research articles reviewed were subjected to swelling tests and the results were used to consider the tablet size and improve drug retention and drug release kinetics.

The swelling characteristic is typically done by subjecting the tablet to hydration in an aqueous solvent used in dissolution or disintegration studies. The degree of hydration may be reported using units, including swelling index (SI), swelling ratio (SR/Q), water uptake ratio (WUR), mass swelling factor (MSF), swelling degree (Q_e), water uptake (WU), swelling percentage, hydration percentage, matrix erosion (ME), and matric dissolution (DS). Although there are various units to report the numerical data of swelling, all of these measure the degree of hydration by the change in weight that occurs in the tablet formulations after hydration. Therefore, it is essential to record the weight of individual tablets prior to hydration. Positive values indicate that the percentage of swelling or weight gain is greater than the initial weight of the dry formulation, while negative values indicate the weight is less than the dry formulations and this can be due to erosion or dissolution of the system in the solvent (11).

From the research articles reviewed, the swelling test was done using three different methods: (i) immersion (49%), (ii) gravimetric (31%), and (iii) water absorbing method (10%) (Tab. VI). The different method describes the different setting

TABLE VI - Swelling assessment conducted by each research article reviewed

Author	Method			Shaking motion	Incubation temperature (°C)	Reporting unit	Swelling witness test
	Immersion	Gravimetric	Water absorbing				
Abidin et al (2020)	√			–	37 ± 1	Swelling index (SI)	–
Abu El-Enin et al (2020)	√			–	Ambient	Swelling index (SI)	–
Baki et al (2009)	–	–	–	–	37 ± 1	–	–
Baloglu et al (2011)		√		–	37 ± 1	Water uptake ratio (WUR)	–
Bartkowiak et al (2018)	√			–	37 ± 1	Mass swelling factor (MSF)	–
Bhat et al (2010)	√			–	37 ± 1	Swelling index (SI)	–
Cazorla-Luna et al (2019)	√			√	37 ± 1	Swelling ratio (SR/Q)	√
Cevher et al (2014)		√		–	37 ± 1	Swelling index (SI)	–
Cevher et al (2008)		√		–	37 ± 1	Swelling index and Matrix erosion (ME)	–
El-Kamel et al (2002)	√			–	37 ± 1	Swelling index (SI)	–
Fitaihi et al (2017)		√		–	37 ± 1	Swelling percentage (%S)	–
Gupta et al (2013)	√			–	37 ± 1	Reweighed	–
Gök et al (2017)		√		–	37 ± 1	Swelling degree (Q_e)	–
Hani et al (2016)	√			–	37 ± 1	Percentage of hydration	–
Hassan et al (2017)	√			–	37 ± 1	Swelling index (SI)	–
Hombach et al (2009)		√		–	37 ± 1	Reweighed	–
Kailasam et al (2010)			√	–	37 ± 1	Swelling index (SI)	–
Kast et al (2002)		√		–	37 ± 1	Reweighed	–
Khan et al (2017)	√			–	Ambient	Swelling index (SI)	–
Khan et al (2014)	√			–	37 ± 1	Swelling index (SI)	–
Lupo et al (2017)		√		–	37 ± 1	Water uptake (WU)	–
Notario-Pérez et al (2019)		√		√	37 ± 1	Swelling ratio (SR)	–
[a]Notario-Pérez et al (2017)		√		√	37 ± 1	Swelling ratio (SR)	√
[b]Notario-Pérez et al (2017)	√			√	37 ± 1	Swelling ratio (SR)	√
Nowak et al (2015)		√		–	37 ± 1	Water uptake percentage	–
Pacheco-Quito et al (2020)	√			√	37 ± 1	Swelling ratio (SR)	√
Paczkowska et al (2020)	–	–	–	–	37 ± 1	–	–
Palade et al (2013)	–	–	–	–	37 ± 1	–	–
Patel A. et al (2012)			√	–	37 ± 1	Swelling percentage (%S)	–
Patel A. et al (2011)			√	–	37 ± 1	Swelling percentage (%S)	–
Patel G.M. et al (2010)			√	–	37 ± 1	Swelling percentage (%S)	–
Pendekal et al (2013)	√			–	37 ± 1	Swelling index (SI)	–
Pendekal et al (2012)	√			–	37 ± 1	Swelling index (SI)	–
Perioli et al (2009)	√			–	37 ± 1	Hydration percentage and Matrix erosion (ME)	–
Perioli et al (2011)	√			√	37 ± 1	Hydration percentage and Matrix erosion (ME)	–
Sánchez et al (2017)	√			–	37 ± 1	Hydration percentage and Matrix dissolution (DS)	–
Szymańska et al (2014)	√			–	37 ± 1	Swelling index (SI)	–
Tunpanich et al (2019)		√		–	37 ± 1	Swelling index (SI) and Matrix erosion (ME)	–
Valenta et al (2001)	–	–	–	–	37 ± 1	–	–

in hydrating the tablets. The immersion method involves total submergence of the tablet into a solvent. Gravimetric method involves fixing the tablet to a needle and suspending it into a solvent. This method is also called the tea bag method in some research articles. There was no specific volume; from the research articles reviewed there was a range of volume from 5 mL up to 1000 mL of solvent used for these methods. In the third method, individual vaginal tablet is placed on 2% agar gel plates to assess the water absorbing capacity of each tablet. Most of these swelling methods were conducted in incubators or water bath, kept at 37 ± 1℃, and some remained stationary or was put in a shaking motion. Two research articles reported to conduct the swelling test in ambient temperature (14,42). Individual tablets are taken out at scheduled time intervals and excess surface water was carefully removed. The swollen tablets were then reweighed for the swelling assessment. Proper swelling characteristic will contribute to effective mucoadhesion and controlled release of the drug (14).

The polymer will start to swell upon hydration, and a viscous gel layer should start to form around the tablet core (26). As it reaches the maximum swelling capacity, erosion occurs until complete dissolving or erosion of tablets (14). The formation of this gel layer has been regarded as an essential first step that would govern the drug release from the vaginal tablet formulation (26).

Swelling witness test

In the past few years, researchers have started to include a complementary technique using prepared swelling witnesses to better understand the swelling behavior of the mucoadhesive polymers used and structural characterization of the tablets (16,43). It is a method that evaluates the tablet's capacity to absorb water or determine the entry pattern of the solvent into the tablet during the hydration process (16). The mobility (movement) of the solvent within the tablet formulation controls the swelling and erosion of the tablets, thus it plays a role in the drug release as well (11).

Tablet formulations were left to hydrate and swell in a chosen solvent in accordance with predetermined time and/or until it reaches the maximum swelling capacity. The swollen tablets were then immediately lyophilized (freeze-dried). Water is removed during freeze-drying, and the space that was originally occupied by the solvent is transformed into pores (hollow gaps), obtaining porous structures similar to a sponge, that are now called swelling witnesses (16,43). The swelling witnesses can then be observed and analyzed by field emission scanning electron microscopy (SEM) (11,16). Corresponding SEM micrographs shows the witness' microstructures which vary depending on the type of polymer and different formulations. Mercury porosimetry can be used to determine the pore size distributions (PSDs) of the witnesses, which reflects the pores (gaps/hollows) that were occupied by the solvent before the freeze-drying process (16). The gaps inside the polymers are related to the swelling ratio, as the gaps get larger the higher its ability to capture water, and the tablet formulation swells more (43). Polymers with good swelling capacity will produce a narrow PSD (the pores are closer together) with high pore sizes. In contrast, polymers with minimal swelling properties produce a wider PSD (the pores are further apart) and smaller pore sizes. The wider PSDs, however, do have an appealing advantage, where it will help the tablet to maintain the shape while the drug diffuses slowly between them (43). Furthermore, formulations capturing less water are expected to be more comfortable for the user.

There were four research articles that included the swelling witness in their research, and they have reported varying microstructures in their witnesses depending on the nature of their polymers and the changes in the swelling witness in different formulations (11,16,29,43). When a solvent enters the polymer during swelling, it creates different microstructures depending on the nature of the polymer. Figure 5 is a compilation of SEM micrographs from the research articles that depict the most distinctive microstructures corresponding to the type of drug release that it can achieve. Figure 5A shows a channeled microstructure (elongated channels) allowing gradual uptake of the surrounding solvent which translates into a moderate swelling behavior (29). The moderate swelling can help maintain the shape of the tablet while the drug diffuses slowly between them (43). Figure 5B shows a sponge-like microstructure with numerous pores, which the solvent can circulate with some difficulty, which would also result in moderate swelling capacity of the formulation (11). Figure 5C shows a microstructure arranged in parallel sheets with the absorbed solvent between them (29). Formulations using this type of polymer will swell the most and can remain swollen the longest, as there is a high capacity for very effectively retaining water between the sheets. However, although the water cannot escape, the drug is able to diffuse through the polymer sheets, thus reducing their ability to retain the drug longer (43). Figure 5D is included to show one of the examples when a formulation was unable to swell (29). This formulation

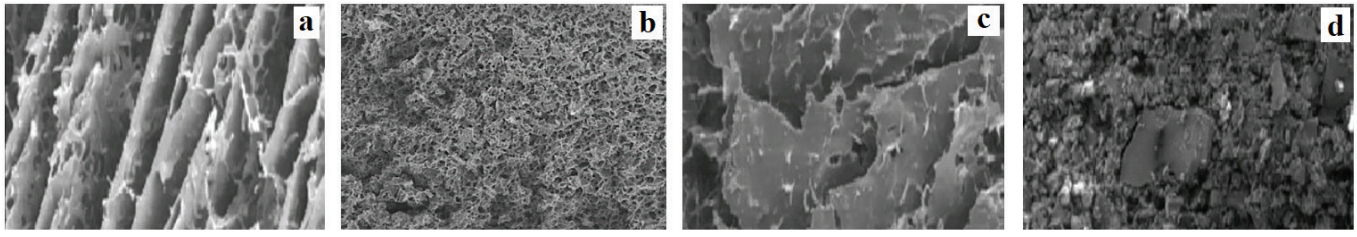

Fig. 5 - Electron microscopy micrographs of distinct swollen witness in different polymers compiled from four research articles reviewed. The different panels show the different type of microstructures observed: A) channeled microstructure (250 µm) (29); B) sponge-like microstructure (1 mm) (11); C) parallel sheets microstructure (250 µm) (29); and D) a material that is unable to swell (250 µm) (29). (Pictures are reproduced from references (11) and (29) under the Creative Common Attributions License.)

showed a grainy microstructure with different-sized particles and was reported to have failed in controlling drug delivery (43). These images are compiled from research articles reviewed and reproduced from references (11) and (29) under the Creative Common Attributions License.

This assessment helps to identify the effects or modification in the microstructure arrangement, WU process, and swelling capability, when different polymers are mixed and/or drugs are incorporated into the formulation. The presence of other materials that do not swell can make it difficult for the polymer to swell as usual. In some cases, materials that do not swell can clog the pores, thus reducing the sizes of the pores or hindering the drug diffusion through the polymer (43). Therefore, WU rates are reduced and can be the cause for slower drug release from the tablet formulations (16). Although the key criteria for selecting the optimum formulation are control over the drug release and mucoadhesion to the vaginal mucosa, the amount of swelling must be taken into account in formulations that can achieve these criteria but capturing less water, as it will be more comfortable (11).

Drug release assessment

The terms "drug dissolution" and "drug release" are not synonyms, although they are often not appropriately distinguished. Drug dissolution refers to the process of diffusion of the drug into the solvent. Unless the drug dissolution process is the factor that can manipulate the release of the drug, the drug dissolution and drug release can be considered synonyms. In all other cases, the drug release is the more appropriate term. Upon contact with the dissolution solvent, water penetrates the tablet formulation and can dissolve the drug content. The dissolved drug substance subsequently diffuses from the tablet due to the concentration gradient. Additionally, the tablet formulation might also undergo several changes including swelling and consequent dissolution in the solvent, all contributing to the overall drug release process (44). Assessing drug dissolution/release from the tablet formulation is extremely important within the absorption process to be effective and it is indicative of the tablet's potential in vivo performance and clinical applications (44,45). Ideally, 100% of drug dissolution is targeted in all formulations as it can increase bioavailability and the efficacy of the formulation. However, it depends on the objective of the therapy to have an immediate or controlled/extended drug release. A constant drug release rate over the targeted time also contributes to the formulation performance (18).

The in vitro dissolution/release test represents an important tool for this purpose as it can be used to assess the dissolution and release profile of the formulation in an artificial vaginal environment (45). Drug releases are primarily recorded at predetermined time intervals to observe the difference in drug concentration over time. It has become an important tool in the drug product development phase and its quality control and the regulatory approval process (44). For many types of mucosal formulations including vaginal tablets, drug testing is performed using the apparatus developed for oral formulations (44). Today four apparatuses for dissolution testing of solid dosage forms are described in pharmacopoeias: paddle apparatus, basket apparatus, reciprocating cylinder, and flow through cell. From the research articles reviewed (Tab. VII), 46% have used the paddle

TABLE VII - Drug release assessment conducted by each research article reviewed

Author	Novel setup/ other	Dissolution medium	pH	Volume (mL)	Sink conditions	RPM	USP paddle	USP basket	Shaking water bath	Still water bath	Disintegration test
Abidin et al (2020)		2% sodium dodecyl sulfate aqueous solution	4.2	900	√	100	√				X
Abu El-Enin et al (2020)		Simulated vaginal fluid	4.5	100	√	50	√				X
Baki et al (2009)		Phosphate buffer	4.6	4	X	X			√		X
Baloglu et al (2011)	√	Simulated vaginal fluid	X	6	X						√
Bartkowiak et al (2018)		Simulated vaginal fluid	5.8	500	√	50	√				X
Bhat et al (2010)		100 mM acetate buffer	6	500	√	25	√				X
Cazorla-Luna et al (2019)		Simulated vaginal fluid	X	80	√	15			√		X
Cevher et al (2014)		Lactate buffer	5	250	X	75	√				X
Cevher et al (2008)		Lactate buffer	5	500	√	75		√			X
El-Kamel et al (2002)		Citrate buffer	5.5	650	√	25	√				X
Fitaihi et al (2017)		McIlvaine's citrate buffer	4.8	250	√	50	√				X

(*Continued*)

TABLE VII - (*Continued*)

Author	Novel setup/ other	Dissolution medium	pH	Volume (mL)	Sink conditions	RPM	USP paddle	USP basket	Shaking water bath	Still water bath	Disintegration test
Gupta et al (2013)		Citrate phosphate buffer	4.5	600	√	100		√			X
Gök et al (2017)		Lactate buffer	X	500	X	75		√			X
Hani et al (2016)		Simulated vaginal fluid	X	900	√	100		√			X
Hassan et al (2017)		Citrate buffer	4.5	900	√	50	√				X
Hombach et al (2009)		Simulated vaginal fluid	4.2	500	√	20	√				X
Kailasam et al (2010)		1 M phosphate buffer	4.0	X	X	50	√				X
Kast et al (2002)		100 mM acetate buffer	6.0	4	√	100			√		√
Khan et al (2017)		Simulated vaginal fluid	4.2	100	√	50	√				X
Khan et al (2014)		Simulated vaginal fluid	4.2	100	√	50	√				X
Lupo et al (2017)	√	Simulated vaginal fluid	4.2	6	√	630					√
Notario-Pérez et al (2019)		Simulated vaginal fluid	X	80	√	15			√		X
[a]Notario-Pérez et al (2017)		Simulated vaginal fluid	X	80	X	15			√		X
[b]Notario-Pérez et al (2017)		Simulated vaginal fluid	X	80	√	15			√		X
Nowak et al (2015		–	–	–	–	–	–	–	–	–	√
Pacheco-Quito et al (2020)		Simulated vaginal fluid	X	80	√	15			√		X
Paczkowska et al (2020)		Phosphate buffer	4.5	150	√	50	√				X
Palade et al (2013)		Acetate buffer	4.2	900	√	60	√				X
Patel A. et al (2012)		Phosphate buffer	4.0	500	√	30		√			X
Patel A. et al (2011)		Phosphate buffer	4.0	500	√	30		√			X
Patel G.M. et al (2010)		Citrate buffer	4.0	500	√	50		√			X
Pendekal et al (2013)		Simulated vaginal fluid	4.2	500	√	50	√				X
Pendekal et al (2012)		Simulated vaginal fluid	4.2	500	√	50	√				X
Perioli et al (2009)		Simulated vaginal fluid	X	900	√	100		√			X
Perioli et al (2011)		Simulated vaginal fluid	X	X	√	100		√			X
Sánchez et al (2017)		Simulated vaginal fluid	5.5	600	√	100	√				√
Szymańska et al (2014)		0.08 M acetic buffer	4.5	900	√	75	√				X
Tunpanich et al (2019)		1% sodium dodecyl sulfate aqueous solution	5.5	900	√	75		√			X
Valenta et al (2001)		100 mM phosphate buffer	6.0	20	√	100			√		X

*NB: X = did not specify; "–" = was not conducted.

apparatus and 26% used the basket method, and others have used alternative dissolution apparatus. Most methods used are designed to mimic the general conditions encountered in the physiological environment of the vagina, including the pH of the dissolution solvent and maintaining a temperature of 37 ± 1℃ during testing. A total of 72% from the reviewed studies have specified the pH of the dissolution medium used and the mean pH was 4.7, which is within the range of the pH of the vaginal fluid (pH 4–5) (46,47).

Although it is acceptable for research articles to use the apparatus mentioned, it should be pointed out that these methods utilize a large volume of dissolution solvent and the rotational movement (36,44). These are generally far from the real in vivo conditions at the vaginal mucosa, thus the method used would not have given an accurate information. Some articles have taken the initiative to use smaller-volume apparatus, and some have designed a novel drug release technique that considers the correct amount of vaginal fluid and its turnover in the vaginal lumen. As reviewed (Tab. VII), 59% of the studies used larger dissolution solvent volumes ranging from 150 to 900 mL; 23% have tried reducing the volume, using 20–100 mL of dissolution solvent; however, even these volumes are significantly higher than those available to the mucoadhesive vaginal tablet on the vaginal mucosa (44). The daily production of vaginal fluid is approximately 6 mL and 0.5–0.75 mL is continually present in the vagina (36). Only 10% of the studies have considered using the correct estimated volumes ranging from 4 to 7 mL to closely mimic the in vivo conditions for vaginal administration. As in the case of the rotational movement, there is no specification. Most studies have chosen a setting of revolutions per minute (rpm) that is appropriate only to their tablet formulations. However, 100 and 50 rpm are commonly used. The rpm used in the research articles reviewed ranges from 15 to 100 rpm and one study (48) used an astounding 630 rpm as its dissolution technique employs a thermomixer. Even though some methods do not represent the correct vaginal environment, the results of these evaluations can still contribute to preliminary findings of the formulation.

An increasing number of research have modified and designed drug release techniques that can better simulate the specific conditions of the vaginal environment for mucoadhesive vaginal tablets. Various parameters must be considered when designing a dissolution apparatus, including selection of apparatus, volume and composition of the dissolution medium, environmental conditions of the absorption site (e.g., agitation), and surface exposure of the tablet (36,44). However, as new technique emerges, there is a need now to further validate and standardize the methods developed (44). For further advancement of drug release techniques, more physiological vaginal conditions should be considered. For instance, different conditions occur during menstrual cycle or at different ages, along with the enzymatic activity of the vaginal microflora. In particular, the pH and composition of vaginal fluid change from low pH values (3.5–4.5) during the ovulation phase to a higher pH during menstruation (49).

Novel release study method by Baloglu et al (36)

In this study, a new simple technique mimicking the vaginal environment was developed to investigate the release behavior of the vaginal tablet formulation. The apparatus mainly consists of a perfusor and syringe which are connected with a thin latex connector and a sample collection vessel as illustrated in Figure 6. (Pictures are reproduced from (36) and have received copyrights permission by the publisher on April 28, 2022.) The syringe used (without needle) has an internal diameter of 20 mm and total length of 75 mm to simulate the vaginal physiology. Tablets were placed at the bottom of the syringe and the assembly was dipped into a water at 37 ± 0.5℃. A perfusor was connected to the top of the syringe to supply a total of 6 mL of vaginal fluid to the tablets in 24 hours. The same amount of sample was collected concurrently from the bottom (36).

Kinetic analysis

To understand the mechanism of drug release from mucoadhesive tablets, some research articles have plotted the in vitro drug release data in kinetic equation models. Many model-dependent approaches can be used to

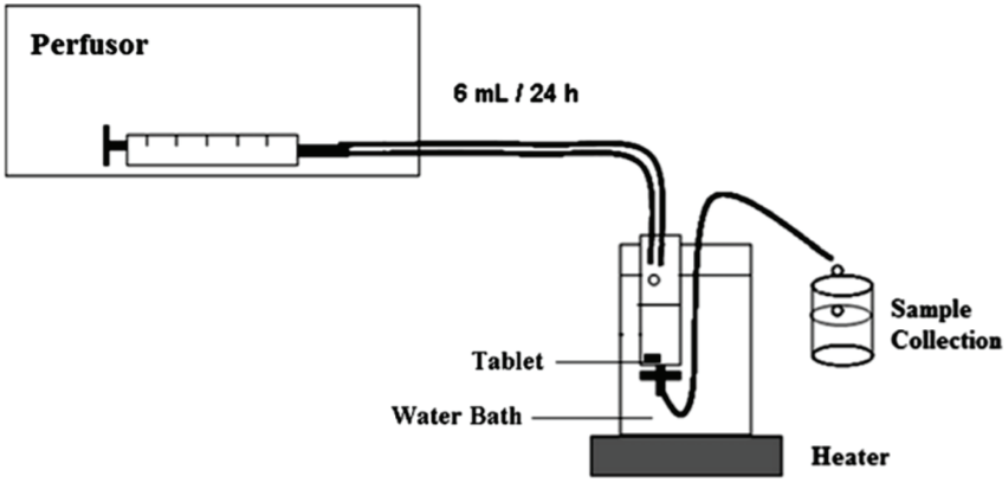

Fig. 6 - Schematic drawing of the in vitro release studies developed by Baloglu et al. (36). (Picture was taken from reference (36) and has received copyrights permission by the publisher on April 28, 2022.)

investigate the best-fit parameters (32). The common equation used in most kinetic studies of the research articles reviewed is the Korsmeyer-Peppas semi-empirical model (Eq. [1]),

$$\frac{M_t}{M_\infty} = k_{KP} t^n \qquad \text{Equation 1}$$

where M_t/M_∞ is the fractional amount of drug release at time t, k_{KP} is the release rate constant, and n is the diffusional exponent that characterizes the type of release mechanism used during the dissolution process (26). The values n and k_{KP} were estimated using a linear regression of log (M_t/M_∞) compared with log t. For the case of cylindrical tablets, n = 0.45 corresponds to a Fickian diffusion release (Case I diffusional), 0.45 < n < 0.89 to a non-Fickian or anomalous diffusion, n = 0.89 to a Case-II transport or typical zero-order release and n > 0.89 to a super Case-II transport (13). Most research articles have obtained diffusional exponent that indicates a non-Fickian or anomalous diffusion, which involves a combination of both diffusion and erosion mechanism (13,26).

There are other types of kinetic release models including Higuchi (32), Weibull (32), Hopfenberg (11), Hixson and Crowell (11), and Moore and Flanner (37) that can be used; however, as mentioned the Korsmeyer-Peppas kinetic model is the most relevant and commonly used.

Mucoadhesion assessment

Defining the mucoadhesive characteristic of the vaginal tablet formulation is of great importance to prevent a decrease in the detachment of the tablet from the vaginal mucosa, prolonging the residence time of the formulation at the site of administration (36). As drug diffuses from the gel layer of the mucoadhesive polymer to the mucus and absorbed by the tissue lining the vaginal cavity, it is essential to quantify the interaction between the mucoadhesive formulation and the vaginal mucosal surface (25,36). This is reflected in Figure 7, showing only 8% of the research articles did not conduct any mucoadhesion assessment.

The main feature of mucoadhesion is the tablet formulation's attachment strength (tensile strength) to the vaginal mucosa. There are numerous tests proposed and adopted in the research articles reviewed to determine the attachment strength. The test methods reviewed can be divided into two major categories: (i) in vitro/ex vivo methods and (ii) in vivo methods. It can be summarized from the research articles reviewed (Fig. 7 and Tab. VIII) that 33% are exclusive to only one type of in vitro/ex vivo assessment method, 41% have used multiple in vitro/ex vivo methods and 18% have assessed the mucoadhesion strength in combination of both in vitro/ex vivo and in vivo methods. The in vitro/ex vivo method is by far the most common as in vivo mucoadhesive studies are costly, time consuming, and ethically sensitive (3). However, when a study does include in vivo mucoadhesive studies, it would normally only involve assessment of the most optimal vaginal tablet formulation.

In vitro/ex vivo mucoadhesive assessment

The in vitro/ex vivo methods can be further divided into forced detachment and residence time methods. In conducting these tests, it is recommended to also test blank formulations and compare it to the drug formulations. It can be useful in confirming and establishing the mucoadhesive properties of the chosen polymers or polymer blends (16). It can also be a control to evaluate if the mucoadhesive properties are altered after the addition of the drug substance with the polymers.

Forced detachment method

This in vitro/ex vivo method is based on measuring the force required for destructing the adhesive bond between the vaginal tablet and the vaginal tissue. It involves a physical act of pulling apart the vaginal tablet from the tissue,

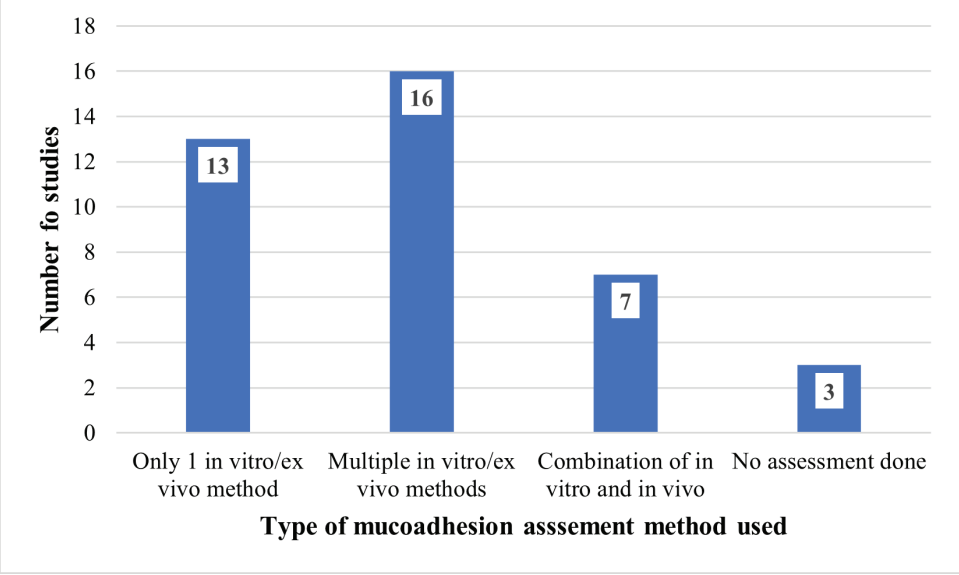

Fig. 7 - Summary of the type of methods in the research articles reviewed in assessing the mucoadhesive property of their vaginal tablet formulations.

TABLE VIII - Mucoadhesion assessments conducted by each research article reviewed

Author	Tensile method to measure force		Methods to measure over time			In vivo
	TA-XT	Novel/modified	Dissolution apparatus	Wash-off/rinsing method	Immersion/ submerged	
Abidin et al (2020)					√	
Abu El-Enin et al (2020)	√				√	√
Baki et al (2009)	–	–	–	–	–	–
Baloglu et al (2011)	√		√			
Bartkowiak et al (2018)	–	–	–	–	–	–
Bhat et al (2010)		√				
Cazorla-Luna et al (2019)	√				√	
Cevher et al (2014)	√					
Cevher et al (2008)	√					
El-Kamel et al (2002)		√				
Fitaihi et al (2017)	√				√	
Gupta et al (2013)		√				√
Gök et al (2017)	√					√
Hani et al (2016)		√			√	√
Hassan et al (2017)		√	√			√
Hombach et al (2009)	√		√			
Kailasam et al (2010)		√				
Kast et al (2002)			√			
Khan et al (2017)		√	√			
Khan et al (2014)		√	√			
Lupo et al (2017)				√		
Notario-Pérez et al (2019)					√	
[a]Notario-Pérez et al (2017)					√	
[b]Notario-Pérez et al (2017)					√	
Nowak et al (2015)		√	√			
Pacheco-Quito et al (2020)	√				√	
Paczkowska et al (2020)	√		√			
Palade et al (2013)	–	–	–	–	–	–
Patel A. et al (2012)		√	√			
Patel A. et al (2011)		√	√			
Patel G.M. et al (2010)		√	√			
Pendekal et al (2013)		√				√
Pendekal et al (2012)		√				√
Perioli et al (2009)		√			√	
Perioli et al (2011)		√			√	
Sánchez et al (2017)		√			√	
Szymańska et al (2014)	√				√	
Tunpanich et al (2019)	√					
Valenta et al (2001)		√				

representing complete detachment (32). This can be done using a tensile tester (e.g., TA-XTplus), where the vaginal tissue is fixed onto the lower clamp/plate and the vaginal tablet can be fixed onto the upper clamp/probe of the tensile tester (16). The vaginal tablet should be wetted with the dissolution medium, then both the tablet and tissue are brought into contact with a slight contact force for a minimum of 30 seconds to allow the formation of the adhesive (13). Next the tablet and the mucosa were pulled apart at a constant speed until complete detachment and the force applied to pull them apart is recorded (14,23,32). There are a total of 31 research articles that have conducted this type of assessment method; 13 research articles have used a tensile tester and the remaining 18 research articles have devised a simple apparatus or have used other alternatives, however, still in keeping with the basic principle of this assessment method.

A simple apparatus called the dynamometer was used to perform the forced detachment test in several studies (30,33,50). Some research articles have devised a simple apparatus by modifying an analytical balance to measure the force required to remove the vaginal tablet from the vaginal tissue. An example of this apparatus setup is shown in Figure 8. Any modifications can be done according to what is available to the researchers. Therefore, only a general apparatus setup will be discussed. One arm of the balance is used to hold the vaginal tablet and contact the vaginal tissue fixed on a plank/platform. A pre-load force is used to ensure adhesion bond formation when contact is made. The other arm is used to place the weights that will be added until the tablet is pulled apart from the vaginal tissue by the weights' gravity (26). In some cases, researchers used addition of water at a constant rate, instead of weights (12,38). The addition of water is stopped when the tablet detaches from the vaginal tissue. The weight required to detach the tablet formulation from the mucosa was noted (26).

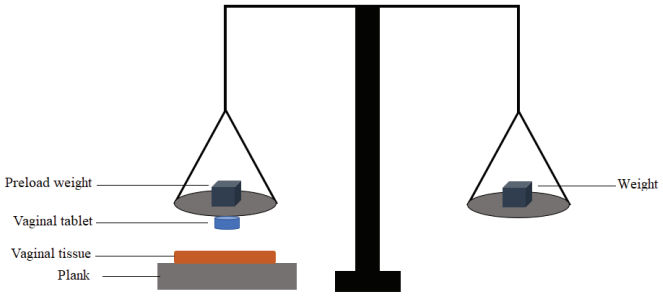

Fig. 8 - A schematic diagram of the modified analytical balance devise to measure the weight required to detach the mucoadhesive vaginal tablet from the vaginal tissue.

Time measurement test

An optimal vaginal tablet formulation must not only be able to have a good drug release control but must also remain adhered to the vaginal mucosa for a similar period of time as the drugs are released to be therapeutically effective (16). A total of 15 research articles in this review have conducted an in vitro/ex vivo test to measure how long the vaginal tablets remain attached to the vaginal mucosa. This assessment was conducted in a few different ways, by (i) total immersion, (ii) modified disintegration apparatus, and (iii) wash-off method. The mucoadhesion time was assessed over time by observation of the samples, including erosion and complete detachment of the tablet from the vaginal mucosa (16). Although there are a few different methods the general principle of a detachment test involves first attaching the vaginal tablet to the vaginal tissue, then immersing them in a dissolution solvent. This then is kept at body temperature of 37 ± 1℃ in an incubator or a water bath with or without agitation depending on the study. Visually, the time taken for erosion and completed detachment of the vaginal tablet is recorded as the mucoadhesion retention time (34).

The method by total immersion involves mounting the vaginal tissue onto a glass slide or stainless steel plate using cyanoacrylate glue. A vaginal tablet was wetted and allowed to attach to the vaginal tissue with a slight force (14). The glass slide can then be inserted in a beaker containing the dissolution solvent, at an angle (Fig. 9) or vertically with an aid of a clamp. Figure 9 shows a schematic diagram of the total immersion method at an angle to measure the mucoadhesion time of a mucoadhesive vaginal tablet formulation. Illustration includes the swelling, formation of gel layer, and detachment of the tablet. (Picture was taken from Pacheco-Quito et al (11), Figure 8, page 12 of 19 and reproduced under the Creative Common Attribution License.)

A study by Hani et al (38) glued the vaginal tissue directly on the inner side of the beaker, attached a vaginal tablet on to the tissue, and filled the beaker with dissolution solvent. This assemble was then left in a shaking incubator (38).

In vivo mucoadhesive assessment

The in vivo mucoadhesive studies include administering the vaginal tablet intravaginally to live healthy animal models,

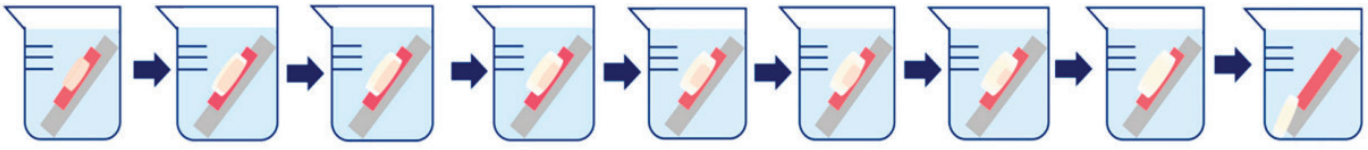

Fig. 9 - A schematic diagram showing the total immersion method at an angle to measure the mucoadhesion time of a mucoadhesive vaginal tablet formulation. Illustration includes the swelling, formation of a gel layer, and detachment of the tablet. (Picture was taken from Pacheco-Quito et al (11), Figure 8, page 12 and 19 and reproduced under the Creative Common Attribution License.)

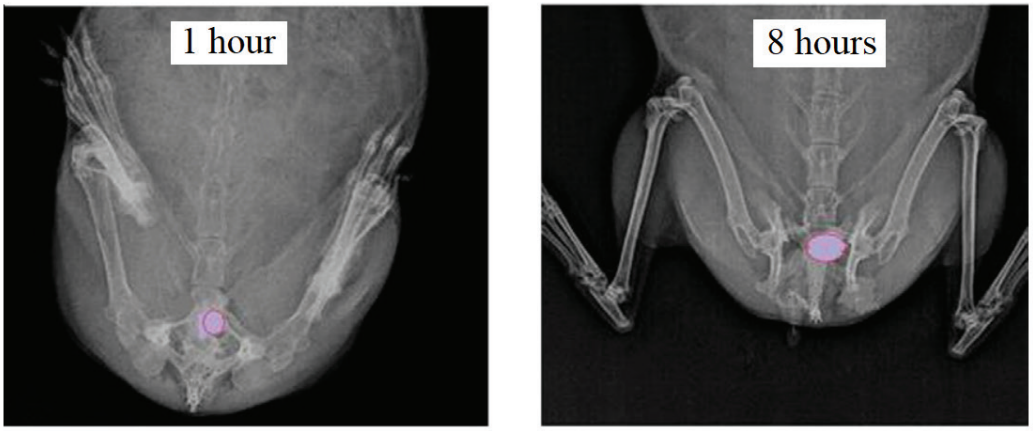

Fig. 10 - X-ray radiographic images of a rabbit's vaginal cavity after 1 and 8 hours of vaginal tablet administration. The images demonstrate that this vaginal tablet formulation was able to swell, remains intact, and adhered to the vaginal mucosa over the time allowed (51). (Pictures are taken from (51), Figure 8, page 184 and reproduced under the Creative Commons license.)

typically rats or rabbits. It is primarily applied to evaluate the behavior of vaginal tablets (appearance, physical changes, swelling behavior, and residence time in vagina) (25). The physical status of the tablets can be observed at certain time intervals using various methods including vaginal speculum (25), pictures taken using laparoscopic probe with a camera (27), and x-ray (51), as shown in Figure 10. The images illustrate that the vaginal tablet was able to swell, remains intact, and adhered to the vaginal mucosa (51). (Pictures are taken from (51), Figure 8, page 184 and reproduced under the Creative Commons license.)

From this assessment, the retention time of the tablet formulation can be established in vivo and evidently show that the tablet can remain intact and adhered to the vaginal mucosa for an expected amount of time (27). Some research articles expanded the in vivo assessment by pharmacokinetic evaluation of the drug substance present in the blood and plasma of the animal taken at different time intervals (12,14) and histological examinations (12). This can then be correlated with the drug release profile of the tablet formulation.

Simulated vaginal fluid

To optimize the formulations destined for the vaginal site, a reliable in vitro method must be put in place that may better mimic the real biological environment in the vagina, in particular in the presence of vaginal fluids. This can be essential as to better understand the behavior of the formulated tablets in the target site (47). The vaginal fluid itself has become an essential tool to evaluate the mucoadhesive strength of the formulation. It demonstrates the interaction between the vaginal fluid to the formulation. Furthermore, it helps to better understand the behavior of the formulated tablets at the target site. Therefore, simulated vaginal fluid (SVF) is used in many methods (e.g., swelling studies, dissolution studies). Vaginal fluid originates from a number of different sources. The fluid is mostly transudate from vaginal and cervical cells and also contains vulvar secretions from sebaceous, sweat, Bartholin, and skene glands, cervical mucus, endometrial and oviductal fluids, microorganisms, and their metabolic products (47). Because of the limited quantity of human vaginal fluid and its rapid degradation once collected from its source, researchers have developed a SVF in order to model the fluid properties originating in the vagina (47). Many research articles had referred and modified the SVF proposed by Owen and Katz's original research (52). A liter of SVF is prepared using NaCl (3.51 g), KOH (1.40 g), $Ca(OH)_2$ (0.222 g), albumin (0.18 g), acetic acid (1.00 g), lactic acid (2.0 g), glycerol (0.16 g), urea (0.4 g), glucose (5.0 g) mixed in 1000 mL water and stirred well until complete dissolution (14). pH of the SVF can be adjusted to 4–5 with either HCl or acetic acid according to different research articles.

Other relevant assessments

There are many other assessment methods that were conducted in the research articles reviewed, as reported in Table IX. We believe they contribute additional information to strengthen the sense of the formulated tablets in terms of stability and performance.

Physicochemical interaction studies

In this type of study, the compatibility of the drug substance and the excipients are assessed. Investigations can be performed using Fourier transform infrared spectrometry (FTIR) and/or differential scanning calorimetry (DSC) on drugs and excipients separately and in a mixed state. The results from these techniques will provide the degree of compatibility between the drug substance–excipient as well as excipient–excipient (38). It can be concluded that there are no chemical interactions in the mixes tested, if there are no changes in the characteristic peaks obtained in the IR spectra and DSC thermograms of individual substances (18,38).

Ex vivo permeation study

A research article by Pendekal et al (51) conducted an ex vivo permeation study that was carried out for the optimized formulation using Franz diffusion cell. The tablet was placed in the donor compartment on the sheep mucosa. The mucosal layer is on donor compartment. The receptor

TABLE IX - Other relevant assessment included in each research article reviewed

Author	DSC	FTIR	NMR	Size analysis	Rheology	SEM
Abidin et al (2020)	√					√
Abu El-Enin et al (2020)	–	–	–	–	–	–
Baki et al (2009)	–	–	–	–	–	–
Baloglu et al (2011)				√		
Bartkowiak et al (2018)	–	–	–	–	–	–
Bhat et al (2010)	√	√				
Cazorla-Luna et al (2019)	–	–	–	–	–	–
Cevher et al (2014)			√			√
Cevher et al (2008)	√	√	√			√
El-Kamel et al (2002)	–	–	–	–	–	–
Fitaihi et al (2017)	–	–	–	–	–	–
Gupta et al (2013)	√	√		√		√
Gök et al (2017)	–	–	–	–	–	–
Hani et al (2016)	√	√				
Hassan et al (2017)	–	–	–	–	–	–
Hombach et al (2009)				√	√	
Kailasam et al (2010)	–	–	–	–	–	–
Kast et al (2002)	–	–	–	–	–	–
Khan et al (2017)		√				
Khan et al (2014)		√				
Lupo et al (2017)			√		√	
Notario-Pérez et al (2019)	–	–	–	–	–	–
[a]Notario-Pérez et al (2017)	–	–	–	–	–	–
[b]Notario-Pérez et al (2017)	–	–	–	–	–	–
Nowak et al (2015)	–	–	–	–	–	–
Pacheco-Quito et al (2020)		√				√
Paczkowska et al (2020)	–	–	–	–	–	–
Palade et al (2013)	–	–	–	–	–	–
Patel A. et al (2012)	–	–	–	–	–	–
Patel A. et al (2011)	–	–	–	–	–	–
Patel G.M. et al (2010)	–	–	–	–	–	–
Pendekal et al (2013)	√	√				
Pendekal et al (2012)	√	√				
Perioli et al (2009)	√					
Perioli et al (2011)	√					
Sánchez et al (2017)	–	–	–	–	–	–
Szymańska et al (2014)	√					√
Tunpanich et al (2019)		√				√
Valenta et al (2001)					√	

DSC = differential scanning calorimetry; FTIR = Fourier transform infrared spectrometry; NMR = nuclear magnetic resonance; SEM = scanning electron microscopy.

compartment was filled with a dissolution solvent and the temperature was maintained at 37 ± 0.5℃ and 50 rpm. The amount of drug substances permeated through the sheep mucosa was determined by taking sample aliquots from the receptor compartment using a syringe and immediately replacing the same volume of solvent (51).

Limitations

The initial strategy was to exclude any unaccessible articles to reduce the limitation in conducting this review. There were no research articles that were unaccessible. The authors also acknowledge the large number of articles selected for this review. However, it was thought that it helps to map out the tests available in evaluating a mucoadhesive vaginal tablet formulation.

Conclusions

Although drug-controlled release profiles, mucoadhesion force, and mucoadhesion residence periods are utilized to determine the optimal formulation, vaginal formulations must be created for women's convenience, which can enhance patient compliance. Because the system must have two unique properties: (i) immobilization and (ii) controlled release characteristics, mucoadhesive drug delivery is quite complex. As a result, the approaches discussed in this study can be used to assess the balance between the two features without sacrificing one. Understanding the goals and concepts of each assessment approach can aid researchers in evaluating experimental formulations and obtaining an optimal formulation more rapidly.

Disclosures

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Conflict of interest: The authors declare no conflict of interest.
Author contributions: I.M. and E.J.M. provided relevant references and resources. I.Z.A. and G.W.F. compiled, interpreted, and analyzed the data. I.Z.A., E.J.M., and I.M. wrote the article. All authors revised the article and gave final approval of the version to be submitted. All authors have read and agreed to the published version of the manuscript.

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REVIEW

Association between cardiovascular diseases and periodontal disease: more than what meets the eye

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ABSTRACT

Cardiovascular diseases (CVDs) are inflammatory diseases of coronary arteries accompanying atheroma formation that can spawn impairment and, in severe cases, death. CVDs are the leading cause of death in the world. In recent decades, investigators have focused their impact on CVD by periodontal disease (PD). PD is a risk factor that can trigger the formation, maturation, and instability of atheroma in the arteries. Two mechanisms have been proposed to explain this relationship: periodontopathic pathogens explicitly invade the circulation or indirectly increase systemic levels of inflammatory mediators. It has been suggested that improvement in disease state has a positive effect on others. This review summarizes evidence from epidemiological studies as well as researches focusing on potential causation channels to deliver a comprehensive representation of the relationship between PD and CVD.

Keywords: Cardiovascular diseases, Periodontal disease, Periodontal therapy, Risk factor, Systematic review, Systemic diseases

Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide, claiming an estimated 17.9 million lives each year. CVD is an encyclopedic term for heart and blood vessel disorders. Atherosclerosis is an underlying cause of CVD. Atherosclerosis is a chronic vascular inflammatory condition characterized by lipid deposition (plaque) in the arterial wall (1). Atherosclerotic formation and its advancement could diminish arterial blood flow and cause ischemia in tissues or organs, as well as endorse clotting.

On the bright side, cardiovascular mortality has decreased. If combating infectious diseases was the public health success story of the first half of the 20th century, then the decline in mortality rates from CVD is the success story of the last four decades: a sharp decline in mortality rates, aided by rapid advances in both areas of prevention and treatment, including drastic reductions in smoking, improvements in the treatment and control of hypertension, and the widespread use of statins.

Periodontitis is the sixth most common disease in humans, affecting 740 million people worldwide. Periodontitis is a bacterially induced chronic tissue destructive inflammation of the teeth. This periodontal microbiota causes the release of proinflammatory mediators both locally and systemically. As the paradigm of chronic infection in dental pathology, periodontal disease (PD) shares several pathogenic pathways with CVDs. As a result of the low-grade state of systemic inflammation posed by periodontitis, it is considered to be strongly associated with CVDs (2).

There is robust association between CVD and PD. The delineating focus of the relationship has been the periodontal pathogens from the oral cavity, which directly exacerbate CVD in which chronic periodontal inflammation at the site of infection increases circulating levels of inflammatory mediators, and bacteria dispersed into the circulation provokes host inflammatory arbiter, which unswervingly alters other systemic diseases.

Several studies have been conducted to determine whether PD is associated with risk factors for CVD (3). C-reactive protein (CRP), homocysteine, fibrinogen, high-density lipoprotein cholesterol (HDL-c), and low-density lipoprotein cholesterol (LDL-c) have all been studied as CVD markers (4,5).

From a public health standpoint, CVD is the most significant of all the systemic conditions associated with PD, accounting for high mortality rates in most countries. Because multiple intervention studies, meta-analyses, and

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systematic reviews have been published in this area, this review aims to answer the following questions: Is there a link between CVD and periodontitis? What does the literature tell us after more than two decades of research, and why is this such a difficult question to answer? What does the Bradford-Hill criteria suggest about this unique association? What are our current circumstances, and what are our prospects for the future?

Mechanism and etiopathogenesis

To explain how PD influences CVD, two mechanisms have been proposed. First, periodontopathic flora directly annex endothelial cells via a direct mechanism (6). Polymerase chain reaction assays for atherosclerotic plaques support this theory. *Streptococcus mutans* was found to be the most common bacteria in cardiovascular specimens containing thrombus tissues (78%), followed by *Aggregatibacter actinomycetemcomitans* (7). Other bacteria found in atherosclerotic lesions in coronary arteries include *Tannerella forsythia*, *Prevotella intermedia*, and predominantly *Porphyromonas gingivalis*. Still, it remains unclear how the existence of periodontopathogenic organisms impacts atherosclerosis intracellularly, but few pathogens, such as *P. gingivalis*, may induce formation of foam cells or tenacity in cells, resulting in tributary inflammation and endothelial dysfunction (8,9).

The second proposed mechanism is the indirect pathway where PD causes increases in the levels of inflammatory cytokines. PD induces an inflammatory response, which results in elevation in levels of various inflammatory mediators, including interleukin 8, interleukin 6, interleukin 1 and tumor necrosis factor, which are also linked to atherosclerotic vascular disease. Some can speed up the production and emission of fibrinogen and CRP. Moreover, bacterial lipopolysaccharides plunge the flow and elicit strong immune response (Fig. 1). These elements influence atherosclerosis by acting on endothelial cells, increase the oxidative stress, and harmonize the lipid metabolism. This is confirmed by a previous study in which endothelial dysfunction was found in patients with periodontitis.

To jot down, it is intelligible that due to PD, inflammation persuades which can immigrate the periodontopathic organisms or leak out the inflammatory cytokines into the circulatory system which might either lead to systemic inflammation or periodontal pathogens may end up in vascular tissues (Fig. 2) which has a final ultimatum – formation, maturation, and exacerbation of atheromatous plaque.

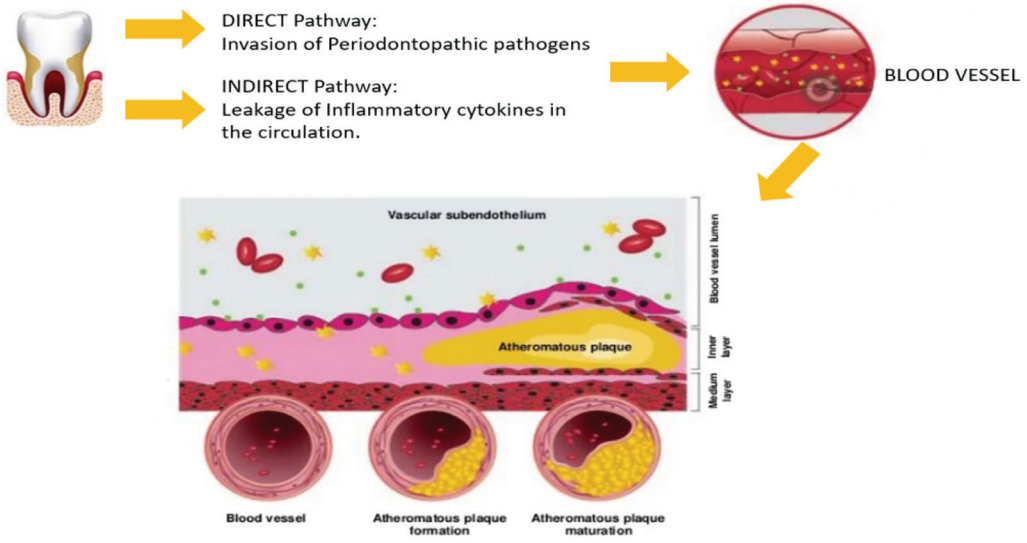

Fig. 1 - Direct and indirect relationship between periodontal disease and cardiovascular disease.

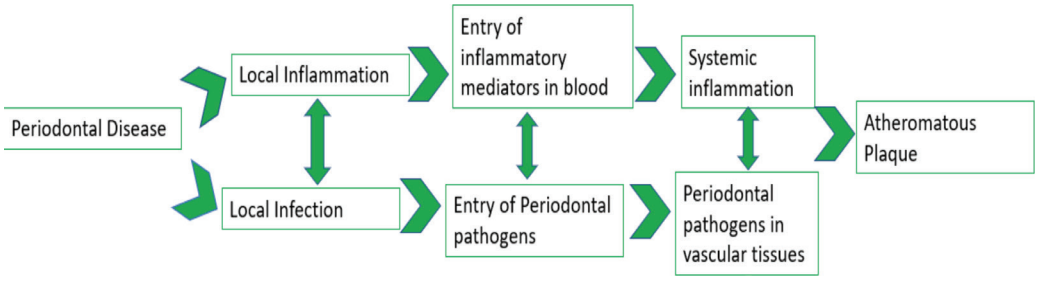

Fig. 2 - Etiopathogenesis flowchart.

Epidemiological studies and literature review of the PD and systemic disease connection

Certainly, contemporary evidences have imparted useful knowledge on common biomarkers of CVD and PD, which has prognostic as well as diagnosing aptitude to crucially decrease the menace of abominable cardiac episode in an untimely manner (Tab. I).

Cross-sectional and case-control studies

Genco and colleagues investigated the link between certain subgingival periodontopathogens and myocardial infarction (MI) (20), comparing 233 controls with 97 nonfatal MI patients. Noninfected individuals were compared with infected individuals. For MI, odds ratio for the presence of *T. forsythia* was 2.99 and for *P. gingivalis* was 2.52. These results support the idea that specific pathogenic bacteria found in PD may also be associated with MI.

Arbes et al (NHANES III) studied the relationship between PD and coronary heart disease (CHD) and found that with increase in the severity of periodontitis the likelihood of suffering an MI increased. The study thus confirmed with other studies the link and displayed an undeviating robust association amidst increased severity of periodontitis and CVD (21).

Longitudinal studies

DeStefano and colleagues reviewed NHANES-I data as well as a 15-year epidemiologic follow-up. They discovered periodontitis was one significant predictor of CVD in 9,760 men and women (22). These relationships were unaffected by age, gender, body mass index, education, marital status, poverty index, race, blood pressure, alcohol consumption, diabetes status, and serum cholesterol levels.

Beck and coworkers conducted a study of 921 men who did not have coronary artery disease at baseline. A total of 40 had stroke, 59 died of coronary artery disease, and 207 men developed coronary artery disease during an 18-year follow-up period. Odds ratios for total periodontal bone loss and CHD, fatal CHD and stroke, CVD risk factors and age were 1.5, 2.8, and 1.9, respectively. Accordingly, the odds of suffering a vascular event or CHD were 0.5-2.8 times higher in individuals with radiographically proven periodontitis (23).

Hujoel et al conducted a longitudinal study that found no link between chronic heart disease and periodontitis (24). These authors evaluated the NHANES-I study and the results of their 21-year follow-up. It is worth noting that DeStefano et al (22) used the same database and discovered a link between CVD and PD in NHANES-I study 15 years later.

Hujoel et al adjusted extensively for potential confounders, which may have explained the lack of a relationship after adjustment (24). Hujoel et al may have adjusted too heavily for factors strongly associated with infection, such as PD. It is also possible that periodontal status of subjects was significantly misclassified over time. In addition, because of treatments and extractions over time, the authors may have misclassified subjects who had PD at baseline. The misclassification being non-differential, which would have been worsened through 21-year follow-up period, could brace the null hypothesis of the research that there is no link between CVD and PD.

Joshipura and colleagues discovered that after controlling for other risk factors, the association between self-reported history of PD and incidence of heart disease was no longer significant (25). However, these researches merit further discussion as they were derived from a well-characterized, large longitudinal study. The majority of studies that found a correlation discovered that the amount of PD was significant. It would be impossible to quantify the extent of PD present in Joshipura's research as they answered a "yes or no" query about PDs. Furthermore, discrepancy based on self-reported PD is possible.

The most recent longitudinal study, published by Howell and colleagues (26), was a double-blind, randomized, placebo-controlled trial of beta-carotene and aspirin for the prevention of CVD and cancer in the United States in 22,071 male physicians. The study outcomes were nonfatal MI, stroke, and death from CVD. After controlling for the treatment and age (beta-carotene and aspirin), the researchers discovered a positive non-significant trend (95% confidence interval [CI], relative risk [RR] = 1.13).

Although most evidence from case-control and longitudinal researches advocates a link between CVD and periodontitis, the link seems to be dwindling. There is insufficient evidence to conclude that the associations are contributory.

Observational studies: from systematic review to meta-analysis

Scannapieco et al (27), in a comprehensive systematic review, concluded that there is moderate evidence of a relation between MI, CVD, atherosclerosis, and PD, but the causation was uncertain.

Khader et al (5), in a meta-analysis, combined two cross-sectional studies and six cohort and found a lower RR of 1.15 (95% CI [1.06-1.25]).

Nine cohort researches compiled by Janket et al (28) in a meta-analysis suggests that, for future CVD and cyclic vomiting syndrome episodes, PD is a determined risk factor and discovered that chronic periodontitis patients had a 19% surged peril for advancing such events. People under the age of 65 were at a higher risk (44%).

Another meta-analysis of observational studies by Alessandra Blaizot et al investigated the relationship between CVD and periodontitis exposure (29). Researches done between 1989 and 2007 were recovered from seven databases via electronic and manual searches. The MOOSE meta-analysis guidelines for observational studies were followed (30). There were 47 observational studies among the 215 epidemiological studies, 29 of which could be combined using meta-analysis methodology. There was increased risk (about 34%) of developing CVD in individuals having periodontitis than in individuals who are not exposed with periodontitis, where 1.34 was the RR of these seven cohort researches ($p = 0.0001$). This finding suggests that people with Parkinson's disease are at higher risk for developing CVD.

Interventional studies

Owing to many factors, for example, financial, ethical, or methodological, the periodontal intervention and its efficacy as primary prevention for CVDs such as Ischemic Heart Disease (IHD) and death haven't yet been studied (50).

Consequently, CVD proxy markers have been thoroughly investigated, and periodontal intervention shows substantial effect on these markers, as shown in Table II. There is limited affirmation on durable effect of periodontal intervention on these proxy markers. In addition, the impact of periodontal intervention on the scientific conclusions of these markers of CVD remains to be still investigated.

Previous research has found that rigorous periodontal intervention transiently impairs the endothelial function role, which raises levels of serum inflammatory markers, probably due to liberation of inflammatory mediators or bacterial organisms in bloodstream. Nevertheless, after a few weeks of periodontal intervention the levels of inflammatory makers and periodontal parameters also seem to lower or decrease (51-54). Furthermore, 6 months after periodontal treatment, carotid intimal-medial thickness is reduced. Several interventions and researches have been done as shown in Table II, which has been published in the past few years, and they all brace up for the hypothesis that periodontal intervention reduces cardiovascular risk factor and thus has an impact on CVD events (55).

Imminent interventional studies are required toward better understanding of the association between PD and CVD, mainly the biological effects of PD on the atherogenic cascade by influencing the vascular endothelium. Further enduring intervention researches are needed, ideally utilizing similar methods to assess CVD events, to determine whether the reported benefits of periodontal intervention can actually decipher the reduction in incidence of CVD.

Microbiological studies

Clinically, it is extremely strenuous to determine the causal agent of atherosclerosis. First, endothelial damage advances by masking the causative agent and progresses without symptoms. Second, multiple contributing factors cause atherosclerosis, and these influencers may coexist, making it difficult to determine the causative factor (56,57). In addition, studies of interventions have shown mixed results. Sometimes after periodontal intervention there is enhancement, whereas sometimes there is brief deterioration of the symptoms and sometimes there is no change.

However, seven rules must be met for promoting atherosclerosis by the periodontal pathogens, which are enlisted below (50).

Evidence 1: Systemic vascular tissues can provide a pathway for periodontal pathogens. Numerous researches have demonstrated that periodontopathic bacteria could cause bacteremia by entering the systemic circulation (50,58-60,62). A previous systematic review found that bacteremia after periodontal procedures could be atop 50% (63,64). Table III summarizes the prevalence of periodontopathic pathogens in systemic circulation after periodontal intervention in atheromatous lesions, with and without periodontal procedures in periodontitis patients. Following periodontal procedures, periodontopathic organisms may infiltrate the circulation, being a determinant of atherosclerosis.

Evidence 2: Affected tissues accommodate periodontal pathogens. Several studies have provided sufficient evidence that DNA, RNA, and antigen sequencing can be used to identify different periodontal species in atheromatous lesions (65-67). Analysis shows that periodontitis patients are at increased risk for emergent atherosclerosis.

Evidence 3: In the affected site, live periodontal pathogens will be present. This proof requires the detection of live periodontopathic bacteria. Live *A. actinomycetemcomitans* and *P. gingivalis* were isolated from atherosclerotic samples in multiple studies (68,69).

Evidence 4: Invasion of the affected cell with in vitro evidence. Several in vitro researches show periodontopathic organisms can invade various types of host cells. According to many studies *P. gingivalis* is responsible for infiltrating the endothelial cells in studies, the significance as well as the mechanism of the specific strain type are being investigated further (70-72).

Evidence 5: Provides indication that periodontal pathogen can encourage atherosclerosis in diseased animal model. In 2012, the European Federation of Periodontology and the American Academy of Periodontology published a review that found proof that periodontal pathogens can endorse atherosclerosis (56). *P. gingivalis* is shown to promote atherosclerosis in murine (73), rabbit (74), and pig models (75). Furthermore, when hyperlipidemic mice were orally infected with *Fusobacterium nucleatum*, *T. forsythia*, *P. gingivalis*, *Treponema denticola*, and other expedient organisms from this specific class were found in aorta, atherosclerotic plaque, and oral epithelium (76,77).

Evidence 6: Pathology is significantly less when caused by noninvasive mutants, according to in vitro and in vivo evidence where the incursion of vascular cells and tissues by bacterial strains is been investigated. Noninvasive *fimA*-deficient mutant of *P. gingivalis* exhibits fewer proinflammatory mediators than the invasive wild-type strain of *P. gingivalis* (73).

Evidence 7: Fulfill a modified version of Koch's postulate to show that a human atheroma isolate causes disease in animal models. To do this, isolate the periodontopathogen from a human atheroma and induce atheroma formation in an animal model after inoculation. *P. gingivalis* were isolated from atherosclerotic samples. There is also evidence that suffused pathogenic bacteria can cause atherosclerosis. The evidence is still, however, considered incomplete as the bacterial strain utilized were not isolated from human atherosclerotic samples (68,76,77).

Apart from Evidence 7, there are abundant researches available to support Evidences 1 to 6. Nonetheless, the first six proofs embrace the notion that periodontal pathogens are linked to CVD.

Neoteric substantiation of CVD and PD

Febbraio et al (88) concluded that there is an association between oral health and CVD, but causality has yet to be established. However, studies show improvements

in cardiovascular risk factors following periodontal treatments, although with relatively short follow-up periods. Rational evidence suggests that good oral health contributes to overall and heart health. The best bet is to continue to remind patients that a healthy mouth contributes to a healthy heart.

Fazal et al (19) concluded that Non Surgical Periodontal Therapy (NSPT) lowers cardiac biomarker concentrations in patients with chronic periodontitis and may reduce the risk of CVD in the future. However, Paul et al (89) suggest a contradictory result in a review that therapeutic periodontal interventions cannot be used to prevent heart disease or stroke.

Larvin et al (90) found an increased risk of CVD in people with PD in a systematic review and meta-analysis. Males and people with severe PD are at the highest risk of developing CVD, indicating potential target populations for future public health interventions and inspection.

In a cross-sectional observational single-center study, Lazureanu et al (91) concluded that increasing patients' awareness of oral healthcare measures resulted in better outcomes and improved oral-health-related quality of life.

In a 13-year follow-up study, Tiensripojamarn et al (92) show that severe periodontitis is linked to a higher incidence of CHD, independent of existing cardiovascular risk factors.

Periodontitis Grade B/C was linked to a higher overall cardiovascular risk, and this association was not explained by smoking confounding in participants aged 65-74 years, according to Petrenya et al (93). They also recommended that patients with periodontitis, particularly those with extensive alveolar bone loss, use NORRISK 2 score for cardiovascular risk assessment. Individuals with a high cardiovascular risk profile should have routine periodontal examinations. In addition to adequate, evidence-based periodontal intervention, cessation of smoking and blood pressure normalization are essential in lowering cardiovascular risk in individuals with PD.

What do the Bradford-Hill criteria imply for the relationship between CVD and PD?

The Canadian Dental Hygienists Association (CDHA) published a position paper that used the Bradford-Hill criteria to determine whether there is sufficient evidence for a causal relationship between PD and CVD (87). The Bradford-Hill criteria analysis found no evidence of a link between PD and CVD. Although the link between PD and CVD is well established, the findings of the CDHA's recent position paper show that, while an association exists, the nature of that link is unknown, and there is insufficient evidence for that association to be causal at this time.

Conclusion

Epidemiologic researches have now substantiated that there may be a link between PD and CVD. Although research continues to point to a connection between CVD and oral health, causality has not yet been established. Despite the fact that the follow-up times in most studies are brief, numerous studies show an improvement of CVD risk factors after periodontal interventions. The association of good oral health and general and heart health is proved by reasonable evidence.

In the coming decades, medical and dental professionals must be capable of better planning of preventive interventions as constant researches approve and account the forte of the consortium between CVD and PD. Scientific data assembled thus far would seem to support the continued value of interventional periodontal therapy not just for oral health but for overall health as well.

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ORIGINAL RESEARCH ARTICLE
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Treatment with levosimendan in an experimental model of early ventilator-induced diaphragmatic dysfunction

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ABSTRACT

Introduction: Mechanical ventilation (MV) is a life-saving approach in critically ill patients. However, it may affect the diaphragmatic structure and function, beyond the lungs. Levosimendan is a calcium sensitizer widely used in clinics to improve cardiac contractility in acute heart failure patients. In vitro studies have demonstrated that levosimendan increased force-generating capacity of the diaphragm in chronic obstructive pulmonary disease patients. Thus the aim of this study was to evaluate the effects of levosimendan administration in an animal model of ventilator-induced diaphragmatic dysfunction (VIDD) on muscle contraction and diaphragm muscle cell viability.

Methods: Sprague-Dawley rats underwent prolonged MV (5 hours). VIDD+Levo group received a starting bolus of levosimendan immediately after intratracheal intubation and then an intravenous infusion of levosimendan throughout the study. Diaphragms were collected for ex vivo contractility measurement (with electric stimulation), histological analysis and Western blot analysis. Healthy rats were used as the control.

Results: Levosimendan treatment maintained an adequate mean arterial pressure during the entire experimental protocol, preserved levels of autophagy-related proteins (LC3BI and LC3BII) and the muscular cell diameter demonstrated by histological analysis. Levosimendan did not affect the diaphragmatic contraction or the levels of proteins involved in the protein degradation (atrogin).

Conclusions: Our data suggest that levosimendan preserves muscular cell structure (cross-sectional area) and muscle autophagy after 5 hours of MV in a rat model of VIDD. However, levosimendan did not improve diaphragm contractile efficiency.

Keywords: Diaphragm contractility, Levosimendan, Mechanical ventilation, Muscle fiber size, Ventilator-induced diaphragmatic dysfunction

Introduction

Mechanical ventilation (MV) is a life support technique used in critically ill patients. However, prolonged MV is also associated with numerous potential complications including a rapid impairment of both lungs (i.e., ventilator-induced lung injury – VILI) and diaphragm (i.e., ventilator-induced diaphragmatic dysfunction – VIDD). MV can also exacerbate a preexisting VILI (1-3). It is also associated with adverse effects on multiple aspects of diaphragmatic structure and function (VIDD) (4). The detrimental impact of prolonged MV on the diaphragm is strictly correlated to problems in weaning patients from the ventilator (5). The incidence level can reach 35% of patients exposed to prolonged MV, with subsequent increase in morbidity and mortality (4,6,7). Furthermore, body composition indexes are known to predict outcome – such as mortality and MV duration itself – in critically ill patients (8). The first evidence on the correlation between MV and VIDD was published almost 35 years ago, in which a study of infant and neonates suggested that MV predisposes diaphragm fibers to atrophy (9). Thenceforth

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numerous experimental and clinical studies documented that prolonged MV induces a rapid muscular atrophy and contractile dysfunction (10).

Skeletal muscle is one of the most significant muscle tissues in the body. Thousands of muscle fibers are encased in connective tissue sheaths to form each skeletal muscle. The size of skeletal muscle fiber is dependent on the balance between the protein synthesis and protein degradation (11,12). Animal studies have demonstrated that protein synthesis declines rapidly within the first 6 hours of MV and remains depressed during the next 12 hours (11). At the same time, the prolonged MV increases the activity of all major proteolytic systems: macroautophagy, calpains, caspases and the ubiquitin-proteasome systems. Moreover prolonged MV results in oxidative damage to the diaphragm – reactive oxygen species and their derivatives have a significant impact on skeletal muscle contractile function (13).

Prolonged MV results in less efficient calcium activation of diaphragm fibers most likely due to oxidative modification of diaphragm contractile proteins (14). Levosimendan is a calcium sensitizer, first described in 1995, that is administered to enhance cardiac contractility in patients with acute heart failure (15,16). In addition, levosimendan improves respiratory muscle function in healthy subjects and patients with chronic obstructive pulmonary disease (COPD) (17), through the improvement of calcium sensitization of the contractile proteins (18). Moreover, it reduces inflammation and oxidative stress (19). A recent study also demonstrated that levosimendan dampens nitrosative stress in the diaphragm of mechanically ventilated endotoxemic mice (20).

Thus, we administered levosimendan to an in vivo model of VIDD to determine if it could alleviate the negative impacts of MV. Therapeutic effects were determined by measuring muscle contraction and diaphragm muscle viability. The aim of the study was to evaluate the effect of the treatment of levosimendan in a rat model on VIDD on:

1. the preservation of diaphragm muscle structure;
2. the level of autophagy activation in the diaphragm;
3. the diaphragm muscular contractile function.

Materials and methods

Animals

Male Sprague-Dawley rats (250-300 g; Envigo RMS S.r.l., Udine, Italy) were used in this study. Animals were housed two per cage in a limited access animal facility, with the room temperature at 20 ± 2°C and the relative humidity set at 55 ± 10%. Artificial lighting provided a 12-hour light/12-hour dark (7 am.–7 pm) cycle. The general condition of the animals before the experiment was assessed daily. The care and husbandry of animals were in conformity with the institutional guidelines in compliance with Italian and European laws and policies. The animal study was reviewed and approved by the Italian Ministry of Health (773/2018-PR) and by the Animal Care Unit of the University of Milano-Bicocca, Monza, Italy. In full respect of the reduction principle of the 3Rs (21), the number of animals/groups was selected to obtain reliable results and enough biological samples to perform the analysis planned.

Experimental protocol

The experimental design is composed of three experimental group of rats:

– Healthy: non-anesthetized and spontaneously breathing rats;
– VIDD: anesthetized and mechanically ventilated rats without any pharmacological treatment;
– VIDD+Levo: anesthetized and ventilated rats with levosimendan infusion.

Rats were anesthetized with ketamine (100 mg/kg) and xylazine (4 mg/kg), orotracheally intubated and ventilated for 5 hours (Harvard Inspira; tidal volume: 10 mL/kg; respiratory rate: 80/min; positive end-expiratory pressure [PEEP]: 2-2.5 cmH_2O; fraction of inspired oxygen [FiO_2: 0.5]). Anesthesia and paralysis were maintained throughout the experiment by infusion in the right femoral artery of propofol (13 mg/kg/h) and ketamine (5 mg/kg/h) and in the right jugular vein of rocuronium bromide (1.5 mg/kg/h) and Ringer acetate (1.8 mL/h). Airway pressure and hemodynamic parameters were monitored through pressure transducers in ventilator and at the arterial catheter. A recruitment maneuver (30 cmH_2O for 10 sec) was performed every 60 minutes, being the plateau pressure, and respiratory system static compliance was measured every hour. Rats belonging to the treatment group (VIDD+Levo) received a dose (24 µg/kg) of levosimendan (Simdax; Orion Pharma) into the tail vein at the beginning of the experiment and then a maintenance of the treatment was achieved by an infusion in the left jugular vein of levosimendan (0.2 µg/kg/min) (22).

Pulmonary function

For the lung mechanical properties, a pressure to volume (PV) curve was calculated. After a recruitment maneuver, five steps of 0.5 mL inspiratory volumes (i.e., total 2.5 mL) were delivered into the lungs. For each step, the plateau pressure was recorded to calculate the static compliance.

Diaphragmatic contractile function

Diaphragmatic contractile function was measured as already described (23), briefly a diaphragm strip was mounted into a tissue bath and, through an electric stimulator, the peak tetanic tension and the force-frequency relationship were evaluated.

Histological analysis: muscular fibers cross-sectional area

A section from the right and one from the left hemidiaphragm tissue was used for histology analysis. The cross-sectional area (CSA) of the muscular fiber was determined by manually tracing the cell contour on digitized

images from hematoxylin and eosin-stained frozen section. The CSA of at least 150 fibers per diaphragm were then averaged.

Western blot analysis

Another section of the diaphragm excised at the sacrifice was immediately frozen in liquid nitrogen and stored at −80°C. The cytoplasmic extraction was prepared using an NE-PER Nuclear Cytoplasmic Extraction Reagent kit (Pierce, Rockford, IL, USA) according to the manufacturer's instruction. Equal amounts of the protein concentrations were quantified by the bicinchoninic acid assay (BCA assay; Pierce, Rockford, IL, USA), and each sample was analyzed according to standard Western blotting protocols. The following antibodies were used: atrogin (AP2041; ECM Biosciences, Versailles, KY, USA) and LC3B (light chain 3, isoform B II, 2775; Cell signaling technology, Danvers, MA, USA). The antibody α-tubulin (#2125; Cell Signaling, Danvers, MA, USA) was used as the reference of internal control.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Differences in variances between groups were assessed by one-way analysis of variance (ANOVA). Post hoc comparisons were performed by Benjamini, Krieger and Yekutieli. Differences in physiological variables between VIDD and VIDD+Levo were performed by unpaired t-test. p-Values < 0.05 were considered as statistically significant (two-tailed). Statistical analysis was performed by GraphPad Prism (version 8.4.2). Some histologic and Western blot analyses could not be performed in some animals because of technical problems.

Results

Systemic response

No rats died during the experiments. In terms of body weight, no differences were found between groups (healthy: 310 ± 6, VIDD: 300 ± 6, VIDD + Levo: 295 ± 7 g; ANOVA p = 0.25). The mean arterial pressure (MAP) was similar between the test groups at the beginning of the experiment and after 3 hours of MV. At the end of the MV, rats without levosimendan treatment showed a significant lower MAP versus VIDD + Levo group, as demonstrated in Table I. The difference in MAP was not influenced by a difference in terms of fluid balance, since it was similar between two groups (VIDD: 18.6 ± 0.6 mL vs. VIDD + Levo: 19.9 ± 1.3 mL, p = 0.31). Regarding the respiratory function, no differences were found between ventilated groups in oxygenation nor in respiratory system static compliance.

Histological analysis

Levosimendan treatment seemed to induce a protection in muscle fiber size from cellular atrophy induced by MV. As shown in Figure 1, MV affects the cross-sectional fiber area in

TABLE I - Mean arterial pressure, heart rate, PaO_2/FiO_2, respiratory system static compliance

		VIDD	VIDD + Levo	p-Values
Mean arterial pressure (mm Hg)	*Start*	90 ± 5	91 ± 11	0.93
	3 hours	94 ± 6	97 ± 9	0.78
	End	66 ± 6	89 ± 10	0.04*
Heart rate (/min)	*Start*	254 ± 10	285 ± 16	0.10
	3 hours	329 ± 12	329 ± 6	0.95
	End	306 ± 12	330 ± 9	0.15
PaO_2/FiO_2	*End*	476 ± 48	529 ± 13	0.25
Respiratory system static compliance (mL/cmH_2O)	*End*	0.34 ± 0.01	0.32 ± 0.01	0.38

All parameters were measured during mechanical ventilation. VIDD: MV + no treatment (n = 12); VIDD+Levo: MV + levosimendan treatment (n = 8).
MV = mechanical ventilation; VIDD = ventilator-induced diaphragmatic dysfunction.
*p < 0.05.

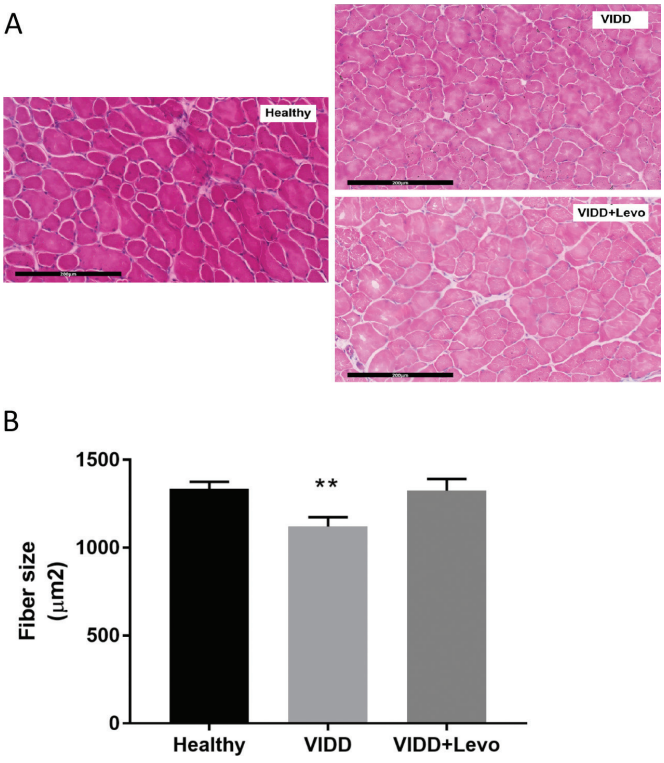

Fig. 1 - Diaphragm fiber size. Cross-sectional diaphragmatic fiber size in histological slice. A) Histological images, hematoxylin-eosin staining, 200×, scale bar = 200 µm. B) ANOVA p < 0.01. Benjamini, Krieger and Yekutieli post hoc test: p = 0.004 vs. healthy and p = 0.01 vs. VIDD+Levo. Healthy: no surgical interventions, no MV, and no treatment (n = 8); VIDD: surgical intervention + MV and no treatment (n = 10); VIDD+Levo: surgical intervention + MV + levosimendan treatment (n = 8). ANOVA = analysis of variance; MV = mechanical ventilation; VIDD = ventilator-induced diaphragmatic dysfunction.

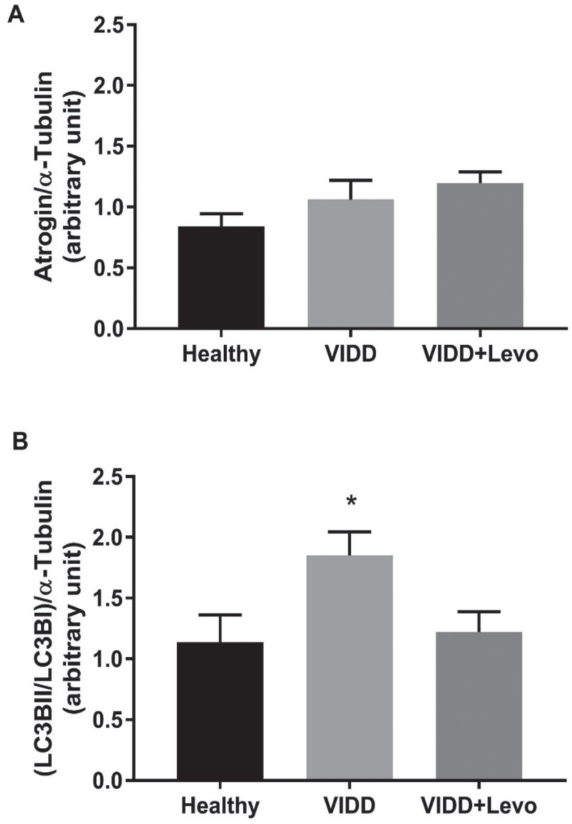

Fig. 2 - Western blot analysis. Atrogin and LC3BII/LC3BI levels on diaphragm tissue. A) ANOVA p = 0.21. B) ANOVA p = 0.03. Benjamini, Krieger and Yekutieli post hoc test: p = 0.02 vs. healthy and p = 0.04 vs. VIDD+Levo. Healthy: no surgical interventions, no MV, and no treatment (n = 8); VIDD: surgical intervention + MV and no treatment (n = 11); VIDD+Levo: surgical intervention + MV + levosimendan treatment (n = 8). ANOVA = analysis of variance; MV = mechanical ventilation; VIDD = ventilator-induced diaphragmatic dysfunction.

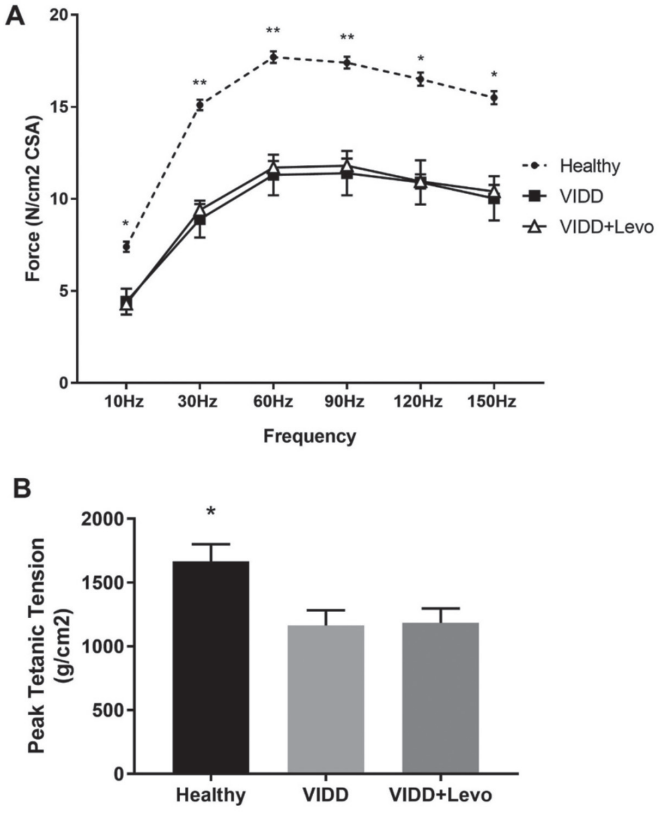

Fig. 3 - Diaphragm contractility. Force-frequency relationship (panel A) and peak tetanic tension (panel B) measured on diaphragmatic strips with electric stimulator at the end of the experiment. A) ANOVA *p < 0.05, **p < 0.01. Benjamini, Krieger and Yekutieli post hoc test: 10 Hz p = 0.03 vs. VIDD and vs. VIDD+Levo, 30 Hz p = 0.001 vs. VIDD and p = 0.004 vs. VIDD+Levo, 60 Hz p = 0.002 vs. VIDD and p = 0.005 vs. VIDD+Levo, 90 Hz p = 0.004 vs. VIDD and p = 0.01 vs. VIDD+Levo, 120 Hz p = 0.006 vs. VIDD and p = 0.01 vs. VIDD+Levo, 150 Hz p = 0.007 vs. VIDD and p = 0.002 vs. VIDD+Levo. B) ANOVA p < 0.05. ANOVA = analysis of variance; VIDD = ventilator-induced diaphragmatic dysfunction. B) ANOVA p < 0.05. Benjamini, Krieger and Yekutieli post-hoc test: p = 0.007 vs VIDD, p = 0.01 vs VIDD+Levo. Healthy: no surgical interventions, no MV, and no treatment (n = 8); VIDD: surgical intervention + MV and no treatment (n = 12); VIDD+Levo: surgical intervention + MV + Levosimendan treatment (n = 8).

VIDD animals if compared to healthy rats. VIDD+Levo group had fiber diameters like unventilated animals.

Western blot analysis

Western blot analysis (Fig. 2) on levels of proteins involved in muscle protein degradation (atrogin) did not reveal any statistical difference between groups. Proteins implicated in autophagy and autophagy-related processes (LC3BI and LC3BII) were significantly higher in the VIDD group, if compared to Healthy and VIDD+Levo groups, while these proteins did not differ in the VIDD+Levo group as compared with the Healthy group.

Diaphragm contractile dysfunction

After 5 hours of MV, rats that underwent ventilation (VIDD and VIDD+Levo) showed a significant reduction in diaphragmatic contractility in response to in vitro electric stimulation, in comparison to unventilated animals. As demonstrated in Figure 3A, increasing the frequency of stimulation the diaphragmatic muscle strip of ventilated rats generated less force than the healthy diaphragm. Levosimendan treatment did not affect the diaphragmatic contractility. The analysis of peak tetanic tension unveiled that ventilated rats (with or without levosimendan treatment) showed a similar diaphragmatic muscular contractility (Fig. 3B).

Discussion

In this experimental model of VIDD, after 5 hours of MV in a rat model of VIDD as compared to control, our data suggest that the administration of levosimendan:

1. preserves the diaphragm muscular cell structure;
2. reduces autophagy-implicated proteins of the diaphragm muscle cells;
3. does not affect diaphragm contractile function.

Levosimendan is a calcium-sensitizing inodilator widely used in the management of acutely decompensated chronic heart failure from over 20 years. In addition, it has been evaluated in a variety of clinical settings for both cardiac and non-cardiac disease (24,25). One possible area of investigation is respiratory muscle dysfunction, because, in addition to cellular atrophy, reduced calcium sensitivity of contraction causes respiratory muscle weakness (26). In vitro studies on isolated muscle fibers showed that levosimendan increased force-generating capacity of diaphragm from COPD and non-COPD patients by improving calcium sensitivity of force generation (17). Clinical studies have produced mixed results, the same investigators discovered that levosimendan increased in vivo diaphragmatic contractile efficiency in healthy participants (27), while it had no effect on diaphragm function in intensive care unit (ICU) patients (28). The role of levosimendan as potential therapeutic intervention of diaphragm dysfunction is an open field of current pharmacological research for VIDD in addition to the one for VILI because of the need of MV in the presence of severe respiratory failure (3,29).

In this study we evaluated the effects of levosimendan treatment in preclinical in vivo rodent model of VIDD. We have previously demonstrated that the model resembles the main features of diaphragmatic dysfunction (22). As levosimendan has a short half-life of 1 hour, a bolus of 24 µg/kg was immediately administered after orotracheal intubation and MV. It is well known that the use of a bolus dose should be avoided due to the risk of hypotension (30), but the animals in this study were healthy at the beginning of the study with physiological hemodynamics, as shown in Table I. In our experimental design, levosimendan treatment, rather than inducing hypotension, maintained higher levels of MAP as compared to untreated animals at the end of experiment (Tab. I).

The diaphragmatic contraction was evaluated by in vitro electric stimulation after 5 hours of MV: both ventilated groups showed a significant reduction in muscular force compared to healthy control rats (Fig. 3A, B). Levosimendan treatment did not improve diaphragm contraction, maybe due to the bolus dose used in the study: 24 vs. 40 µg/kg, which was used in similar investigations (27). However, histological analysis of muscular diaphragmatic fiber revealed that levosimendan administration preserved significant cellular diameter from atrophy. Prolonged MV led to a reduction in cross-sectional muscular fiber area of 16% (Fig. 1), whereas levosimendan-treated rats showed conserved cellular structure when compared to the VIDD group. The levels of atrogin, a protein belonging to the ubiquitin-proteasome system of proteolysis, did not differ between groups (Fig. 2), whereas microtubule-associated protein light chain 3 I and II (LC3BI and II), autophagosome marker, were significantly modified by MV. It has been previously reported that prolonged MV can lead to upregulation of atrogin and LC3B (4,31). We hypothesize that, in our experimental protocol, the hours of MV should influence the protein expression, maybe 5 hours MV were not sufficient to stimulate atrogin production.

This study has some limitations that should be acknowledged. First, the duration of MV (5 hours) potentially did not allow enough time to evaluate all the parameters (such as atrogin levels). However, the purpose of this study was to determine the early effects of levosimendan. Early administration ensured that possible side effects on hemodynamics were avoided. It has been previously demonstrated that MV can have detrimental effects on muscle fibers. Therefore, future studies will identify more specifically the different types of muscle fibers after MV with and without treatment. Second, we decided to administer the dose of levosimendan used routinely in the clinical setting (decompensated heart failure). We cannot exclude that the use of a higher dose of levosimendan might provide a benefit in terms of diaphragmatic contraction. A dose-response study may be a useful potential step to further explore the role of levosimendan during VIDD. Third, during the MV period, all rats received a continuous infusion of rocuronium bromide that may probably affect the diaphragmatic muscle contraction that was assessed ex vivo after animal euthanasia. Indeed, it is known that, simultaneously with MV, some drugs – such as neuromuscular blocking agents – may worsen the diaphragm dysfunction, albeit data on their effects on diaphragm are not debated (32-35).

In conclusion, in an experimental animal model of VIDD, levosimendan prevented autophagy allowing to preserve diaphragm muscular cellular structure as compared with VIDD untreated group. However, levosimendan did not improve the diaphragm contractile efficiency.

Disclosures

Conflict of interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH ARTICLE

Lipid profiles of people with human immunodeficiency virus with dyslipidemia after switching from efavirenz to dolutegravir

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ABSTRACT

Introduction: Human immunodeficiency virus (HIV) infection and the long-term use of antiretroviral therapy, especially efavirenz (EFV)-based regimens, impact lipid profiles due to insulin resistance and lead to a higher risk of metabolic diseases. Dolutegravir (DTG) is an integrase inhibitor with better lipid profiles than EFV. However, data on treatment experience in Thailand are limited. The primary outcome was lipid profile changes at 24 weeks after switching therapy.

Methods: We conducted a prospective, open-label, cohort study in people with HIV aged ≥18 years who had undergone at least 6 months of EFV-based therapy, had HIV-1 ribonucleic acid levels <50 copies/mL for ≥6 months before switching, and were diagnosed with dyslipidemia or had risk factors for atherosclerosis cardiovascular disease based on modified National Cholesterol Education Program Adult Treatment Panel III guidelines.

Results: Sixty-four patients were enrolled. The mean age (standard deviation [SD]) was 48.20 ± 10.46 years, and 67.19% were male. At week 24, there were decreases from baseline in mean total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglycerides. However, mean body weight and waist circumference had increased.

Conclusions: DTG resulted in better lipid profiles after switching from EFV-based therapy, suggesting that this switch could benefit patients with a high risk of cardiovascular disease. However, it is essential to note that weight gain and increased waist circumference were also observed.

Keywords: ARV, Dolutegravir, Dyslipidemia, Efavirenz, Switching treatment

Introduction

People with human immunodeficiency virus (HIV) typically have the potential to live for a considerable length of time after receiving highly active antiretroviral therapy (HAART). The virus triggers an inflammatory response that can result in metabolic issues such as diabetes mellitus, hypertension, and dyslipidemia. The prevalence of people with HIV with dyslipidemia is as high as 51% (1).

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Long-term use of antiretroviral treatment regimens may lead to dyslipidemia, which is a significant risk factor for cardiovascular disease (2-5). Previously, people with HIV in Thailand were typically prescribed a first-line antiretroviral regimen that included efavirenz (EFV) along with two nucleoside reverse transcriptase inhibitors (NRTIs) (6).

As a long-acting non-nucleoside reverse transcriptase inhibitor (NNRTI), EFV is an effective form of HIV treatment in clinical settings. However, there are side effects such as drug rash, hepatitis, and long-term metabolic diseases (3,4). This can raise the likelihood of developing hyperglycemia and subsequent insulin resistance while also affecting lipid metabolism. In addition, it can hinder the breakdown of fat, leading to an increase in triglycerides, very-low-density lipoprotein (VLDL), and low-density lipoprotein (LDL) levels, and a decrease in high-density lipoprotein (HDL) levels. This ultimately results in dyslipidemia, which can eventually cause cardiovascular disease (2).

The US Food and Drug Administration approved dolutegravir (DTG) in 2013 as an integrase strand transfer inhibitor (INSTI)-based regimen, which works by inhibiting integrase, an enzyme that HIV needs to insert its deoxyribonucleic acid (DNA) into the DNA of host lymphocytes (7). It is highly effective, has few side effects compared to other drugs, and only needs to be taken once per day. However, there have been some reports of patients gaining weight after taking this drug long term (8). It is currently the first-line antiretroviral regimen administered in Thailand (9).

A randomized controlled trial in naive people with HIV compared the levels of lipids between an EFV group and DTG group and found that the latter had less of an increase in cholesterol (10-12).

A comprehensive approach is necessary for dyslipidemia management in people with HIV, which may involve lifestyle modification including controlled calories intake, exercise, and maintaining a healthy body weight or wight reduction. Another approach is to choose antiretroviral drugs that do not worsen dyslipidemia, and to modify antiretroviral therapy when necessary to control lipid levels. The use of lipid-lowering agents, such as statin agents and fibrates, may also be essential to reduce the risk of cardiovascular disease. At present, there are limited data available on switching from EFV to DTG in people with HIV who have dyslipidemia in Thailand.

The main goal of this study was to examine alterations in the lipid profile of people with HIV who have dyslipidemia, specifically at the 24-week mark following the switch from EFV to DTG. Secondary objectives were to evaluate the efficacy of DTG in maintaining HIV-1 ribonucleic acid (RNA) levels at <50 copies/mL after 24 weeks of switching treatment, as well as its safety, tolerability, body weight, body mass index (BMI), and waist circumference.

Methods

A prospective, open-label cohort study was conducted at Srinagarind Hospital, a tertiary university hospital in northeastern Thailand, between April 2021 and April 2022. The patients were eligible for the study if they met all the following criteria: (1) age over 18 years, (2) having received EFV-based therapy for at least 6 months, (3) HIV-1 RNA <50 copies/mL for ≥6 months before switching therapies, (4) diagnosis with dyslipidemia or risk factors for atherosclerosis cardiovascular disease (ASCVD) based on modified National Cholesterol Education Program (NCEP) Adult Treatment Panel (ATP) III guidelines (13). In brief, dyslipidemia was defined as either (1) LDL-cholesterol ≥130 mg/dL with at least one of the following coronary heart disease (CHD) risk factors: age >45 years if male or age >55 years if female, hypertension (blood pressure ≥140/90 mmHg or on antihypertensive medication), current cigarette smoking, or family history of premature CHD and/or diabetes; (2) LDL-cholesterol ≥160 mg/dL regardless of CHD risk factors; or (3) previous diagnosis of dyslipidemia and on lipid-lowering drugs. Exclusion criteria were pregnancy or breastfeeding, active opportunistic infections, or taking metformin >1,000 mg/day, rifampicin, St. John's wort, antiarrhythmic drugs (e.g., dofetilide, pilsicainide), antiepileptic drugs (e.g., carbamazepine, oxcarbazepine, phenytoin, phenobarbital), or medications or supplements containing polyvalent cations (e.g., magnesium, aluminum, cation-containing antacids or laxatives, sucralfate, buffered medications).

Patient evaluation was performed at baseline, week 12, and week 24. Data collected for each participant included age, sex, body weight, height, BMI, waist circumference, backbone regimen, CHD risks, current lipid-lowering agents, duration from HIV diagnosis to enrollment, duration of first treatment with antiretroviral agents to enrollment, and duration of EFV treatment to that with lipid-lowering agents. Clinical laboratory testing was performed at a local laboratory. Laboratory tests included HIV-1 RNA, absolute CD4 cell count, %CD4, and lipid profiles including total cholesterol, LDL-cholesterol, HDL-cholesterol, and triglycerides. The safety of the studied regimens was assessed using patient interviews, medical history, physical examination, and clinical laboratory test results.

Study procedure

Upon approval to undertake the project by the Human Research Committee at Khon Kaen University, the patients were screened and provided informed consent to be enrolled into this study. Blood tests were obtained on the date of enrollment according to protocols. Patients were changed from an EFV-based to a DTG-based regimen and received dosing instructions from the investigators. Patients had two follow-up appointments at 12 (±1) and 24 (±1) weeks.

The study protocol was reviewed and approved by the Khon Kaen University Center of Ethics in Human Research (HE641043).

Sample size calculation

Assuming a change in LDL-cholesterol level of 10.67, a standard deviation (SD) of ±30.37 mg/dL extrapolated from a study with 80% power, and a one-sided type 1 error of 0.05, a sample size of 64 patients was necessary. We calculated a 10% loss to follow-up, making the total required population 70 patients.

Statistical analysis

The data were analyzed using Statistical Package for the Social Sciences (SPSS) version 26. Categorical data were expressed as proportions, and continuous data were expressed as mean and SD, 95% confidence interval (CI), or median (range), as appropriate. The data depended on whether the distribution was normal or non-normal. Comparisons between values before and after changing medications were performed using a paired dependence t-test or proportional McNemar test, as appropriate.

Results

A total of 64 patients with dyslipidemia were enrolled in the study at baseline, followed up on for 12 weeks, and attended study visits for 24 weeks (Fig. 1).

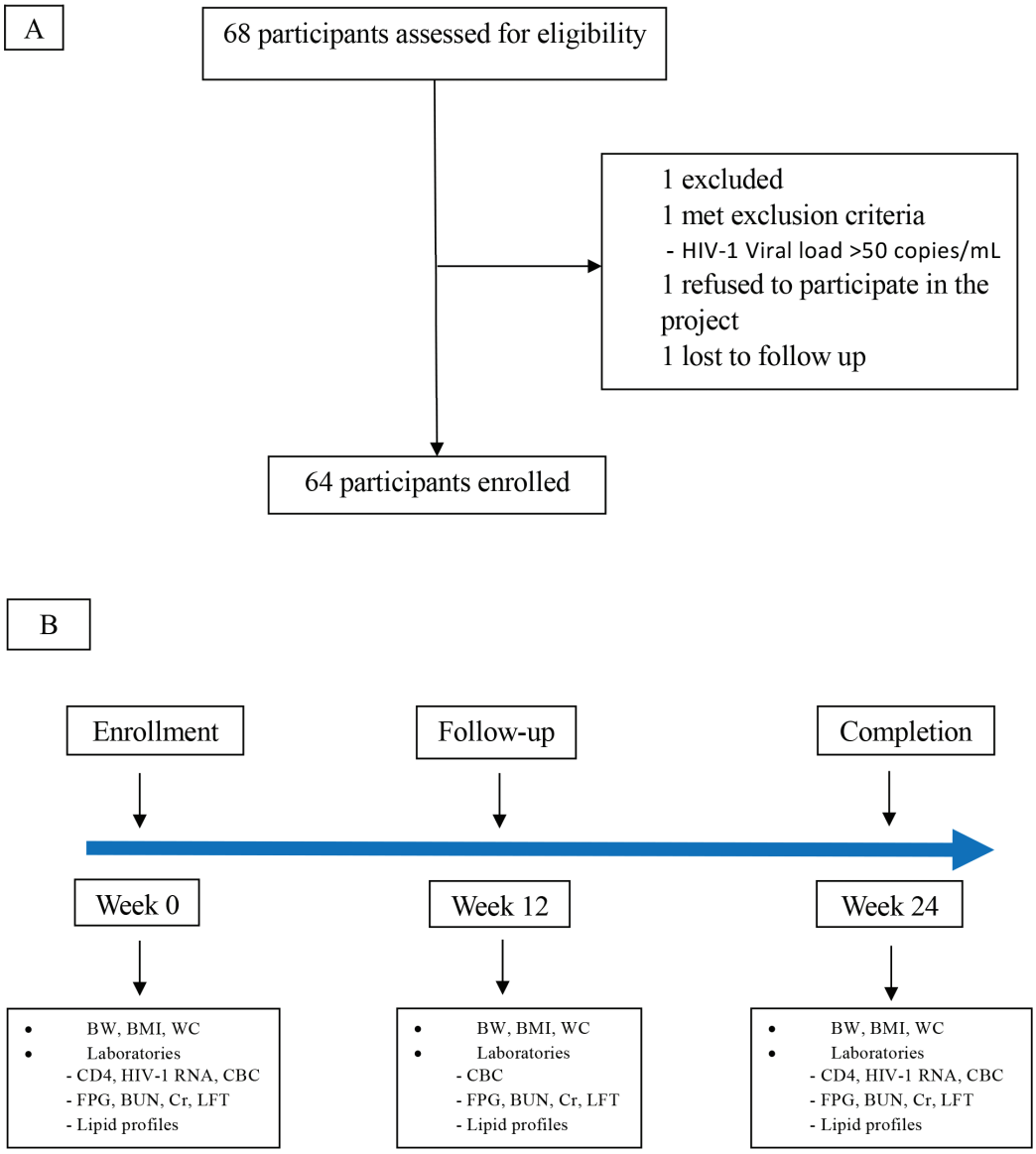

Figure 1 - Panel A shows the flow diagram. Panel B shows the protocols from eligible week until follow-up at week 12 and week 24. BMI = body mass index; BUN = blood urea nitrogen; BW = body weight; CBC = complete blood count; CD4 = cluster of differentiation 4; Cr = creatinine; FPG = fasting plasma glucose; HIV RNA = human immunodeficiency virus ribonucleic acid; LFT = liver function test; WC = waist circumference.

The majority of patients were male (67.19%), and mean age (SD) was 48.20 ± 10.46 years. Mean absolute CD4 count was 603.27 ± 237.08 cells/mm^3. Mean duration from diagnosis of HIV and from first antiretroviral agents until switching therapy were 103.44 ± 57.79 and 88.81 ± 44.53 months, respectively.

Mean body weight, height, BMI, and waist circumference were 66.0 ± 12.02 kg, 165.56 ± 8.80 cm, 23.99 ± 3.51 kg/m^2, and 87.79 ± 10.82 cm, respectively.

The most common NRTI backbones were tenofovir disoproxil fumarate (TDF)/emtricitabine (FTC; 84.38%), followed by abacavir (ABC)/lamivudine (3TC; 14.06%) and TDF/3TC (1.56%). CHD risk factors were dyslipidemia (75.00%), hypertension (12.50%), and diabetes mellitus (3.13%). None of the patients were current smokers.

Of 64 patients, 45 (70.31%) received lipid-lowering agents for dyslipidemia before switching to a DTG-based regimen. The mean duration to initiation of lipid-lowering agents after starting EFV was 35.22 ± 49.16 months. The most common lipid-lowering agents were simvastatin (34.38%), atorvastatin (21.88%), and rosuvastatin (6.25%). Patient demographics and baseline characteristics are summarized in Table I.

At week 12

Mean total cholesterol decreased significantly from baseline (−38.81 mg/dL, 95% CI −32.35 to −12.00, p < 0.001), as did LDL-cholesterol (−25.70 mg/dL, 95% CI −31.53 to −19.88, p < 0.001), HDL-cholesterol (−6.24 mg/dL, 95% CI −8.12 to −4.36,

TABLE I - Demographic and baseline characteristics

Characters	Total N = 64
Age, mean (SD) years	48.20 ± 10.46
Male, n (%)	43 (67.19)
Bodyweight, mean (SD) kg	66.0 ± 12.02
Height, mean (SD) cm	165.56 ± 8.80
Body mass index, mean (SD) kg/m^2	23.99 ± 3.51
• Underweight, < 18.5, n (%)	0 (0.0)
• Normal, ≥18.5 to <25, n (%)	42 (65.62)
• Overweight, ≥25 to <30, n (%)	19 (29.69)
• Obese, ≥30, n (%)	3 (4.69)
Waist circumference, mean (SD) cm	87.79 ± 10.82
Backbone regimen	
• TDF/FTC, n (%)	54 (84.38)
• ABC/3TC, n (%)	9 (14.06)
• TDF/3TC, n (%)	1 (1.56)
Current CD4, mean (SD)	
• Absolute CD4, cells/mm^3	603.27 ± 237.08
• %CD4	26.03 ± 8.22
Coronary heart disease risk, n (%)	
• Dyslipidemia	48 (75.00)
• Hypertension	8 (12.50)
• Diabetes mellitus	2 (3.13)
• Current smoking	0 (0.0)
Current lipid-lowering agent, n (%)	
• None	19 (29.69)
• Simvastatin	22 (34.38)
• Atorvastatin	14 (21.88)
• Rosuvastatin	4 (6.25)
• Fenofibrate	2 (3.12)
• Simvastatin plus gemfibrozil	2 (3.12)
• Atorvastatin plus fenofibrate	1 (1.56)
Duration of HIV diagnosis to enrollment, mean (SD) months	103.44 ± 57.79
Duration of the first antiretroviral agents to enrollment, mean (SD) months	88.81 ± 44.53
Duration of efavirenz to lipid-lowering agents, mean (SD) months	35.22 ± 49.16
Laboratory parameters	
• Hemoglobin, g/dL	13.84 ± 2.09
• Fasting plasma glucose, mg/dL	98.88 ± 12.41
• Creatinine, mg/dL	0.96 ± 0.16
• eGFR, mL/min/1.73 m^2	85.46 ± 22.16
• Albumin, g/dL	4.76 ± 0.42
• Alanine aminotransferase, U/L	36.78 ± 23.79
• Aspartate aminotransferase, U/L	32.13 ± 19.66
• Alkaline phosphatase, U/L	105.86 ± 31.35

3TC = lamivudine; ABC = abacavir; eGFR = estimated glomerular filtration rate; FTC = emtricitabine; TDF = tenofovir disoproxil fumarate; SD = standard deviation.

p < 0.001), and triglycerides (−36.17 mg/dL, 95% CI −58.62 to −13.71, p = 0.002). Mean changes in fasting lipid parameters from baseline are presented in Table II and Fig. 2.

There were statistically significant increases from baseline in mean body weight (0.97 kg, 95% CI 0.49 to 1.44, p < 0.001), BMI (0.32 kg/m^2, 95% CI 0.16 to 0.49, p < 0.001), and waist circumference (1.53 cm, 95% CI 0.88 to 2.18, p < 0.001; Table III and Fig. 3).

At week 24

Mean total cholesterol had decreased significantly from baseline (−32.78 mg/dL, 95% CI −41.16 to −24.39, p < 0.001), LDL-cholesterol (−21.00 mg/dL, 95% CI −28.34 to −13.65, p < 0.001), HDL-cholesterol (−4.21 mg/dL, 95% CI −6.24 to −2.18, p < 0.001), and triglycerides (−49.70 mg/dL, 95% CI −66.54 to −32.86, p < 0.001). Mean changes in fasting lipid parameters from baseline are presented in Table II and Fig. 2.

There were significant increases from baseline in mean body weight (1.39 kg, 95% CI 0.77 to 2.01, p < 0.001), BMI (0.49 kg/m^2, 95% CI 0.27 to 0.73, p < 0.001), and waist circumference (2.6 cm, 95% CI 1.53 to 3.68, p < 0.001; Table III and Fig. 3).

Of 64 patients, 61 (95.31%) had HIV-1 RNA <50 copies/mL at week 24. The HIV-1 RNA of the remaining three were 52, 67, and 62 copies/mL. Nonstatistically significant changes were seen in absolute CD4 (24.09 cells/mm^3, 95% CI −9.60 to 57.79, p = 0.158).

Mean changes in other laboratory parameters are as follows: fasting blood sugar = 0.45 mg/dL, 95% CI −5.70 to 4.79, p = 0.864, creatinine = 0.15 mg/dL, 95% CI 0.11 to 0.18, p < 0.001, and estimated glomerular filtration rate (eGFR) = −9.50 mL/min/1.73 m^2, 95% CI −11.97 to −7.04, p < 0.001 (Table IV).

Discussion

The use of DTG-based regimen is currently widespread as a first-line antiretroviral treatment globally, including in Thailand. This study found that switching to DTG-based regimen in people with HIV with dyslipidemia resulted in improved lipid profiles.

The SCOTA study is a large observational cohort study that examined patients who switched from EFV to DTG, EFV to elvitegravir (EVG), or EFV to rilpivirine (RPV). It was found that total cholesterol significantly decreased in the EFV to DTG and EFV to RPV groups but not in the EFV to EVG group. At month 12, total cholesterol/HDL had significantly decreased in the EFV to RPV group but not in the EFV to DTG and EFV to EVG groups. The study results showed that significant reductions in triglycerides were observed only in the group that switched from EFV to RPV. Furthermore, the decrease in total cholesterol, LDL-cholesterol, triglycerides, and total cholesterol/HDL over 1 year was higher in patients with higher baseline levels (14).

The STRATEGY-NNRTI trial examined the effects of switching from an NNRTI-based regimen (EFV, NVP, or RPV) combined with TDF and FTC to coformulated EVG/cobicistat (c),

TABLE II - Mean change in fasting lipid parameters from baseline

Lipid profiles (mg/dL)	N = 64; mean (SD)											
	Week 0	Week 12	Week 24	Change from week 0 vs. 12		p-Value	Change from week 0 vs. 24		p-Value	Change from week 12 vs. 24		p-Value
				Diff	95% CI		Diff	95% CI		Diff	95% CI	
Total cholesterol	209.69 ± 38.99	170.88 ± 36.43	176.91 ± 35.14	−38.81 ± 25.86	−32.35, −12.00	<0.001	−32.78 ± 33.55	−41.16, −24.39	<0.001	6.03 ± 33.56	−2.35, 14.41	0.156
LDL-cholesterol	131.88 ± 36.17	106.17 ± 31.37	110.88 ± 30.72	−25.70 ± 23.31	−31.53, −19.88	<0.001	−21.00 ± 29.41	−28.34, −13.65	<0.001	4.71 ± 26.78	−1.98, 11.39	0.165
HDL-cholesterol	54.45 ± 13.56	48.20 ± 12.48	50.23 ± 13.23	−6.24 ± 7.52	−8.12, −4.36	<0.001	−4.21 ± 8.12	−6.24, −2.18	<0.001	2.03 ± 6.13	0.49, 3.56	0.010
Triglycerides	181.64 ± 94.12	145.47 ± 77.75	131.94 ± 75.28	−36.17 ± 89.88	−58.62, −13.71	0.002	−49.70 ± 67.40	−66.54, −32.86	<0.001	13.53 ± 67.79	−30.46, 3.40	0.115
Cholesterol/HDL	4.00 ± 0.93	3.69 ± 1.01	3.69 ± 1.06	−0.31 ± 0.61	−0.46, −0.15	<0.001	−0.31 ± 0.85	−0.52, −0.09	0.005	−0.003 ± 0.81	−0.21, 1.9	0.973

CI = confidence interval; HDL = high-density lipoprotein; LDL = low-density lipoprotein; SD = standard deviation.

TABLE III - Mean change in body weight, body mass index, waist circumference, and ASCVD risk score

	N = 64; mean (SD)											
	Week 0	Week 12	Week 24	Change from week 0 vs. 12		p-Value	Change from week 0 vs. 24		p-Value	Change from week 12 vs. 24		p-Value
				Diff	95% CI		Diff	95% CI		Diff	95% CI	
Bodyweight, kg	66.00 ± 12.02	66.97 ± 12.46	67.39 ± 12.58	0.97 ± 1.88	0.49, 1.44	<0.001	1.39 ± 2.49	0.77, 2.01	<0.001	0.42 ± 1.85	−0.04, 0.88	0.073
Body mass index, kg/m^2	23.99 ± 3.51	24.32 ± 3.55	24.49 ± 3.67	0.32 ± 0.67	0.16, 0.49	<0.001	0.49 ± 0.92	0.27, 0.73	<0.001	0.17 ± 0.68	−0.001, 0.34	0.051
Waist circumference, cm	87.79 ± 10.82	89.33 ± 11.38	90.39 ± 10.95	1.53 ± 2.60	0.88, 2.18	<0.001	2.60 ± 4.27	1.53, 3.68	<0.001	1.06 ± 3.65	0.15, 1.97	0.023
ASCVD risk score*	4.56 ± 4.30 (N = 42)	3.99 ± 3.71 (N = 39)	4.69 ± 4.67 (N = 44)	0.464 ± 1.43	−0.04, 0.97	0.07	0.14 ± 1.92	−0.49, 0.77	0.66	−0.11 ± 1.48	−0.59, 0.38	0.66

ASCVD = atherosclerotic cardiovascular disease; LDL = low-density lipoprotein; SD = standard deviation.

*Calculated score from patient age above 40 years old and LDL level above 70 mg/dL.

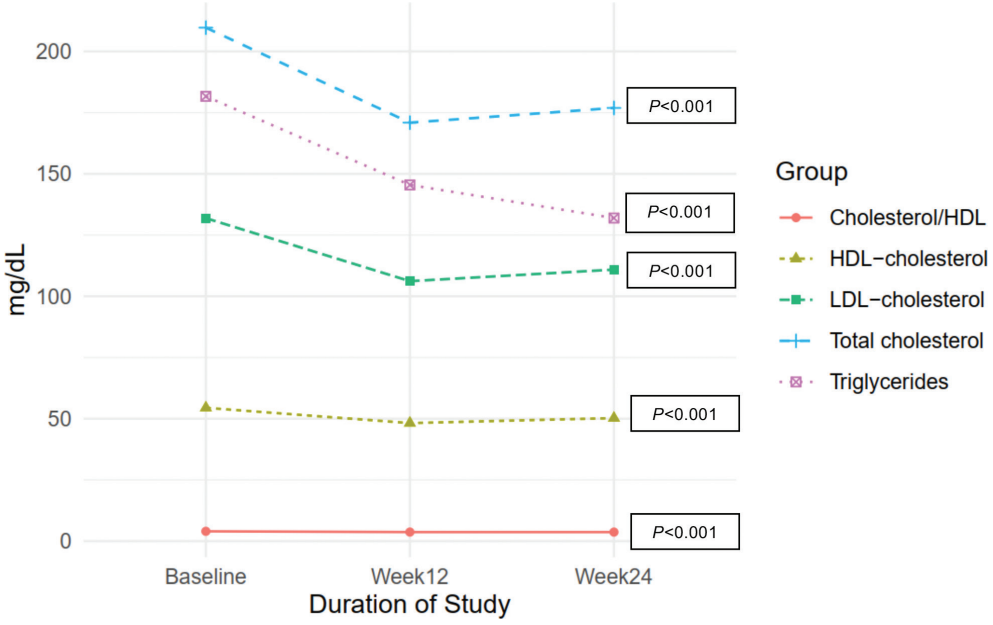

Figure 2 - Change in mean total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, and cholesterol/HDL from baseline through week 24. HDL = high-density lipoprotein; LDL = low-density lipoprotein.

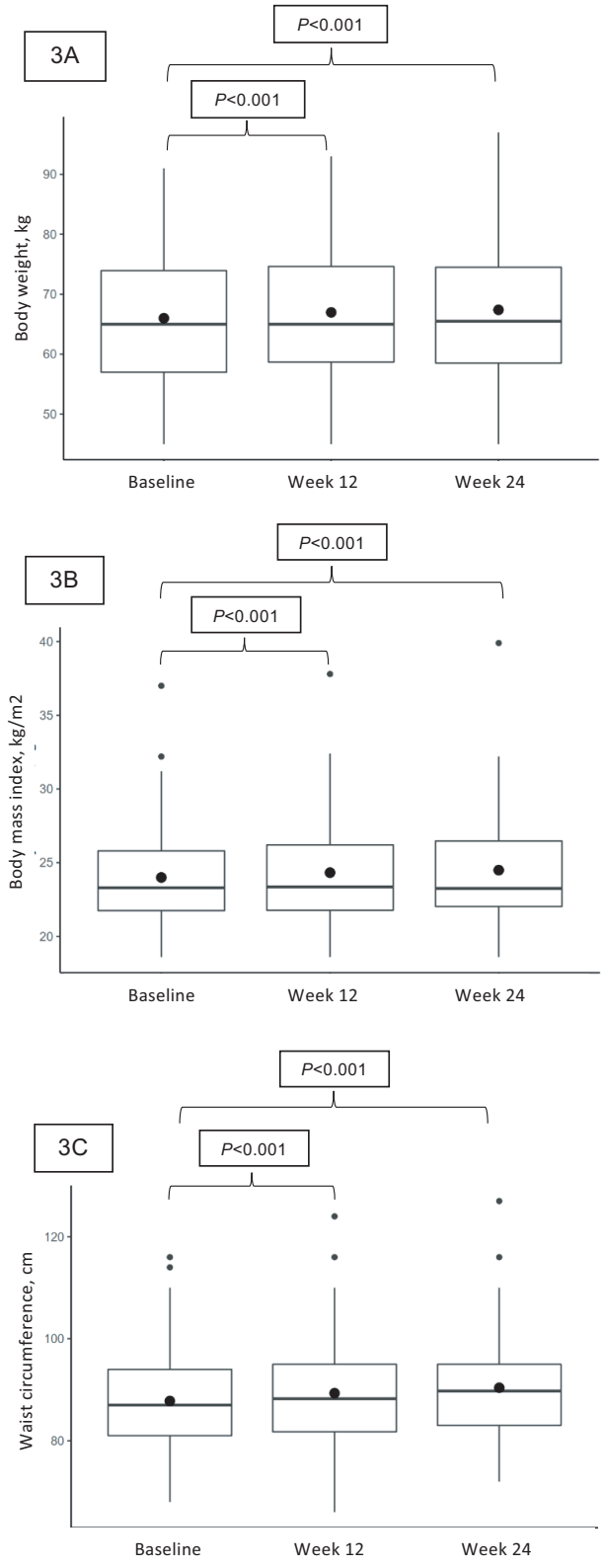

Figure 3 - Box plot of body weight, body mass index, and waist circumference change from baseline. The horizontal line in the box interior represents the group median. The large black dot represents the group mean. (3A) body weight, (3B) body mass index, (3C) waist circumference.

TDF, or FTC or continuing the NNRTI-based regimen. At 48 weeks, the only significant reduction in plasma lipid levels was observed in HDL-cholesterol levels in patients who switched to the EVG/c-based regimen compared to those who continued in the NNRTI-based regimen. The changes in lipid levels varied based on the type of NNRTI. Switching from EFV to the EVG/c-based regimen led to a significant decrease in total cholesterol and LDL-cholesterol and a slight decrease in HDL-cholesterol compared to those who continued EFV. Switching from NVP or RPV to EVG/c led to substantial increases in LDL-cholesterol and the cholesterol/HDL ratio compared to continuing with NVP or RPV (15). However, other recent studies have shown that switching to RPV or a once-daily integrase regimen can improve lipid profiles and reduce dyslipidemia without causing virological failure (14-16).

Virological failure is the primary issue to consider when changing treatments for patients who are already experiencing viral suppression. The cause of viral blips, which were observed in three patients within 24 weeks of transitioning to DTG in our study, is still unknown. Regimes based on INSTIs have been associated with a low frequency of viral blips and do not appear to be linked to virologic failure. However, the occurrence of these blips may increase the clinical workload (17). Therefore, these three patients must undergo further follow-up.

The potential for weight gain is another significant concern when transitioning to a DTG-based regimen (18-20). Our study found a substantial increase in body weight, BMI, and waist circumference after the switch. While INSTI-based regimens are generally recommended as the first-line treatment for HIV (21), recent studies have shown that people receiving these regimens for initial therapy may experience greater weight gain compared to those on protease inhibitors (PIs) or NNRTI-based regimens. For example, a cohort from Brazil found that individuals on RAL-based regimens had a sevenfold higher likelihood of developing obesity than those on NNRTI- or PI-based regimens (22). Additional observational studies have indicated that INSTI-based regimens, particular DTG-based regimens, may be linked to more significant weight gain (23-26). The NAMSAL study, which involved 613 people with HIV in Cameroon randomized to either TDF/3TC with DTG or EFV, revealed that those on the DTG-based regimen gained more weight compared to those on EFV at 48 weeks, and this weight gain was most prominent in women (27).

Furthermore, a recent analysis of eight phase III clinical trials, including 5,680 ART-naive participants, reported that 17.3% of them had a weight gain of ≥10% from baseline, and the weight gain was greater among those taking INSTIs (3.24 kg) than NNRTIs (1.93 kg) and PIs (1.72 kg) (28). Female gender and African origin were factors associated with weight gain (29). The studies conducted in the Asian population reported that factors such as low initial CD4 counts and starting treatment with DTG/TAF/FTC were associated with weight gain (30). These findings suggest that racial diversity may influence changes in body weight among people with HIV.

DTG is generally well-tolerated and appear to have less long-term adverse effects than other regimens. Some

TABLE IV - Mean change in other laboratory parameters

Laboratory parameters	N = 64; mean (SD)										
	Week 0	Week 12	Week 24	Change from week 0 vs. 12		p-Value	Change from week 0 vs. 24		p-Value	Change from week 12 vs. 24		p-Value
				Diff	95% CI		Diff	95% CI		Diff	95% CI	
Fasting blood sugar, mg/dL	98.88 ± 12.41	94.23 ± 12.61	98.42 ± 21.35	−4.64 ± 12.32	−7.72, −1.56	0.004	−0.45 ± 21.01	−5.70, 4.79	0.864	4.18 ± 17.07	−0.07, 8.45	0.054
Creatinine, mg/dL	0.95 ± 0.16	1.11 ± 0.19	1.11 ± 0.19	0.15 ± 0.11	0.12, 0.18	<0.001	0.15 ± 0.13	0.11, 0.18	0.001	−0.01 ± 0.13	−0.04, 0.02	0.660
eGFR, mL/min/ 1.73 m^2	85.46 ± 22.16	74.92 ± 20.36	75.96 ± 20.47	−10.54 ± 8.24	−12.59, −8.47	<0.001	−9.50 ± 9.86	−11.97, −7.04	<0.001	1.03 ± 8.41	−1.07, 3.13	0.330
Hemoglobin, g/dL	13.84 ± 2.09	13.94 ± 1.97	14.13 ± 2.04	0.11 ± 0.88	−0.11, 0.32	0.340	0.29 ± 0.84	0.08, 0.51	0.007	0.19 ± 0.71	0.01, 0.37	0.038
Albumin, g/dL	4.76 ± 0.41	4.68 ± 0.25	4.63 ± 0.28	−0.07 ± 0.35	−0.16, 0.01	0.079	−0.12 ± 0.35	−0.21, −0.03	0.006	−0.04 ± 0.21	−0.10, 0.01	0.090
Globulin, g/dL	3.05 ± 0.36	2.91 ± 0.41	3.02 ± 0.39	−0.14 ± 0.30	−0.21, −0.06	<0.001	−0.02 ± 0.29	−0.10, 0.04	0.442	0.11 ± 0.34	0.03, 0.20	0.009
Alanine aminotransferase, U/L	36.78 ± 23.78	34.38 ± 27.68	31.17 ± 16.46	−2.41 ± 23.99	−8.4, 3.58	0.425	−5.61 ± 23.52	−11.48, 0.26	0.061	−3.20 ± 21.72	−8.62, 2.22	0.243
Aspartate aminotransferase, U/L	32.13 ± 19.65	29.08 ± 15.17	28.61 ± 10.29	−3.04 ± 17.52	−7.42, 1.33	0.169	−3.51 ± 18.94	−8.24, 1.21	0.143	−0.46 ± 12.18	−3.51, 2.57	0.759
Alkaline phosphatase, U/L	105.86 ± 31.35	86.11 ± 25.72	85.92 ± 26.25	−19.75 ± 17.17	−24.04, −15.46	<0.001	−19.93 ± 17.94	−24.42, −15.45	<0.001	−0.18 ± 10.81	−2.88, 2.51	0.890

eGFR = estimated glomerular filtration rate; SD = standard deviation.

patients in this cohort had elevated creatinine values and a slight decrease in eGFR after switching to DTG, and these were significant compared to baseline. DTG has been found to cause a predictable, early increase in serum creatinine of approximately 10% of baseline values in treatment-naive patients and 14% in treatment-experienced patients. This increase is caused by the inhibition of tubular creatinine secretion through the organic cation transporter 2 (OCT2) receptor, but it does not result in a genuine decline in the eGFR (31,32).

This is the first prospective cohort study to examine the consequences of switching from EFV to DTG in people with HIV with dyslipidemia in Thailand. Our data confirm that the use of DTG is safe and adverse effects are rare in this population.

There were a few limitations to this study. Firstly, it was a single-arm, monocentric study, and open-label study. Additionally, the sample size was relatively small. Furthermore, since the follow-up duration was brief, some effects may not have been detectable yet. Finally, as patients were aware when their blood lipids were high, they may have engaged in lifestyle modification, such as diet and exercise, regardless of any adjustments to their medication regimen.

Conclusions

The study showed that switching from EFV-based therapy to DTG improved lipid profiles, suggesting that this switch could benefit patients with a high risk of cardiovascular disease. However, it is essential to note that weight gain and increased waist circumference were also observed.

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Disclosures

Conflict of interest: The authors declare no competing interests.

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Authors' contributions: All authors participated in the study, contributed to manuscript preparation, and reviewed the manuscript. PC contributed to the conception and design. SK, AM, and WS designed the study, analyzed data, and drafted the manuscript.

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Are calcium channel blockers related to lung cancer?

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ABSTRACT

Background: Calcium channel blocker (CCB) is a common antihypertensive agent for the treatment of hypertension. There are inconsistent data of an association of CCB and lung cancer in the literature. This study aimed to evaluate this association by a case-control design.

Methods: The inclusion criteria were adult patients 18 years or over, diagnosed with hypertension, lung cancer or pulmonary tuberculosis, and presenting with one of the suggestive symptoms of lung cancer. Those who were pregnant or had a diagnosis of lung cancer or pulmonary tuberculosis prior to the diagnosis of hypertension were excluded. Diagnosis of lung cancer was made pathologically, while tuberculosis was made by positive acid-fast bacilli on sputum examination, sputum culture positive for *Mycobacterium tuberculosis*, or polymerase chain reaction positive for *M. tuberculosis* with a chest x-ray compatible with tuberculosis. Cases were those diagnosed with lung cancer, while controls were those diagnosed with tuberculosis. Factors associated with lung cancer were calculated by logistic regression analysis.

Results: There were 178 patients who met the study criteria. Of those, 69 patients (38.8%) were in the case group. The lung cancer group had *EGFR* gene mutation in 21 patients (52.5%) and adenocarcinoma was the most common cell type of lung cancer (55 patients; 79.7%). There were two factors independently associated with lung cancer including dyslipidemia and family history of lung cancer.

Conclusions: CCB was not associated with lung cancer in patients with hypertension but dyslipidemia and family history of lung cancer were independently associated with lung cancer in this setting.

Keywords: Dyslipidemia, Family history, Risk factors

Introduction

Hypertension is a common disease, with an estimated 1.39 billion adults diagnosed with hypertension (1). Generally, the prevalence of hypertension was 31.1% worldwide in 2010. The main goal of hypertension treatment is to achieve blood pressure control. A previous report found that only 23% of women and 18% of men with hypertension had a good blood pressure control globally (2). Blood pressure lowering may result in reduction of stroke by up to 40%, and reduction in heart failure by up to 50% (3). Additionally, treatment of conditions or comorbid diseases of hypertension is also crucial (4-8).

Antihypertensive agents are effective in blood pressure lowering including calcium channel blockers (CCBs). Even though CCB is recommended as the first-line treatment for hypertension (9), it has been reported to be increasing the risk of cancer (10). Several studies including a meta-analysis showed that CCB was associated with lung cancer with adjusted odds ratio of 1.13 (95% confidence interval 1.06, 1.21) (11-13), while other studies did not find this association (14,15). As there are inconsistent and inconclusive data on this issue, this study aimed to evaluate the additional association of CCB and lung cancer.

Methods

This was a case-control study conducted at Srinagarind Hospital, Khon Kaen University, Thailand. The inclusion criteria were adult patients 18 years or over, diagnosed with lung cancer or pulmonary tuberculosis, and presenting with one of the following symptoms: hemoptysis, chronic cough, constitutional symptoms, lung mass, and a checkup. Those who were pregnant or with diagnosis of lung cancer

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or pulmonary tuberculosis prior to the diagnosis of hypertension were excluded. Diagnosis of lung cancer was made pathologically, while tuberculosis was made by positive acid-fast bacilli on sputum examination, sputum culture positive for *Mycobacterium tuberculosis*, or polymerase chain reaction (PCR) positive for *M. tuberculosis* with a chest x-ray compatible with tuberculosis. Cases were those diagnosed with lung cancer, while controls were those diagnosed with tuberculosis. The study period was between 2007 and 2019.

Eligible patients were recorded for baseline characteristics, medications, and risk factors for lung cancer. Baseline characteristics included age, sex, body mass index, comorbid diseases, alcohol consumption, and smoking history. Risk factors of lung cancer were also recorded: family history of lung cancer, occupational exposure, or chest wall radiation. Antihypertensive medications particularly CCB were retrieved as well as duration of CCB use. The primary outcome of this study was to evaluate the association of lung cancer and CCB.

Sample size calculation

Based on a probability of event in the case and control group of 0.6 and 0.3, the required sample size for case group was 49 patients with a confidence of 95% and power of 80%. The control group was enrolled for a ratio of case:control of 1:1-2.

Statistical analyses

Patients were categorized into two groups: lung cancer (cases) and tuberculosis (controls). Descriptive statistics were used to compare differences between both groups: independent t-test or Wilcoxon rank sum test for numerical variables and chi-square or Fisher's exact test for categorical variables. Factors associated with lung cancer were calculated by logistic regression analysis. Multicollinearity was checked in the logistic regression analysis. The final model was executed for goodness of fit by Hosmer-Lemeshow test. Statistical analyses were calculated by STATA software version 10.1 (College Station, Texas, USA).

Results

There were 178 patients who met the study criteria. Of those, 69 patients (38.8%) were in the case group or lung cancer group. The lung cancer group had tested for gene mutation in 40 patients (56.0%). *EGFR* was the most common gene mutation (21 patients; 52.5%), followed by no mutation (15 patients; 37.5%), and *ALK* mutation (4 patients; 10%). Adenocarcinoma was the most common cell type of lung cancer (55 patients; 79.7%), followed by squamous cell carcinoma (8 patients; 11.6%). Table I shows baseline characteristics and CCB treatment of the case and control groups. There were three significant factors between both groups, namely dyslipidemia, obstructive sleep apnea, and family history of lung cancer. The case group had significantly higher proportions of these three factors than the control group such as family history of lung cancer (15.9% vs. 0.9%; $p < 0.001$).

TABLE I - Baseline characteristics and CCB treatment of patients with hypertension categorized by lung cancer (case group) and tuberculosis (control group)

Factors	Cases (n = 69)	Controls (n = 109)	p value
Age, years*	67 (61,71)	66 (59,71)	0.603
Male	44 (63.8)	77 (70.6)	0.338
Female	25 (36.2)	32 (29.4)	
BMI			0.676
<19	13 (18.8)	17 (15.9)	
19-24.5	16 (23.2)	25 (23.4)	
24.5-30	5 (7.2)	4 (3.7)	
>30	35 (50.7)	61 (57.0)	
Smoking	40 (58)	64 (58.7)	0.922
Alcohol drinking	28 (40.6)	53 (48.6)	0.294
Comorbidities			
CVD	10 (14.5)	17(15.6)	0.841
Dyslipidemia	29 (42)	27 (24.8)	0.016
DM	27 (39.1)	48 (44)	0.518
OSA	5 (7.2)	1 (0.9)	0.033
CKD	10 (14.5)	23 (21.1)	0.269
Ischemic stroke	6 (8.7)	2 (1.8)	0.057
Autoimmune	2 (2.9)	4 (3.7)	>0.999
HBV infection	2 (2.9)	3 (2.8)	>0.999
HCV infection	0	5 (4.6)	0.158
Hyperthyroidism	1 (1.4)	0	0.388
Family history of lung cancer	11 (15.9)	1 (0.9)	<0.001
Occupational exposure	1 (1.4)	2 (1.8)	>0.999
Chest wall radiation	0	0	NA
CCB used	45 (65.2)	62 (56.9)	0.268
CCB duration, years*	5 (3-10)	6 (2.8-8.2)	0.642

Data presented as number (percentage) except * indicated as median (1st-3rd quartile range).
BMI = body mass index; CCB = calcium channel blocker; CKD = chronic kidney disease; CVD = cardiovascular disease; DM = diabetic mellitus; HBV = hepatitis B virus; HCV = hepatitis C virus; NA = not available; OSA = obstructive sleep apnea.

There were five factors in the final model for prediction of lung cancer (Tab. II). Two factors were independently associated with lung cancer including dyslipidemia and family history of lung cancer. These factors had an adjusted odds ratio (95% confidence interval) of 2.12 (1.07, 4.23) and 22.43 (2.76, 182.36), respectively. The Hosmer-Lemeshow chi-square was 4.88 ($p = 0.559$). CCB use had an adjusted odds ratio of 1.50 (95% confidence interval of 0.53, 2.01).

Discussion

This study found that CCB was not associated with lung cancer but dyslipidemia and family history of lung cancer did.

TABLE II - Factors associated with lung cancer in patients with hypertension by logistic regression analysis

Factors	Unadjusted OR	95% CI	Adjusted OR	95% CI
Smoking	0.97	0.53, 1.79	1.03	0.53, 2.01
Dyslipidemia	2.20	1.15, 4.20	2.12	1.07, 4.23
Obstructive sleep apnea	8.44	0.96, 73.84	7.50	0.79, 71.17
Family history of lung cancer	20.48	2.58, 162.61	22.43	2.76, 182.36
Calcium channel blocker used	1.42	0.76, 2.67	1.50	0.53, 2.01

CI = confidence interval; OR = odds ratio.

CCB-related cancer is still inconclusive and needs further studies to evaluate this issue (16). The possible mechanisms of CCB-related cancer include cell growth, cell proliferation, and cell apoptosis (17,18). However, some studies found that CCB may reduce risks of lung cancer as CCB may suppress lung tumorigenesis and suppression of *CACNA2D2*, a link to non–small cell lung cancer (19,20). Previous studies and meta-analyses that reported on the risk of CCB on lung cancer were mainly from database study with asymptomatic controls, while this study evaluated this association with a case-control study using controls who presented with similar symptoms of lung cancer.

Rotshild et al published one meta-analysis and one nested case-control study. Both studies reported that CCB was associated with lung cancer with an adjusted odds ratio of 1.13 (95% confidence interval 1.06, 1.21) and a risk ratio of 1.15 (95% confidence interval 1.01-1.30) (12,13). However, there are some limitations in both studies. The case-control study did not state how lung cancer diagnosis was made and significant factors in this study, namely dyslipidemia and family history of lung cancer, were not studied (12). The results of this study were compatible with the large population cohort study in Hong Kong (21). Among 84,116 patients with lung cancer, CCB did not increase the risk of lung cancer regardless of aspirin therapy. The hazard ratios for both settings were 0.89 (95% confidence interval of 0.73, 1.08) for CCB and 0.82 (95% confidence interval of 0.64, 1.06) for CCB plus aspirin. As data regarding CCB and lung cancer are still conflicting, further studies are required.

This study found that dyslipidemia was associated with lung cancer, which was supported by a previous study from China (22). High-density lipoprotein cholesterol (HDL-C) reduced the risk of non–small cell lung cancer by 77% (adjusted odds ratio of 0.233; 95% confidence interval of 0.134, 0.407). There are several biological explanations for the reduction of cancer and high HDL-C, such as suppression of signaling via lipid raft formation and reduction of viability and proliferation (23,24). Regarding family history of lung cancer, several studies support this finding conducted in twins, women, and nonsmokers (25-27). This association may be explained by second-hand smokers or genetic factors such as *HER2* gene or *EGFR* variant (28-32).

Study Limitations

There are some limitations in this study. First, this was a study conducted in a single, referral university hospital resulting in a small sample size. Second, some factors were not completely evaluated such as genetic factors, sleep apnea, or aspirin therapy (21,33-38). Similar to previous published articles, smoking history may be underestimated as smoking status may not be recorded in the medical records (12,13,21). Additionally, selection bias may have existed as this study enrolled only those with pathological diagnosis of lung cancer. This may also result in small sample size. Finally, some factors such as alcohol consumption may not be studied or may be missing due to retrospective design.

Conclusions

CCB was not associated with lung cancer in patients with hypertension but dyslipidemia and family history of lung cancer were independently associated with lung cancer in this setting.

Disclosures

Conflict of interest: The authors declare that they have no conflicts of interest.
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ORIGINAL RESEARCH ARTICLE

Network analysis for identifying potential anti-virulence targets from whole transcriptome of *Pseudomonas aeruginosa* and *Staphylococcus aureus* exposed to certain anti-pathogenic polyherbal formulations

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ABSTRACT

Introduction: Antimicrobial resistance (AMR) is a serious global threat. Identification of novel antibacterial targets is urgently warranted to help antimicrobial drug discovery programs. This study attempted identification of potential targets in two important pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus.*

Methods: Transcriptomes of *P. aeruginosa* and *S. aureus* exposed to two different quorum-modulatory polyherbal formulations were subjected to network analysis to identify the most highly networked differentially expressed genes (hubs) as potential anti-virulence targets.

Results: Genes associated with denitrification and sulfur metabolism emerged as the most important targets in *P. aeruginosa*. Increased buildup of nitrite (NO_2) in *P. aeruginosa* culture exposed to the polyherbal formulation *Panchvalkal* was confirmed through *in vitro* assay too. Generation of nitrosative stress and inducing sulfur starvation seemed to be effective anti-pathogenic strategies against this notorious gram-negative pathogen. Important targets identified in *S. aureus* were the transcriptional regulator *sarA*, immunoglobulin-binding protein Sbi, serine protease *SplA*, the *saeR/S* response regulator system, and gamma-hemolysin components *hlgB* and *hlgC*.

Conclusion: Further validation of the potential targets identified in this study is warranted through appropriate *in vitro* and *in vivo* assays in model hosts. Such validated targets can prove vital to many antibacterial drug discovery programs globally.

Keywords: AMR (antimicrobial resistance), Anti-virulence, Network Analysis, Novel antibacterial targets, Polyherbal, Protein-Protein Interaction (PPI)

Introduction

Despite wide recognition of antimicrobial resistance (AMR) as a major global health threat, the progress on discovery and development of new antibiotics in the last three to four decades clearly has fallen short from being satisfactory. For a variety of reasons, for example, lack of interest among major pharmaceutical firms, rapid emergence and spread of resistance among pathogenic bacterial populations, dearth of new validated cellular and molecular targets, the list of effective antimicrobials available for treatment of resistant infections remains short. The status of antibiotic discovery research has been reviewed thoroughly (1-4). Since most currently available antibiotics target a narrow range of bacterial traits, that is, cell envelope synthesis, protein or nucleic acid synthesis, or folic acid synthesis, a truly new class of antibiotics will be discovered only if we have a longer list of validated targets. Development of new bactericidal antibiotics is not the only way of tackling the slow pandemic of AMR infections; discovery of resistance modifiers and non-antibiotic virulence-attenuating agents can also be of great

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value (5,6). Hence identification of new potential targets for both bactericidal antibiotics as well as antibiotic adjuvants is useful. There is a clear need for antibiotics with previously unexploited new targets and wide target diversity in the discovery pipeline. One of the major challenges in antibacterial discovery is associated with the proper target selection, for example, the requirement of pursuing molecular targets that are not prone to rapid resistance development (7).

Various public health agencies like CDC (Centers for Disease Control and Prevention, USA), WHO (World Health Organization), and DBT (Department of Biotechnology, India) have published lists of priority pathogens against which novel antimicrobials need to be discovered urgently. Antibiotic-resistant strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus* commonly appear on all such lists. As per CDC's Vital Signs report (https://www.cdc.gov/vitalsigns/index.html) more than 33% of the bloodstream infections in patients on dialysis in the United States in 2020 were caused by *S. aureus.* This gram-positive human commensal has been recognized as an important opportunistic pathogen responsible for a wide range of infections (8). *P. aeruginosa* is the primary cause of gram-negative nosocomial infections. Its ability to adapt to a wide range of environmental niches combined with its nutritional versatility and genome plasticity, along with a multitude of intrinsic and acquired resistance mechanisms make it one of the most notorious pathogens of critical clinical importance. Efforts for finding perturbants capable of targeting the *P. aeruginosa* pathogenicity and antibiotic resistance are highly desired (9).

We had previously studied the anti-virulence effect of certain polyherbal formulations against *S. aureus* or *P. aeruginosa* at the whole transcriptome level of the target pathogen, wherein we gained some insight into the molecular mechanisms associated with the virulence-attenuating potential of the test formulations, which was largely independent of any growth-inhibitory effect. Pathogens exposed to the test formulations were compromised in their ability to kill the model host *Caenorhabditis elegans*. The current study attempted network analysis of the differentially expressed genes (DEG) of *P. aeruginosa* and *S. aureus* exposed to the anti-pathogenic polyherbal formulations *Panchvalkal* (10) and Herboheal (11), respectively, reported in the previous studies, with an aim to identify highly networked genes as potential anti-virulence targets. *Panchvalkal* is a mixture of bark extracts of five different plants – *Ficus benghalensis, Ficus religiosa, Ficus racemosa, Ficus lacor*, and *Albizia lebbeck.* Herboheal comprised of extracts of six different plants. Its full composition can be seen at: https://downloads.hindawi.com/journals/aps/2019/1739868.f1.pdf

Methods

Network analysis

We accessed the list of DEG for *Panchvalkal* (Pentaphyte-P-5®)-exposed *P. aeruginosa* (NCBI Bioproject ID 386078) and Herboheal-exposed *S. aureus* (NCBI Bioproject ID 427073). The *P. aeruginosa* used was a multidrug-resistant strain. Network analysis for both the studies was carried out independently, wherein only the DEG fulfilling the dual filter criteria of log fold change ≥2 and False Discovery Rate (FDR) ≤0.01 were selected for further analyses. The list of such DEG was fed into the database STRING (v. 11.5) (12) for generating the PPI (Protein-Protein Interaction) network. Then the genes were arranged in decreasing order of 'node degree' (a measure of connectivity with other genes or proteins), and those above a certain threshold value were subjected to ranking by cytoHubba (v. 3.9.1) (13). Since cytoHubba uses 12 different ranking methods, we considered the DEG being top-ranked by more than six different methods (i.e., 50% of the total ranking methods) for further analysis. These top-ranked shortlisted proteins were further subjected to network cluster analysis through STRING and those which were part of multiple clusters were considered 'hubs' which can be taken up for further validation of their targetability. Here 'hub' refers to a gene or protein interacting with many other genes/proteins. Hubs thus identified were further subjected to co-occurrence analysis to see whether an anti-virulence agent targeting them is likely to satisfy the criterion of selective toxicity (i.e., targeting the pathogen without harming the host). This sequence of analysis allowed us to end with a limited number of proteins which satisfied various statistical and biological significance criteria simultaneously, that is, (i) log fold change ≥2; (ii) FDR ≤0.01; (iii) relatively higher node degree; (iv) top-ranking by at least six cytoHubba methods; (v) (preferably) member of more than one local network cluster; and (vi) high probability of the target being absent from the host. A schematic presentation of the methodology employed for network analysis is presented in Figure 1.

Nitrite estimation

Nitrite estimation in *P. aeruginosa* culture supernatant was done through Griess assay (14). *P. aeruginosa* strain studied by us is a multidrug-resistant strain, which is resistant to ampicillin (10 µg), augmentin (30 µg), nitrofurantoin (300 µg), clindamycin (2 µg), chloramphenicol (30 µg), cefixime (5 µg), and vancomycin (30 µg). This bacterium was grown in *Pseudomonas* broth (HiMedia, Mumbai) with or without *Panchvalkal* (547 µg/mL; dried extract powder without any bulking agent was procured from Dr. Palep's Medical Education and Research Foundation Pvt. Ltd., Mumbai, India, and dissolved in dimethylsulfoxide (DMSO) for assay purpose) at 35°C for 21±1 hour. Following incubation, cell density was quantified at 764 nm (15), and then the bacterial culture suspension was centrifuged at 13,600 *g* for 10 minutes. Resulting supernatant was mixed with Griess reagent (Sigma-Aldrich) in 1:1 ratio and incubated for 15 minutes in the dark at room temperature. Absorbance of the resulting pink color was quantified at 540 nm (Agilent Technology Cary 60 UV-Vis). These optical density (OD) values were plotted on standard curve prepared using $NaNO_2$ to calculate the nitrite concentration. To nullify any effect of variation in cell density between control and experimental culture, nitrite unit (i.e., nitrite produced per unit of growth) was calculated by dividing the nitrite concentration values by cell density. Sodium nitroprusside (Astron chemicals, Ahmedabad) being

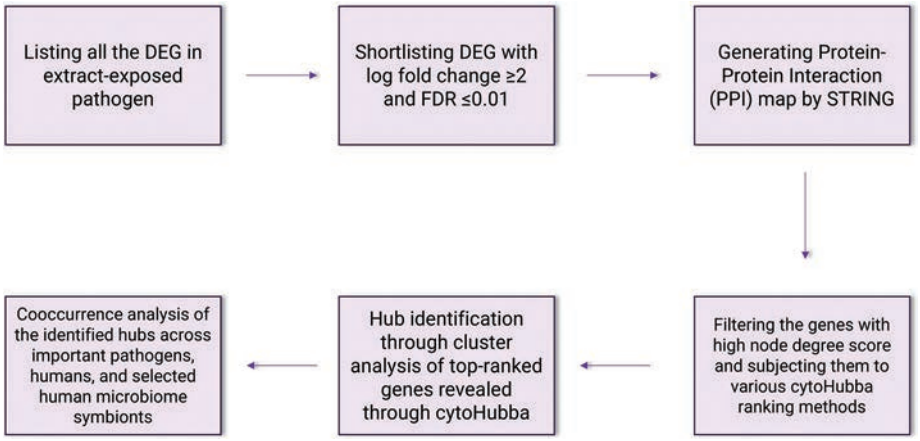


Fig. 1 - A schematic of methodology for network analysis and hub identification.

a chemical known to be capable of generating nitrosative stress in bacteria (16-18) was used as a positive control. Appropriate vehicle control (i.e., bacteria grown in the presence of 0.5% v/v DMSO (Merck)), negative control (deionized water), and abiotic control (*Panchvalkal*-supplemented *Pseudomonas* broth) were included in the experiment. Griess reagent was added in all these controls in the same proportion as that in extract-exposed or not-exposed bacterial culture samples.

Results

Network analysis of DEG in Panchvalkal-exposed P. aeruginosa

Our original experimental study exposed *P. aeruginosa* to *Panchvalkal* at 567 µg/mL, wherein the extract-exposed pathogen could kill 90% lesser host worms than its extract-not-exposed counterpart. Whole transcriptome study revealed that approximately 14% of the *P. aeruginosa* genome was expressed differently under the influence of *Panchvalkal*. The total number of DEG satisfying the dual criteria of log fold change ≥2 and FDR ≤0.01 was 228, of which 105 were downregulated (Tab. S1) and 123 were upregulated (Tab. S4). We created PPI network for up- and downregulated genes separately (Figs. 5 and 2, respectively). PPI network for downregulated genes generated through STRING is presented in Figure 2, which shows 101 nodes connected (105 genes were fed to string, out of which 101 were shown in the PPI network) through 86 edges with an average node degree of 1.7. Since the number of edges (86) in this PPI network is 3.18-fold higher than expected (27) with a PPI enrichment *p* value <1.0e-16, this network can be said to possess significantly more interactions among the member proteins than what can be expected for a random set of proteins of identical sample size and degree distribution. Such an enrichment can be taken as an indication of the member proteins being at least partially biologically connected. When we arranged the 105 downregulated genes in decreasing order of node degree, 52 nodes were found to have a nonzero score (Tab. S2), and we selected top 13 genes with a node degree ≥6 for further ranking by different cytoHubba methods. Then we looked for genes which appeared among the top-10 ranked candidates by ≥6 cytoHubba methods, and 10 such shortlisted genes (Tab. S3) were further checked for interactions among themselves followed by cluster analysis (Fig. 3), which showed them to be strongly networked as the average node degree score was 8. This network possessed 40 edges as against expected (zero) for any such random set of proteins (PPI enrichment *p* value <1.0e-16). The PPI network generated through STRING showed these 10 important genes to be distributed among three different local network clusters. Five (norB, norC, norD, nirS, and nirQ) of the predicted hubs were part of each of the three clusters, and they have a role in denitrification (19). Of the remaining five predicted hub proteins, one more (norE) is also associated with nitrogen metabolism, and two (nosL and nosY) have a role in denitrification as well as copper homeostasis. These three proteins were members of two out of three clusters. The eight proteins (Tab. I) found to be members of minimum two clusters can be said to be potential hubs, whose downregulation can be hypothesized to attenuate *P. aeruginosa* virulence.

Since all the targets mentioned in Table I are known to play an important role in *P. aeruginosa* with respect to detoxification of reactive nitrogen species, we hypothesized that *Panchvalkal*-treated *P. aeruginosa*'s ability to detoxify reactive nitrogen species is compromised. To check this hypothesis, we quantified nitrite concentration in extract-treated *P. aeruginosa* culture, wherein it was found to have 31% higher nitrite concentration in supernatant as compared to control (Fig. 4). This higher accumulation of nitrite can be taken as an indication of compromised denitrification efficiency as nitrite is an intermediate of denitrification pathway (22).

PPI network for upregulated genes in *Panchvalkal*-exposed *P. aeruginosa* generated through STRING is presented in Figure 5, which shows 121 nodes connected through 70 edges with an average node degree of 1.16. Though empirically the centrality of the upregulated genes appeared to be lesser than those downregulated in *Panchvalkal*-exposed *P. aeruginosa*, since the number of edges (70) in this PPI network is 1.89-fold higher than expected (37) with a PPI enrichment *p* value of 1.27e-06, this network can be said to possess significantly more interactions among the member proteins than what can be expected for a random set of proteins of this much sample size and degree distribution. Such an enrichment can be

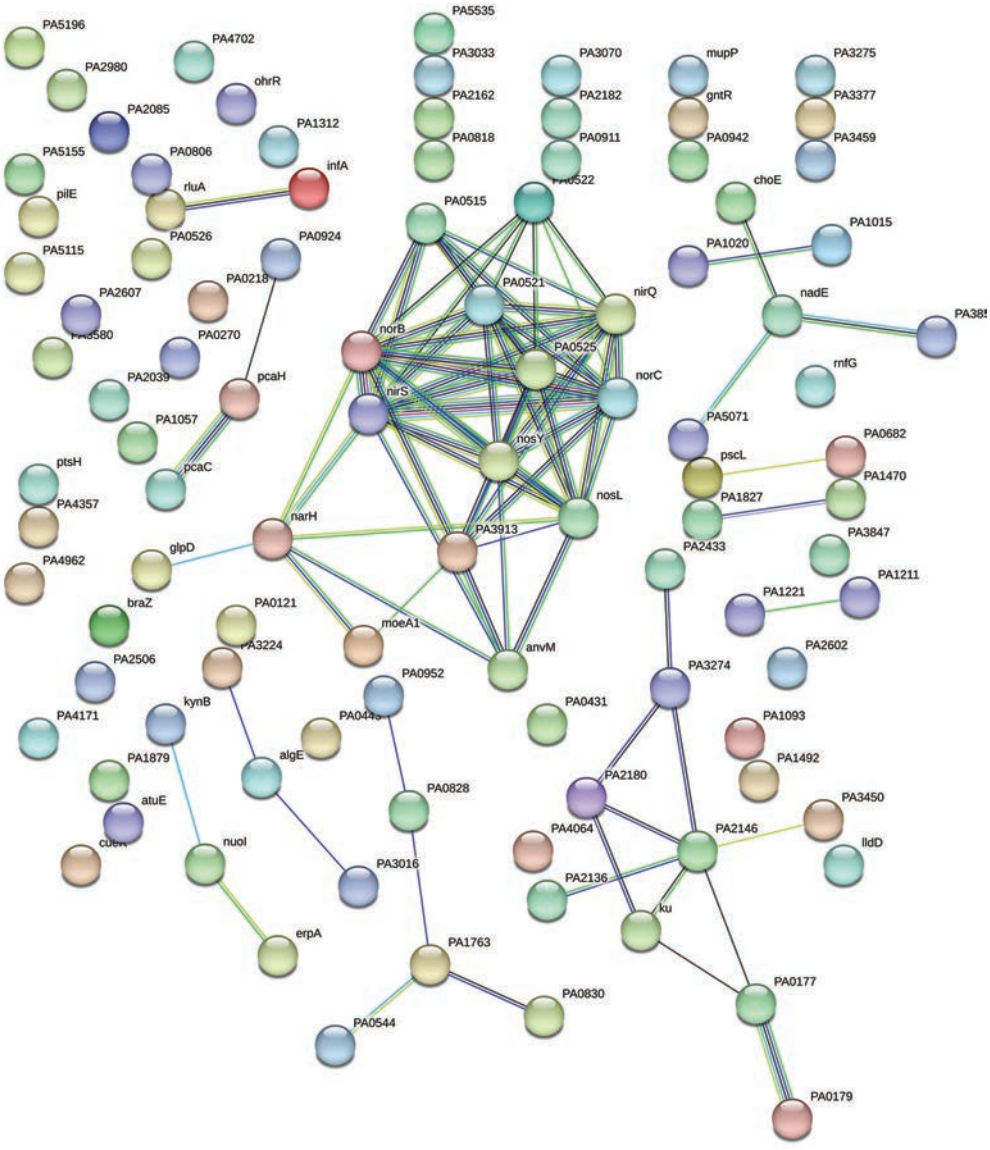
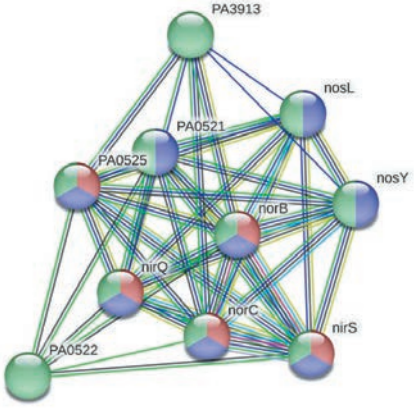


Fig. 2 - Protein-Protein Interaction (PPI) network of downregulated genes in *Panchvalkal*-exposed *Pseudomonas aeruginosa.* Edges represent protein-protein associations that are meant to be specific and meaningful, that is, proteins jointly contribute to a shared function; this does not necessarily mean they are physically binding to each other. Network nodes represent proteins. Splice isoforms or post-translational modifications are collapsed, that is, each node represents all the proteins produced by a single, protein-coding gene locus.



Symbolic representation	Cluster	Description	Count in network	Strength of the enrichment effect	FDR
	CL: 3517	Mixed, incl. oxidoreductase activity, acting on other nitrogenous compounds as donors, cytochrome as acceptor and quinone metabolic process	10 of 27	2.31	5.65e-20
	CL: 3520	Mixed, incl. oxidoreductase activity, acting on other nitrogenous compounds as donors, cytochrome as acceptor and signal anchor	8 of 13	2.54	1.70e-16
	CL: 3523	oxidoreductase activity,acting on other nitrogenous compounds as donors, cytochrome as acceptor and signal anchor	5 of 6	2.67	4.18e-10

Fig. 3 - Protein-Protein Interaction (PPI) network of top-ranked genes revealed through cytoHubba among downregulated differentially expressed genes (DEG) in *Panchvalkal*-exposed *Pseudomonas aeruginosa*.

Table I - Hubs identified as potential targets from among the downregulated genes in *Panchvalkal*-exposed *Pseudomonas aeruginosa*

No.	Gene ID	Gene name	Functional role
1	PA0520	*nirQ*	Denitrification regulatory protein NirQ
2	PA0519	*nirS*	Heme d1 biosynthesis protein, which is important for denitrification (20)
3	PA0524	*norB*	Nitric oxide reductase subunit B
4	PA0523	*norC*	Nitric oxide reductase subunit C
5	PA0525	*NorD*	Nitric oxide reductase NorD protein
6	PA0521	*NorE*	Nitric oxide reductase NorE protein
7	PA3395	*nosY*	Nitrous oxide reductase; a Cu-processing system permease protein having role in denitrification pathway (21)
8	PA3396	*nosL*	A lipoprotein attached to the outer membrane described as a copper-binding protein. Regulator of *nos* operon, NosR also associates with NosL. This protein is probably responsible for the insertion and coordination of the multicopper center within NosZ (22).

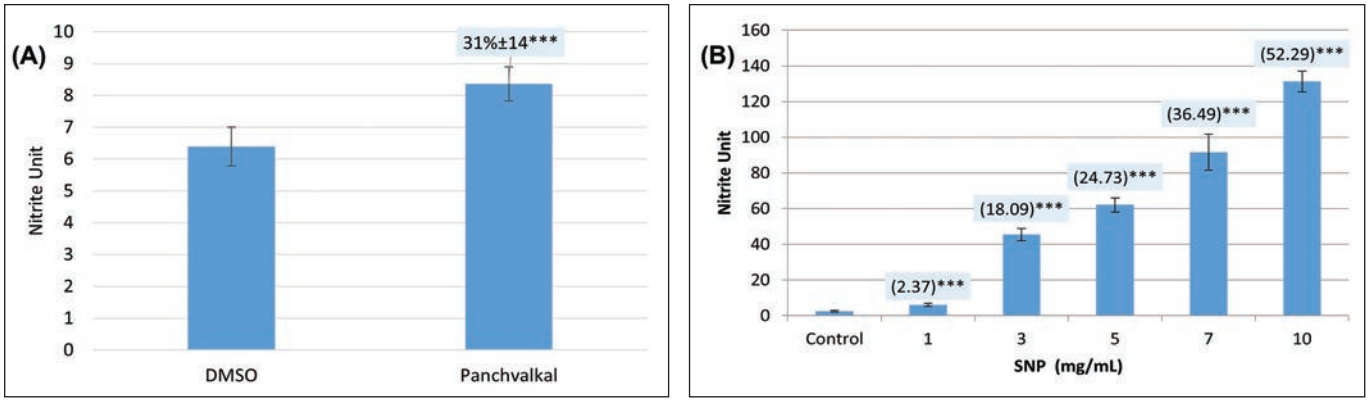


Fig. 4 - *Panchvalkal*-treated *Pseudomonas aeruginosa* culture has higher extracellular accumulation of nitrite. While nitrite concentration in vehicle control (*P. aeruginosa* incubated in media supplemented with 0.5% v/v dimethylsulfoxide (DMSO)) was at par to that without DMSO, *Panchvalkal* caused nitrite concentration in *P. aeruginosa* culture supernatant to rise (A). Sodium nitroprusside used as positive control caused a dose-dependent 2.37 to 52.29-fold higher nitrite buildup in *P. aeruginosa* culture (B). Nitrite unit (i.e., nitrite concentration:cell density ratio) was calculated to nullify any effect of cell density on nitrite production. ***p<0.001.

taken as an indication of the member proteins being at least partially biologically connected. When we arranged the 121 upregulated genes in decreasing order of node degree, 62 nodes were found to have a nonzero score, and we selected the top 26 genes with a node degree ≥3 (Tab. S5) for further ranking by different cytoHubba methods. Then we looked for genes which appeared among top-ranked candidates by ≥6 cytoHubba methods, and 14 such genes (Tab. S6) were identified for further cluster analysis. Interaction map of these 14 important genes (Fig. 6) showed them to be networked with the average node degree score of 2.29. Number of edges possessed by this network was 16 as against expected 1 for any such random set of proteins. These 14 genes were found to be distributed among five different local network clusters. Strength score for each of these clusters was >1.5. While three of the proteins (*atsB, msuE,* and *ssuB1*) were common members of three different clusters, one gene (*tauA*) appeared in two clusters**.** All these four highly networked upregulated genes (Tab. II) are involved in sulfur metabolism in *P. aeruginosa* (23). Hence it may be speculated that *Panchvalkal* has induced sulfur starvation in *P. aeruginosa*, to overcome which the pathogen is forced to upregulate genes involved in sulfur transport and metabolism.

Network analysis of DEG in Herboheal-exposed S. aureus

Herboheal is a folk-inspired wound-healing formulation, and we had earlier demonstrated its anti-virulence potential against multiple bacterial pathogens including *S. aureus*. Pretreatment of *S. aureus* with Herboheal (0.1% v/v) could attenuate its virulence toward the surrogate host *C. elegans* by 55%. This concentration had a moderate growth-inhibitory effect (32%) on *S. aureus*, while heavily inhibiting staphyloxanthin production (79%). Whole transcriptome study revealed that approximately 17% of the *S. aureus* genome was expressed differently under the influence of Herboheal. The total number of DEG satisfying the dual criteria of log fold change ≥2 and FDR ≤0.01 was 113, of which 57 were upregulated and 56 were downregulated (Tab. S7). Since the number of genes amenable to mapping by STRING turned out to be

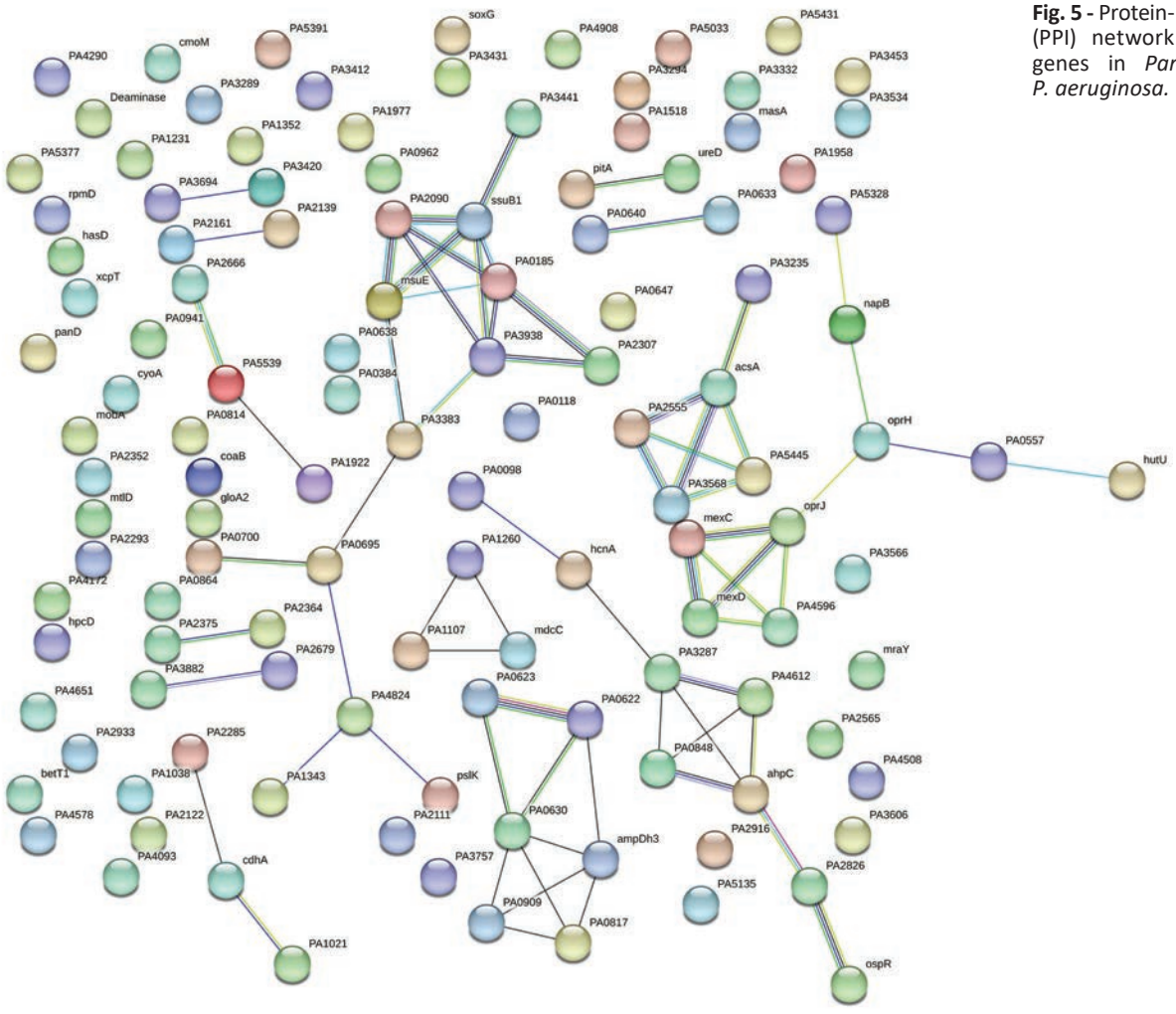
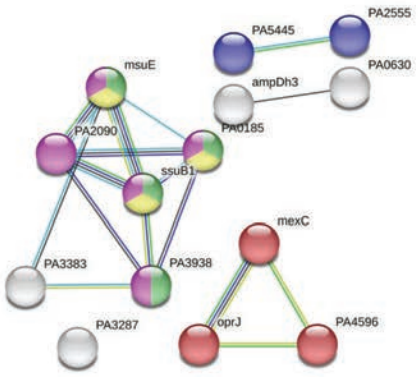


Fig. 5 - Protein-Protein Interaction (PPI) network of up-regulated genes in *Panchvalkal*-exposed *P. aeruginosa.*



Symbolic representation	Cluster	Description	Count in network	Strength of the enrichment effect	False discovery rate
	CL:9698	Beta-lactamase resistance	3 of 12	2.0	0.0012
	CL:2228	Acetyl-CoA metabolic process and, acylphosphatase activity	2 of 10	1.9	0.0380
	CL:8737	Alkanesulphonate transport, and sulphur metabolism	4 of 26	1.79	0.00055
	CL: 8738	Mixed, incl. alkanesulfonate transmembrane transporter activity, and alkane sulfonate monooxygenase activity	3 of 21	1.75	0.0036
	CL: 8733	Mixed, incl. Sulfur metabolism, and siderophore uptake transmembrane transporter activity	5 of 63	1.5	0.00055

Fig. 6 - PPI network of top-ranked genes revealed through cytoHubba among up-regulated DEG in *Panchvalkal*-exposed *P. aeruginosa*.

Table II - Hubs identified as potential targets from among the upregulated genes in *Panchvalkal*-exposed *Pseudomonas aeruginosa*

No.	Gene ID/name	Codes for	Remarks
1	PA2357/*msuE* (*slfA*)	FMN reductase	Involved in riboflavin metabolism and sulfur metabolism pathways
2	PA3442/*ssub1*	Aliphatic sulfonates import ATP-binding protein SsuB 1	Aliphatic sulfonates import ATP-binding protein SsuB 1; part of the ABC transporter complex SsuABC involved in aliphatic sulfonate import. Responsible for energy coupling to the transport system
3	PA0185/*atsB*	Serine-modifying enzyme (24); probable permease of ABC transporter	*atsB* is a member of a *cys* regulon in *P. aeruginosa*, which constitutes a general sulfate ester transport system (25)
4	PA3938/*tauA*	TauA (sulfonate transport system ATP-binding protein)	This probable periplasmic taurine-binding protein precursor is part of *tau* operon involved in sulfur metabolism

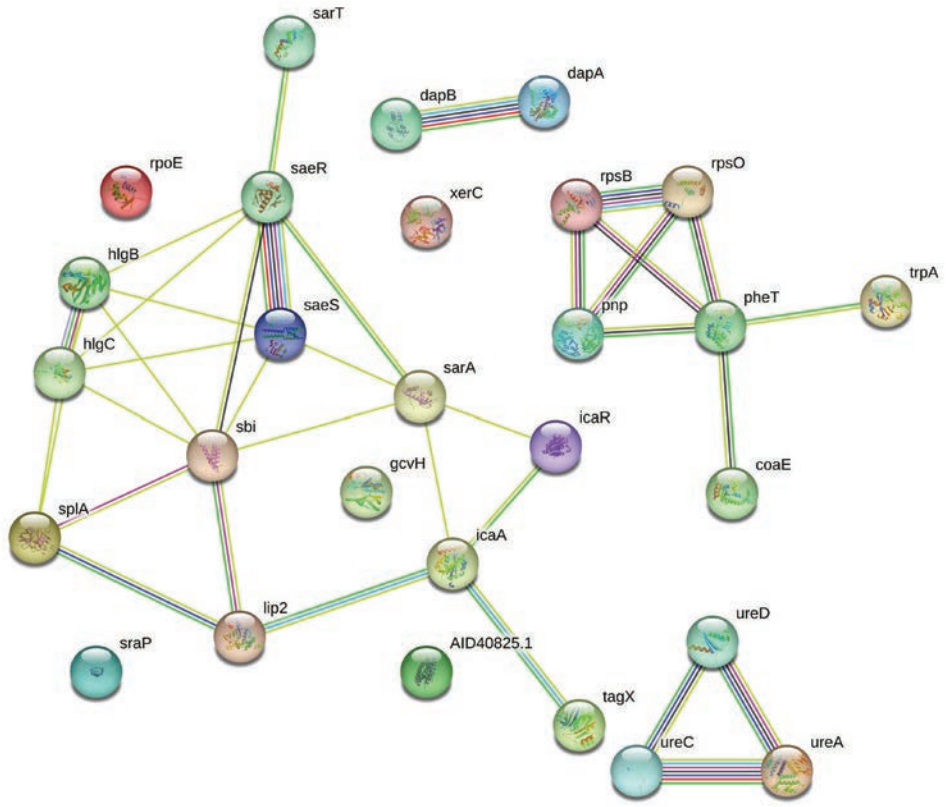
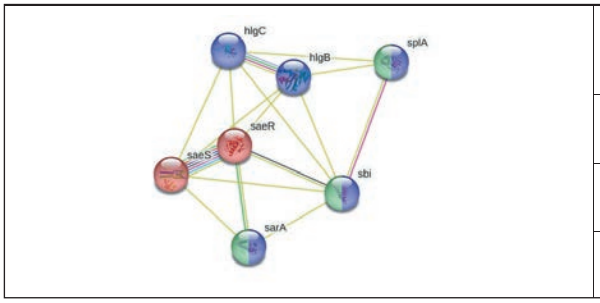


Fig. 7 - Protein-Protein Interaction (PPI) network of upregulated and downregulated genes in Herboheal-exposed *Staphylococcus aureus.*

only 28 of these 113, we went for a combined PPI network (Fig. 7) of all these DEG instead of preparing separate PPI map of upregulated or downregulated genes. The said PPI network had 28 nodes connected through 36 edges with an average node degree of 2.57. Since the number of edges (36) in this PPI network is threefold higher than expected (12) with a PPI enrichment *p* value of 1.02e-08, this network can be said to possess significantly more interactions among the member proteins than what can be expected for a random set of proteins having identical sample size and degree distribution. Such an enrichment is suggestive of the member proteins being at least partially biologically connected.

When we arranged all the 28 nodes in decreasing order of node degree, 23 nodes were found to have a nonzero score, and we selected the top 13 genes with a node degree ≥3 (Tab. S8) for further ranking by different cytoHubba methods. Then we looked for genes which appeared among top-ranked candidates by ≥6 cytoHubba methods. Of such 12 genes, 8 (Tab. S9) which were ranked among top 10 by ≥11 cytoHubba methods were taken for further cluster analysis. Interaction map of these eight important genes (Fig. 8) showed them to be networked with the average node degree score of 4. Number of edges possessed by this network was 16 as against expected 1 for any such random set of proteins. These eight genes were found to be distributed among three different local network clusters. Strength score for each of these clusters was >1.46. While three of the proteins (*sarA, sbi*, and *splA*) were common members of two different clusters, four proteins were part of any one cluster, while *pnp* was not shown to be connected to the remaining seven genes.



Symbolic representation	Cluster	Description	Count in network	Strength of the enrichment effect	False discovery rate
	CL: 4867	Two component regulatory system, and DM13 domain	2 of 7	2.09	0.0188
	CL: 2906	Secreted, and LPXTG cell wall anchor motif	5 of 45	1.68	1.46e-05
	CL: 2908	Secreted, and LPXTG cell wall anchor motif	3 of 39	1.52	0.0116

Fig. 8 - Protein-Protein Interaction (PPI) network of top-ranked genes revealed through cytoHubba among differentially expressed genes (DEG) in Herboheal-exposed *Staphylococcus aureus*.

Table III - Hubs identified as potential targets from among the up- and down-regulated genes in Herboheal-exposed *Staphylococcus aureus*

No.	Gene ID	Gene name	Codes for	Function
1	SAXN108_0683	*sarA*	Transcriptional regulator SarA	Probably activates the development of biofilm by both enhancing the *ica* operon transcription and suppressing the transcription of either a protein involved in the turnover of PIA/PNAG or a repressor of its synthesis, whose expression would be sigma-B-dependent
2	SAXN108_2673	*sbi*	Immunoglobulin-binding protein Sbi	Plays a role in the inhibition of both the innate and adaptive immune responses
3	SAXN108_1846	*splA*	Serine protease SplA	Poorly characterized secreted protein probably involved in virulence
4	SAXN108_0774	*saeR*	Response regulator transcription factor SaeR	The saeR/S system plays a role in regulating such virulence factors which decrease neutrophil hydrogen peroxide and hypochlorous acid production following *S. aureus* phagocytosis
5	SAXN108_0773	*saeS*	Histidine kinase	
6	SAXN108_2677	*hlgB*	Gamma-hemolysin component B precursor	Toxins that seem to act by forming pores in the membrane of the cell; has a hemolytic and a leukotoxic activity
7	SAXN108_2676	*hlgC*	Gamma-hemolysin component C precursor	

PIA = polysaccharide intercellular adhesin; PNAG = poly-*N*-acetyl-β-(1-6)-glucosamine.

Since in case of *S. aureus*, we analyzed up- and downregulated genes together, instead of considering only the multicluster proteins as hubs, we took all of those which appeared to be part of PPI network as shown in Figure 7. Functions of these seven potential hubs are listed in Table III.

Discussion

Panchvalkal-exposed *P. aeruginosa* appears to suffer from sulfur starvation and nitrosative stress. Compromised nitric oxide (NO) detoxification can render bacteria more susceptible to the NO produced by the host immune system (19). Mutant *P. aeruginosa* deficient in NO reductase was shown to register a reduced survival rate in NO-producing macrophages (26). NO has a strategic role in the metabolism of microorganisms in natural environments and also during host-pathogen interactions. NO as a signaling molecule is able to influence group behavior in microorganisms. Downregulation of the denitrification pathway can disturb the homeostasis of the bacterial biofilms. NO levels can also affect motility, attachment, and group behavior in bacteria by affecting various signaling pathways involved in the metabolism of 3′,5′-cyclic diguanylic acid (c-di-GMP). Suppressing bacterial detoxification of NO can be an effective anti-pathogenic strategy, as NO is known to modulate several aspects of bacterial physiology, including protection from oxidative stress and antimicrobials, homeostasis of the bacterial biofilm, etc. (27-29). From this *in silico* exercise, nitric oxide reductase (NOR) has emerged as the most important target of *Panchvalkal* in *P. aeruginosa*. NOR is one of the important detoxifying enzymes of this pathogen, which is crucial to its ability to withstand nitrosative stress, and has also been reported to be important for virulence expression of this pathogen, and thus can be a plausible potential target for novel anti-virulence agents (19). NOR inhibitors can be expected to compromise the pathogen's ability to detoxify nitric oxide (NO), not allowing its virulence traits (e.g., biofilm formation, as NO has been indicated to act as a biofilm-dispersal signal) to be expressed fully. NOR inhibitors can be expected to be effective not only against *P. aeruginosa* but against multiple other pathogens too, as NO is reported to be perceived as a dispersal signal by

various gram-negative and gram-positive bacteria (30). This is to say, NOR inhibitors may be expected to have broad-spectrum activity against multiple pathogens. Major function of NOR is to detoxify NO generated by nitrite reductase (NIR). NO is a toxic byproduct of anaerobic respiration in *P. aeruginosa*. NO-derived nitrosative species can damage DNA and compromise protein function. Intracellular accumulation of NO is likely to be lethal for the pathogen. It can be logically anticipated that *P. aeruginosa*'s ability to detoxify NO will be compromised under the influence of potent NOR inhibitors like *Panchvalkal.* Since NO seems to have a broad-spectrum anti-biofilm effect, NOR activity is essential for effective biofilm formation by the pathogens. NOR activity and NO concentration can modulate cellular levels of c-di-GMP, which is a secondary messenger molecule recognized as a key bacterial regulator of multiple processes such as virulence, differentiation, and biofilm formation (31). In the mammalian pathogens, the host's macrophages are a likely source of NO. NOR expressed by the pathogen provides protection against the host defense mechanism (26). Since NOR activity is known to be important in multiple pathogenic bacteria (e.g., *P. aeruginosa*, *S. aureus*, *Serratia marcescens*) for biofilm formation, virulence expression, combating nitrosative stress, and evading hose defense, NOR seems to be an important target for novel broad-spectrum anti-pathogenic agents. A potential NOR inhibitor besides troubling the pathogen directly may also boost its clearance by the host macrophages (32).

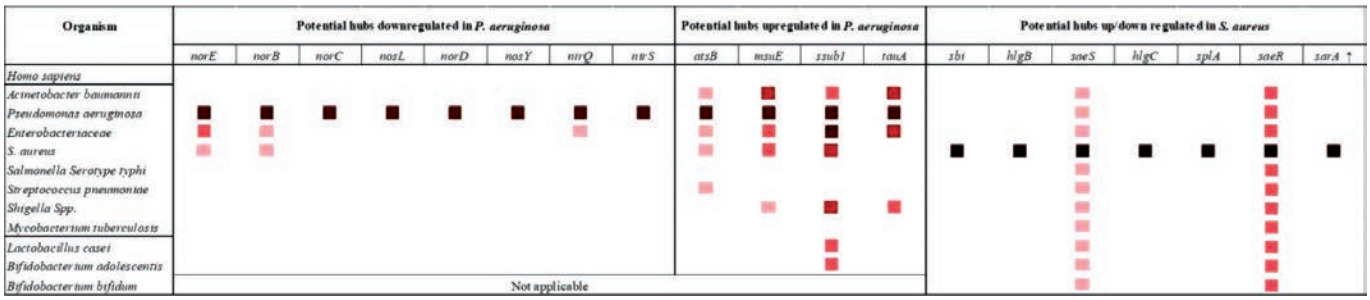
Based on the analysis of differently expressed upregulated genes, sulfur-starved culture of *P. aeruginosa* can be expected to experience compromised virulence. Upregulation of organic sulfur transport and metabolism genes has been reported in *P. aeruginosa* facing sodium hypochlorite-induced oxidative stress (33). Two of the upregulated hubs mentioned in Table II are part of *tau* or *ssu* gene clusters, which are reported in gram-negative bacteria like *Escherichia coli* too for being necessary for the utilization of taurine and alkane sulfonates as sulfur sources. Since these genes are exclusively expressed under conditions of sulfate or cysteine starvation (34), one of the multiple effects exerted by *Panchvalkal* on *P. aeruginosa* can be said to be sulfur starvation. Upregulation of n-alkane sulfonates or taurine (sources of carbon and organic sulfur) utilization genes in *P. aeruginosa* suggests that the sulfur in these compounds was used to counter *Panchvalkal*-induced sulfur starvation, and that the neutrophilic amines and alpha-amino acids formed by catabolization of n-alkane sulfonates may guard the cell against oxidative stress (35). Thus, depriving *P. aeruginosa* of sulfur can be viewed as a potential anti-virulence strategy.

Among the potential targets identified in *S. aureus* in this study, first we discuss two such downregulated genes which are common members of two different clusters. Of them, *splA* is a serine protease, exclusively specific to *S. aureus*, and thought to have a role in the second invasive stage of the infection (36). Another potential hub *sbi* is an IgG-binding protein, which has a role in the inhibition of the innate as well as adaptive immune responses. Its secreted form acts as a potent complement inhibitor of the alternative pathway-mediated lysis. *sbi* helps mediate bacterial evasion of complement via a mechanism called futile fluid-phase consumption (37). Among the remaining potential hubs listed in Table III, *SaeR/S* two-component system is recognized as a major contributor to *S. aureus* pathogenesis and neutrophil evasion. *SaeR/S* also plays a role in regulating such virulence factors which decrease neutrophil hydrogen peroxide and hypochlorous acid production following *S. aureus* phagocytosis (38). *S. aureus* escapes from the antimicrobial protein's neutrophil extracellular traps (NETs), which is dependent on its secreting nuclease (*nuc*), and the latter in turn is regulated by *SaeR/S*. The *SaeR/S* system also modulates neutrophil fate by inhibiting interleukin (IL)-8 production and nuclear factor (NF)-κB activation. *SaeR/S* deletion mutant of *S. aureus* was shown to be inferior than its wild-type counterpart in causing programmed neutrophil death (39). The *SaeR/S* system regulates expression of many important virulence factors in *S. aureus*, and some of them do appear in our list of important targets such as *sbi, hlgB*, and *hlgC*. Thus, inhibiting *SaeR/S* from sensing its environment can be expected to prevent expression of a multitude of *S. aureus* virulence factors in response to host signals. *hlgB* and *hlgC* are hemolytic proteins, and such proteins are used by many pathogens to fulfill their iron requirement as the concentration of free iron in human serum is much lesser than that required by the bacteria (40). Downregulation of bacterial hemolytic machinery may push them toward iron starvation, thus compromising their fitness for in-host survival. This corroborates well with our earlier report (11) describing reduced hemolytic potential of *S. aureus* under the influence of Herboheal. Among all the potential hubs identified in Herboheal-exposed *S. aureus*, only one (*sarA*) was upregulated, and its upregulation seems to be a response from *S. aureus* to compensate the Herboheal-induced downregulation of many important virulence traits. For example, *sarA* regulates expression of *ica* operon, which is required for biofilm formation in *S. aureus*. It can be said that *S. aureus*'s ability to adhere to surfaces and biofilm formation was compromised in the presence of Herboheal as suggested by downregulation of adhesion/biofilm-relevant genes (*SaeR/S* and *sarA*), and as an adaptation to such challenge the pathogen is trying to upregulate *SarA*. This corroborates well with our previous report describing 56% reduced biofilm formation by *S. aureus* in the presence of Herboheal (11).

This study has identified certain potential hubs in *P. aeruginosa* (Tabs. I and II) and *S. aureus* (Tab. III) which should further be investigated for their candidature as potential anti-pathogenic targets. The most suitable targets in bacterial pathogens would be the ones which are absent from their host, as this will allow the criteria of selective toxicity to be satisfied for a newly discovered drug. We did a gene co-occurrence pattern analysis of gene families across genomes (through STRING) with respect to the major hubs identified in each of the pathogens (Tab. IV). Of the 19 hubs identified in either of the pathogen, none was shown to be present in *Homo sapiens*, and hence drugs causing dysregulation of one or more of these genes in pathogens are less likely to be toxic to humans.

If any target gene is present among multiple pathogens, then it can be considered suitable for a broad-spectrum

Table IV - Co-occurrence analysis of genes coding for potential targets in *Pseudomonas aeruginosa* and *Staphylococcus aureus*



The darker the shade of the squares, higher is the homology between the genes being compared.

antibacterial. We analyzed the co-occurrence of identified hubs among some of the important pathogens listed by CDC and WHO. From among those listed in Table IV, *atsB, msuE, ssub1, norE,* and *norB* seemed to be present in multiple gram-negative as well as gram-positive pathogens, and thus suitable to be targeted by a broad-spectrum anti-pathogenic discovery program. On the other hand, *tauA* and *nirQ* seemed to be present only among gram-negative pathogens. They can prove to be important targets in light of the fact that discovery of novel antimicrobials against gram-negative bacteria is relatively more challenging (41).

One of the issues with conventional antibiotics is that they cannot differentiate between the 'good' (symbionts in human microbiome) and 'bad' (pathogens) bacteria, and hence their consumption may lead to gut dysbiosis. An ideal antimicrobial agent should target pathogens exclusively without causing gut dysbiosis. In this respect, a target in pathogenic bacteria absent from symbionts of human microbiome will be the most suitable candidate for antibiotic discovery programs. To gain some insight on this front regarding the targets identified by us, we run a gene co-occurrence analysis with some representative 'good' bacteria reported to be part of healthy human microbiome. *Bifidobacterium* species showed presence of no other target except *SaeR/S. SaeR/S* being widely distributed among bacteria can be considered a valid target; however, an antibacterial agent targeting it may lead to gut dysbiosis too. All downregulated targets in *P. aeruginosa* were absent from the selected symbionts, which further adds value to their potential candidature as anti-virulence targets. However, *atsB* and *ssub1* appeared to be present in *Lactobacillus casei*.

Conclusion

This study has identified certain potential targets in two important pathogens. Such *in silico* studies being predictive in nature, further work is warranted on wet-lab validation of the identified targets. Deletion mutants of the identified hub genes should be assessed for their expected attenuated virulence in appropriate host models. Next-generation pathoblockers targeting any one of these genes may not always be effective as stand-alone therapeutic, and simultaneous targeting of more than one of these genes may be required for an effective therapy. They can also prove to be useful adjuvants to conventional antibiotics allowing use of bactericidal antibiotics at lower concentrations.

Besides indicating generation of nitrosative stress, inducing sulfur starvation, and disturbing regulation of bacterial virulence as potentially effective anti-pathogenic strategies, this study also demonstrates the relevance of the polyherbalism concept of the Traditional Medicine systems, and utility of the network analysis approach in elucidating the multiple modes of anti-pathogenic action exerted by the multicomponent natural extracts.

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Abbreviations

AMR = antimicrobial resistance; DEG = differentially expressed genes; NO = nitric oxide; NOR = nitric oxide reductase; PPI = protein-protein interaction

Disclosures

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Author's contribution: Conceptualization: VK; Data Curation: FR, SS, JP, NT, GG; Formal Analysis: FR, SS, JP, NT, GG, VK; Funding Acquisition: VK; Investigation: FR, SS, JP, NT, GG; Methodology: VK, GG, NT; Project Administration: VK; Resources: VK; Supervision: VK; Writing – Original Draft: VK, FR; Writing – Review & Editing: VK, GG, NT.

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REVIEW

Licorice as a herbal extract in periodontal therapy

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ABSTRACT

Periodontal disease is caused by specific pathogens which results in inflammation of the tooth-supporting structures and subsequently causes the continued breakdown of alveolar bone and periodontal ligament. Licorice (*Glycyrrhiza glabra*) is a perennial herb with substantial medicinal value. Licorice extract is derived from dried, unpeeled stolons and roots of *Glycyrrhiza uralensis* and *G. glabra*. The bioactive ingredients in licorice extract such as glycyrrhizin, licoricidin, glabridin, licochalcone A, and licorisoflavan A have anti-inflammatory, antimicrobial, and anti-adherence effects that are beneficial against periodontal disease. Since periodontal disease has a complex etiology that includes the host response and microorganisms, licorice phytochemicals offer a therapeutic advantage due to their dual functionality. The aim of this review was to enumerate the bioactive compounds present in herbal licorice extract and to elucidate the beneficial effects of licorice and its derivatives in periodontal therapy. Literature review and clinical trials evaluating the effect of licorice on periodontopathogens and periodontal disease are included in this article.

Keywords: Gingivitis, *Glycyrrhiza glabra*, Herbal therapy, Periodontitis

Introduction

Periodontal disease is caused by specific pathogens which results in inflammation of the tooth-supporting structures and subsequently causes the continued breakdown of alveolar bone and periodontal ligament. The pathogenesis of periodontal diseases involves two major causative factors. The first is the microbial factor, that is, the presence of increased levels of periodontopathogenic bacteria in subgingival tissues, which causes periodontal destruction by producing proteinases and toxins (1,2). The pathogens associated with periodontitis are *Porphyromonas gingivalis, Treponema denticola*, *Aggregatibacter actinomycetemcomitans,* and *Tannerella forsythia*. The other factor is the immune response of the host to the periodontal pathogens, which is the over-production of mediators of inflammation such as cytokines, prostaglandins, and matrix metalloproteinases (MMPs), which regulate the continuance of periodontal disease (3).

Periodontal therapy primarily includes scaling and root planing to eliminate the local factors, that is, plaque and calculus, and to maintain satisfactory oral hygiene. Since periodontal disease is known to be an inflammatory condition with a microbial etiology, the adjunctive use of locally applied or systemic administration of antimicrobials and/or host response-modulating medications has been suggested.

Conventional synthetic agents such as chlorhexidine products which are used therapeutically and prophylactically in dentistry have some disadvantages such as altered taste sensation, tooth staining, and resistance to bacteria, which limit their usage over a long term (4). Therefore, innovative strategies need to be developed against periodontal diseases, such as exploring the extensively available medicinal plants. The active ingredients in medicinal plants restore health, with maximum efficiency and minimal side effects. Herbal extracts incorporated into medications have been found to be safe and efficacious for the treatment of several oral health conditions such as gingival bleeding, dental caries, halitosis, and mouth ulcers. The extracts obtained from aloe vera, green tea plant, neem, tulsi, propolis, rosemary, meswak, turmeric, chamomile, tea tree oil, peppermint oil, cranberry, clove, ginger, etc. have been used commonly for

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the prevention and treatment of different oral diseases. Herbal extracts contain phytochemicals that are responsible for the desired anti-inflammatory and antimicrobial effects (5). Herbal formulations are gaining widespread attention as they do not contain artificial preservatives, alcohol, colors, or flavors, which are commonly found in other drugstore products.

One such herb with medicinal properties is licorice (*Glycyrrhiza glabra*). Licorice, synonym being sweet wood, is found in the Mediterranean region and in a few regions of Asia. Licorice is a perennial herb that holds a sweet taste and has widespread pharmacological effects on human beings. Licorice extract is derived from the dried, unpeeled stolons and roots of *G lycyrrhiza uralensis* and *G. glabra*.

However, limited information is available on the use of this herb in periodontal therapy. The mechanism of action by which licorice works against periodontal diseases has not been elucidated in previous studies. The aim of this review was to enumerate the bioactive compounds present in herbal licorice extract and to elucidate the beneficial effects of licorice and its derivatives in periodontal therapy.

Methodology

The literature search was performed using three databases which included PubMed, Google Scholar, and Cochrane, in addition to searching reference lists of original and review articles. The combination of the following keywords was used to search for relevant articles: "licorice," "Glycyrrhiza glabra," "periodontal therapy," and "periodontal disease." Relevant studies published between 1985 and 2022 were selected. Only articles in English language were considered, and unpublished data were not sought. Two reviewers obtained information on the quality and characteristics of the included studies.

Components in licorice extract

Licorice is a potential source of natural anti-inflammatory agents. Its major active component is glycyrrhetinic acid (GA) that is derived from licorice root extract. Major phytochemicals found in licorice are shown in Table I (Fig. 1) (6).

Safe usage of licorice

The Food and Drug Administration (FDA) has labeled licorice as "Generally Recognized as Safe." It has been suggested to be safe when used in minimal quantities by people who are not allergic to glycyrrhizin (7,8). Intake of excessive quantity, that is, over 200 mg, of licorice may cause hypertension, hypokalemia, rhabdomyolysis, respiratory impairment, muscle paralysis, hyperparathyroidism, acute renal failure, and encephalopathy (9). According to the World Health Organization (WHO), 100 mg/day of licorice can be used safely without adverse effects. A potential risk of excessive bleeding may be seen in patients using medications for anti-clotting for cerebrovascular or cardiovascular diseases in conjunction with licorice-containing herbal medications due to its antiplatelet and anticoagulant effects (10).

TABLE I - Important phytochemicals in licorice root

Group	Bioactive compounds
Aurones	Licoagroaurone
Benzofurans	Licocoumarone
Chalcones	Isoliquiritigenin, licochalcone A
Coumarins	Glycerol, glabrocoumarone, glycocoumarin, licofuranocoumarin, glabrocoumarin
Flavonoids	Glabrol, liquiritigenin
Isoflavonoids	Glabridin, glabrone, licoricidin, licoisoflavones A and B, licorisoflavan A
Pterocarpenes	Glycyrrhizol A
Saponins	Glycyrrhizin, glycyrrhizic acid, 18β-glycyrrhetinic acid, liquiritic acid, glabrolide
Stilbenes	Gancaonin G

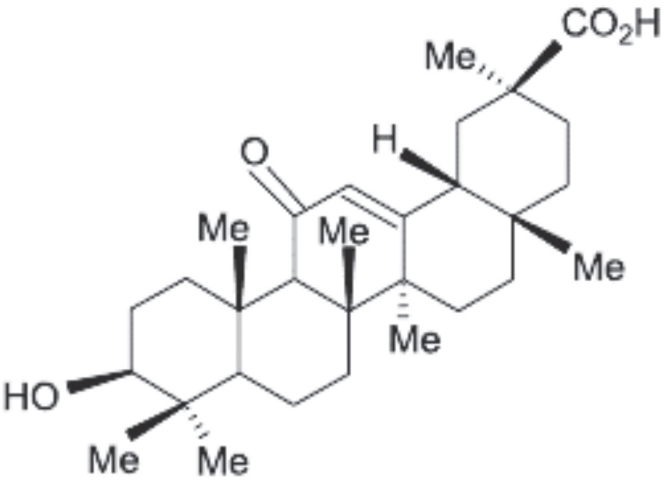

Fig. 1 - Chemical structure of glycyrrhetinic acid.

Effects of licorice

Licorice constituents have shown antimicrobial (11), antiviral (12), anti-inflammatory (13), antidiabetic, antitumor, immunoregulatory (14), sedative (15), antidepressive (16), estrogenic (17,18), antioxidant (19), hepatoprotective (20), neuroprotective activities (21), and skin effects (22). The active constituents of licorice extract have a potential role on the oral microorganisms as well as the host response involved in orodental diseases like periodontitis, dental caries, recurrent aphthous ulcers, and candidiasis.

Antiviral effects – Licorice extracts inhibit the growth of viruses such as herpes simplex, influenza virus, and vesicular stomatitis virus. Glycyrrhizin prevents the replication of viruses and interferes with viral binding (12).

Antidiabetic activity – Glycycoumarin, glycerin, etc., present in *G. glabra* extracts lower blood glucose level by binding to peroxisome proliferator-activated receptor (PPAR) gamma. Glabridin helps in efficient glucose utilization and prevents glucose intolerance by translocation of GLUT-4 (14).

Antitumor activity – 18-β-GA and glycyrrhizic acids induce mitochondrial permeability transition causing tumor cell apoptosis (14).

Immunoregulatory effect – Glycyrrhiza extracts stimulate the immune system by production of macrophages and lymphocytes, and increasing the phagocytic capacity of neutrophils. It prevented the accumulation of immune complexes involved in autoimmune diseases such as systemic lupus erythematosus (14).

Sedative effect – Glabridin shows sedative and hypotonic effects by positively modulating the gamma-aminobutyric acid (GABA) receptors (15).

Antidepressive effect – Licorice shows antidepressive effects by inhibition of monoamine oxidase and increasing epinephrine and dopamine levels in the brain (16).

Estrogenic effect – Licorice extracts show estrogenic activity through uterine retention and vaginal opening. Isoflavones present in licorice can influence sexual development, impair estrus cycling, and alter the proper functioning of the ovarian, hypothalamus, and pituitary glands. Glabridin can be used as a treatment for menopausal symptoms (17,18).

Hepatoprotective activities – Glycyrrhizin has shown improved liver histology and reduced serum aminotransferases. It shows hepatoprotective effect against CCl4-induced oxidative stress, prevents oxidative and hepatic damage caused due to aflatoxin, and improves liver function (20).

Neuroprotective activities – Licorice has an antioxidant activity that can reduce brain damage by eliminating or utilizing the free radicals and improving neural function and memory (21).

Skin effects – Licorice is popular in treating dermatitis, pruritus, cysts, and eczema. It is also used for cosmetic formulation as a depigmenting agent to inhibit the tyrosinase enzyme (22).

Mechanism of action

The beneficial effects of licorice can be due to various mechanisms.

Antimicrobial activity

Microbial growth is selectively inhibited by the isoprenoid phenols present in *G. glabra*. The presence of secondary metabolites, such as alkaloids, saponins, flavonoids, and alkaloids, is responsible for the antibacterial activity (23,24). The reduction in bacterial gene expression, decrease in growth of bacteria, and inhibition of production of bacterial toxins are suggested as the mechanism behind this (24,25).

Licorice extract showed antimicrobial effects against *P. gingivalis* with minimum inhibitory concentration (MBC) and minimum bactericidal concentration (MIC) of 25 and 62.5 µg/mL, respectively (26). Glycyrrhizol A showed a strong antibacterial effect against *Streptococcus mutans* with MIC of 1 µg/mL. GA at an appropriate concentration has good efficacy against isolated periodontopathogenic and capnophilic bacteria (27). The MICs of GA were 8, 16, and 8 mg/L for *A. actinomycetemcomitans*, *Eikenella corrodens*, and *Capnocytophaga*, respectively, and the MBC was 16 mg/L for all species (28).

Antioxidant activity

Licorice phytochemicals exhibit significant antioxidant activity. Licorice hinders the synthesis of reactive oxygen species (ROS) by neutrophils at the site of inflammation. *G. glabra* contains licochalcones B and D, which show powerful scavenging activity on DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) radical and also have the ability to prevent the peroxidation of microsomal lipids (29,30).

Anti-inflammatory activity

GA activates signaling of glucocorticoid receptors since its chemical structure resembles the glucocorticoids, and it also inhibits the classical complement pathway, both of which are responsible for its anti-inflammatory properties.

Preclinical studies have also shown that licorice inhibits synthesis of prostaglandins and cyclooxygenase activity, and also indirectly inhibits aggregation of platelets as well as the components of the inflammatory cascade (31). Licorice extract prevents the phosphorylation of proteins involved in intracellular signaling of macrophages, such as the transcription factors, nuclear factor-kappa B, and activator protein (AP) 1, which play an important role in the pathways of inflammatory signaling (32).

Licorice in periodontal disease

The bioactive ingredients in licorice like glycyrrhizin, glabridin, licorisoflavan A, licochalcone A, and licoricidin are effective against periodontal disease. Table II summarizes the studies which suggest the potential use of licorice in periodontal therapy.

These ingredients exhibit antimicrobial, anti-inflammatory, and anti-adherence effects (Fig. 2). Since the etiology of periodontal diseases is complex involving the periodontal pathogens and host immune response, dual functionality compounds such as the phytochemicals in licorice offer therapeutic superiority. Phytochemicals are structurally distinct from the conventional microbial-derived antibiotics, and so are advantageous as antimicrobials. The phytochemicals act against different bacterial strains by inhibiting the efflux pumps, inhibiting the cell wall biosynthesis by interacting with the cell membrane, and by inhibition of enzymes such as dihydrofolate reductase, urease, and sortase A (51). Thus, their mechanisms of action are different from classic substances and against which microbial resistance does not develop.

The ability of licorice to inhibit the formation of dental plaque enhances its significance in the treatment of periodontal disease. At high concentrations of licorice extract (5%-10%), there was a slight reduction in bacterial growth and formation of plaque was completely inhibited. No effect was seen on either adherence or growth with lower concentrations. At high concentrations (0.5%-1%) of its pure active component, that is, glycyrrhizin, there was a complete inhibition in the adherence, whereas partial inhibition was observed at lower concentrations. The surface activity of glycyrrhizin could be responsible for its inhibitory effect, as

TABLE II - List of studies suggesting the potential use of licorice in periodontal therapy

Authors	Study design	Findings
Sharma et al 2022 (33)	Randomized controlled trial	Both licorice and chlorhexidine mouthwash inhibited the accumulation of plaque and inflammation of the gingiva. The herbal mouthwash was shown to be effective as a self-care treatment since chemical formulations are associated with adverse effects with long-term usage.
Madan et al 2019 (34)	Randomized clinical trial	Bleeding of the gingiva, probing pocket depth, and attachment loss were significantly decreased in patients using *Glycyrrhiza glabra* gum paint in 10% concentration. It can be used for longer periods to prevent and treat periodontal disease as they do not have any side effects, and thus, it is also effective as an alternative for synthetic agents.
Takamori et al 2018 (35)	Animal study	Loss of attachment, immune complex formation, and inflammatory cell infiltration were greater in the lipopolysaccharide (LPS) group than in the control, and were completely reduced in the glycyrrhetinic acid (GA) groups. Increased alveolar bone destruction was seen in the LPS group than in the GA or control groups. Hence, in the experimentally induced periodontitis model in rats, GA had the ability to reduce periodontal destruction.
Suwannakul and Chaibenjawong 2017 (26)	In vitro study	Licorice extract showed antimicrobial effects against *Porphyromonas gingivalis* with MBC and MIC of 25 and 62.5 µg/mL respectively. It also reduced the quantity of biofilm and the activities of Arg- and Kgp-proteases.
Salehi et al 2017 (36)	Double-blind clinical trial	Mucoadhesive tablets containing licorice extract can relieve pain, decrease the diameter of the ulcer and the inflammation around it, and improve the recovery in aphthous stomatitis.
Jain et al 2017 (37)	Randomized clinical trial	Licorice mouthwash reduced the accumulation of plaque and inflammation of gingiva, without any tooth discoloration or unpleasant taste sensation.
Shivprasad et al 2017 (38)	Randomized controlled trial	Subgingivally delivered licorice as an adjunctive treatment modality to scaling and root planing showed clinical and microbiological benefits in periodontal therapy, with a reduction in the prevalence of *P. gingivalis.*
Ali and Mohammed 2016 (39)	Comparative human study	Licorice extract based mouthwash inhibits plaque formation and inflammation of gingiva without any adverse effects. Therefore, it can be used as an adjunctive to scaling and root planing in periodontal treatment.
Hamdon et al 2014 (40)	Human study	Licorice extract showed antibacterial effects against *Aggregatibacter actinomycetemcomitans,* based on antimicrobial sensitivity tests. The antibacterial effect was greater against planktonic cells as compared to the cells within the biofilm. It produced an inhibition zone similar to tetracyclines with a concentration of 250 µg.
Kim et al 2013 (41)	Invitro study	18α-GA is effective in the treatment of vascular diseases caused by *P. gingivalis*. It reduces vascular permeability induced by LPS by inhibiting IL-8 production from the endothelium.
Farhad et al 2013 (42)	Experimental human study	A significant reduction of MMP-8 concentration was seen in both licorice and doxycycline groups than in the placebo group. The licorice group showed better reduction of MMP-8 concentration than doxycycline group, which was not statistically significant. Hence, licorice extract can be as potent as antibiotics such as doxycycline to treat periodontal diseases by preventing the MMP production by host cells.
Kim et al 2012 (43)	In vitro study	Glabridin inhibits activation of signaling molecules induced by RANKL and other transcription factors of osteoclast precursors, and so it can be used to inhibit osteoclastogenesis.
Zhu et al 2012 (44)	Animal study	Isoliquiritigenin (ISL) inhibits osteoclastogenesis induced by RANKL and bone loss by various signaling pathways. Hence, it has the potential to be used as a therapeutic or preventive agent for the treatment of lytic bone diseases.
Feldman et al 2012 (45)	In vitro study	Licochalcone A inhibits the two primary causative factors of periodontitis, i.e., formation of biofilm with *P. gingivalis* and the immune response of the host.
La et al 2011 (46)	In vitro study	Licoricidin (LC) and licorisoflavan A (LIA) inhibited the production of IL-6, MMP-7, -8, and -9 in macrophages. They can be used to treat MMP and cytokine-mediated conditions such as periodontal disease, and are potent host-modulating agents.
Bodet et al 2008 (32)	In vitro study	Licorice extract showed anti-inflammatory effects by reducing the IL-1b, -6, -8 and TNF-α responses of macrophages induced by LPS. It is a potential therapeutic agent to prevent or treat the tissue destruction caused due to periodontal disease.
Wittschier et al 2006 (47)	In vitro study	The polysaccharides in *G. glabra* inhibit bacterial adhesion and thus can be potential therapeutic agents against bacterial infection.
He et al 2006 (27)	In vitro study	Glycyrrhizol B and gancaonin G showed moderate antibacterial effect against *Streptococcus mutans,* while Glycyrrhizol A showed strong antibacterial effect with MIC of 1 µg/mL. Hence, the roots of *Glycyrrhiza uralensis* contain isoflavones which exhibit antibacterial effects.

Authors	Study design	Findings
Choi 2005 (48)	Animal study	Glabridin, an estrogenic plant product, stimulates the in vitro formation of bone in cultured osteoblasts. Thus, glabridin can be a potent agent in the management of osteoporosis.
Salari et al 2003 (49)	Human study	Enoxolone with the mentioned concentrations is effective against isolated periodontopathogenic and capnophilic bacteria. Its MICs were 8 µg/mL for *A. actinomycetemcomitans and Capnocytophaga* species, and 16 µg/mL for *Eikenella corrodens*. The MBC was also 16 µg/mL for all the microorganisms.
Salari and Kadkhoda 2003 (28)	In vitro study	GA at an appropriate concentration has good efficacy against isolated periodontopathogenic and capnophilic bacteria. The MICs of GA were 8, 16, and 8 mg/L for *A. actinomycetemcomitans, Eikenella corrodens* and *Capnocytophaga*, respectively, and the MBC was 16 mg/L for all species.
Saeedi et al 2003 (50)	Randomized, controlled trial	Edema, erythema, and itching were more effectively reduced with 2% licorice topical gel than with 1% gel in 2 weeks.

IL = interleukin; MBC = minimum inhibitory concentration; MIC = minimum bactericidal concentration; MMP = matrix metalloproteinase; TNF = tumor necrosis factor.

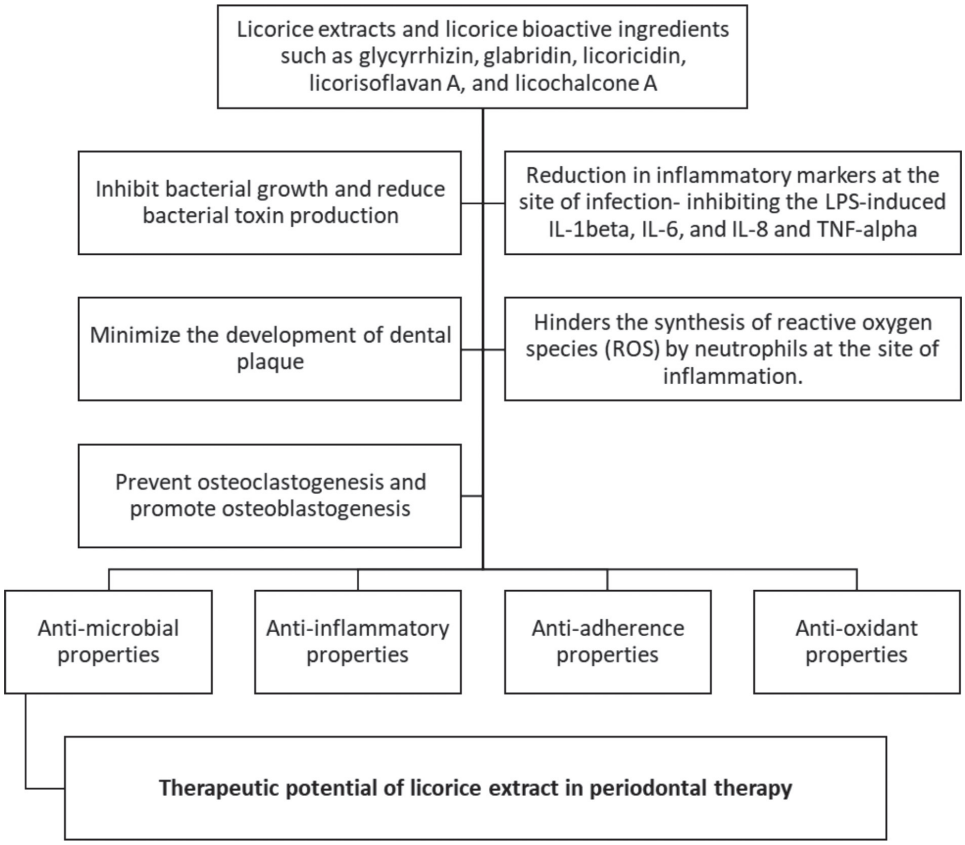

Fig. 2 - Mechanism of action of licorice in periodontal therapy.

bacterial adherence and growth are known to be affected by surfactants. Bacterial adherence can also be inhibited by the adsorption of glycyrrhizin onto smooth surfaces. Licorice extract shows minimal antibacterial activity along with its effect on inhibition of plaque. Glycyrrhizin as a vehicle can be effective for topical agents used orally due to its sweetness, good dispersing properties, and the ability to remain stable in the form of aqueous gels. This suggests that the balance of the oral microbial flora will not be affected on using glycyrrhizin as an oral medication (52).

Phytochemicals of *G. glabra* reduce bacterial growth and inhibit the mediators of inflammation at the infection site. It also inhibits the activity of osteoclasts responsible for destruction of alveolar bone in periodontal disease and promotes the formation of bone by stimulating osteoblastogenesis. High amounts of inflammatory markers like interleukin (IL)-1β, IL-2, IL-6, IL-8, tumor necrosis factor (TNF)-α, and RANKL are present in patients with periodontal disease. Licorice extract showed potent anti-inflammatory properties by inhibiting these proinflammatory mediators stimulated by lipopolysaccharide (LPS) from *A. actinomycetemcomitans* and *P. gingivalis* (32).

Resorption of alveolar bone is an important feature of periodontitis. The differentiation, activation, and survival of

osteoclasts are regulated by RANKL leading to bone resorption. Glabridin can be used to inhibit osteoclastogenesis by preventing the activation of signaling molecules induced by RANKL and subsequent transcription factors for osteoclast precursors, suggesting its therapeutic potential (34).

Recommendations for future research

Though the use of herbals for medicinal purpose is traced back to several centuries, it is only in recent evidence-based era that methodical and systematic approaches to study their properties have been reinstituted. This has sparked a wide interest for their application in all healthcare specialties including periodontal therapy. The beneficial phytochemicals in licorice must be studied so as to incorporate these herbal extracts in oral care products that may be useful in dental therapy.

In vitro studies have shown the capability of licorice and its bioactive components in periodontal therapy; however, most of the clinical studies have limitations pertaining to the design of the study and the number of participants included in the study, which makes them statistically insignificant. Thus, further clinical studies need to be carried out to investigate the oral care products containing licorice extracts, in the forms of toothpaste, mouthwash chewing gum, and gel to be able to validate its beneficial effects. The local application of these bioactive compounds would be more suitable. For example, the local application of a licorice-based gel into sites with periodontal disease permits the bioactive ingredients to be released slowly, which will act locally on the periodontal pathogens and the host immune response, the two contributory factors in the destruction of periodontal tissues.

Further research focusing on the in vivo anti-inflammatory/antimicrobial effects of licorice is required on larger sample sizes to better understand its specific role in the management of periodontitis. Clinical trials evaluating the effect of licorice extract on periodontopathogens and inflammatory cytokines would be recommended.

Conclusion

The usage of herbal agents for the treatment of periodontal disease is considered as an intriguing alternative to conventional antibiotics due to their lesser negative effects and to overcome drug resistance during treatment. Licorice extracts exhibit a wide range of biological effects such as anti-inflammatory, antioxidant, and antimicrobial activities. It has the ability to prevent the release of proinflammatory mediators and MMPs from host cells, and hence it is a potent agent for periodontal therapy. The bioactive ingredients present in this herb help in reducing loss of alveolar bone, which is commonly associated with periodontal disease. It should also be emphasized that no adverse effects have been seen with the use of licorice extracts. Hence, local application of licorice-containing agents into the diseased periodontal sites can be beneficial, which would act locally on periodontopathogens and the host inflammatory response.

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REVIEW

Efficacy of LAMP assay for Mycobacterial spp. detection to prevent treatment delays and onset of drug resistance: a systematic review and meta-analysis

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ABSTRACT

Background: Tuberculosis (TB) *re*mains a deadly disease affecting one-third population globally. Long turnaround time and poor sensitivity of the conventional diagnostics are the major impediments for faster diagnosis of *Mycobacterial spp* to prevent drug resistance. To overcome these issues, molecular diagnostics have been developed. They offer enhanced sensitivity but require sophisticated infrastructure, skilled manpower and remain expensive.
Methods: In that context, loop-mediated isothermal amplification (LAMP) assay, recommended by the WHO in 2016 for TB diagnosis, sounds as a promising alternative that facilitates visual read outs. Therefore, the aim of the present study is to conduct a meta-analysis to assess the diagnostic efficiency of LAMP for the detection of a panel of *Mycobacterium spp*. following PRISMA guidelines using scientific databases. From 1600 studies reported on the diagnosis of *Mycobacterium spp*., a selection of 30 articles were identified as eligible to meet the criteria of LAMP based diagnosis.
Results: It was found that most of the studies were conducted in high disease burden nations such as India, Thailand, and Japan with sputum as the most common specimen to be used for LAMP assay. Furthermore, *IS6110* gene and fluorescence-based detections ranked as the most used target and method respectively. The accuracy and precision rates mostly varied between 79.2% to 99.3% and 73.9% to 100%, respectively. Lastly, a quality assessment based on QUADAS-2 of bias and applicability was conducted.
Conclusion: LAMP technology could be considered as a feasible alternative to current diagnostics considering high burden for rapid testing in low resource regions.

Keywords: Diagnosis, LAMP, Meta-analysis, Mycobacteria, Therapeutics, Tuberculosis

Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB) remains a deadly disease affecting millions of people worldwide. It is estimated to affect approximately one-third of the global population and is becoming one of the most fatal infectious diseases. MTB usually attacks the lungs, but TB bacteria can infect any part of the body such as kidney, spine, or brain (1). Worldwide, TB is the 13th leading cause of death and the second raging infectious killer after human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) (2). In 2020, an estimated 10 million people got ill with TB worldwide, the infection being divided as 5.6 million men, 3.3 million women, and 1.1 million children. TB affects most of the countries among all age groups and can be fatal if not treated properly. Moreover, the emergence of drug-resistant strains has further complicated the problem and has become a rising obstacle against efficient therapeutics (3). Therapeutics are available but the effective control of the disease is impeded due to the lack of rapid and accurate diagnostics. Under such significant circumstances, there is an urgent need for rapid, accurate, and cost-effective diagnostic test for TB to identify new cases and reduce the time-to-treatment and prevent its further transmission.

The current available methods are primarily based on smear microscopy (acid-fast staining), culture, and nucleic

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acid amplification. Although methods based on acid-fast staining are sensitive, they pose problems in low-resource places and are time-consuming (4). The solid culture method requires around 4-8 weeks, while liquid-based culture methods also require around 10-14 days (4). Nucleic acid amplification techniques are based on polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP). Although hemi-nested PCR based on GeneXpert for MTB detection is rapid, sensitive, and specific, it also poses challenges of high cost and high end equipment dependency, which limits its implementation in low-resource regions (5). LAMP is an isothermal DNA amplification method that relies on four or six pairs of primers to amplify minute quantities of DNA within a shorter period with simple operation, making it more suitable for low-resource regions (6). Thus, research in TB diagnostics aims to find an efficient, reproducible, cost-effective tool with minimal infrastructure requirements. LAMP is a popularly adopted new age technology for rapid nucleic acid amplification which is widely used for pathogen (virus, bacteria, and malaria) detection including severe acute respiratory syndrome coronavirus 2 (SARS CoV-2) (7-9). LAMP-based detection methods have been proved to be more sensitive than GeneXpert assay. In fact, the World Health Organization (WHO) has endorsed LAMP for TB as a replacement for smear microscopy for peripheral settings (10).

In pursuit of developing better diagnostics, which are crucial for achieving global elimination of TB, we performed a systematic review and meta-analysis to access the diagnostic accuracy of LAMP to detect mycobacteria. Even if couple of studies have depicted the efficacy of LAMP during the last decade, an updated version is missing. Moreover, most of these studies were specific to either pulmonary or extrapulmonary TB. Therefore, the present study not only offers an up-to-date diagnostic performance of LAMP for TB detection but also covers other *Mycobacterium* spp. The pooled sensitivity and specificity of LAMP were analyzed against different references. Further, diagnostic efficiency was determined based on reference methods, target genes, and detection methods of LAMP. Taken together, we aimed to evaluate the diagnostic potency of LAMP as a tool for detection of mycobacteria to address the current TB diagnosis burden in low-resource places.

Methods

The Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA) guidelines (11) were followed for identification of eligible studies in the present systematic review and meta-analysis.

Search strategy

Diverse scientific databases, for example, PubMed, Google Scholar, Science Direct, Scopus, BioRxiv, and MedRxiv, were searched to screen for studies performed using LAMP for TB diagnostics from the year 2000 till March 2022. The terms such as LAMP, Tuberculosis, *Mycobacterium* and mycobacteria were used in various combinations during our research without any limitations: "LAMP + Tuberculosis" or "LAMP + *Mycobacterium*" or "LAMP + mycobacteria" or "LAMP + TB" or "LAMP + Tuberculosis + *Mycobacterium*" or "LAMP + Tuberculosis + mycobacteria" for PubMed, Science Direct, and Google Scholar without using any language restriction. The retrieved results were screened for duplication and conformity with the prespecified eligibility criteria.

Study eligibility criteria

Inclusion criteria

This systematic review and meta-analysis included: (1) both peer-reviewed and preprint original articles on LAMP technology used for detection of any mycobacterial species such as MTB, *M. bovis*, and *M. africanum*; (2) only full-text articles written in English language; and (3) articles that contain data on true-positive (TP), false-positive (FP), false-negative (FN), and true-negative (TN) values for the assay or have sufficient data so that the number of TP, FP, FN, and TN (performed on clinical samples) could be determined.

Exclusion criteria

Exclusion was made for: (1) studies based on non-isothermal amplification; (2) studies where data are irretrievable; (3) review articles, editorials, commentaries, proceedings, etc.; (4) foreign language articles (other than English) based on LAMP-mediated detection of mycobacteria.

Data extraction

Potential articles after reviewing titles and abstracts followed by full text for inclusion were extracted by two authors (G.S.B. and Z.H.). Consultation from two independent authors (S.J. and S.H.) was made to eliminate the doubt about any discrepancy. The extracted information from included studies had authors, year of publication, location of study, sample size, types of specimens, target genes, detection method, and standard reference method. The data extracted for evaluation of diagnostic accuracy for LAMP were performed by using either respiratory or non-respiratory specimens with any of the reference methods such as smear microscopy, culture, and GeneXpert. The important parameters in this meta-analysis such as TP, TN, FP, and FN of all studies were either extracted or calculated to provide their sensitivity and specificity values. The included studies (n = 30) were then assessed for their methodological quality to reduce systematic biases and inferential errors from the collected data.

Statistical analysis

The quantitative analysis of the included studies (n = 30) from the data extracted such as the values of TP, FP, TN, FN and sample size was performed. Furthermore, the values of sensitivity and sensitivity were mined or calculated from the available data. Moreover, pooled sensitivity and specificity of LAMP associated with 95% confidence interval (CI) were estimated. To maintain the accuracy and precision, the formulas: Accuracy = [TP + TN/TP + TN + FP + FN]* 100 and Precision = [TP/TP+FP]* 100 (12,13) were used. Accuracy and precision are important characteristics of any measurement.

Accuracy is the degree of closeness of measured value to a standard value. However, precision provides the information regarding the closeness of multiple measured values to each other. Accuracy and precision are independent of each other. Forest plot for sensitivity and specificity were plotted using R-software along with summary receiver operating characteristic (SROC) for the given study.

Quality assessment

Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) tool was used to assess the methodological quality of the eligible studies. The risk of bias in the included studies (n = 30) was assessed from four areas of bias, for example, patient selection, index test, reference standards, and flow timing (14,15). For each QUADAS-2 domain specific yes/no questions were tailored. Following these criteria, the eligible studies were then refereed for low, unclear, or high risks of bias. Furthermore, we also judged to generate low, unclear, or high-risk applicability.

Results

Literature survey

We followed the PRISMA guidelines (11) to search the literature for the present study (Fig. 1). The major scientific databases viz. PubMed, Science Direct, Scopus, BioRxiv, and MedRxiv have been extensively searched applying the above inclusion criteria and around 1,600 articles were extracted. From the 1,600 articles, we included the ones that were published after the year 2000 since the inception of LAMP technology (6) and thus excluded 22 articles. Further, only articles written in English language were considered and thus excluded 44 articles. Reading the titles and abstracts of these studies allowed to exclude further 1,029 articles comprising the review articles, editorials, proceedings etc. Following this exclusion, we removed the duplicated articles and further excluded 390 articles. Additional 73 articles were irrelevant as they didn't use LAMP technology for the diagnosis of any mycobacterial species and were excluded, leaving a panel composed of 42 eligible studies. Lastly, from the 42 included articles, further 12 articles were also eliminated because their TP, FP, TN, and TN values were either not specified in these articles or the sensitivity and specificity values could not be calculated. Altogether, we observed that only 30 articles were eligible for detailed meta-analysis (Fig. 1) considering all the exclusion criteria.

Study characteristics and meta-analysis

Table I shows the data extracted from the eligible studies mentioning the details of authors, year of publication, country of study, types of specimens, target genes, detection method, and reference methods. Figure 2 shows the country-wise distribution of 30 identified articles included in the present study. Most of the studies (43.3%; n = 13) were conducted in the high TB burden nations such as India followed

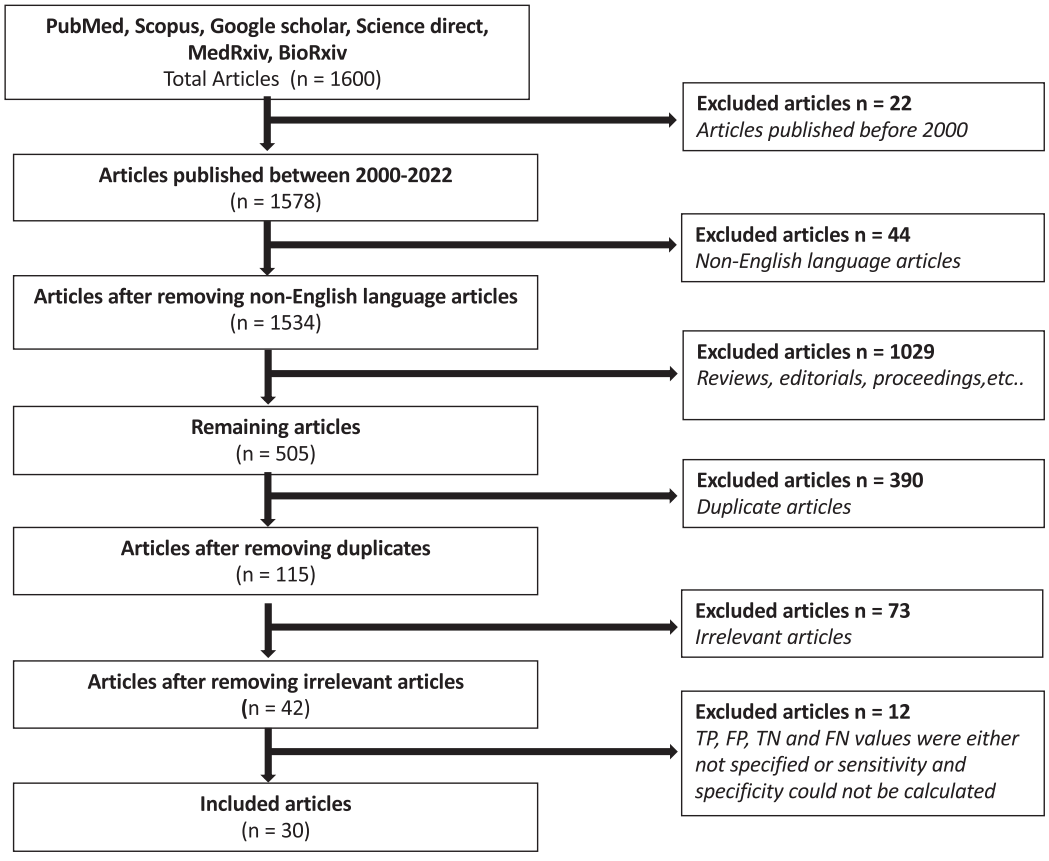


Fig. 1 - Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA) flowchart depicts search of the literature and screening strategy for meta-analysis.

Table I - Characteristics and outcomes of the included studies (n = 30)

S. No.	Author	Journal	Country	Reference Method	Specimen	Target Gene	Detection Method	TP	TN	FP	FN	Size	Sensitivity	Specificity
1	Boehme et al (2007)	J Clin Microbiol	Switzerland	Culture, smear microscopy	Sputum	gyrB	Fluorescence, turbidity	173	500	4	5	682	97.70%	99%
2	Pandey et al (2008)	J Med Microbiol	Japan	Acid-fast staining, bacterial culture, radiology	Sputum	16S rRNA	Fluorescence	90	98	6	6	200	94%	94.20%
3	Poudel et al (2009)	Kathmandu Univ Med J (KUMJ)	Nepal	Smear microscopy, culture, radiology	Sputum	16S rRNA	Fluorescence	97	96	6	3	202	97%	94.12%
4	Geojith et al (2011)	J Microbiol Methods	India	Culture, PCR reverse-hybridization line probe assay, genotype MTBE assay	Sputum	rimM	Colorimetry, gel electrophoresis	17	17	1	21	56	44.70%	94.40%
5	George et al (2011)	PLoS One	India	Fluorescence smear microscopy, culture	Sputum	rimM	Colorimetry, gel electrophoresis	31	36	2	2	71	93.90%	94.70%
6	Mitarai et al (2011)	Int J Tuberc Lung Dis	Japan	Culture, smear microscopy, nucleic acid amplification (NAA)	Sputum	gyrB	Fluorescence, turbidity	196	88	9	27	320	87.90%	90.70%
7	Nagdev et al (2011)	J Clin Microbiol	India	PCR	Cerebrospinal fluid	IS6110	Turbidity	15	8	2	2	27	88.23%	80%
8	Sethi et al (2013)	J Clin Lab Anal	India	Smear microscopy, culture, PCR	Sputum	16S rRNA, IS6110	Colorimetry, gel electrophoresis	87	30	0	16	133	84.50%	100%
9	Cao et al (2015)	J Microbiol Methods	China	Smear microscopy, culture, PCR	Sputum	IS6110	Fluorescence	98	18	5	2	123	98.00%	78.30%
10	Joon et al (2015)	Int J Tuberc Lung Dis	India	Smear microscopy, culture, PCR	Endometrial fluid, urine, blood, semen, cerebrospinal fluid, pleural fluid, pus, pericardial fluid, peritoneal fluid, intestinal and lymph node biopsy tissue	IS6110, MPB64, sdaA	Colorimetry	28	262	23	2	315	93.30%	91.90%
11	Moon et al (2015)	J Med Microbiol	Korea	Culture, smear microscopy	Sputum	hspX	Colorimetry, turbidity, gel electrophoresis	32	255	3	13	303	71.10%	98.80%
12	Bojang et al (2016)	J Infect	Gambia	Smear microscopy, culture, GeneXpert MTB/RIF	Sputum	16S rRNA	Fluorescence	98	157	10	1	266	99.00%	94.00%
13	Gray et al (2016)	J Clin Microbiol	Switzerland	Culture, smear microscopy	Sputum	gyrB	Fluorescence	331	###	52	61	1777	84.40%	96.60%
14	Kaku et al (2016)	Jpn J Infect Dis	Japan	Smear microscopy, culture	Sputum	gyrB, IS6110	Fluorescence	134	312	5	21	472	86.50%	98.40%
15	Modi et al (2016)	Int J Tuberc Lung Dis	India	Culture, radiology, staining, PCR	Cerebrospinal fluid	IS6110, MPB64	Fluorescence, gel electrophoresis, turbidity	144	100	0	6	250	96.00%	100.00%

(*Continued*)

Table I - (*Continued*)

16	Sharma et al (2016)	Tuberculosis (Edinb)	India	PCR, culture, smear microscopy	Needle aspirate	IS6110, MPB64	Fluorescence, gel electrophoresis, turbidity	108	50	0	12	170	90.00%	100.00%
17	Sharma et al (2016)	J Orthop Res	India	Culture, staining, PCR	Synovial fluid, pus	IS6110, MPB64	Fluorescence, gel electrophoresis, turbidity	81	50	0	9	140	90.00%	100.00%
18	Joon et al (2017)	J Microbiol Methods	India	PCR, culture, smear microscopy	Sputum	IS6110, MPB64	Colorimetry, gel electrophoresis	17	212	6	1	236	94.40%	97.20%
19	Reddy et al (2017)	Int J Tuberc Lung Dis	South Africa	Culture, smear microscopy, Xpert	Sputum	gyrB	Fluorescence	119	514	17	45	695	72.60%	96.80%
20	Yadav et al (2017)	Int J Tuberc Lung Dis	India	Culture, smear microscopy, GeneXpert	Sputum	gyrB, IS6110	Fluorescence	82	368	3	0	453	100.00%	99.20%
21	Kim et al (2018)	Ann Lab Med	Korea	Culture, smear microscopy, PCR	Sputum	gyrB, IS6110	Fluorescence, turbidity	87	186	0	17	290	83.60%	100.00%
22	Nguyen et al (2018)	Diagn Microbiol Infect Dis	Vietnam	Smear microscopy, culture, Xpert MTB/RIF	Sputum	gyrB, IS6110	Colorimetry, fluorescence	15	445	23	18	501	45.50%	95.10%
23	Perera et al (2018)	Ceylon Med J	Sri Lanka	Smear microscopy, culture	Culture isolates	rimM	Colorimetry	31	10	5	0	46	100.00%	66.67%
24	Joon et al (2019)	J Microbiol Methods	India	Culture, smear microscopy, GeneXpert MTB/RIF assay, PCR, LAMP-LFD assay	Sputum	sdaA	Colorimetry	13	92	2	0	107	100.00%	97.87%
25	Phetsuksiri et al (2019)	Jpn J Infect Dis	Thailand	Culture, immuno-chromatographic test	Sputum	mpt64	Colorimetry	144	5	1	1	151	99.31%	83.33%
26	Punati et al (2019)	Braz J Microbiol	India	Culture, PCR	Fecal samples	IS900	Turbidity, gel electrophoresis, colorimetry, lateral flow device	86	294	9	0	389	100.00%	97.02%
27	Rajput et al (2019)	J Microbiol Methods	India	Culture, smear microscopy, PCR	Fluids, urine, pus	IS6110, Pab, MPB64	Colorimetry, fluorescence	90	32	9	23	154	79.65%	78.05%
28	Han et al (2020)	BMC Infect Dis	China	Xpert MTB/RIF, SAT-TB assay	Pleural fluids	gyrB, IS6110	Fluorescence	59	41	1	164	265	26.50%	97.60%
29	Phetsuksiri et al (2020)	Jpn J Infect Dis	Thailand	Microscopy, culture, PCR, radiology	Sputum	16S rRNA	Colorimetry, fluorescence, gel electrophoresis, immuno-chromatography	119	102	24	7	252	94.44%	80.95%
30	Phetsuksiri et al (2020)	Rev Inst Med Trop Sao Paulo	Thailand	Xpert MTB/RIF, culture, smear microscopy	Sputum	16S rRNA	Colorimetry, fluorescence, gel electrophoresis	126	71	0	7	204	94.74%	100.00%

LAMP = loop-mediated isothermal amplification; LFD = lateral flow dipstick; PCR = polymerase chain reaction.

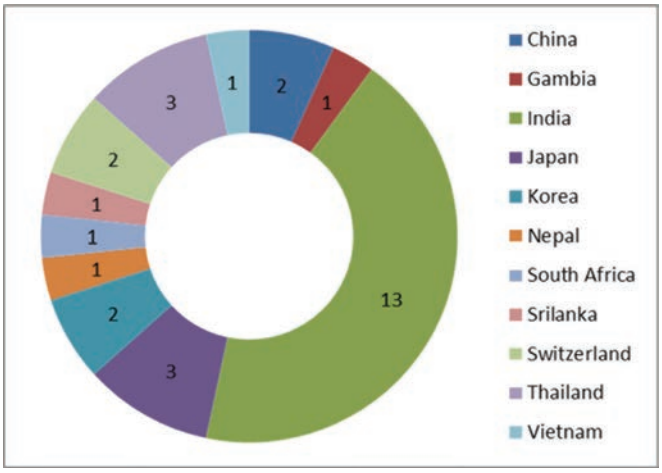


Fig. 2 - Country-wise distribution of included studies (n = 30) reported in the present investigation.

by Thailand and Japan (each 10%; n = 3). Two studies each were also conducted in countries such as China, Korea, and Switzerland (6.3%; n = 2). Apart from this, one study each, that is, 3.3%, was from countries included such as Gambia, Nepal, South Africa, Sri Lanka, and Vietnam. Although most of the included articles do not mention about the patient details, the type of specimen (Fig. 3) used in most of the studies was sputum (42.8%; n = 21). In addition, some studies have been tested on other specimens such as cerebrospinal fluid (n = 4), fecal samples (n = 1), urine (n = 3), blood (n = 2), and pleural fluid (n = 3) for the detection of mycobacteria by using LAMP. Furthermore, the standard smear microscopy, culture assay, and PCR-based methods were used as references either alone or in combination (n = 30). Of note, radiology was also used (n = 4) to validate LAMP results as a reference standard (Tab. I), with one study using immunochromatography (16). Next, we examined the various target genes used for the eligible studies. Ten different types of target genes including hspX, *IS900*, mpt64, Pab, sdaA, rimM, 16SrRNA, *MPB64*, gyrB, and *IS6110* were used in the included studies (n = 30). IS6110 gene was most frequently used in the included studies (n = 14; 31.18%) followed by gyrB (n = 9, 20.45%), 16SrRNA (n = 6, 13.63%), and *MPB64* (n = 6, 13.63%) genes (Fig. 4). Furthermore, while analyzing detection methods used for these 30 studies, fluorescent method (n = 19, 32.39%) was the most frequently performed followed by colorimetry (n = 14, 25.35%), gel electrophoresis (n = 11, 20.00%) and turbidity (n = 9, 16.36%) methods (Fig. 5). In 53.33% (n = 16) of studies, more than one detection method was used. In 16.66% (n = 5) of studies, combination of three methods was used while in only two studies (6.66%), combination of four different methods was reported (17,18).

Among all the eligible studies, 4 studies showed 100% sensitivity, while for 16 studies this parameter was higher than 90%. Similarly, 6 studies exhibited 100% specificity while 90% or more specificity was observed in 24 studies (Tab. I). Furthermore, upon analysis of sensitivity and specificity using forest plot at 95% CI, we found that the sensitivity values varied between 0.26 and 1.00 and the specificity values ranged from 0.67 to 1.00 (Fig. 6). A total of 27 out of the 30 included studies showed pooled sensitivity greater than 70%. Only three studies reported sensitivity values of 26% and 45% each (19-21). In terms of FP rate (1-specificity), 27 included studies showed a pooled FP rate higher than 80% (Fig. 7). Additionally, the accuracy and precision rates of included studies were calculated and varied between 37.73% and 99.33%. The analysis proved that 22 studies displayed more than 90% accuracy with only 4 studies depicting less than 80% accuracy (Tab. II). Likewise, the precision rates varied between 39.47% and 100%. The analysis showed that 21 studies exhibited more than 90% precision rate with only 3 studies depicting less than 80%. Of note, we observed that six studies displayed 100% precision rate.

Table II - Accuracy and precision of the included studies (n = 30)

S. No.	Study	Accuracy	Precision
1	Boehme et al (2007)	98.68	97.74
2	Pandey et al (2008)	94.00	93.75
3	Poudel et al (2009)	95.54	94.17
4	Geojith et al (2011)	60.71	94.44
5	George et al (2011)	94.36	93.93
6	Mitarai et al (2011)	88.75	95.60
7	Nagdev et al (2011)	85.18	88.23
8	Sethi et al (2013)	87.96	100.00
9	Cao et al (2015)	94.30	95.14
10	Joon et al (2015)	92.06	54.90
11	Moon et al (2015)	94.71	91.42
12	Bojang et al (2016)	95.86	90.74
13	Gray et al (2016)	93.64	86.42
14	Kaku et al (2016)	94.49	96.40
15	Modi et al (2016)	97.60	100.00
16	Sharma et al (2016)	92.94	100.00
17	Joon et al (2017)	97.03	73.91
18	Reddy et al (2017)	91.07	87.50
19	Sharma et al (2016)	93.57	100.00
20	Yadav et al (2017)	99.33	96.47
21	Kim et al (2018)	94.13	100.00
22	Nguyen et al (2018)	91.81	39.47
23	Perera et al (2018)	89.13	86.11
24	Joon et al (2019)	98.13	86.66
25	Phetsuksiri et al (2019)	98.67	99.31
26	Punati et al (2019)	97.68	90.52
27	Rajput et al (2019)	79.22	90.90
28	Han et al (2020)	37.73	98.33
29	Phetsuksiri et al (2020)	87.69	83.21
30	Phetsuksiri et al (2020)	96.56	100.00

Quality assessment of the study

Almost two-thirds of the included studies (22 out of 30 studies) have a high risk of patient selection bias due to

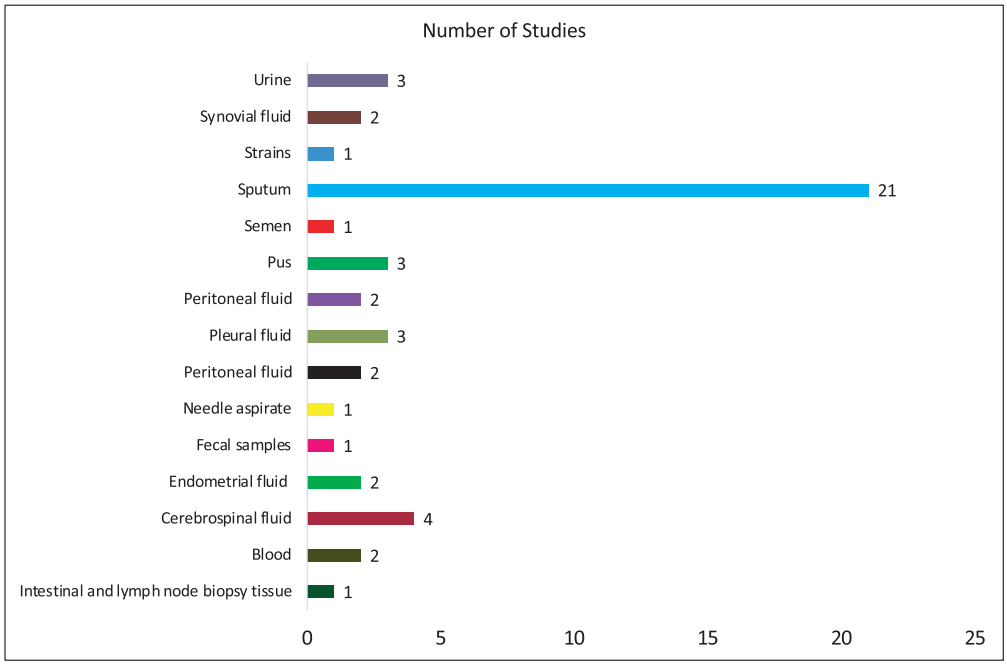


Fig. 3 - Distribution of type of specimen for detection of mycobacteria in the included studies (n = 30).

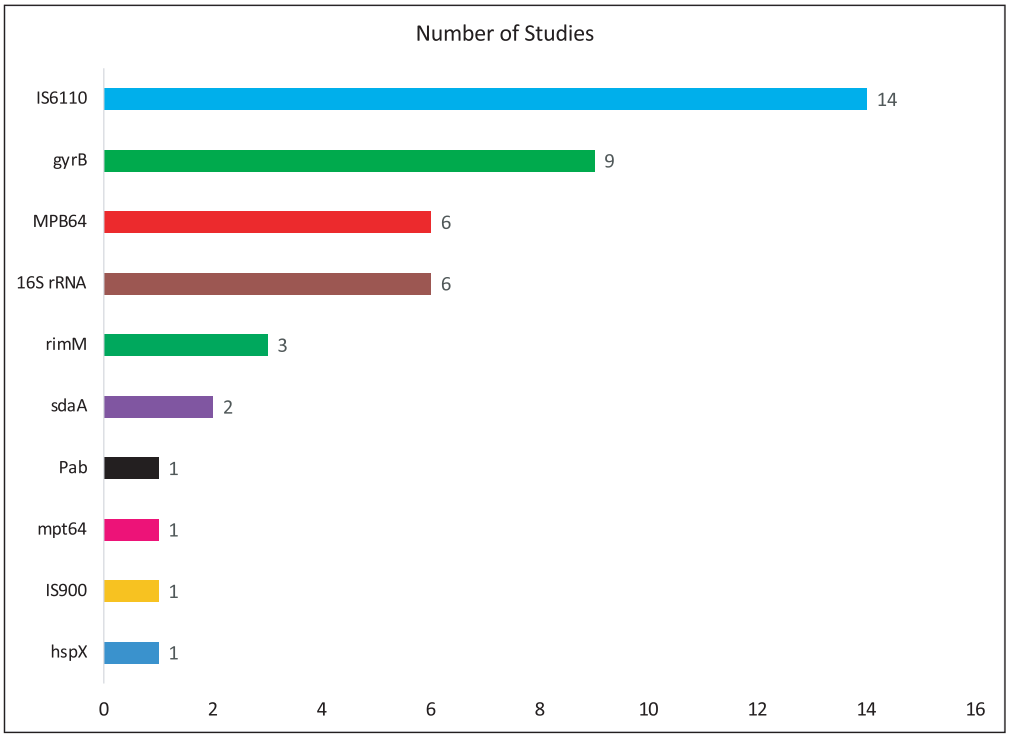


Fig. 4 - Distribution of target genes reported in the included studies (n = 30).

non-random patient selection and case-control study design (Fig. 8, Tab. I). Around 26% (8 out of 30) of the included studies have low risk of patient selection bias because these studies provided sufficient details about patient inclusion/exclusion criteria; 86% of included articles (26 out of 30 studies) present low risk of index test bias because these tests clearly stated the quantitative detection read-outs with reported thresholds. Moreover, these studies explicitly declared that their index and reference tests were done simultaneously in parallel to each other or that testing was blinded from each other. Two studies (19,22) were reported without defined detection thresholds. One study (23) had unclear risk of index test bias as the quantitative detection thresholds were not explained. It was either unclear whether index test results were interpreted with knowledge of reference test results or if only qualitative read-out was used for

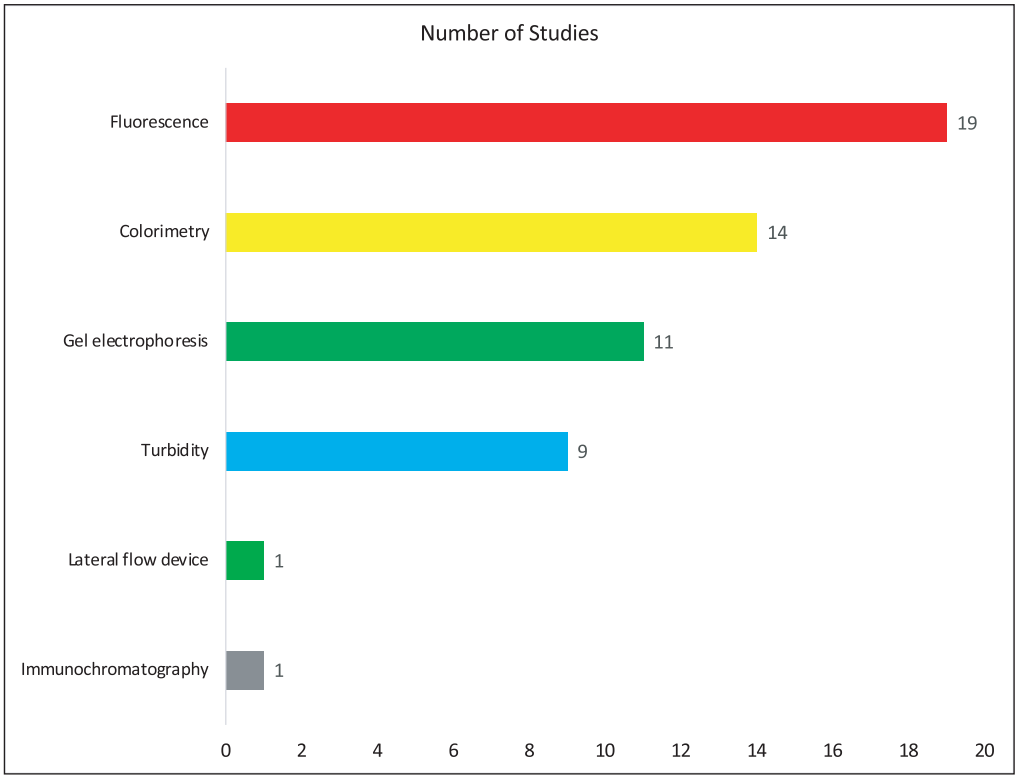
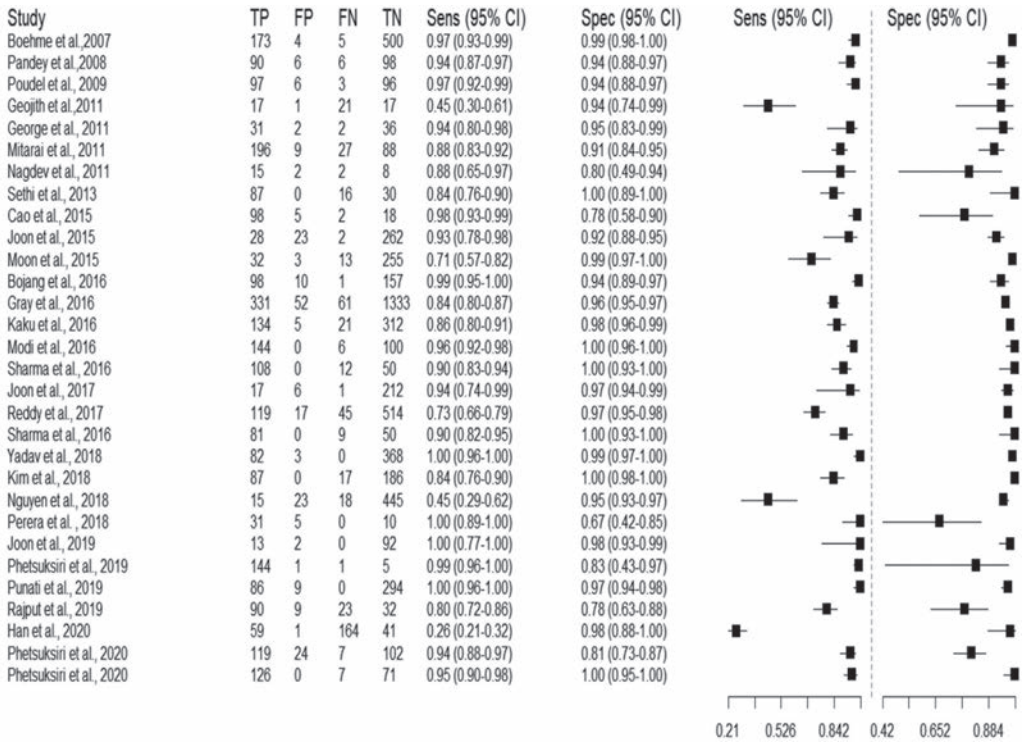


Fig. 5 - Distribution of type of detection method for mycobacteria in the included studies (n = 30).



Study	TP	FP	FN	TN	Sens (95% CI)	Spec (95% CI)
Boehme et al.,2007	173	4	5	500	0.97 (0.93-0.99)	0.99 (0.98-1.00)
Pandey et al.,2008	90	6	6	98	0.94 (0.87-0.97)	0.94 (0.88-0.97)
Poudel et al., 2009	97	6	3	96	0.97 (0.92-0.99)	0.94 (0.88-0.97)
Geojith et al.,2011	17	1	21	17	0.45 (0.30-0.61)	0.94 (0.74-0.99)
George et al., 2011	31	2	2	36	0.94 (0.80-0.98)	0.95 (0.83-0.99)
Mitarai et al., 2011	196	9	27	88	0.88 (0.83-0.92)	0.91 (0.84-0.95)
Nagdev et al., 2011	15	2	2	8	0.88 (0.65-0.97)	0.80 (0.49-0.94)
Sethi et al., 2013	87	0	16	30	0.84 (0.76-0.90)	1.00 (0.89-1.00)
Cao et al., 2015	98	5	2	18	0.98 (0.93-0.99)	0.78 (0.58-0.90)
Joon et al., 2015	28	23	2	262	0.93 (0.78-0.98)	0.92 (0.88-0.95)
Moon et al., 2015	32	3	13	255	0.71 (0.57-0.82)	0.99 (0.97-1.00)
Bojang et al., 2016	98	10	1	157	0.99 (0.95-1.00)	0.94 (0.89-0.97)
Gray et al., 2016	331	52	61	1333	0.84 (0.80-0.87)	0.96 (0.95-0.97)
Kaku et al., 2016	134	5	21	312	0.86 (0.80-0.91)	0.98 (0.96-0.99)
Modi et al., 2016	144	0	6	100	0.96 (0.92-0.98)	1.00 (0.96-1.00)
Sharma et al., 2016	108	0	12	50	0.90 (0.83-0.94)	1.00 (0.93-1.00)
Joon et al., 2017	17	6	1	212	0.94 (0.74-0.99)	0.97 (0.94-0.99)
Reddy et al., 2017	119	17	45	514	0.73 (0.66-0.79)	0.97 (0.95-0.98)
Sharma et al., 2016	81	0	9	50	0.90 (0.82-0.95)	1.00 (0.93-1.00)
Yadav et al., 2018	82	3	0	368	1.00 (0.96-1.00)	0.99 (0.97-1.00)
Kim et al., 2018	87	0	17	186	0.84 (0.76-0.90)	1.00 (0.98-1.00)
Nguyen et al., 2018	15	23	18	445	0.45 (0.29-0.62)	0.95 (0.93-0.97)
Perera et al., 2018	31	5	0	10	1.00 (0.89-1.00)	0.67 (0.42-0.85)
Joon et al., 2019	13	2	0	92	1.00 (0.77-1.00)	0.98 (0.93-0.99)
Phetsuksiri et al., 2019	144	1	1	5	0.99 (0.96-1.00)	0.83 (0.43-0.97)
Punati et al., 2019	86	9	0	294	1.00 (0.96-1.00)	0.97 (0.94-0.98)
Rajput et al., 2019	90	9	23	32	0.80 (0.72-0.86)	0.78 (0.63-0.88)
Han et al., 2020	59	1	164	41	0.26 (0.21-0.32)	0.98 (0.88-1.00)
Phetsuksiri et al., 2020	119	24	7	102	0.94 (0.88-0.97)	0.81 (0.73-0.87)
Phetsuksiri et al., 2020	126	0	7	71	0.95 (0.90-0.98)	1.00 (0.95-1.00)

Fig. 6 - The Forest plot of sensitivity and specificity of included studies (n = 30) on the diagnostic performance of loop-mediated isothermal amplification (LAMP) technique.

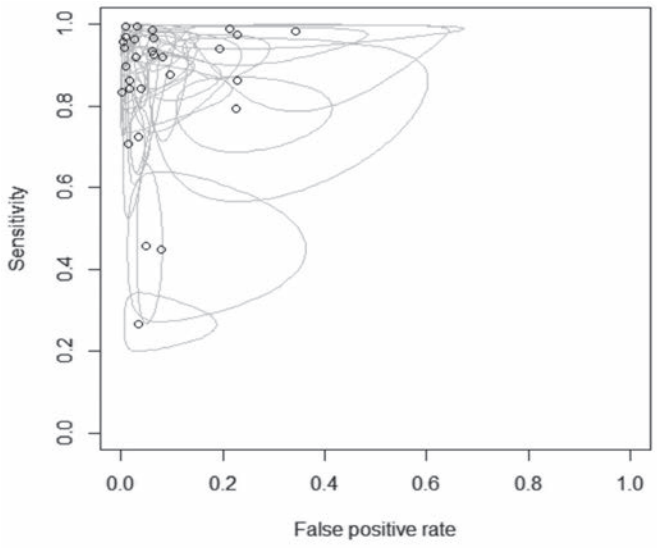


Fig. 7 - Summary receiver operating characteristic (SROC) depicts loop-mediated isothermal amplification (LAMP) diagnostic performance in mycobacteria diagnosis.

reading the results. Hence, index test bias of these studies was unclear. For the rest of the included studies, almost all (n = 30) have low risk of reference standard bias because they provided enough information about the standard reference test used in the study.

Half of the studies (15 out of 30) have an unclear risk of flow and timing bias as there is not enough information, whether reference standard results were interpreted with the knowledge of the results of the index test. One study (24) was at high risk as it did not provide any information on whether the samples for a reference test and the index test were taken at the same time. Our review question did not focus on any patient demographics. None of the included studies attempted to exclude patients based on demographics and thus had no "concern of patient selection applicability" (Fig. 8, Tab. I). Index tests of all studies have generally been used for Point-of-care test (POCTs) and thus have low concern of index test applicability. Reference standard tests of nearly all studies were culture, smear microscopy, Xpert test, PCR, or combinations of them. Thus, we graded these studies as having low concern of standard test applicability.

Discussion

Early and correct diagnosis of all the TB forms is pertinent for effective treatment of the disease and prevention of the spread of infection, particularly in nations which have high burden. The currently available diagnostics rely mostly on smear microscopy, culture, and PCR-based methods which are not only time-consuming and low sensitive but cumbersome and costly (25,26). LAMP assay provides a faster and innovative point-of-care diagnostic alternative as it is cost-effective, sensitive, and gives results in less than 1 hour due to amplification under isothermal condition by strand displacement activity of Bst DNA polymerase and visual readouts (27-30). In fact, the efficiency of LAMP in diagnosis of pulmonary TB is evident from wide ranges of studies (31-35). Additionally, LAMP has been successfully deployed for diagnosis of other forms of TB such as tuberculous meningitis (36,37), osteoarticular TB (38), and tubercular lymphadenitis (39). Although a few studies have evaluated the diagnostic validity of LAMP by meta-analysis for diagnosis of MTB (40),

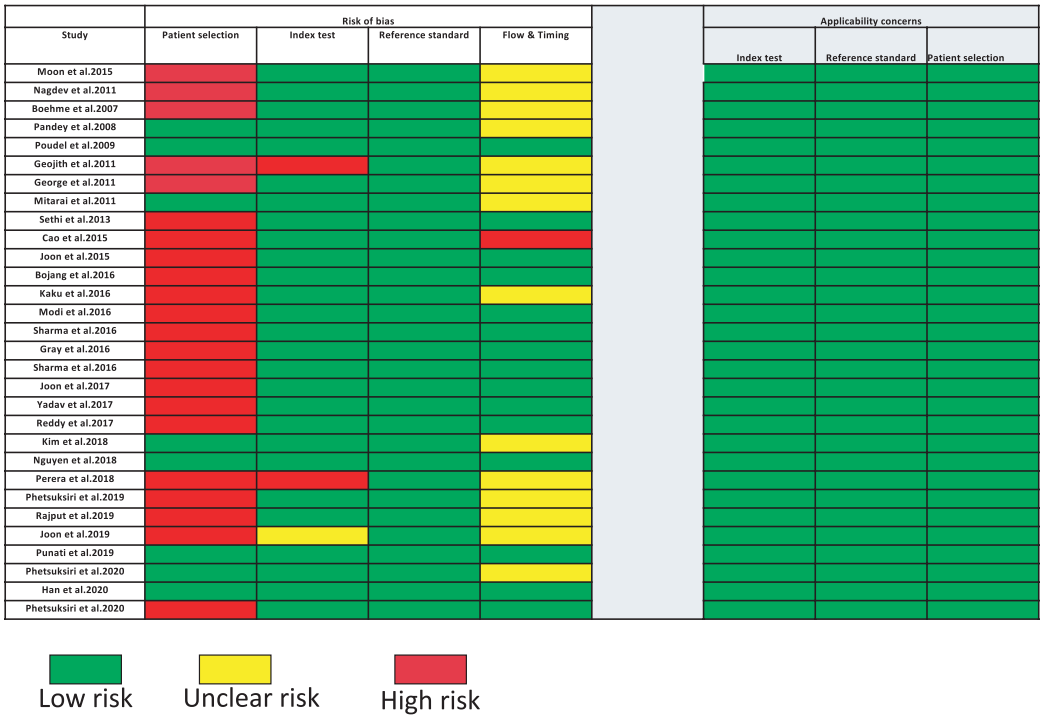


Fig. 8 - Quality Assessment of Diagnostic Accuracy Studies 2 (QUADAS-2) summary of items for risk of bias and applicability in included studies (n = 30). Green color depicts the low risk of biasedness, yellow color depicts the unclear risk of biasedness, and red color depicts the high risk of biasedness.

pulmonary TB (41), and extrapulmonary TB (42), an updated meta-analysis covering all forms of mycobacteria was still missing. Hence, the aim of the present study was to systematically review and perform the meta-analysis to assess the diagnostic accuracy of the LAMP assay for detection of all forms of mycobacteria.

This meta-analysis revealed that most of the studies were conducted in high TB burden countries such as India, Thailand, and Japan (Fig. 2). We observed that for the detection of mycobacteria sputum could be considered as the most chosen sample (Fig. 3). When considering the target genes, we found a variety of genes that were used in the included studies. However, IS6110 ranked first among all evaluated genes in the included studies (Fig. 4). This occurrence could be due to the presence of multiple copies of IS6110 present in the MTB genome (43). However, other target genes such as 16s rRNA and gyrB were also prominent. Next, we considered the detection method that was used for assessing the LAMP results. Most of the studies used fluorescence-based methods followed by colorimetry, gel electrophoresis, and turbidity, with no justification of their choices (Fig. 5). The prominence of fluorescence methods could be due to their increased sensitivity for the detection. Exceptionally, only one study mentioned lateral flow-based detection method despite market applicability.

Forest plot was used to calculate the sensitivity and specificity. The pooled sensitivity values of meta-analysis ranged between 0.26 and 1.0 (Fig. 6) and forest plot and SROC curve revealed a pooled specificity value between 0.67 and 1.0 (Fig. 7) with 95% CI. The accuracy and precision were calculated for the included studies and for 16 studies we found that the accuracy rate was higher than their corresponding precision rates and vice versa for 14 articles upon intra-comparison of accuracy with precision (Tab. II).

The current study also exhibited few limitations. Firstly, we observed high risk of patient selection bias or index test bias in almost two-thirds of the eligible studies (Fig. 8). Therefore, the use of unbiased patient cohorts and double-blinded index test may be recommended for future studies. Secondly, few studies showed the highest performance with 100% sensitivity and specificity, respectively, hence displaying the lowest QUADAS risk and concerns in all the domains. Furthermore, lack of subgroup analysis and the use of solely peer-reviewed English language articles were also additional limitations. Hence, although the current meta-analysis should be interpreted with caution, however, we believe that it will not impact the robustness of the analysis leading to further improved studies and reviews. Particularly considering the growing significance of LAMP-based detection for TB comparable to other methods, such studies may be encouraged (43-45).

Conclusion

Despite suffering from few disadvantages, like false positivity due to heavy reliance on indirect detection methods such as turbidity and nonspecific dyes and not providing any additional benefits like information on mutations, drug resistance etc., the LAMP technique could be a promising molecular test to enhance case detection before conventional time-consuming culture. Its simplicity, less turnaround time, and cost-effectiveness are major attractions for clinical laboratories. Also, it will be unjust to rely on single point-of-care test for TB successfully in various kinds of populations and resource availability. Although the unit cost is higher than smear microscopy and culture-based methods, it is likely to offer good value for money relative to conventional methods. In a nutshell, the present study endorses the use of LAMP assay as a promising alternative for detection of mycobacteria, particularly in regions which are financially compromised, where drug-resistant strains are not prevalent and PCR-based tests cannot be done so frequently. The faster diagnosis through LAMP could provide an alternative solution for failed medications to current therapeutics due to delayed diagnosis and subsequent development of drug resistance, thereby providing an opportunity to employ this new information in improving treatment strategies. However, the LAMP assay still must be improved to turn to a strong and competitive alternative to other molecular diagnostic methods.

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Author contributions

G.S.B.: search, data extraction, validation. G.S.B., S.J.: data analysis. Z.F. and S.H.: supervision. G.S.B. and S.H.: writing, original draft. Z.F. and S.H. contributed to the conception and design of the study and review and editing of the manuscript.

Disclosures

Conflict of interest: The authors declare no conflicts of interest, financial or otherwise.

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CASE REPORT

Activity of sotorasib against brain metastases from NSCLC harboring *KRAS* p.G12C mutation: a case report

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ABSTRACT

In the CodeBreaK 100 phase 2 study, sotorasib was active for patients with metastatic non-small cell lung cancer (NSCLC) harboring Kirsten rat sarcoma viral oncogene homologue (KRAS) p.G12C mutation. However, patients with untreated and/or active brain metastases were excluded from the trial, and the activity of sotorasib in the setting of brain metastases should be further investigated. Here we report the case of a KRAS p.G12C mutant NSCLC patient with three brain metastases, of whom one was untreated and the other two had progressed after radiotherapy with symptoms requiring steroids, that responded to sotorasib. Our report suggests that sotorasib may be active against untreated or progressive brain metastases, supporting further evaluation of sotorasib in this setting.

Keywords: Brain metastases, Central nervous system, KRAS, NSCLC, Sotorasib

Background

About 25-30% of non-small cell lung cancers (NSCLCs) harbor a mutation in the Kirsten rat sarcoma viral oncogene homologue (*KRAS*) gene. Particularly, *KRAS* p.G12C mutation is the most frequent *KRAS* mutation and it is found in approximatively 13% of NSCLC (1). Sotorasib, a specific inhibitor of *KRAS* p.G12C mutation, has demonstrated activity in pretreated metastatic NSCLC patients. In the CodeBreaK 100 trial, a single-group phase 2 study on 126 pretreated patients with metastatic, *KRAS* p.G12C mutant NSCLC, sotorasib led to a response rate of 37.1% (95% confidence interval (CI), 28.6-46.2), a median progression-free survival of 6.8 months (95% CI, 5.1-8.2), and a median overall survival of 12.5 months (95% CI, 10.0-not reached), with an acceptable safety profile (2).

Brain metastases represent a frequent complication of NSCLC. They are associated with deterioration of quality of life, poor prognosis, and low response rates to chemotherapy (3). For patients with brain metastases from oncogene addicted NSCLC, such as tumors with *EGFR* mutations or *ALK* rearrangements, target therapy achieves high intracranial response rate (4,5). However, there is still paucity of data regarding the activity of sotorasib against brain metastases from *KRAS* p.G12C mutant NSCLC. In fact, although in the CodeBreak 100 trial approximately 20% of patients had brain metastases at baseline, patients with active untreated brain metastases were excluded from the trial. More recently, a retrospective study reported six patients with active untreated brain metastases receiving sotorasib. Among four patients evaluable for response, confirmed intracranial response to sotorasib was observed in three patients, with a median duration of response of 4.1 months, and a median intracranial progression-free survival of 4.7 months (6).

Here we report a case of a patient with metastatic, *KRAS* p.G12C mutant NSCLC with both treated and untreated active brain metastases receiving sotorasib as second-line therapy.

Case report

In June 2017, a 72-year-old former Caucasian female smoker underwent upper right lung lobectomy with regional nodal dissection for lung adenocarcinoma, stage pT2a pN1. Comorbidities were: previous left nephrectomy for clear cell renal carcinoma, hypertension, type 2 diabetes, and meningioma. Molecular profile of NSCLC was: *EGFR* wild type, *ALK* negative, ROS1 negative, programmed death ligand (PDL) tumor proportion score (TPS) <1%, KRAS mutant p.G12C. At baseline staging, patient also had two synchronous intracranial metastases, in right parietal lobe and in right cerebellar hemisphere, both treated with stereotactic radiosurgery (21 Gy as single fraction). The patient also received

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first-line chemotherapy with carboplatin plus paclitaxel for four courses, from August to October 2017.

During the following surveillance program, the patient developed two lung metastases in the right middle lobe (March 2018) both treated with stereotactic radiotherapy (70 Gy in 10 fractions), a further histology-proven, adenocarcinoma lung metastasis *KRAS* mutant p.G12C in upper left lobe (September 2019) also treated with stereotactic radiotherapy (60 Gy in 10 fractions), and progressive right parietal and right cerebellar metastases treated with further radiation therapy, respectively 21 Gy in three fractions and 27 Gy in three fractions.

In March 2021 the patient experienced symptomatic intracranial disease progression, with a new brain metastasis in the right frontal lobe and increase in size of the other two known metastases and appearance of surrounding edema in the right parietal lobe, requiring steroid therapy. Positron emission tomography (PET)/computed tomography (CT) scan did not show extracranial disease. The patient was started on sotorasib, and the brain magnetic resonance imaging (MRI) after 2 months of treatment showed stability of the cerebellar metastasis, reduction in size of the previously treated parietal right metastasis with improvement of the surrounding edema, reduction in size of the previously untreated right frontal lobe metastasis, and no appearance of new brain metastases (Fig. 1). In March 2022 posterior fossa hemorrhage occurred due to bleeding of the cerebellar metastasis, which was treated with surgical evacuation of the hemorrhagic focus and metastasectomy. Histology examination of the cerebellar metastasis revealed radionecrosis with no residual viable cancer tissue. Treatment with sotorasib was continued and the disease remained stable until July 2022 when brain MRI showed oligoprogression due to increase in size of the right frontal metastasis, which was treated with stereotactic radiotherapy (24 Gy in three fractions). Sotorasib was continued and, after 27 months (May 2023), treatment is still ongoing, without safety concerns, and with stable intracranial disease at brain MRI and still no evidence of extracranial metastases at the PET/CT scan.

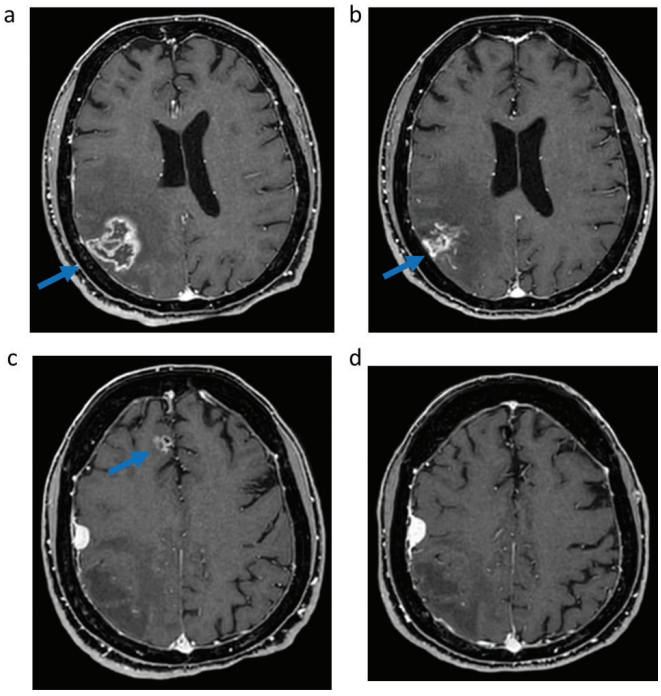

Fig. 1 - Intracranial response to sotorasib assessed with magnetic resonance imaging. T1-weighted imaging of right parietal metastasis before (A) and after (B) 6 months of sotorasib; Fluid-attenuated inversion recovery of right parietal metastasis surrounding edema before (C) and after (D) 6 months of sotorasib; T1-weighted imaging of right frontal metastasis before (E) and after (F) 6 months of sotorasib; T1-weighted imaging of right cerebellar metastasis before (G) and after (H) 6 months of sotorasib.

Conclusion

We have reported a case of intracranial response to sotorasib in a patient with both pretreated and untreated symptomatic brain metastases from *KRAS* p.G12C mutant NSCLC, with a duration of intracranial response of 16 months. An oligoprogressive brain metastasis was successfully managed with stereotactic radiotherapy while continuing sorafenib, with a time to treatment failure exceeding 27 months.

This report supports further investigation of sotorasib in the setting of *KRAS* p.G12C mutant NSCLC with untreated brain metastases.

Disclosures

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ORIGINAL RESEARCH ARTICLE
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ESBL and carbapenemase-producing Enterobacteriaceae in infectious pleural effusions: current epidemiology at Hôpital du Mali

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ABSTRACT

Background: Antimicrobial resistance (AMR) is a global health concern, with extended-spectrum β-lactamases (ESBLs) and carbapenemases being major contributors. Pleural infection (PI) is a severe condition in West Africa, complicated by AMR. This study aimed to investigate the prevalence and molecular characteristics of ESBL and carbapenemase-producing enterobacteria in pleural effusions in Mali.

Materials and methods: Pleural fluid samples from 526 patients with pleuritis were analyzed. Enterobacterial species were isolated and identified, and the prevalence of resistance genes (bla_{OXA-48}, bla_{NDM-1}, bla_{KPC}, bla_{TEM}, bla_{SHV}) and virulence factors was determined.

Results: Among the patients, 110 were diagnosed with enterobacterial pleuritis. *Escherichia coli, Klebsiella pneumoniae, and Proteus mirabilis* were the main pathogens identified. Resistance to β-lactams and cephalosporins was high, while carbapenems showed good activity. ESBL production was detected in 33.6% of isolates, with bla_{TEM} being the most common gene. Carbapenemase gene (bla_{NDM-1}) was found in three isolates.

Conclusion: The study highlights the high prevalence of multidrug-resistant bacteria and the need for appropriate antibiotic selection based on local resistance patterns. Understanding the molecular characteristics of resistance is crucial for optimizing patient care and developing effective therapeutic strategies. Further research is needed to monitor and control AMR in PIs in Mali.

Keywords: Carbapenem-resistant Enterobacteriaceae, ESBL, Mali, Pleural effusions

Introduction

Infectious pleural effusion (IPE) is a severe clinical issue. Often secondary to a pre-evolving pulmonary infection, the condition presents with an increasing incidence worldwide (1,2). Care of IPE involves pleural drainage in most of the cases and antibiotic therapy; however, treatment proves inadequate in substantial cases, resulting in mortality rate ranging from 10.7% to 22% (3-5). Few studies have evaluated IPE management, status, and microbiology in Mali. A recent study by Tapia et al in 2021 reported 13.3% mortality rate (6). Among causal factors predictive of treatment failure is the alarming emergence of antimicrobial resistance (AMR).

AMR has emerged as a significant global health concern, undermining the effectiveness of antibiotics and exacerbating the burden of infectious diseases. Major contributors to AMR include extended-spectrum β-lactamases (ESBLs) and carbapenemases produced by Enterobacteriaceae (7,8). Both enzymes are β-lactamases with proven ability to degrade β-lactam antibiotics. The ESBLs exhibit hydrolytic activity against penicillins and all cephalosporins, yet are suppressed by β-lactam inhibitors (9). Mutation-wise, ESBLs encoding genes can be grouped into several variants: bla_{TEM}, bla_{SHV}, bla_{IRT}, etc (10). On the other hand, another important

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enzyme is carbapenemase, which degrade carbapenem antibiotics and include gene variants like bla_{KPC}, bla_{IMP}, bla_{OXA-1}, etc (9). Thus, their inclusion in World Health Organization list as top priority pathogens underscores their potential to pose significant challenge in clinical settings (11). Data from recent studies found an association between extended-spectrum β-lactamases and carbapenemases producing Enterobacteriaceae (ESBL-E/CPE) infections and risk of mortality (12,13). Moreover, prior to antibiotherapy, comorbidities as well as persistent colonization were found to be strong predictors of infection and subsequent treatment failure (14,15). Yet to date, detailed reports on the magnitude of ESBL-E/CPE in West Africa are scarce (16). Although there are data on phenotypic and genotypic distribution of AMR in pathogens responsible for various clinical infections in Mali, there are still unanswered questions related to the genotypic distribution of these AMR genes in IPEs (17-19). Understanding the extent of resistance and identifying the underlying resistance mechanisms is crucial for designing effective therapeutic strategies and optimizing patient care in this region.

This study has mainly focused on the epidemiology of bla_{OXA-48}, bla_{NDM-1}, bla_{KPC} genes responsible for the induction of carbapenem resistance; bla_{TEM}, bla_{SHV} genes that are responsible for ESBL production, as well as genes associated with bacterial adhesins (*bfp, eae, ipah, eagg*) and toxin genes (*slt1, slt2, lt, sta*). The primary objective of this study was to isolate, identify, and analyze the diversity of enterobacterial species found in pleural fluid samples from patients with IPE. A better knowledge of IPE in Mali could facilitate the management of the condition and thus reduce the level of mortality.

Material and methods

Study design and population

The present research is a prospective cross-sectional study conducted between October 2021 and December 2022, in the thoracic surgery and pediatrics departments of CHU "Hôpital du Mali" in Bamako. The study included all 6,096 hospitalized patients with pleurisy in these two departments.

The inclusion criteria were: (1) clinical diagnosis of pleural infection (PI) with subsequent diagnostic thoracentesis and microbiological confirmation (pleural fluid culture positive to at least one microorganism). PI was defined as the presence of positive pleural fluid culture and/or purulent pleural fluid and clinically manifesting as complex parapneumonic effusion (CPPE) or empyema (20); (2) patients who provided written informed consent to participate in the study.

The exclusion criteria were: (1) patients who had non-purulent or no growth from pleural fluid; (2) patients with pleural effusion caused by noninfectious etiologies such as malignancy and congestive heart failure.

The data were collected using Microsoft Excel through questionnaires administered to consenting patients by physicians prior to sample collection. Subsequently, laboratory data were obtained and combined with the questionnaire responses.

Microbiological processing and identification

All pleural fluid samples were processed within an hour after collection at the microbiology laboratory of CHU Hôpital du Mali. Samples were inoculated on brain-heart infusion (BHI) and an anaerobic blood culture flask and incubated for 18 to 24 hours at 35±2°C. From these broth cultures, fresh blood agar, enriched chocolate agar, and Sabouraud agar were plated. The colonies were characterized and identified using Gram stain, biochemical tests, and the Phoenix M50 automated system (panel 449044-NMIC/ID-435).

Susceptibility to antibiotics

All isolates were subjected to susceptibility testing against 19 antibiotics (Tab. I). Table I provides an overview of the different antibiotic classes and specific antibiotic used for susceptibility tests. The antibiogram was conducted using the Phoenix M50 automated system (panel 449044-NMIC/ID-435) and was complemented by disk diffusion technique for antibiotics not covered by the automated panel. The results were expressed as susceptible or resistant in accordance with the guidelines previously described (21).

ESBL production was detected using the combination disk test method with the following combinations: ceftazidime-clavulanate, cefepime-clavulanate, and cefotaxime-clavulanate (21). *Klebsiella pneumoniae* ATCC 700603 was used as quality control strain.

TABLE I - Antibiotic classes and specific antibiotics used in this study

Family	Antibiotics
Beta-lactam antibiotics	Amoxicillin (20 µg)
	Amoxicillin (20 µg) + clavulanic acid (10 µg)
	Piperacillin (30 µg)
	Piperacillin (30 µg) + tazobactam (6 µg)
	Ticarcillin (75 µg)
	Cefuroxime (30 µg)
	Cefoxitin (30 µg)
	Ceftazidime (10 µg)
	Ceftriaxone (30 µg)
	Cefepime (30 µg)
	Aztreonam (30 µg)
	Imipenem (10 µg)
	Meropenem (10 µg)
	Ertapenem (10 µg)
Aminosides	Amikacin (30 µg)
	Gentamicin (10 µg)
	Tobramycin (10 µg)
Quinolones	Ciprofloxacin (05 µg)
Other	Trimethoprim (1.25 µg) + sulfamethoxazole (23.75 µg)

Molecular analysis

DNA was extracted according to the method described previously (22). Briefly, pure colonies were suspended in 200 µL of Tris-ethylenediamine tetraacetic acid (EDTA) solution, heated at 100°C for 10 minutes, and then immediately placed at −20°C for 5 to 10 minutes. After centrifugation at 12,000 rpm for 10 minutes, the obtained supernatant was used as DNA template. Quality control of the extraction was carried out using Thermo Scientific NanoDrop One/One^{C} instrument at the molecular biology unit of the University Centre for Clinical Research (UCRC) in Bamako.

Using conventional polymerase chain reaction (PCR), eight virulence factor genes from *Escherichia coli*, namely *bfp, eae, eagg, ipah, slt1, slt2, lt* and *sta*, as well as ESBL coding genes, namely bla_{TEM} and bla_{SHV}, were characterized. In addition, the following genes were screened for their role in conferring antibiotic resistance: the *catA1* gene responsible for encoding chloramphenicol acetyltransferase, mutations on genes encoding for topoisomerase IV, and DNA gyrase protective proteins targeted by quinolones (*qnrA, qnrB, qnrS*), as well as class 1 (*int1*), class 2 (*int2*), and class 3 (*int3*) integrons, which carry resistance genes for multiple antibiotics. We screened bacterial isolates for the presence of carbapenemase genes, namely: bla_{KPC}, bla_{NDM-1}, and bla_{OXA-48}.

PCR was performed using ABI 9700 thermocycler (Applied Biosystems, USA) with the following cycling parameters: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at variable temperature for each primer set for 39 seconds, and extension at 72°C for 60 seconds (Tab. II). Table II provides essential information about selected genes, including their nucleotide sequences, hybridization temperatures, amplicon sizes. The final extension step was carried out at 72°C for 5 minutes.

TABLE II - Primer sequences and characteristics of selected genes

Gene names	Nucleotide sequences (5′ → 3′)	Hybridization temperature	Amplicon size	Reference
Adhesin genes				22
bfp	GAC ACC TCA TTG CTG AAG TCG	57°C	324 bp	
	CCA GAA CAC CTC CGT TAT GC			
eae	TCA ATG CAG TTC CGT TAT CAG TT	65°C	494 bp	
	GTA AAG TCC GTT ACC CCA ACC TG			
ipaH	GAA AAC CTC CTG GTC CAT CAG G	53°C	424 bp	
	GCC GGT CAG CCA CCC TCT GAG AGT AC			
Eagg	ACG CAG AGT TGC CTG ATA AAG	53°C	630 bp	
	AAT ACA GAA TCG TCA GCA TCA GC			
Toxin genes				
slt1	TTT ACG ATA GCA TTC TCG AC	56°C	130 bp	
	CAC ATA TAA ATT ATT TCG CTC			
slt2	CTT CAC GTC ACC ATA CAT AT	56°C	346 bp	
	ACG ATG TGG TTT ATT CTG GA			
lt	GGC GAC AGA TTA TAC CGT GC	56°C	707 bp	
	CCG AAT TCT GTT ATA TAT GTC			
sta	TTA ATA GCA CCC GGT ACA AGC AGG	43°C	146 bp	
	CTT GAC TCT TCA AAA GAG AAA ATT AC			
Antibiotic resistance genes				
int1	ACATGTGATGGCGACGCA CGA	57°C	580 bp	
	ATTTCTGTCCTGGCTGGC GA			
int2	GTAGCAAACGACTGACGAAAT G	62°C	806 bp	
	CACGGATATGCGACAAAA AGG T			
int3	GCC CCG GCA GCG ACT TTC AG	62°C	1200 bp	
	ACG GCT CTG CCA AAC CTG ACT			
SHV	TTATCTCCCTGTTAGCCACC	55°C	800 bp	
	GATTTGCTGATTTCGCTCGG			
Tem	ATAAAATTCTTGAAGACGAAA	55°C	850 bp	
	GACAGTTACCAATGCTTAATC			

Gene names	Nucleotide sequences (5' → 3')	Hybridization temperature	Amplicon size	Reference
catA1	CCTGCCACTCATCGCAGTAC	57°C	450 bp	
	CTGCCTGGACAACATTGCTT			
QnrA	TCAGCACAAGAGGATTTCTC	55°C	657 bp	
	GGCAGCACTATTACTCCCA			
QnrB	GATCGTGAAAGCCAGAAAGG	55°C	469 bp	
	ACGATGCCTGGTAGTTGTCC			
QnrS	ACGACATTCGTCAACTGCAA	55°C	417 bp	
	TAAATTGGCACCCTGTAGGC			
Carbapenemase				27
bla_{KPC}	CATTCAAGGGCTTTCTTGCTGC	55°C	538 bp	
	ACGACGGCATAGTCATTTGC			
bla_{OXA-48}	GCTTGATCGCCCTCGATT	55°C	281 bp	
	GATTTGCTCCGTGGCCGAAA			
bla_{NDM-1}	ATGGAATTGCCCAATATTATGCAC	55°C	813 bp	
	TCAGCGCAGCTTGTCGGC			

Each reaction was carried out in a 25 µL mixture prepared as described previously, with modifications (22). In all reactions, a negative control (water) was included alongside a positive control consisting of reference isolates (E2348-69, M90T, EDL 933, EDL 1493, R3, R4, R5, R6, and R7).

The sequence of primers used for amplification and the expected amplicon size are detailed in Table II. PCR products were visualized by transillumination after migration in 1.5% Tris base, acetic acid and EDTA (TAE) buffer.

Statistical analysis

The data were analyzed using IBM SPSS Statistics for Windows, Version 23.0. Student's t-test was used to compare mean values of continuous variables, while chi-square test was employed to analyze categorical variables. A p-value of less than 0.05 was considered to indicate statistical significance. Statistical analysis of AMR data was performed using the software R (version 4.3.0) and the integrated development environment R Studio (version 2023.03.1+446). The package "AMR" was employed for AMR data processing (23).

Ethical approval

Written consent was obtained from all included participants, and the study protocol was subjected to review and approval by the Ethics Committee of the University of Sciences, Techniques, and Technologies of Bamako. The approval was granted under reference number 2021/228/USTTB on June 9, 2021.

Results

Sociodemographic characteristics of patients

The study specifically analyzed pleural fluid samples obtained from 526 patients with pleurisy, out of which 110 were diagnosed with enterobacterial pleuritis (Tab. III). Table III provides an overview of patient characteristics and their distribution within the thoracic surgery and pediatrics' ward. It allows for a comparison between the two groups and helps identify any statistically significant differences in

TABLE III - Patient characteristics and distribution by thoracic surgery and pediatric wards

	Thoracic surgery	Pediatrics	Total	P
Characteristics	n = 92 (%)	n = 18 (%)	n = 110 (%)	0.000
Gender				1.000
Male gender	59 (64.1)	12 (66.7)	71 (64.5)	
Female gender	33 (35.9)	6 (33.3)	39 (35.5)	
Median age	42	8.5	37.5	
Age group				
0–4	–	13 (72.2)		0.000
5–9	–	5 (27.8)		
10–14	–			
15–19	3 (3.3)	–		
20–24	3 (3.3)	–		
25–29	16 (17.4)	–		0.000
30–34	12 (13.0)	–		
35–39	6 (6.6)	–		
40–44	10 (10.9)	–		
45–49	6 (6.5)	–		
50–54	10 (10.9)	–		
55–59	9 (9.8)	–		
60–64	8 (8.7)	–		
65–69	4 (4.3)	–		
70–90	5 (5.4)	–		

gender, age, and age group distribution. A total of 71 (64.5%) patients were men and 39 (35.5%) were women with a male-to-female sex ratio of 1.8. The majority of the patients (76/110, 69.1%) resided in urban areas. Pediatric patients aged 0-4 years, and young adults (25-29 years) constituted 72.2% of the cases, highlighting the vulnerability of these age groups to the condition. The observed distribution within these age groups was found to be statistically significant (p = 0.000).

Bacterial diversity and antibiotics resistance profile

The three main pathogens isolated in this study were *Escherichia coli* (44.5%; n = 49), *Klebsiella pneumoniae* (11.8%; n = 13), and *Proteus mirabilis* (13.6%; n = 15). Antibiotic resistance profile of *E. coli*, *P. mirabilis*, and *K. pneumoniae* was assessed against several antibiotics (Tab. I). Results showed marked differences in antibiotic susceptibility between β-lactams, cephalosporins, and carbapenems. β-Lactams showed no activity against the three Enterobacteriaceae; second-generation cephalosporins had shown moderate activity against *E. coli* and *K. pneumoniae* (65.3% and 53.8% respectively) but had no activity against *P. mirabilis*, while third-generation cephalosporins showed moderate activity against all three types of isolates, with susceptibility rates ranging from 8.2% to 91.8%. In contrast, all carbapenems had high activity against all three types of isolates. In terms of efficacy, *E. coli* and *P. mirabilis* isolates exhibited higher susceptibility to the combination of penicillins and β-lactamase inhibitors (piperacillin/tazobactam [TZP]); however, they were moderately to highly resistant to trimethoprim/sulfamethoxazole (Tab. IV). Table IV provides valuable information on the susceptibility patterns of *E. coli, P. mirabilis,* and *K. pneumoniae* to various antibiotics. It assists in understanding the effectiveness of different antibiotics against these bacterial species, aiding in the selection of appropriate treatment options.

The combination disk test method revealed that 33.6% (n = 37) of all isolates were ESBL producing. *E. coli* had the highest prevalence at 24.5% (n = 27), followed by *K. pneumoniae*, *Enterobacter cloacae*, and others.

It is noteworthy that a substantial proportion of the patients, precisely 77.3% (85/110), had previously undergone at least one course of antibiotic treatment before the sampling procedure. Moreover, an alarming 94.5% of the isolates displayed multidrug-resistant (MDR) profiles, as per the guidelines set forth by Magiorakos et al (24).

Molecular characterization of genes

Table V lists the frequencies of the virulence genes identified in *E. coli* isolates. Table V provides valuable information on the presence and distribution of genes related to adhesins, enteroxins, antibiotic resistance, and carbapenemases in *E. coli* and related species. It helps in understanding the genetic characteristics and potential resistance patterns of these bacterial strains.

Eagg was found in 6.1% (n = 3) of the *E. coli* isolates while *bfp* and *eae* were not detected; the gene *ipah* was found in a higher percentage (n = 27; 55.1%). Among isolates, bla_{TEM} was the most common ESBL, being present in 29.7% of *E. coli.* In contrast, bla_{SHV}, *int2, int3, qnrS, qnrA,* and *qnrB* genes were not detected. In terms of carbapenemase genes, bla_{NDM-1} was detected (Tab. V).

TABLE IV - Antibiotic susceptibility profiles of *Escherichia coli, Proteus mirabilis*, and *Klebsiella pneumoniae*

Antibiotics	*E. coli*		*P. mirabilis*		*K. pneumoniae*	
	S%	R%	S%	R%	S%	R%
Amoxicillin	0	49 (100)	0	15 (100)	0	13 (100)
Amoxi + clavulanic acid	4 (8.2)	45 (91.8)	0	15 (100)	2 (15.4)	11 (84.6)
Piperacillin	0	49 (100)	0	15 (100)	0	13 (100)
Piperacillin + tazobactam	46 (93.9)	3 (6.1)	12 (80)	3 (20)	10 (76.9)	3 (23.1)
Ticarcillin	0	49 (100)	2 (13.3)	13 (86.7)	0	13 (100)
Aztreonam	46 (93.9)	3 (6.1)	11 (73.3)	4 (26.7)	12 (92.3)	1 (7.7)
C2G	4 (8.2)	45 (91.8)	0	15 (100)	3 (23.1)	10 (76.9)
C3G	4 (8.2)	45 (91.8)	2 (13.3)	13 (86.7)	5 (38.5)	8 (61.5)
Cefoxitin	32 (65.3)	17 (34.7)	0	15 (100)	7 (53.8)	6 (46.2)
Ertapenem	48 (98.0)	1 (2.0)	14 (88.2)	1 (6.7)	11 (84.6)	2 (15.3)
Imipenem	46 (93.9)	3 (6.1)	11 (73.3)	4 (26.7)	11 (84.6)	2 (15.3)
Amikacin	39 (79.6)	10 (20.4)	10 (66.7)	5 (33.3)	9 (69.2)	4 (30.8)
Gentamicin	12 (24.5)	37 (75.5)	2 (13.3)	13 (86.7)	5 (38.5)	8 (61.5)
Tobramycin	13 (26.5)	36 (73.5)	2 (13.3)	13 (86.7)	5 (38.5)	8 (61.5)
Ciprofloxacin	5 (10.2)	44 (89.8)	6 (40)	9 (60)	4 (30.7)	9 (69.2)
Trimethoprim + sulfamethoxazole	2 (4.1)	47 (95.9)	6 (40)	9 (60)	3 (23.1)	10 (76.9)

TABLE V - Distribution of genes of adhesins, enteroxins, antibiotic resistance, and carbapenemases in *Escherichia coli* and related species

Genes of adhesins	***Escherichia coli* n = 49 (%)**			
Eagg	3 (6.1)			
Bfp	0			
eae	0			
ipaH	27 (55.1)			
Enteroxin genes	***E. coli* n = 49 (%)**			
lt	1 (2.0)			
slt1	1 (2.0)			
slt2	0			
Sta	1 (2.0)			
Antibiotic resistance genes N = 37	***E. coli* n = 27**	***Klebsiella pneumoniae* n = 4**	***Enterobacter cloacae* n = 2**	***Others**** **n = 4**
Tem	11 (29.7)		1 (2.7)	0
SHV	0	–	–	0
Int1	10 (27.0)	4 (10.8)	1 (2.7)	0
Int2	0	0	0	0
Int3	0	0	0	0
QnrS	0	0	0	0
QnrA	0	0	0	0
QnrB	0	0	0	0
CatA1	12 (32.4)	0	0	0
Carbapenemase N = 13	***Proteus penneri* n = 1 (%)**	***K. pneumoniae* n = 2 (%)**	***Providencia rettgeri* n = 2 (%)**	***Others*** ** n = 8**
KPC	0	0	0	0
OXA-48	0	0	0	0
NDM-1	1 (7.7)	1 (7.7)	1 (7.7)	0

Based on molecular screening, only one carbapenemase gene, bla_{NDM-1}, was detected in three different isolates only with single occurrence, namely *K. pneumonia, Providencia rettgeri,* and *Providencia penneri.*

Discussion

The results reported in this study provide valuable insights into the bacteriology of PIs in Mali. By addressing resistance rates toward various antibiotics, and the frequency of ESBL, carbapenemases, and MDR bacteria, this study is the first to comprehensively analyze these factors and lay the groundwork for future clinical studies to determine whether improved bacterial diagnosis and antibiotic selection can positively impact the outcomes of PIs. According to the British Thoracic Society (BTS) Guidelines for Pleural Disease, PI was clinically addressed in this discussion as a case of CPPE or empyema and literature was searched accordingly (20).

Out of the 526 samples analyzed, 244 were positive to culture; of which 110 cultures tested positive for Enterobacteriaceae, indicating a significant number of negative results. These results could potentially be attributed to alternative etiologies or could be a result of previous antibiotic utilization (as 77.3% of our patient's population have admittedly taken at least one antibiotic prior to sampling).

In the current study, *E. coli, K. pneumoniae*, and *P. mirabilis* were the main bacteria identified, indicating their significant role as causative agents of PI. These findings align with previous studies that have also identified the above-mentioned pathogens as the primary contributors to PI (25,26). Furthermore, the study revealed that those pathogens exhibited higher rate of resistance to third-generation cephalosporins, indicating their classification as ESBL phenotypes. The prevalence rates of ESBL reported in this study were significantly lower than a study from Burkina Faso, which reported 70% of ESBL-producing isolates in hospitalized patients (27). Genotypically, the most common ESBL gene was bla_{TEM}. This finding was also reported by Sonda et al (28). We did not detect bla_{SHV} gene.

The observed high resistance among the most common isolates to penicillin, quinolones, cephalosporins, and others is likely attributed to the higher utilization of those antibiotic classes in hospital settings. The existence of such association between antibiotic prescriptions and susceptibility pattern was previously highlighted in another hospital setting in Eritrea (29). Clinicians' choice of broad-spectrum antibiotics or combination therapy may be suggestive of the infection being acquired in a hospital setting. It could be inferred that despite the treatment recommendations as per European Respiratory Society (ERS) and American Association for Thoracic Surgery (AATS), the selection of the treatment regimen must typically depend on infection setting, the local prevalence of microorganisms, and antibiotic resistance patterns (30,31).

Additionally, our study reported a high prevalence of MDR bacteria. This MDR rate aligns with previous study in Turkey reporting the widespread occurrence of MDR bacteria (26). The study findings revealed a higher prevalence of carbapenem resistance (7.2%) compared to the previous report by Dwomoh et al (32), which documented a resistance rate of 5.6%. Hackman et al (33) reported a similar rate of carbapenem resistance. However, our study observed significant disparities in the resistance rates of individual carbapenems (ertapenem, imipenem, meropenem) as well as the TZP combination compared to their study. These variances can be attributed to our study's focus on the three predominant pathogens isolated (*E. coli, K. pneumoniae, P. mirabilis*). On the basis of available data, TZP and carbapenems appear to maintain significant effectiveness against the tested pathogens; the results were in accordance with those of a Malaysian observational cohort study (34). The recorded prevalence aligns with other studies conducted in Africa, such as those conducted in Nigeria (35) and South Africa (36). However, Egypt has recently reported a notably higher prevalence of carbapenem resistance (37).

The relatively low abundance of carbapenem resistance genes further supports this notion, indicating that the isolates in this study may not possess robust mechanisms of resistance to carbapenems. We did not detect cases of bla_{KPC} and bla_{OXA-48} type of carbapenemase but 7.7% of bla_{NDM-1}. In 2022, a study investigating the global epidemiology of *OXA-48*-like β-lactamases, treatment option and pipeline development were conducted by Sara E. Boyd et al (38). The study found that the enzymes of bla_{OXA-48} type are the most common carbapenemases among Enterobacteriaceae in much of western Europe. In Africa, the same study reported circulation of these types of β-lactamase in Tunisia, Algeria, Egypt, and South Africa. In West Africa, cases have been reported in Senegal and Nigeria. Mali did not provide data probably due to lack of adequate health care infrastructure and limited molecular diagnostic capabilities (38). Nabi Jomehzadeh et al found higher levels of bla_{NDM-1} (31%) (39). In 2020, Muggeo et al reported the first description of bla_{NDM-5} in Mali (18). Overall, β-lactam/β-lactam inhibitor combinations and carbapenems are suitable choices, as presented previously in observational studies and guidelines (40-42). However, it is crucial to interpret these results within the context of the provided data. The analysis is limited to the specific isolates and may not be representative of the broader population or other geographical regions.

According to this study, the presence of virulence factor genes in *E. coli* isolates was limited to a specific set. The occurrence of these factors varied, ranging from 2% for genes *sta, slt*, and *lt*, to 55.1% for the *ipah* gene. The *ipah* gene encodes Invasion Plasmid Antigen and is closely linked to immune system modulation in the host and bacterial survival. It is commonly detected in the majority of EIEC isolates (43).

The study has certain limitations. Firstly, it was conducted in a single center, which might restrict the applicability of the results to other populations or settings. Additionally, the sample size was relatively small, warranting larger multicenter studies to validate the findings. The study also lacked detailed information on patients' antibiotic exposure history and prior hospitalizations, which could have influenced the prevalence of antibiotic resistance. Despite these limitations, the study emphasizes the significant prevalence of antibiotic resistance in the examined setting and underscores the ongoing need for surveillance and efforts in antibiotic stewardship to address this critical public health concern. Further research involving larger sample sizes, multicenter designs, and comprehensive patient data would greatly enhance our understanding and approach toward combating antibiotic resistance in our region.

In countries with limited resources such as those in West Africa, more specific socioeconomic and behavioral factors contribute to exacerbating this threat, among others: (i) certain common societal practices such as self-medication; (ii) a failing medical sector with insufficiently trained prescribers and inefficient diagnostic tools; or (iii) an uncontrolled drug chain with over-the-counter, improperly stored, counterfeit, and/or expired antibiotics favor the emergence of resistance. This study on the prevalence and characteristics of ESBL and carbapenemase-producing Enterobacteriaceae in pleural effusion in Mali holds great significance for low- and middle-income countries. By providing critical insights into the extent of resistance and molecular epidemiology, this research will facilitate the development of effective strategies to combat AMR, improve patient outcomes, and safeguard public health in Mali.

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ORIGINAL RESEARCH ARTICLE

Identification of anti-pathogenic activity among *in silico* predicted small-molecule inhibitors of *Pseudomonas aeruginosa* LasR or nitric oxide reductase (NOR)

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ABSTRACT

Introduction: Antibiotic-resistant *Pseudomonas aeruginosa* strains cause considerable morbidity and mortality globally. Identification of novel targets in this notorious pathogen is urgently warranted to facilitate discovery of new anti-pathogenic agents against it. This study attempted to identify small-molecule inhibitors of two important proteins LasR and nitric oxide reductase (NOR) in *P. aeruginosa*. 'Las' system can be said to be the 'master' regulator of quorum sensing in *P. aeruginosa*, whose receptor protein is LasR. Similarly, NOR is crucial to detoxification of reactive nitrogen species.

Methods: *In silico* identification of potential LasR or NOR inhibitors was attempted through a virtual screening platform AtomNet® to obtain a final subset of <100 top scoring compounds. These compounds were evaluated for their *in vivo* anti-pathogenic activity by challenging the model host *Caenorhabditis elegans* with *P. aeruginosa* in the presence or absence of test compounds. Survival of the worm population in 24-well assay plates was monitored over a period of 5 days microscopically.

Results: Of the 96 predicted LasR inhibitors, 11 exhibited anti-*Pseudomonas* activity (23%-96% inhibition of bacterial virulence as per third-day end-point) at 25-50 µg/mL. Of the 85 predicted NOR inhibitors, 8 exhibited anti-*Pseudomonas* activity (40%-85% inhibition of bacterial virulence as per second-day end-point) at 25-50 µg/mL.

Conclusion: Further investigation on molecular mode of action of compounds found active in this study is warranted. Virtual screening can be said to be a useful tool in narrowing down the list of compounds requiring actual wet-lab screening, saving considerable time and efforts for drug discovery.

Keywords: Antimicrobial resistance (AMR), Nitric oxide, Nitrosative stress, Priority pathogen, *Pseudomonas aeruginosa*, Quorum sensing (QS), Virulence

Introduction

Antimicrobial resistance (AMR) among infectious bacteria has emerged as a healthcare challenge of global concern (Online). The World Health Organization (WHO) has also published a global action plan on AMR in 2015. As per the Indian National Action Plan on Antimicrobial Resistance (NAP-AMR: 2017-2021), India is among the nations with the highest burden of bacterial infections. The crude mortality from infectious diseases in India today is 417 per 100,000 persons. The situation in other developing countries is equally grave. Murray et al estimated 1.27 million deaths attributable to bacterial AMR in 2019 (1). Of the six leading pathogens identified by them responsible for maximum death toll, one is *Pseudomonas aeruginosa*. It is among the most notorious pathogenic bacteria, and its carbapenem-resistant phenotype has been listed by the WHO among priority pathogens for the development of new antibiotics (Online). Antibiotic-resistant *P. aeruginosa* has been listed as an important pathogen by the U.S. Centers for Disease Control and Prevention (CDC) (Online) as well as the Department of Biotechnology, India (DBT) (Online) against which new antimicrobials are urgently required.

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The pipeline for new antibiotics does not contain sufficient number of promising candidates (2). There is a dearth of novel antibacterial leads as well as targets (3,4). Identification and validation of new targets in important pathogens is of utmost significance (5,6). Conventional bactericidal antibiotics attack a very narrow range of targets in susceptible bacteria, for example, cell wall synthesis, protein synthesis, nucleic acid synthesis, etc. Since the past decade, there has been much interest in the research community regarding discovery of anti-virulence molecules, which may attenuate the bacterial virulence without necessarily killing them. Such 'pathoblockers' are expected to compromise the ability of the pathogen to damage the host, by exerting their effect on nonessential targets such as bacterial quorum sensing (QS) (7), stress-response machinery (8), metal homeostasis (9,10), etc. The next-generation antibacterials preferably should be attacking such hitherto unexplored/underexplored targets in pathogenic bacteria (11-13).

Proper functioning of the chemical signal-based intercellular communication, known as QS, is crucial for sufficient expression of virulence in pathogens like *P. aeruginosa*, as QS is an effective mechanism for regulating expression of multiple genes associated with a multitude of functions (14). Interrupting bacterial QS can be an effective strategy to combat pathogens (15,16). QS consists of two components: signal generation and signal response, respectively, encoded by LuxI and LuxR homologues (17,18). Inhibiting the 'signal response' component of QS (e.g. LasR in *P. aeruginosa*) can notably compromise their ability to exert collective behaviour in response to environmental changes or host defence. *P. aeruginosa* regulates its drug resistance and pathogenicity through multiple QS mechanisms including the LasI/R, RhlI/R, and PQS/MvfR systems. Targeting one or more of these QS systems may prove an effective way of dealing with *P. aeruginosa* infections (19). Owing to the important role of Las system in overall QS circuit of *P. aeruginosa*, its receptor protein LasR is believed to be a plausible anti-virulence target (20). LasR is a transcriptional activator of various virulence-associated genes in *P. aeruginosa*, which recognizes a specific signal molecule, namely *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3O-C12-HSL) (21). The LasR-3O-C12-HSL complex triggers the expression of multiple QS-regulated genes. Potential LasR inhibitors either may prevent its binding with its natural ligand or compromise its ability to affect expression of target genes (22). QS not being essential for bacterial survival, its inhibitors are expected to exert lesser selection pressure on bacterial population with respect to development of resistant phenotypes (23). Additionally, due to the overlap in QS systems among various gram-negative bacteria (24-25), inhibitors effective against one gram-negative species may have broad-spectrum activity against multiple other gram-negative pathogens.

Pathogens striving to survive inside a host body are forced to face a variety of stresses such as iron deprivation, oxidative stress, nitrosative stress, etc. Bacteria employ antioxidant enzymes to counter reactive oxygen species, and similarly certain other enzymes to counter reactive nitrogen species (26). From the work done in our lab as well as literature survey, we consider the components of *P. aeruginosa* genome involved in responding to nitrosative stress to be potential targets. Among the components of nitrosative stress response in *P. aeruginosa*, one important enzyme is nitric oxide reductase (NOR). This protein was one of the major targets of an anti-infective polyherbal formulation (*Panchvalkal*) investigated by us in the recent past (6,27). NOR also emerged among the top differentially expressed genes in *P. aeruginosa* treated by us with other anti-virulence polyherbal formulations Enteropan (SRX15248092) or colloidal silver (SRX14392191) at sub-lethal levels.

NOR is an important detoxifying enzyme in *P. aeruginosa*, which is crucial to its ability to withstand nitrosative stress (e.g. in the form of nitric oxide [NO]). NOR has also been reported (28,29) to be important for virulence expression of this pathogen, and thus can be a plausible target for novel anti-virulence agents. Molecules capable of inhibiting NOR can be expected to compromise the pathogen's ability to detoxify NO, not allowing its virulence traits (e.g. biofilm formation, as NO has been indicated to act as a biofilm-dispersal signal) to be expressed fully (30). The test molecules capable of inhibiting NOR may emerge as novel anti-biofilm agents not only against *P. aeruginosa* but against multiple pathogens as NO is reported to be perceived as a dispersal signal by various gram-negative and gram-positive bacteria (31). Thus, NOR inhibitors may be expected to have a broad-spectrum activity against multiple pathogens. Major function of NOR is to detoxify NO generated by nitrite reductase (NIR). NO is a toxic by-product of anaerobic respiration in *P. aeruginosa*. NO-derived nitrosative species can damage deoxyribonucleic acid (DNA) and compromise protein function. Intracellular accumulation of NO is likely to be lethal for the pathogen (32). It can be logically anticipated that *P. aeruginosa*'s ability to detoxify NO will be compromised under the influence of a potent NOR inhibitor. Since NO seems to have a broad-spectrum anti-biofilm effect, NOR activity is essential for effective biofilm formation by the pathogens. NOR activity and NO concentration can modulate cellular levels of cyclic di-GMP, which is a secondary messenger molecule recognized as a key bacterial regulator of multiple processes such as virulence, differentiation, and biofilm formation (33). In mammalian pathogens, the host's macrophages are a likely source of NO. NOR expressed by the pathogen provides protection against the host defence mechanism. Since NOR activity is known to be important in multiple pathogenic bacteria (e.g. *P. aeruginosa, Staphylococcus aureus, Serratia marcescens*) for biofilm formation, virulence expression, combating nitrosative stress, and evading hose defence, NOR seems to be an important target for novel anti-pathogenic agents. Any molecule capable of interfering with bacterial NOR activity is likely to be an effective anti-pathogenic agent, since bacterial populations require NOR for various purposes including detoxification and evasion of host defences (34). A potential NOR inhibitor besides troubling the pathogen directly may also boost its clearance by the host macrophages.

Proteins such as LasR and NOR important to the pathogens and whose structure is known can be useful starting point for a drug discovery programme. *In silico* virtual screening tools can be used to screen large chemical libraries

to predict inhibitors of the target proteins. In the recent past, quite a few potent anti-pathogenic compounds have been identified using this approach. For example, one such virtual screening study by Abelyan et al (35) identified benzamides, synthetic derivatives of flavones, as potential inhibitors of LasR. Another *in silico* effort by Narayanaswamy et al (36) identified potent inhibitors of enzymes involved in nitrogen metabolism in various bacteria including *P. aeruginosa*, nitrous oxide reductase (N_2OR), and NIR, from among a library of synthetic and natural compounds.

This study aimed at screening 96 compounds identified through a virtual screen as potential LasR inhibitors, and 85 compounds predicted to be NOR inhibitors *in silico* for their possible anti-virulence activity against *P. aeruginosa* in the model host *Caenorhabditis elegans*. Any such potent NOR or LasR inhibitors identified through this study may prove to be useful lead(s) for novel anti-pathogenic drug development. They can be useful either as standalone therapy or in combination with conventional antibiotics.

Methods

Virtual screening

The virtual screening from a library of approximately 3 million compounds was conducted using AtomNet® screening platform (37). AtomNet® is a proprietary deep learning neural network useful for structure-based drug design and discovery through its small-molecule binding affinity prediction capacity.

Screening for LasR binding ligands

There are a number of available crystal structures of LasR in complex with small molecules, including the endogenous ligand and other agonists. Although discovery of a potent antagonist is preferable, the virtual screen attempted to find novel chemical matter that binds at the desired site and the mode of binding may be analysed later. All of the available crystal structures were considered as receptor templates for virtual screening. The highest resolution structure, PDB 3IX3, chosen as the ligand binding pocket is deep and solvent excluded and appears well-suited for binding small molecules (Fig. 1). The screening volume was restricted to the binding pocket surrounding the existing ligand. A screening library of approximately 3 million compounds was exhaustively pose-sampled and scored, followed by filtering for drug-like properties, and selection of the top 96 compounds for ordering in physical form.

Screening for NOR binding ligands

The structure of *P. aeruginosa* NOR bound to an antibody fragment has been determined by crystallography (PDBID 3O0R). This structure reveals NOR composed of two subunits: NORB containing 12 transmembrane helices and NORC with a transmembrane helix and hydrophilic periplasmic domain. Three haem cofactors are complexed in NOR. Two haems are deep in the NORB transmembrane region, and the third haem spans the NORB-NORC interface.

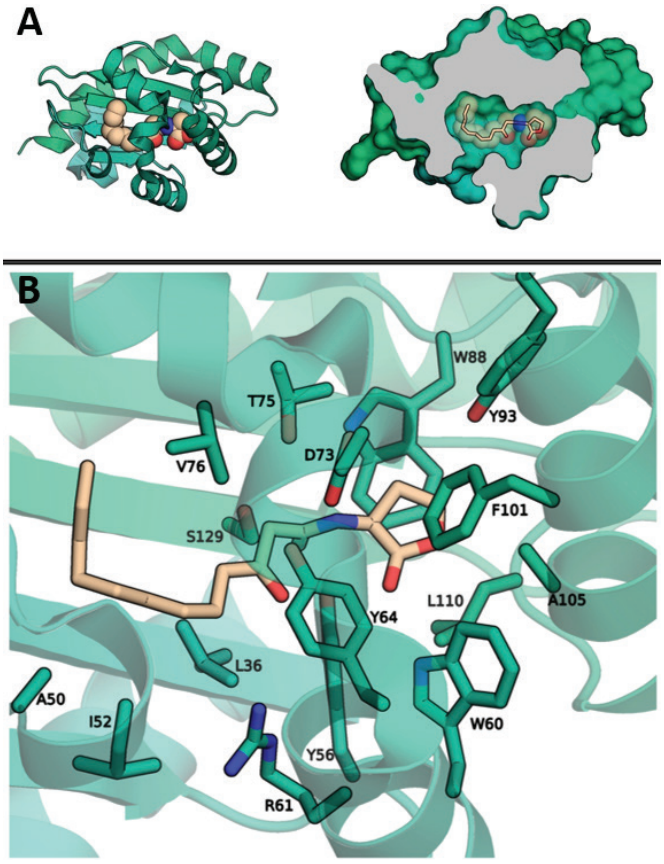

Fig. 1 - Quorum sensing receptor LasR. **A)** The structure of LasR (green) in complex with its natural ligand 3O-C12-HSL (tan). Coordinates taken from PDB 3IX3. **B)** The binding site of LasR with the 3O-C12-HSL ligand is present. Residues expected to make direct contacts with ligands are labelled.

No drug-like small-molecule inhibitors have been reported for NOR, and no druggable pocket is obvious from the reported structure. To identify potential targetable sites, the structure of NOR was analysed by fPocket. This analysis revealed a pocket at the interface of NORB and NORC and near one of the NORB haem cofactors. This positioning suggests that small-molecule binding to this pocket may disrupt norB-norC interactions and/or inhibit haem-mediated electrochemistry. The virtual screen therefore sought to identify small molecules that bind to this pocket in NOR and potentially disrupt enzymatic function. A library of approximately 3 million compounds was screened for identifying compounds capable of virtual binding at the selected target site on NOR (Fig. 2) using AtomNet®. Top scoring compounds were clustered and filtered to arrive at a final subset of 85 deliverable compounds.

Test compounds

Ninety-six compounds showing *in silico* affinity to LasR (Supplementary table S1, List of predicted LasR inhibitors subjected to wet-lab assay) and 85 compounds showing *in silico*

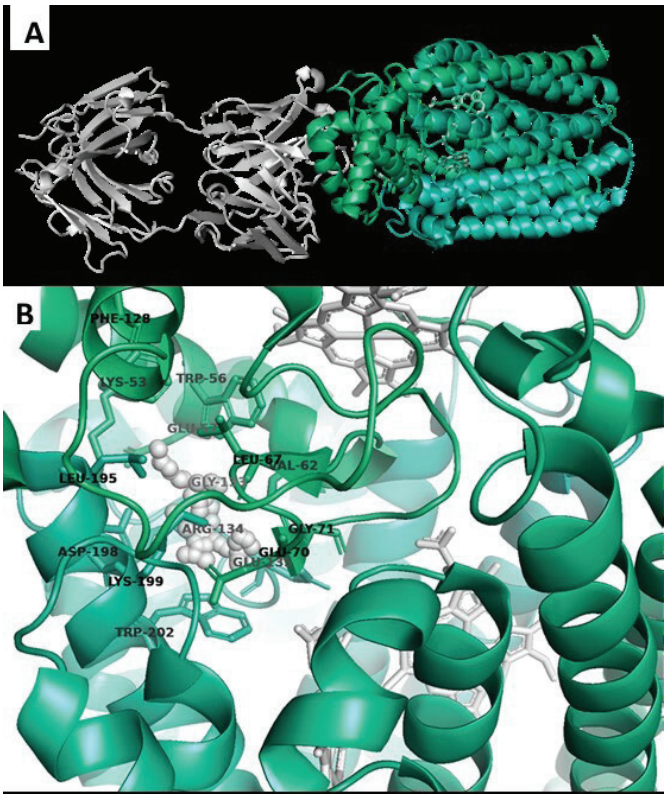

Fig. 2 - Nitric oxide reductase (NOR). **A)** Crystal structure of NOR complexed with an antibody fragment (white cartoons) and with three haem cofactors (white sticks). **B)** Proposed target for virtual screen (white spheres) with surrounding residues from NOR chains B and C showing the proximity to the haem cofactors.

affinity to NOR (Supplementary table S2, List of predicted NOR inhibitors subjected to wet-lab assay) were ordered in physical form from Enamine (Kyiv, Ukraine) and Mcule (Budapest, Hungary), respectively. Test compounds were stored in the refrigerator and reconstituted in dimethyl sulfoxide (DMSO; 500-1000 µL) (Merck) on the day of assay.

Bacterial strain

The *P. aeruginosa* strain used in this study was sourced from our internal culture collection, which has been characterized by us for its antibiotic susceptibility/resistance, pigment production, and certain other virulence traits. Its antibiogram (Online) generated through a disc-diffusion assay performed as per the National Committee for Clinical Laboratory Standards (NCCLS) guidelines revealed it to be resistant to eight antibiotics (cotrimoxazole, augmentin, nitrofurantoin, ampicillin, chloramphenicol, clindamycin, cefixime, and vancomycin) belonging to five different classes. Hence it can be described as a multidrug-resistant (MDR) strain. As reported in our earlier publications (27,38) with this strain, it is a haemolytic strain capable of producing the QS-regulated pigments (pyocyanin and pyoverdine), and also of biofilm formation. We maintained this bacterium on *Pseudomonas* agar (HiMedia). While culturing the bacteria for *in vivo* assay, they were grown in *Pseudomonas* broth (magnesium chloride 1.4 g/L, potassium sulphate 10 g/L, peptic digest of animal tissue 20 g/L, pH 7.0 ± 0.2).

Nematode host

C. elegans (N2 Bristol) was used as the model host in this study. Worms were maintained on nematode growth medium (NGM): 3 g/L NaCl (HiMedia, MB023-500G), 2.5 g/L peptone (HiMedia), 17 g/L agar-agar (HiMedia), 1 M $CaCl_2$ (HiMedia), 1 M $MgSO_4$ (Merck), 5 mg/mL cholesterol (HiMedia), 1 M phosphate buffer of pH 6, agar plate with *Escherichia coli* OP50 (LabTIE B.V., the Netherlands) as food. For synchronization of the worm population, adult worms from a 4- to 5-day-old NGM plate were first washed with distilled water, and then treated with 1 mL of bleaching solution (water + 4% NaOCl [Merck] +1 N NaOH [HiMedia] in 3:1:1 proportion), followed by centrifugation (1,500 rpm at 22°C) for 1 min. Eggs in the resultant pellet were washed multiple times with sterile distilled water, followed by transfer onto a new NGM plate seeded with *E. coli* OP50. L3-L4 stage worms appearing on this plate after 2-3 days of incubation at 22°C were used for further experimentation.

Virulence assay

P. aeruginosa grown in *Pseudomonas* broth (at 35±1°C for 21 hours with intermittent shaking) was allowed to attack *C. elegans* (L3-L4 stage) in a 24-well plate (HiMedia) in the presence or absence of test compounds, and their capacity to kill the worm population was monitored over a period of 5 days. In each well, there were 10 worms in M9 buffer (3 g/L KH_2PO_4, 6 g/L Na_2HPO_4, 5 g/L NaCl), which were challenged with *P. aeruginosa* by adding 100 µL (OD_{764} = 1.30) of bacterial culture grown in *Pseudomonas* broth. Appropriate controls, that is, worms, exposed neither to test compound nor to bacteria; worms exposed to test compound, but not to bacterial pathogens (toxicity control); worms challenged with bacteria in the presence of 0.5% v/v DMSO (vehicle control); and worms challenged with bacteria in the presence of 0.5 µg/mL ofloxacin (positive control) were also included in the experiment. Incubation was carried out at 22°C. The number of dead vs. live worms was counted every day for 5 days by putting the plate with lid under a light microscope 4× objective. Straight non-moving worms were considered to be dead. Plates were gently tapped to confirm lack of movement in the apparently dead worms. On the last day of the experiment, when plates could be opened, their death was also confirmed by prodding them with a straight wire, wherein no movement was taken as confirmation of death.

Statistics

Results reported are means of three replicates. Statistical significance was assessed through a t-test performed using Microsoft Excel. Values of $p \leq 0.05$ were considered to be significant.

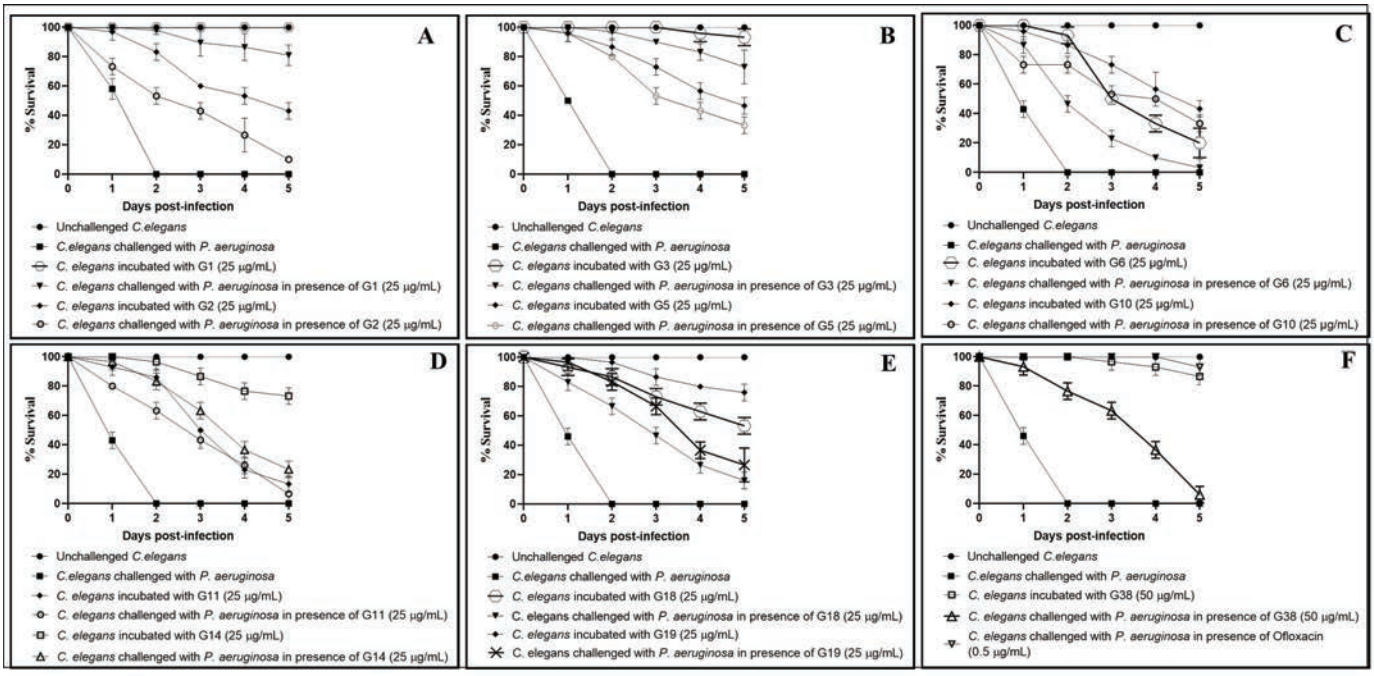

Fig. 3 - *Pseudomonas aeruginosa*'s virulence towards the host worm gets attenuated in the presence of certain predicted LasR inhibitors. **A)** *P. aeruginosa* could kill 90%±9.19%** and 43%±5.7%*** lesser worms in the presence of G1 (Z30981775) and G2 (Z65195564), respectively. **B)** *P. aeruginosa* could kill 90%±0%*** and 53%±5.7%*** lesser worms in the presence of G3 (Z212728858) and G5 (Z354444420), respectively. **C)** *P. aeruginosa* could kill 23%±5.7%*** and 53%±5.7%*** lesser worms in the presence of G6 (Z400859658) and G10 (Z1426094174), respectively. **D)** *P. aeruginosa* could kill 43%±5.7%*** and 63%±5.7%*** lesser worms in the presence of G11 (Z1625994950) and G14 (Z104586200), respectively. **E)** *P. aeruginosa* could kill 46%±5.7%*** and 67%±5.7%*** lesser worms in the presence of G18 (Z1084397894) and G19 (Z1212781307), respectively. **F)** *P. aeruginosa* could kill 63.3%±5.7%*** and 100%±0%*** lesser worms in the presence of G38 (Z89293640) and ofloxacin (0.5 µg/mL), respectively. Later it was employed as a positive control at its sub-minimum inhibitory concentration (MIC) level, and it did allow progeny formation in worm population from the third day onwards.
Dimethyl sulfoxide (DMSO; 0.5% v/v) present in the 'vehicle control' neither affected virulence of the bacterium towards *Caenorhabditis elegans,* nor did it show any effect on worm survival. **p<0.01, ***p<0.001. The percent values reported pertain to the worm survival 3 days post-infection.

Results

Anti-pathogenic activity of potential LasR inhibitors

Results of anti-virulence assay for all active compounds are presented in Fig. 3. Since *P. aeruginosa* strain used by us could kill all worms within 18-36 hours, any end-point beyond that can be taken as valid for labelling any compound as 'active' or 'inactive'. However, to have more robust interpretation, we continued worm counting in assay plates till 5 days for comparing number of live worms in experimental vs. control wells.

Eleven of the 88 DMSO-soluble compounds (i.e. 12.5%) assayed exhibited anti-*Pseudomonas* activity (23%-96% as per third-day end-point) at 25-50 µg/mL (Tab. I). These 11 compounds (G1, G2, G3, G5, G6, G10, G11, G14, G18, G19, and G38) should be tested at still lesser concentrations to find out minimum effective concentration (MEC). Eight of the test compounds were found to possess dual activity, that is, anti-pathogenic as well as anthelmintic. Eight of the active anti-*Pseudomonas* compounds (G2, G5, G6, G10, G11, G14, G18, G19) identified in this study were also toxic to the host worm at concentrations employed. Hence, they should be tested at still lower concentrations. It is possible that their lower concentrations may exhibit anti-pathogenic

TABLE I - List of predicted LasR inhibitors found to possess *in vivo* anti-*Pseudomonas aeruginosa* activity

Lab code	Manufacturer's code	Conc. (µg/mL)	% reduction in bacterial virulence	
			3rd day end-point	5th day end-point
G1	Z30981775	25	90 ± 9.19**	82 ± 7.07***
G2	Z65195564	25	43 ± 5.7***	10 ± 0***
G3	Z212728858	25	90 ± 0***	73 ± 11.5***
G5	Z354444420	25	53 ± 5.7***	33 ± 5.7***
G6	Z400859658	25	23 ± 5.7***	3 ± 5.7
G10	Z1426094174	25	53 ± 5.7***	33 ± 5.7***
G11	Z1625994950	25	43 ± 5.7***	6 ± 11.5
G14	Z104586200	25	63 ± 5.7***	23 ± 5.7***
G18	Z1084397894	25	46 ± 5.7***	16 ± 5.7**
G19	Z1212781307	25	67 ± 5.7***	26 ± 11.5**
G38	Z89293640	50	63.3 ± 5.7***	6 ± 5.7

**p<0.01, ***p<0.001.

TABLE II - Anti-pathogenic activity of potential LasR inhibitors may be masked by their anthelmintic activity

Lab code	Manufacturer's code	Conc (µg/mL)	% anti-pathogenic activity based on fifth day end-point	
			Without nullifying compound's toxicity towards worms	After nullifying compound's toxicity towards worms
G2	Z65195564	25	10 ± 0***	67 ± 0***
G5	Z354444420	25	33 ± 5***	87 ± 5***
G6	Z400859658	25	3 ± 5.7	83 ± 5.7***
G10	Z1426094174	25	33 ± 5***	90 ± 5***
G11	Z1625994950	25	6 ± 11.5	93 ± 11.5***
G14	Z104586200	25	23 ± 5.7**	50 ± 5.7***
G18	Z1084397894	25	16 ± 5.7**	63 ± 5.7***
G19	Z1212781307	25	26 ± 11.5**	53 ± 11.5***
G38	Z89293640	50	6 ± 5.7	20 ± 5.7**

**p<0.01, ***p<0.001.

activity without exerting any toxicity towards the eukaryotic host. Masking of the anti-pathogenic activity by anti-worm activity of the same compound (Tab. II) needs to be paid attention while interpreting the results. Compounds found to possess anti-pathogenic activity in our study were effective at 25-50 ppm, which seems to be good enough to warrant further investigation, while comparing with effective concentrations reported for other LasR inhibitors. For example, a potent LasR inhibitor, LasR-IN-4, was shown to possess inhibitory activity against *P. aeruginosa* with MIC of 56.25 µg/mL (39). Another LasR inhibitor, naringenin, was reported to inhibit QS response in *P. aeruginosa* by competing with *N*-(3-Oxo-dodecanoyl)-L-homoserine lactone for LasR binding at 136 µg/mL (40). O'Brien et al (41) reported Br-HSL to antagonize LasR with IC_{50} of 5 µg/mL.

Further, *in vitro* incubation of bacteria with the compounds identified in this study to possess anti-*P. aeruginosa* activity is required to find out whether these compounds exhibit bactericidal/bacteriostatic/anti-virulence activity. While evaluating any compound(s) for their anti-virulence activity, it should be kept in mind that even compounds capable of curbing bacterial virulence partially can be potentially useful in combination with conventional antibiotics. Such compounds may be potential resistance modifiers. Even as a standalone therapy, they may be of indirect help to host immune system by reducing the overall bacterial load to be cleared by the immune system (42,43). Three of these anti-pathogenic compounds (G1, G3, G38) did not exhibit any notable toxicity towards the host worm, and hence seem to be the most logical candidates for further investigation. These compounds should be tested against multiple species of antibiotic-resistant bacteria to know whether they are broad-spectrum antimicrobials. Additionally, their effect on bacterial gene expression at whole transcriptome level should also be investigated to elucidate the underlying molecular mechanisms.

Anti-pathogenic activity of potential NOR inhibitors

Of the total 85 compounds received, 10 were insoluble in the vehicle solvent DMSO. Remaining 75 compounds were assayed for their possible anti-pathogenic activity by challenging the host worm with *P. aeruginosa* in the presence or absence of test compounds. Eight (~11% of all the compounds tested) of the test compounds were able to rescue the worm population from the pathogen-induced death by 40%-85% (second-day end-point) (Fig. 4; Tab. III).

Five of the test compounds were found to possess dual activity, that is, anti-pathogenic as well as anthelmintic. Five of the active anti-*Pseudomonas* compounds (N4, N36, N37, N61, N65) identified in this study were also toxic to the host worm at concentrations employed. Hence, they (excluding N61) should be tested at still lower concentrations. It is possible that their lower concentrations may exhibit anti-pathogenic activity without exerting any toxicity towards the eukaryotic host. Masking of the anti-pathogenic activity by anti-worm activity of the same compound (Tab. IV) needs to be paid attention while interpreting the results.

Conclusion

This study is a preliminary demonstration of the utility of virtual screening approach for discovery of potentially novel anti-pathogenic agents. Virtual screening can reduce the number of compounds required to be actually subjected to wet-lab assays, and thus reducing the investment of labour, time, and money. Among the top 181 compounds predicted through virtual screening to be capable of binding to NOR or LasR of *P. aeruginosa*, we could detect *in vivo* anti-*P. aeruginosa* activity in 19 (i.e. 10.4% of all compounds tested) of them in the model host *C. elegans*. As per our search on PubChem on 1 June 2023, these 19 compounds have yet not been reported to possess any kind of biological activity, and hence we believe this to be the first report of anti-pathogenic activity in these compounds. Further investigation on these active compounds with respect to their mode of action is warranted, which besides confirming their anti-bacterial activity will also provide additional validation to the targetability of NOR and LasR.

Limitations

The anti-virulence assay performed in this study is not specific to LasR or NOR, hence precise mode of action of active anti-pathogenic compounds warrants further confirmatory assays. We could not carry out *in vitro* MIC/MBC assay for active compounds owing to limited quantity at our disposal, hence it was not possible to distinguish between growth-inhibitory and virulence-inhibitory (i.e. non-antibiotic action)

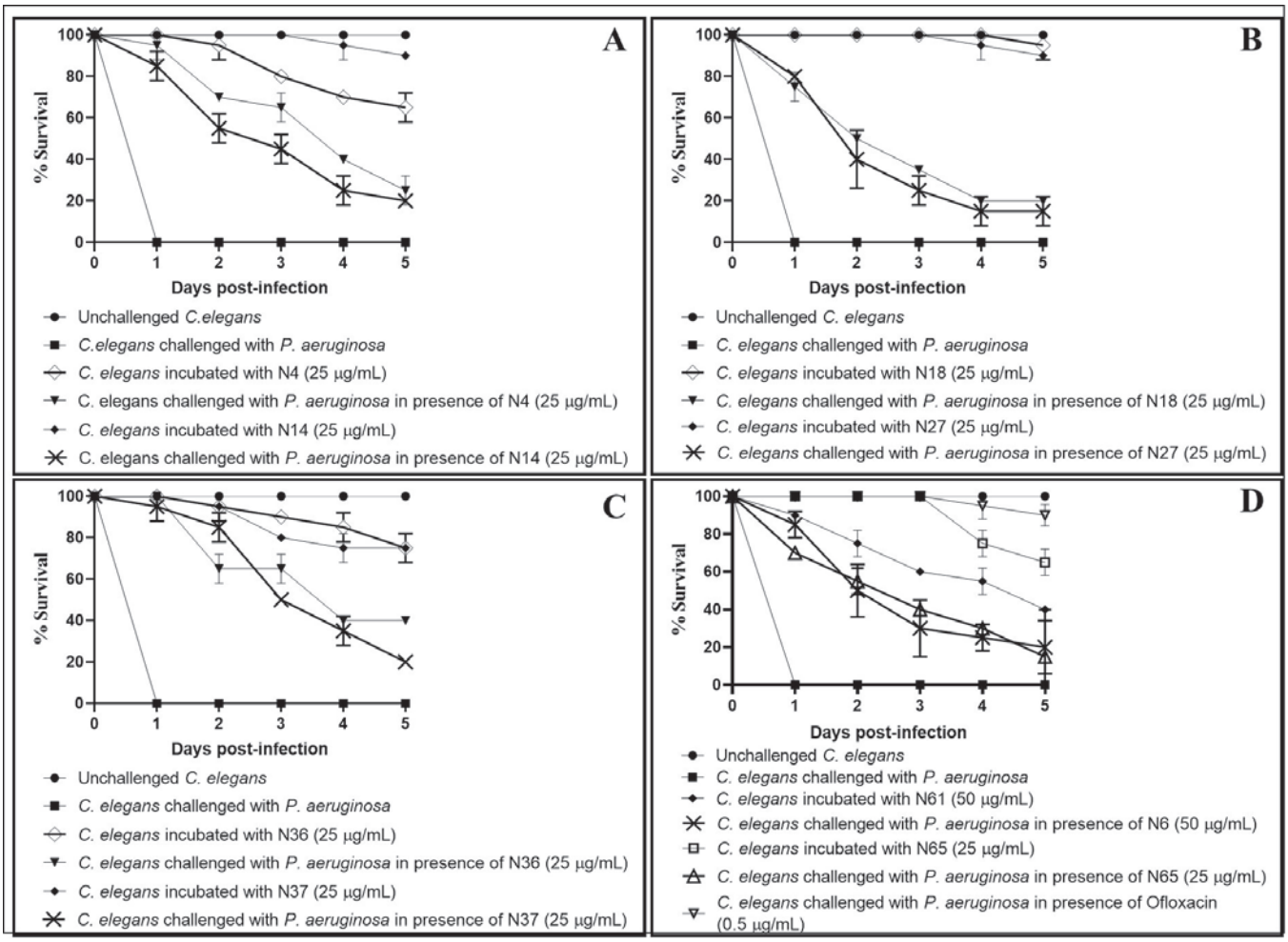

Fig. 4 - *Pseudomonas aeruginosa*'s virulence towards the host worm gets attenuated in the presence of certain predicted nitric oxide reductase (NOR) inhibitors. **A)** *P. aeruginosa* could kill 70%±0%*** and 55%±7%** lesser worms in the presence of N4 (Z954454636) and N14 (Z1765101069), respectively. **B)** *P. aeruginosa* could kill 50%±0%*** and 40%±14%** lesser worms in the presence of N18 (Z110018576) and N27 (Z1611882500), respectively. **C)** *P. aeruginosa* could kill 65%±7%* and 85%±7%** lesser worms in the presence of N36 (Z397755956) and N37 (Z1190350270), respectively. **D)** *P. aeruginosa* could kill 50%±14%**, 55%±0.7%**, and 100%±0%*** lesser worms in the presence of N61 (Z2740017161), N65 (Z1874308288), and ofloxacin (0.5 µg/mL), respectively. Later it was employed as a positive control at its sub-minimum inhibitory concentration (MIC) level, and it did allow progeny formation in worm population from the third day onwards. Dimethyl sulfoxide (DMSO; 0.5% v/v) present in the 'vehicle control' neither affected virulence of the bacterium towards *Caenorhabditis elegans,* nor did it show any effect on worm survival. **p<0.01, ***p<0.001. The percent values reported pertain to the worm survival 2 days post-infection.

TABLE III - List of predicted NOR inhibitors found to possess *in vivo* anti-*Pseudomonas aeruginosa* activity

Lab code	Manufacturer's code	Conc. (µg/mL)	% reduction in bacterial virulence	
			1st day end-point	2nd day end-point
N4	Z954454636	25	95 ± 7***	70 ± 0***
N14	Z1765101069	25	85 ± 7**	55 ± 7**
N18	Z110018576	25	75 ± 7**	50 ± 0***
N27	Z1611882500	25	80 ± 0***	40 ± 14*
N36	Z397755956	25	100 ± 0***	65 ± 7*
N37	Z1190350270	25	95 ± 7**	85 ± 7**
N61	Z2740017161	50	85 ± 7**	50 ± 14**
N65	Z1874308288	25	70 ± 0***	55 ± 0.7**

**p<0.01, ***p<0.001.

NOR = nitric oxide reductase.

Table IV - Anti-pathogenic activity of potential NOR inhibitors may be masked by their anthelmintic activity

Lab code	Manufacturer's code	Conc (µg/mL)	% anti-pathogenic activity based on fifth day end-point	
			Without nullifying compound's toxicity towards worms (A)	After nullifying compound's toxicity towards worms (B)
N4	Z954454636	25	25 ± 7**	60 ± 7**
N36	Z397755956	25	40 ± 0**	65 ± 0***
N37	Z1190350270	25	20 ± 0***	45 ± 0***
N61	Z2740017161	50	20 ± 14	80 ± 14**
N65	Z1874308288	25	15 ± 21	40 ± 21**

**p<0.01, ***p<0.001.

NOR = nitric oxide reductase.

activity. Further, anti-pathogenic activity of some of the compounds might be masked by their anthelmintic activity (Tabs. II-IV).

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Authors contribution: GG: Carried out experiments; participated in manuscript preparation; literature survey; NH and BC: Executed *in silico* part of the study; arranged procurement of test compounds in physical form; VK: Conceptualization, data analysis, and manuscript preparation

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CASE REPORT

First-line tepotinib for a very elderly patient with metastatic NSCLC harboring *MET* exon 14 skipping mutation and high PD-L1 expression

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ABSTRACT

Optimal treatment for metastatic non-small cell lung cancer (NSCLC) with mesenchymal epithelial transition gene (*MET*) exon 14 skipping mutation has not been established yet. MET inhibitors were demonstrated to be effective and tolerated in patients with this condition, while evidence on safety and efficacy of immunotherapy and/or chemotherapy in this population is limited. Here we report the case of an 86-year-old male with metastatic NSCLC harboring *MET* exon 14 skipping mutation and with high programmed cell death ligand 1 (PD-L1) expression (tumor proportion score ≥50%). The patient received the MET inhibitor tepotinib as first-line treatment, achieving a partial response, with G2 peripheral edema as adverse event that was successfully managed with temporary discontinuation, dose reduction, diuretics and physical therapy. After 31 months, the patient is still receiving tepotinib, with an ongoing response. Tepotinib is a valuable therapeutic option for first-line treatment of older patients with NSCLC harboring *MET* exon 14 skipping mutation, even in the presence of high PD-L1 expression.

Keywords: Elderly, First-line treatment, *MET* 14-exon skipping mutation, MET inhibitor, Non-small cell lung cancer, Tepotinib

Introduction

Mesenchymal epithelial transition gene (*MET*) exon 14 (*MET*ex14) skipping mutations occur in approximately 3-4% of non-small cell lung cancer (NSCLC) (1). In the VISION trial, a phase 2, non-randomized open-label study, the MET inhibitor tepotinib achieved a response rate of 51.4% and a median duration of response (DOR) of 18 months among 313 patients with *MET*ex14 mutant advanced/metastatic NSCLC, both treatmet-naïve (n = 164) and previously treated (n = 149) patients (2,3). Based on these results, tepotinib was approved by the Food and Drug Administration for the treatment of patients with metastatic NSCLC harboring *MET*ex14 skipping mutation regardless of the line of therapy (4), whereas the European Medicines Agency (EMA) approved tepotinib only for patients previously treated with immunotherapy and/or platinum-based chemotherapy (5).

Here we report the case of an 86-year-old male with *MET*ex14-mutated metastatic NSCLC and concomitant high programmed cell death ligand 1 (PD-L1) expression treated with first-line tepotinib that achieved a deep and durable response with manageable toxicity.

Case description

Following an accidental fall, a former 86-year-old male smoker underwent a head and chest computed tomography (CT) scan, which showed a lung tumor in the right lower lobe. The 18F-fluorodeoxyglucose (FDG) positron emission tomography (PET)/CT confirmed the presence of a lung tumor and showed bone, left adrenal and peritoneal metastases (Fig. 1). Stage was cT1c N0 M1c, IVB according to the American

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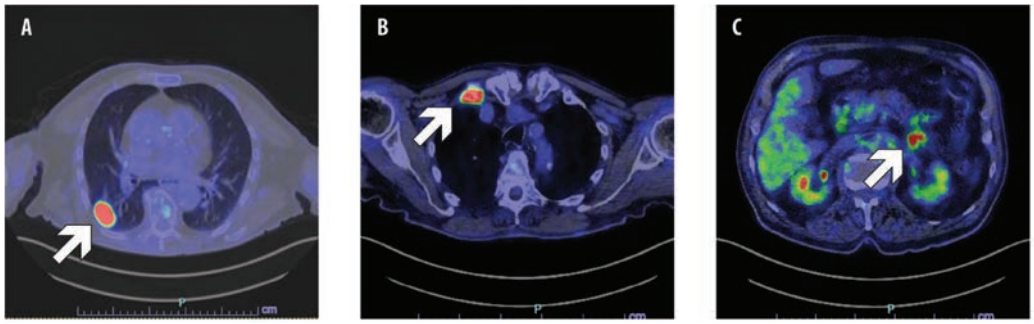

Fig. 1 - Positron emission tomography-computed tomography (PET-CT) scan showing primary lung tumor (A), bone metastasis in the first right rib (B), and peritoneal metastasis (C).

Joint Committee on Cancer (AJCC) 8th edition. The pathology examination of a CT-guided biopsy of the lung mass diagnosed lung adenocarcinoma with sarcomatoid transformation. The immunohistochemical evaluation showed high PD-L1 expression with a tumor proportion score (TPS) ≥50%, and next-generation sequencing (NGS) found *MET*ex14 skipping mutation. Comorbidities included trigeminal neuralgia, prostatic hypertrophy, eye maculopathy, and bilateral knee and right hip replacement.

First-line tepotinib 500 mg/day was started through an Early Access Program. The patient achieved partial response at the first tumor assessment after 3 months of treatment. Further response was observed at months 6, 9 and 12, then stable disease was observed (Fig. 2). During the treatment, the patient was diagnosed also with a prostate carcinoma (stage cT2 cN0, Gleason Score 4+4), therefore androgen deprivation therapy was added, without safety concerns. After 6 months of treatment with tepotinib, the patient developed treatment-related G2 peripheral edema; therefore, tepotinib was temporarily discontinued, and diuretics were administered, with complete regression of edema. Tepotinib was then restarted at 250 mg/day. After 16 months of treatment, however, tepotinib was discontinued again due to recurrent G2 peripheral edema. The patient received a further course of diuretics combined with physical therapy (i.e., compression stockings, retrograde massage), achieving edema improvement at G1; then, tepotinib was restarted at 250 mg/day. After 23 months of treatment, tepotinib was reduced at 250 mg every other day because of worsening edema, and it is currently ongoing (after 31 months).

Discussion

For patients with metastatic NSCLC without driver molecular alterations, immunotherapy with or without chemotherapy represents the standard of care in the first-line setting (6). Particularly, for patients with high PD-L1 expression (TPS ≥50%), immunotherapy alone with anti-PD-(L)1 antibodies as single agent represents the treatment of choice. Indeed, in the phase III KEYNOTE-024 trial, the anti-PD-1 antibody pembrolizumab as single agent achieved a median overall survival (OS) of 26.3 months, and a 5-year OS rate of 31.9%, compared respectively with 13.4 months and 16.3% of platinum-based chemotherapy as first-line treatment of metastatic

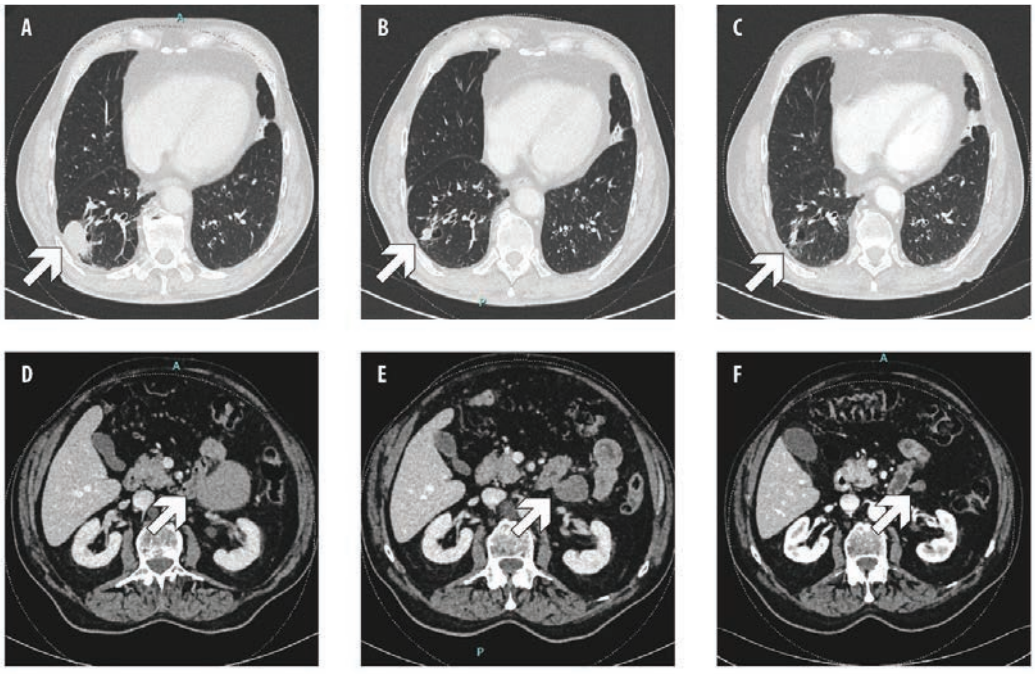

Fig. 2 - Computed tomography (CT) scan showing, respecively, primary lung tumor and peritoneal metastasis at baseline (A, D), after 12 weeks (B, E) and 1 year (C, F) of tepotinib.

NSCLC with TPS ≥50% (7). Similar results are available also for other anti-PD-(L)1 agents (8,9). Moreover, the addition of immunotherapy to the first-line platinum-based chemotherapy improved survival over chemotherapy alone, regardless of PD-L1 expression levels (10).

For patients with NSCLC harboring *MET*ex14 skipping mutations, however, the optimal treatment strategy has not yet been established. In fact, there are no data from randomized studies on the efficacy of immunotherapy and/or chemotherapy in the specific population of patients with *MET*ex14 mutant NSCLC. Retrospective studies showed conflicting results, with some studies suggesting a limited activity of immunotherapy in this population regardless of PD-L1 expression level (11,12), whereas other studies reported a similar activity of immunotherapy among *MET* mutant and wild-type cancers (13,14). However, no randomized comparisons of immunotherapy, either with or without chemotherapy, vs. MET inhibitors are available for patients with *MET*ex14 mutant metastatic NSCLC as front-line treatment.

Of note, *MET*ex14-mutated NSCLC is mainly found in elderly subjects (15). Elderly patients were generally underrepresented in clinical trials investigating immunotherapy with or without chemotherapy in NSCLC; therefore, the benefit/risk balance of these regimens should be carefully assessed individually when treating older patients in daily clinical practice. In fact, a recent pooled analysis showed that addition of chemotherapy to immunotherapy in patients with PD-L1 ≥50% older than 75 years was not beneficial (16). Chemotherapy alone may represent another option for elderly patients with advanced NSCLC. However, a joint analysis of two randomized trials on NSCLC older than 70 years reported limited activity of chemotherapy in this population, with a median progression-free survival (PFS) of 3 months for single-agent chemotherapy (either gemcitabine or pemetrexed) and 4.6 months with the addition of cisplatin. Interestingly, in this joint analysis the addition of cisplatin to single-agent chemotherapy did not significantly prolong OS (17). Thus, the use of platinum-based chemotherapy, with or without immunotherapy, may be questionable in elderly patients.

In clinical trials, selective MET inhibitors including tepotinib, capmatinib and savolitinib have recently shown meaningful activity against *MET*ex14-mutated NSCLC. Particularly, in the phase II VISION study, among 152 *MET*ex14-mutated NSCLC patients treated with tepotinib, the response rate by independent review was 46%, and median PFS was 8.5 months. This trial enrolled both treatment-naive and preatreted patients. Interestingly, in treatment-naive patients (n = 164), objective response rate (ORR) was 57.3% and median DOR was 46.4 months, whereas among pretreated patients, ORR was 45.0% and median DOR was 12.6 months, suggesting that tepotinib might be more beneficial as front-line treatment. In this study, the toxicity profile of tepotinib was acceptable, with peripheral edema reported as main grade 3 toxicity. In this study, 43% of patients received tepotinib as the first-line treatment (2,3). In the phase II GEOMETRY mono-1 study, among 97 *MET*ex14-mutated NSCLC patients treated with capmatinib, the ORR was observed in 41% of 69 patients who had previously received one or two lines of therapy and in 68% of 28 previously untreated patients; the median DOR was 9.7 months and 12.6 months, respectively (18). A Chinese phase II study evaluated savolitinib in 70 patients with *MET*ex14-mutated NSCLC after ≥1 line of standard treatment or deemed unsuitable for standard treatment, reporting an ORR of 42.9% (19).

Our patient achieved a partial response to tepotinib, with a PFS of 31+ months and DOR of 28+ months, that is consistent with data from VISION trial and compares favorably with data of first-line immunotherapy or chemotherapy. Based on this observation, we believe that MET inhibitors represent an effective and well-tolerated therapy for metastatic, *MET*ex14 mutant NSCLC in the first-line setting. Unfortunately, the EMA approval of MET inhibitors only for patients with progressive disease after chemotherapy and/or immunotherapy is likely to limit across Europe the access to treatment for very elderly patients unfit to receive first-line chemotherapy and/or immunotherapy before MET inhibitors.

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ORIGINAL RESEARCH ARTICLE

Prevalence of antibiotic misuse in cases of pneumonia and diarrhea in Saudi Arabia

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ABSTRACT

Background: Antibiotic misuse is a major public health issue with long-term repercussions.
Objective: The purpose of this investigation was to evaluate the effects of pneumonia and diarrhea, with an emphasis on antibiotic misuse.
Methodology: This study included 410 participants (217 fathers and 193 mothers), of whom 239 purchased antibiotics for their children without a prescription, whereas 171 had a prescription or were unsure if one was required.
Results: Antibiotics were used incorrectly by 58.1% of respondents. About 51.2% of participants said they were taking two antibiotics at the same time. Around 30% of people admitted to using antibiotics inefficiently. The most prevalent reason for use was "viral and bacterial," followed by "viral," and then "bacterial," with 35%, 21%, and 20%, respectively. In addition, 22.4% of patients have used antibiotics for an unknown reason.
Conclusion: Saudi parents of children with pneumonia and diarrhea abuse antibiotics. Saudi legislation banning medications without a prescription has helped reduce antibiotic abuse, but more community-based education and awareness are needed.

Keywords: Antibiotic abuse, Antibiotic misuse, Diarrhea, Pneumonia, Saudi Arabia

Introduction

Abuse of antibiotics is a major global public health hazard, needing concerted efforts to control its spread (1). Antimicrobial resistance threatens global food, health, and development. Antibiotic resistance is caused by antibiotic misuse. Maximizing antibiotic use improves medical outcomes, reduces toxicity, and prevents resistance (2). Antibiotic resistance genes (ARGs) have plagued antibiotic treatment and antimicrobial chemotherapy worldwide due to overuse and misuse (3).

Like any health issue, affluence and antibiotic usage vary depending on the type of antibiotic abuse and the country's health level. Understanding the social and economic factors could help create antibiotic misuse prevention programs and policies (4).

Because of the country's high rates of antimicrobial administration without a prescription, antimicrobial abuse and misuse have reached pandemic proportions in Saudi Arabia. Implementation of the regulation in Saudi Arabia's public pharmacies led to a slight decrease in the use of antimicrobials without prescriptions (5). Most antibiotics are misused or overused in Saudi Arabia for the treatment of upper respiratory tract infections (URTIs), which are predominantly viral infections (6). The current study investigates antibiotic usage in Saudi Arabia after the restriction on antibiotic sales without a prescription, with pneumonia and diarrhea being the most common indicators of antibiotic misuse.

Materials and methods

Between May 2022 and December 2022, 410 local inhabitants participated in a community-based survey in Hail, which is located in northern Saudi Arabia. When selecting participants at random, no consideration was paid to their age, social standing, degree of education, or monthly income. To be eligible, a child's parents had to show that they had already purchased antibiotics for their child's pneumonia or diarrhea. Antibiotic misusers are parents who give antibiotics to their children without first obtaining a doctor's prescription or doing a culture and sensitivity test. Individuals whose antibiotic use could be proven were considered not to be misusing the medications. A poll found that 239 parents bought antibiotics for their children without a prescription, whereas 171 had a prescription or were unsure if they needed one (including 217 fathers and 193 mothers).

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Data analysis

The data was analyzed using SPSS, which generated cross-tabulations, frequencies, and statistically significant results. We utilized a 95% confidence interval (CI) chi-square test to determine significance. Data with a p-value less than 0.05 was considered significant.

Results

This study comprised parents aged 20 to 60 years; 217/410 (52.9%) fathers and 193/410 (47.1%) mothers were polled. A total of 239/410 (58.1%) respondents admitted to using antibiotics inappropriately; an additional 12/410 (3.1%) were unsure. About 210 (51.2%) of the 410 participants reported using two antibiotics at the same time, while 17 (4.4%) were unsure; 123/410 people (30%) said they didn't use antibiotics enough, while 28/410 (6.8%) weren't sure. The most common category was "viral and bacterial," followed by "viral," and then "bacterial," with relative frequencies of 35% (144/410), 22% (89/410), and 20% (85/410). Furthermore, approximately 92/410 (22.4%) patients have used antibiotics for an unknown reason, as indicated in Table I, Figures 1 and 2.

TABLE I - Distribution of study participants based on their antibiotic use status

Variable	Yes	No	Don't know	Total
Antibiotic misuse	239	159	12	410
Antibiotic double use	210	183	17	410
Antibiotic complete use	259	123	28	410
Treatment indication	Viral	Bacterial	Viral and bacterial	Unknown
Frequencies	89	85	144	92

About 176 out of 299 participants (59%) chose the wrong antibiotic to treat pneumonia, while 114 out of 299 (38%) chose the right antibiotic and 9 out of 299 (3%) were not sure.

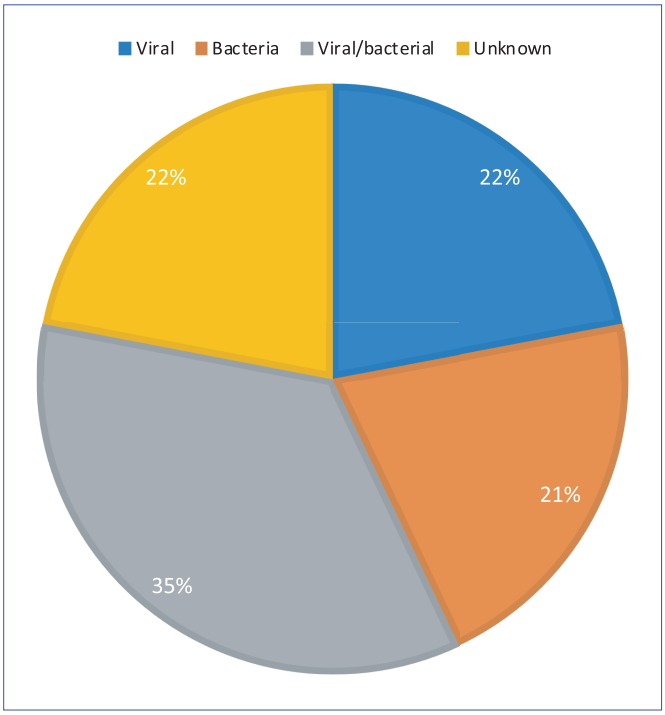

Fig. 2 - Indications of antibiotic use by study participants.

Approximately 68/112 (60.7%) children used the antibiotic incorrectly for diarrhea, 42/112 (37.5%) used it appropriately, and only 2/112 (1.8%) were uncertain. Only 114/299 (38%) of the 299 children with pneumonia had used the right antibiotics, as indicated in Table II.

In comparison to 117/193 (60%) mothers, around 122/217 (56.2%) fathers purchased improper antibiotics. The relative risk (RR) and 95% CI for moms purchasing unsuitable antibiotics were RR (95% CI) = 1.0783 (0.9157 to 1.2697), p = 0.3662.

Antibiotic abuse was most prevalent in the 35–44 age range, followed by 25–34 and 45 years, with 100/239 (42%), 88 (37%), and 42 (17.6%), respectively.

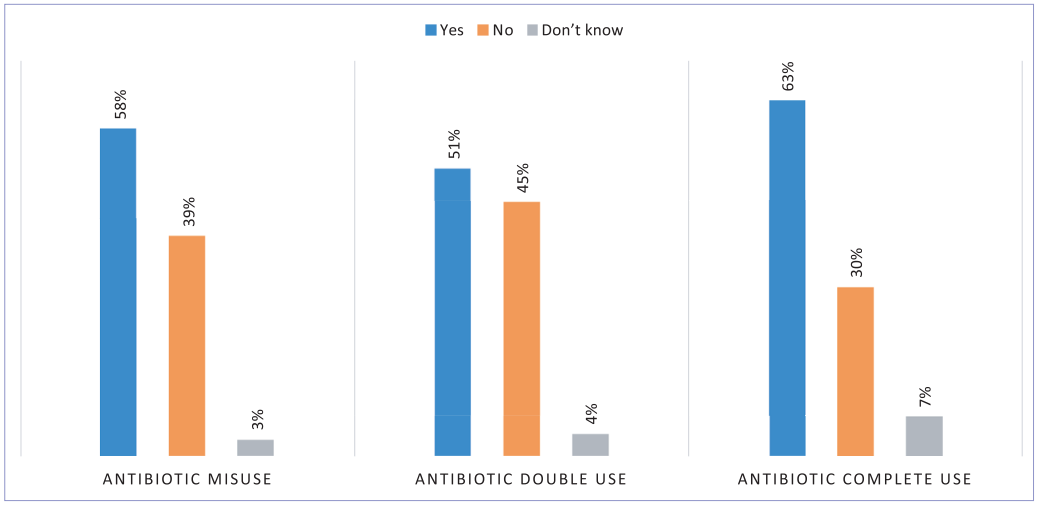

Fig. 1 - Description of the participants by antibiotic use status.

TABLE II - Distribution of antibiotic use status in relation to pneumonia and diarrhea

Antibiotic misuse	Yes	No	Unsure	Total
Pneumonia				
Yes	176	114	9	299
No	38	33	0	71
Unsure	25	12	3	40
Total	239	159	12	410
Diarrhea				
Yes	68	42	2	112
No	143	101	6	250
Unsure	28	16	4	48
Total	239	159	12	410

TABLE III - Distribution of antibiotic use status in relation to sociodemographic characteristics of the parents

Antibiotic misuse	Yes	No	Don't know	Total
Parent				
Father	122	91	4	217
Mother	117	68	8	193
Total	239	159	12	410
Age (years)				
≤24	9	7	1	17
25–34	88	76	6	170
35–44	100	52	2	154
≥45	42	24	3	69
Total	239	159	12	410
Social status				
Married	226	152	12	390
Divorced	12	4	0	16
Widow	1	3	0	4
Total	239	159	12	410
Family members				
<5 members	107	96	4	207
≥5 members	132	63	8	203
Total	239	159	12	410

Antibiotic misuse was reported by 58% of married people, 75% of divorcees, and 25% of widows. The probability that children of divorced parents will abuse antibiotics is RR (95% CI) = 1.2305 (0.9179 to 1.6495), p = 0.1654, and the *z* statistic is 1.387.

Around 132/203 (65%) of those with ≥5 family members reported antibiotic abuse, compared to 107/207 (51.7%) of those with <5 family members. The RR of antibiotic abuse was 1.2580 (1.0656 to 1.4850), with a 95% CI of 1.0656 to 1.4850 (p < 0.001) (see Tab. III, Fig. 3).

The status of antibiotic use in relation to the parents' level of education, occupation, and income was summarized in Table IV and Figure 4. About 151/275 (55%) parents with a college degree used antibiotics wrongly, compared to 68/107 (64%) parents with a secondary education and 20/28 (71%) parents with a primary education. Reduced education increased the risk of antibiotic abuse: RR (95% CI) = 0.7187 (0.5915–0.8731), p = 0.0009, *z* statistic = 3.326.

About 158/287 (55%) government employees, 41/63 (65%) self-employed, and 40/60 (67%) unemployed reported antibiotic abuse. The risk associated with self-employed and unemployed antibiotic usage is RR (95% CI) = 1.1962 (1.0146 to 1.4104), p = 0.0330, and the *z* statistic = 2.132.

About 33/56 (59%), 83/139 (60%), 87/145 (60%), and 36/70 (51%) of parents with monthly incomes of less than 5,000, 5,000 to 9,000, 10,000 to 14,000, and >15,000 SAR, respectively, misused antibiotics.

Table V summarizes the negative effects of using antibiotics in various situations. Approximately 150/230 (65%) of the children of individuals who were exposed to antibiotic overuse later had negative effects; 129/210 (61.4%) of the

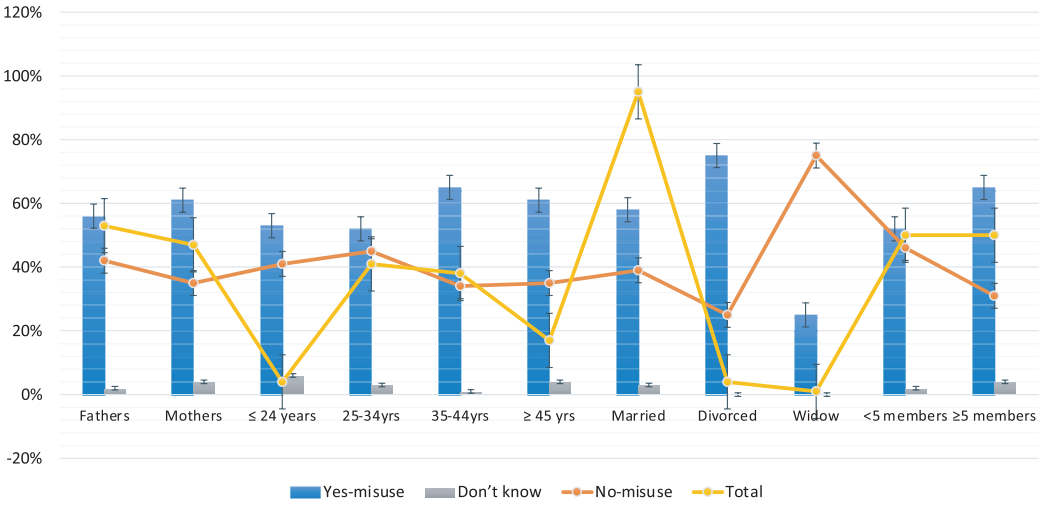

Fig. 3 - Description of status of antibiotic use for each of the categories of social and demographic factors.

TABLE IV - Distribution of antibiotic use status in relation to education, occupation, and income of the parents

Antibiotic misuse	Yes	No	Don't know	Total
Education				
Primary	20	8	0	28
Secondary	68	33	6	107
Universal	151	118	6	275
Total	239	159	12	410
Occupation				
Government employees	158	120	9	287
Self-employed	41	22	0	63
Unemployed	40	17	3	60
Total	239	159	12	410
Monthly income (SAR)				
<5,000	36	29	5	70
5,000-9,000	87	55	3	145
10,000-14,000	83	53	3	139
≥15,000	33	22	1	56
Total	239	159	12	410

TABLE V - The distribution of antibiotic abuse based on drug adverse effects

Side effects	Yes	No	Unsure	Total
Antibiotic misuse				
Yes	150	33	56	239
No	101	28	30	159
Unsure	3	4	5	12
Total	254	65	91	410
Antibiotic double use				
Yes	129	31	50	210
No	120	29	34	183
Unsure	5	5	7	17
Total	254	65	91	410
Pneumonia				
Yes	194	44	61	299
No	39	15	17	71
Unsure	21	6	13	40
Total	254	65	91	410
Diarrhea				
Yes	75	12	25	112
No	156	43	51	250
Unsure	23	10	15	48
Total	254	65	91	410

210 antibiotics that were used twice had negative effects; 194/299 (65%) of the 299 pneumonia patients experienced adverse symptoms. About 75/112 (67%) of the 112 children who had diarrhea experienced adverse symptoms.

Discussion

The findings of this study reveal that despite Saudi Arabia's ban on acquiring antibiotics without a prescription, a considerable number of people continue to misuse antibiotics. According to the Saudi Law Compendium, pharmacists are not permitted to provide therapeutic advice. The Saudi Ministry of Health (MOH) revised the rule to include financial penalties, license revocation, business closure, and a 6-month prison sentence for the pharmacist. In May 2018, antibiotic distribution regulations were validated (7,8).

Substantial rates of antibiotic misuse (58.1%) and overuse (51%) were found in the present investigation, both of which have undesirable effects such as the emergence of

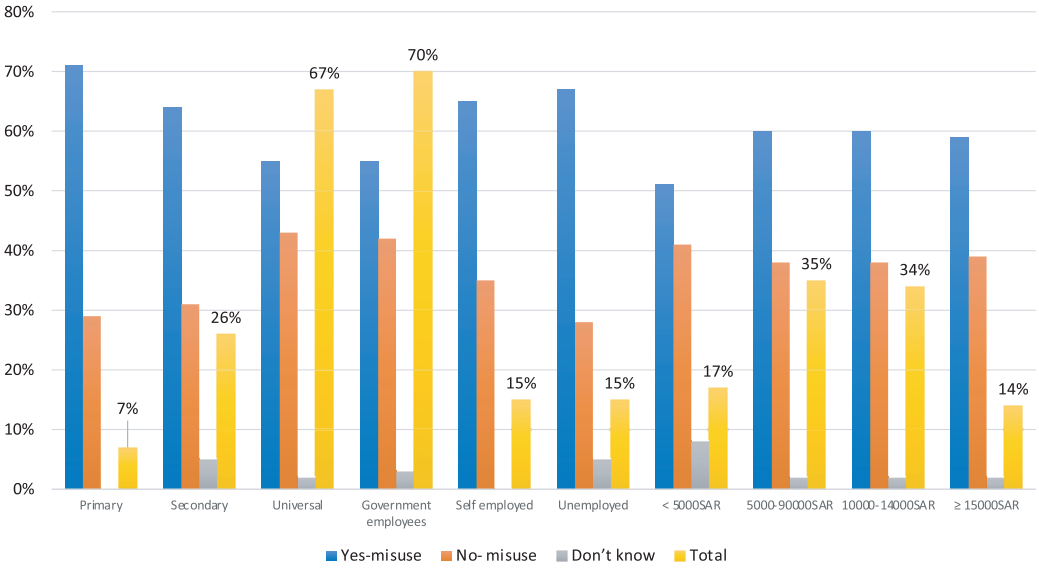

Fig. 4 - Description of antibiotic use status within education, occupation, and income categories.

antimicrobial resistance and financial burdens. Antibiotic resistance is a major public health concern that has received a great deal of attention in recent years from physicians and researchers. Misusing or overusing antibiotics can lead to resistance, which is caused by natural selection and the evolution of bacteria (9).

Physicians are struggling to improve patient outcomes due to rising infection rates, multidrug-resistant bacteria, and antibiotic use. Optimizing antimicrobial use can enhance patient outcomes, avoid resistance, and reduce drug abuse and overuse (10,11). A recent study in Saudi Arabia evaluated the antibiotic consumption after the adoption of a new prescription-only antimicrobial dispensing policy in community pharmacies. Generally, non-prescription antimicrobial use has dropped slightly (5). Although effective prescription limitations must be implemented, community-based activities are deemed essential.

Approximately 30% of this study's participants reported using an inadequate antibiotic dose, and 6.8% were unclear. Clinicians and other health providers should advise patients to finish the entire course of prescribed antibiotics, even if their symptoms have subsided, in order to stop the spread of antibiotic resistance and avoid a resurgence of illness (12).

According to the findings of this investigation, many drugs were abused in cases of viral ailments. Furthermore, around 59% of patients were given the wrong drug to treat pneumonia. The overuse of antibiotics to treat viral community-acquired pneumonia is a major public health issue. According to one study, antibiotics were given to 98.3% of patients who had viral pneumonia (13). Antibiotic overuse, especially for viral, self-limiting respiratory tract infections like sore throats, increases community-wide antimicrobial resistance. Approximately 80% of sore throats are viral and resolve without medicine. Although there are over-the-counter topical sore throat medicines, antibiotics are still administered inappropriately (14). However, whereas viral illnesses are more prevalent in children, adults with viral respiratory tract infections are more likely to overuse antibiotics (15).

In this study, approximately 60.7% of children used the antibiotic incorrectly for diarrhea. Over a million people die annually from the consequences of infectious diarrhea, which affects more than four billion people worldwide. A consistent geographic surveillance system would help fight the worrying rise in worldwide resistance caused by antimicrobial overuse and misuse (16). However, there is a scarcity of data on how Saudi eating habits may be linked to diarrhea in young children. Diarrhea was shown to be common among children aged 0 to 2 years, and it was also discovered that exclusive breastfeeding was not often followed in Saudi Arabia (17). About 40.3% of Saudi mothers thought it was a major problem in the Saudi community, but nearly 23% couldn't identify any critical sign of severe diarrhea, and 66% incorrectly stated that diarrhea is caused by teething (18).

According to the current study, mothers, particularly divorced mothers, are more likely to abuse antibiotics. Antibiotic misuse is more common in families with five or fewer members, and additional research is needed in this context. Furthermore, the current study's findings demonstrate that antibiotic overuse is inversely associated with education level. Furthermore, self-employed individuals receive more antibiotics that are misused than government employees. In this study, however, there was no correlation between monthly income and antibiotic misuse. Parents must accurately observe the use of antibiotics in their children. Although parental antibiotic awareness, attitude, and practice have received little attention, most parents were uninformed, pessimistic, and did not administer antibiotics to their children correctly. Children's antibiotic use was influenced by their parents' socioeconomic position, education, occupation, knowledge, and viewpoint (19).

Saudi researchers sought to refute common beliefs among primary school parents regarding when and how to give their children URTI medications. Antibiotics were unnecessary for nasal congestion (62.5%) and fever (74%). Compared to 39.4% for ear pain and 26% for throat discomfort, 61% of respondents correctly identified URTI as a virus, while only 20% believed antibiotics could not immediately treat it (20). Recent systematic reviews and meta-analyses included only 57 of 702 publications. ASM was highest in the Middle East (34%), Africa (22%), Asia (20%), and South America (17%). ASM is more prevalent in children who live far from a hospital or whose families are poor due to having multiple children. Parents may overuse antibiotics when their children develop fevers or recurrent coughs. Antimicrobial pharmaceutical sales should be rigorously restricted to reduce self-medication.

Bert et al revealed that antibiotic self-medication (ASM) among children was highest in the Middle East (34%), Africa (22%), Asia (20%), and South America (17%) and lowest in Europe (8%). ASM risk in children is increased by a long distance from the hospital, a low income, and several children. Antibiotics are sometimes abused by parents due to fever and cough. Self-medication can be reduced by focusing on antimicrobial drug control (21).

Participants in the current study indicated that their children experienced negative side effects because of antibiotic usage for both pneumonia and diarrhea. Antibiotic abuse increases antimicrobial resistance and causes gastrointestinal, neurologic, and mental issues. Amoxicillin-clavulanate hepatotoxicity can be deadly. Overprescribing antibiotics for self-limiting infections increases patient readmission rates (22). However, in an era when new agents are required to combat multiresistant bacteria, balancing the dangers and advantages of existing antimicrobials is an intriguing challenge.

The past decade has significantly influenced global antibiotic stewardship. High-level policy conversations, regulations, and legislation have focused on antibiotic use improvements, and antibiotic stewardship infrastructure has grown rapidly in hospitals, nursing homes, and ambulatory settings (23). Antibiotic stewardship programs (ASPs) effectively reduced antibiotic misuse, decrease antibiotic resistance, and improve treatment outcomes. The Saudi MOH devised a national antimicrobial stewardship plan to implement ASPs in hospitals, but little is known about its success or factors. According to a statewide cross-sectional poll that included all MOH hospitals, the utilization of ASP was only verified in 26% of hospitals (24).

Even though this study was an important update on antibiotic abuse after Saudi Arabia banned antibiotics without a prescription, it had some limitations, such as a cross-sectional design and a low level of acceptability for data collection.

Conclusion

Antibiotic misuse is still prevalent among parents caring for children with pneumonia and diarrhea in Saudi Arabia. Even though laws in Saudi Arabia that say antibiotics can't be given out without a prescription have helped cut down on antibiotic abuse, it is thought that more community-based education and awareness efforts are needed.

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Disclosures

Conflict of interest: The authors declare no conflict of interest.
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Informed consent: Participants in this study provided both written and verbal informed consent.
Ethical approval: The research plan received approval from the university's ethics board. HERC 0137/CM.UOH/5/20 is the approval number.
Data availability: The data presented in this study is available on request from the corresponding author.

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Mortality rate and factors associated with mortality of carbapenem-resistant Enterobacteriaceae infection

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ABSTRACT

Background: Carbapenem-resistant Enterobacteriaceae (CRE) is a serious pathogen with high mortality. Recognition of factors associated with mortality and treating these modifiable factors are crucial to reducing mortality.
Objective: To determine the 30-day mortality and factors associated with a 30-day mortality of CRE infection.
Methods: A retrospective cohort study was conducted between January 1, 2015, and December 31, 2019. All patients diagnosed with CRE infection aged ≥18 years were included. Multivariate logistic regression was used for evaluating the factors associated with 30-day mortality and presented as adjusted odds ratio (aOR) with 95% confidence interval (CI).
Result: One hundred and ninety-four patients were enrolled. The 30-day mortality occurred in 75 patients (38.7%). The common antibiotic regimen was monotherapy and combination of carbapenem, colistin, amikacin, tigecycline, and fosfomycin. CRE isolates were susceptible to tigecycline (93.8%), colistin (91.8%), fosfomycin (89.2%), and amikacin (89.2%). The independent factors associated with 30-day mortality were an increasing simplified acute physiology (SAP) II score (aOR 1.11, 95% CI 1.05-1.16, p < 0.001), sepsis at time of CRE infection diagnosis (aOR 7.93, 95% CI 2.21-28.51, p = 0.002), pneumonia (aOR 4.48, 95% CI 1.61-12.44, p = 0.004), monotherapy (aOR 4.69, 95% CI 1.71-12.85, p = 0.003), and improper empiric antibiotic (aOR 5.13, 95% CI 1.83-14.40, p = 0.002).
Conclusion: The overall 30-day mortality of CRE infection was high. The factors associated with mortality were an increasing SAP II score, sepsis at time of CRE infection diagnosis, pneumonia, monotherapy, and improper empiric antibiotic. The study suggested that proper empiric antibiotic and combination antibiotics might reduce mortality from CRE infection.

Keywords: 30-Day mortality, Carbapenem-resistant Enterobacteriaceae, Factors

Introduction

Carbapenems are broad-spectrum antibiotics and have a good potency against gram-positive and gram-negative bacteria by penetrating the cell walls of bacteria, binding with penicillin-binding proteins (PBPs), and resulting in inhibiting cell wall synthesis, ultimately killing the bacteria. They are used as antibiotics of mostly last resort for fighting drug-resistant gram-negative pathogens (1,2). Carbapenem-resistant Enterobacteriaceae (CRE) have emerged and become a major problem of nosocomial infection after extensive use of carbapenems and its spread, with the consequent change in local epidemiology continuing to evolve rapidly worldwide (3-6). Among hospitalized patients, asymptomatic gastrointestinal colonization of CRE is challenging, which oversteps and significantly increases the risk of subsequent infections caused by these pathogens. The prevalence of CRE infection was shown to be 1.3 per 10,000 hospital admissions (1).

The mechanisms of resistance to carbapenems include β-lactamase production, efflux pumps, and mutations that alter the expression and/or function of porins and PBPs.

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Certain bacteria have combinations of these mechanisms that cause high levels of resistance to carbapenems (1,2). Cefiderocol and new beta-lactam-beta-lactamase inhibitors (BLBIs), that is, ceftazidime-avibactam, meropenem-vaborbactam, and imipenem-cilastatin-relebactam, have been developed to fight with CRE infection (7). The Infectious Diseases Society of America (IDSA) and the European Society of Clinical Microbiology and Infectious Diseases (ESMID) have published updated guidance on the treatment of antimicrobial-resistant gram-negative infections (2,8). The new BLBIs and cefiderocol are preferred treatment options for CRE infection. New BLBIs and cefiderocol, however, are not widely available including in our center; therefore, monotherapy or combination of colistin, fosfomycin, tigecycline, amikacin, gentamicin, and carbapenem is usually used to combat CRE infection (9,10).

The mortality rate of CRE infection is high as shown in many studies, varying from 31% to 53% (11-14). Recognition and identification of factors associated with mortality of CRE infection are important in clinical practice. Treatment of modifiable risk factors is useful for reducing the mortality of CRE infection. Previous reports demonstrated age, sepsis, shock, chronic renal failure, dialysis, neutropenia, high Acute Physiology And Chronic Health Evaluation (APACHE) scores, monotherapy, and inadequate empiric antibiotic were the factors associated with mortality (12,13,15-17). The study of the mortality rate and factors associated with mortality in CRE infection are still limited in Thailand. Hence, the study was conducted for evaluating the mortality rate and factors associated with CRE infection.

Methods

This was a retrospective cohort study that was conducted between January 1, 2015, and December 31, 2019, at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, which is a 1,466-bed tertiary center in Northeast Thailand. The study was approved by the local Human Research Ethics Committee (approval number HE631252).

Patients and data collection

The study included patients aged ≥18 years who had been diagnosed with CRE infection by criteria from the Clinical and Laboratory Standards Institute (CLSI) 2015. In brief, CRE is defined as resistant to at least one carbapenem or producing a carbapenemase enzyme (18). The exclusion criteria were the patients who were colonized with CRE organisms without clinical signs and symptoms of infection.

The medical records of demographic data, laboratory results, microbiological and sensitivity profiles, treatment regimen, and 30-day mortality were reviewed. The simplified acute physiology (SAP) II score and sepsis at time of CRE infection diagnosis were obtained.

Definition and outcomes

The outcome was the 30-day mortality and factors associated with 30-day mortality. The 30-day mortality was death for any reason after CRE infection diagnosis within 30 calendar days. The empiric antibiotic regimen was selected depending on the gram stain of the specimen from source of infection, local data, and antibiogram of pathogens. The improper empiric antibiotic was defined as any antibiotic in the empiric treatment regimen for the pathogens that were not susceptible to antibiotic in the empiric regimen. The result of culture and drug susceptibility test was reported 72-96 hours after specimens were collected. The drug susceptibility test of microbiology was interpreted by CLSI 2015 (18). The treatment regimen was adjusted by the drug susceptibility test. The common antibiotic treatment is monotherapy or a combination of carbapenem, colistin, amikacin, tigecycline, and fosfomycin. The administration dose of these antibiotics was as follows: meropenem 1000 mg intravenous every 8 hours, imipenem-cilastatin 1000 mg intravenous every 8 hours, colistin 300 mg intravenous loading then 150 mg intravenous every 12 hours, fosfomycin 4 g intravenous every 8 hours, amikacin 750 mg intravenous every 24 hours, tigecycline 200 mg intravenous loading then 100 mg intravenous every 12 hours, sitafloxacin 100 mg oral every 12 hours, cotrimoxazole 15-20 mg of trimethoprim/kg/day intravenous divided every 8 hours. The renal dosage was adjusted where appropriate.

Statistical analyses

The categorical data were presented with numbers and percentages. The normal distributed continuous data are presented as mean and standard deviation (SD) while the non-normal distributed data were presented with median and interquartile range (IQR). A comparison of category data used the Chi-square test and Fisher's exact test depending on data. The nonparametric data used the Mann-Whitney U-test for comparison. The factors associated with 30-day mortality were evaluated by univariate logistic regression analysis. The stepwise backward multiple logistic regression analysis including factors with a p-value <0.2 on univariate analysis or factors with previous reports of clinical significance was performed. Crude odds ratio (cOR) and adjusted odds ratio (aOR) with their 95% confidence intervals (95% CI) were demonstrated. A p-value <0.05 was considered statistically significant. The statistical analysis was performed by Stata version 10.1 (StataCorp, Texas, USA).

Results

A total of 194 patients were included in the study. Of these, 110 patients (56.7%) were male. The mean age (SD) was 61.6 (16.7) years. The overall 30-day mortality occurred in 75 patients (38.7%). The most common source of infection was pneumonia (90 cases, 46.4%), intra-abdominal infection (43 cases, 22.2%), and urinary tract infection (41 cases, 21.1%). The nonsurviving patients had a significantly greater proportion of lung disease, sepsis at time of CRE infection diagnosis, and a higher SAP II score ($p < 0.05$). The nonsurviving patients had a significantly lower proportion of urinary tract infection and intra-abdominal infection ($p < 0.05$). The demographic data of patients are shown in Table I.

Table II shows the CRE pathogens and in vitro susceptibility. The most common pathogens were *Klebsiella pneumoniae*

TABLE I - Demographic data of patients

Parameters	Surviving group n = 119	Nonsurviving group n = 75	p-Value
Mean age in years (SD)	61.6 (16.0)	61.6 (17.8)	0.98
Male, n (%)	62 (52.1)	48 (64.0)	0.10
BMI (kg/m^2), mean (SD)	21.1 (4.0)	20.8 (3.5)	0.57
Comorbidity, n (%)	111 (93.3)	72 (96.0)	0.43
Diabetes mellitus, n (%)	36 (30.3)	21 (28.0)	0.74
Hypertension, n (%)	47 (39.5)	30 (40.0)	0.94
Dyslipidemia, n (%)	15 (12.6)	6 (8.0)	0.32
Neurological disease, n (%)	24 (20.2)	11 (14.7)	0.33
Cardiovascular disease, n (%)	18 (15.1)	18 (24.0)	0.12
Lung disease, n (%)	3 (2.5)	8 (10.7)	0.02
Liver disease, n (%)	11 (9.2)	12 (16.0)	0.16
Renal disease, n (%)	15 (12.6)	16 (21.3)	0.11
Malignancy, n (%)	45 (37.8)	18 (24.0)	0.05
Sepsis*, n (%)	38 (31.9)	69 (92.0)	<0.001
SAP II score*, mean (SD)	29.5 (11.6)	47.9 (13.4)	<0.001
Source of infection			
Pneumonia, n (%)	32 (26.9)	58 (77.3)	<0.001
Urinary tract infection, n (%)	36 (30.3)	5 (6.7)	<0.001
Intra-abdominal infection, n (%)	35 (29.4)	8 (10.7)	0.002
SSI, n (%)	8 (6.7)	3 (4.0)	0.43

BMI = body mass index; SAP = simplified acute physiology; SD = standard deviation; SSI = skin and soft tissue infection.
*Status at time of CRE infection diagnosis.

TABLE II - Pathogens and in vitro sensitivity

Parameters	Surviving group (n = 119)	Nonsurviving group (n = 75)	p-Value
Pathogens			
Klebsiella pneumoniae	90 (75.6)	63 (84.0)	0.16
Escherichia coli	18 (15.1)	7 (9.3)	0.24
Enterobacter spp.	8 (6.7)	4 (5.3)	0.70
Others*	3 (2.5)	1 (1.3)	0.57
In vitro sensitivity, n (% sensitive)			
Meropenem	32 (26.9)	16 (21.3)	0.38
Imipenem	24 (20.2)	15 (20.0)	0.98
Amikacin	105 (88.2)	68 (90.7)	0.60
Fosfomycin	110 (92.4)	63 (84.0)	0.07
Colistin	111 (93.3)	67 (89.3)	0.33
Tigecycline	114 (95.8)	68 (90.7)	0.15

Data were presented as n (%).
*Others: *Proteus mirabilis* (n = 1), *Citrobacter* spp. (n = 2) in surviving group, *P. mirabilis* (n = 1) in the nonsurviving group.

TABLE III - Treatment regimen of CRE infection

Regimen	Surviving group (n = 119)	Nonsurviving group (n = 75)	p-Value
Monotherapy	61 (51.3)	57 (76.0)	0.001
Meropenem/ imipenem-cilastatin	22 (18.5)	13 (17.3)	0.84
Colistin	19 (16.0)	40 (53.3)	<0.001
Fosfomycin	3 (2.5)	3 (4.0)	0.68
Amikacin	15 (12.6)	1 (1.3)	0.005
Tigecycline	0 (0.0)	2 (2.7)	0.15
Combination therapy	58 (48.7)	18 (24.0)	0.001
Fosfomycin/colistin	29 (24.4)	7 (9.3)	0.009
Fosfomycin/amikacin	7 (5.9)	1 (1.3)	0.16
Meropenem/colistin	14 (11.8)	3 (4.0)	0.06
Fosfomycin/tigecycline	1 (0.8)	1 (1.3)	1.00
Fosfomycin/meropenem	4 (3.4)	0 (0.0)	0.16
Fosfomycin/others*	2 (1.7)	0 (0.0)	0.52
Tigecycline/colistin	0 (0.0)	2 (2.7)	0.15
Tigecycline/meropenem	0 (0.0)	2 (2.7)	0.15

Data were presented as n (%)
CRE = carbapenem-resistant Enterobacteriaceae; fosfomycin/others = fosfomycin/sitafloxacin (n = 1), fosfomycin/cotrimoxazole (n = 1) in the surviving group.

(153 patients, 78.9%), *Escherichia coli* (25 patients, 12.9%), and *Enterobacter* spp. (12 patients, 6.2%). The CRE isolates were susceptible to 24.7% of meropenem, 20.1% of imipenem, 89.2% of amikacin, 91.8% of colistin, 89.2% of fosfomycin, and 93.8% of tigecycline.

Table III shows treatment regimen of CRE infection. One hundred and eighteen patients (60.8%) were treated with monotherapy and 76 patients (39.2%) were treated with combination therapy. The surviving patients had a significantly greater proportion that was treated with combination antibiotics than nonsurviving patients (p = 0.001). An improper empiric antibiotic was used in 107 patients (55.2%), 60 patients (50.4%) in the surviving group and 47 patients (62.7%) in the nonsurviving group (p = 0.09).

Table IV shows the factors associated with 30-day mortality that were analyzed by univariate and multivariate analysis. With univariate analysis, sepsis at time of CRE infection diagnosis (cOR 24.51; 95% CI 9.78-61.44; p < 0.001), increasing SAP II score (cOR 1.13; 95% CI 1.09-1.17; p < 0.001), pneumonia (cOR 9.28; 95% CI 4.72-18.22; p < 0.001), and monotherapy (cOR 3.01; 95% CI 1.59-5.71; p = 0.001) were significantly associated with 30-day mortality. With backward stepwise logistic regression analysis, sepsis at time of CRE infection diagnosis (aOR 7.93; 95% CI 2.21-28.51; p = 0.002), increasing SAP II score (aOR 1.11; 95% CI 1.05-1.16; p < 0.001), pneumonia (aOR 4.48; 95% CI 1.61-12.44; p = 0.004), monotherapy (aOR 4.69; 95% CI 1.71-12.85; p = 0.003), and improper empiric antibiotic (aOR 5.13; 95% CI 1.83-14.40; p = 0.002) were independent factors associated with 30-day mortality.

TABLE IV - Factors associated with 30-day mortality of CRE infection

Parameters	cOR (95% CI)	p-Value	aOR (95% CI)	p-Value
Age >60 years	0.76 (0.42-1.36)	0.35		
Sepsis*	24.51 (9.78-61.44)	<0.001	7.93 (2.21-28.51)	0.002
Increasing SAP II score*	1.13 (1.09-1.17)	<0.001	1.11 (1.05-1.16)	<0.001
Pneumonia	9.28 (4.72-18.22)	<0.001	4.48 (1.61-12.44)	0.004
Urinary tract infection	0.16 (0.61-0.44)	<0.001		
Monotherapy	3.01 (1.59-5.71)	0.001	4.69 (1.71-12.85)	0.003
Improper empiric antibiotic	1.65 (0.91-2.98)	0.09	5.13 (1.83-14.40)	0.002

aOR = adjusted odds ratio; cOR = crude odds ratio; CI = confidence interval; CRE = carbapenem-resistant Enterobacteriaceae; SAP = simplified acute physiology.
*Status at time of CRE infection diagnosis.

Discussion

CRE infection has been an important health problem in recent decades (19). This study revealed that the most common CRE pathogens were *K. pneumoniae* (78.9%), *E. coli* (12.9%), and *Enterobacte*r spp. (6.2%), which are similar to previous reports (12,13,16,20-22). The mortality rate of CRE infection from several studies is high, from 31% to 53% (11-14). Similar to this current study, the overall 30-day mortality was 38.7%. The optimal antibiotic regimen that is the most effective with lowest side effects is still unknown, particularly for pneumonia treatment (2,8,19,23). The recent guidelines prefer new BLBIs and cefiderocol for the treatment of CRE infection (2,8). Furthermore, a growing body of evidence demonstrated new BLBIs and cefiderocol has a lower mortality in CRE infection than treatment regimen used in this study (23-27). These antibiotics were not available during the period of this current study. The best available regimen used in this study included monotherapy and a combination of carbapenem, colistin, amikacin, tigecycline, and fosfomycin. This is the one possible explanation that might contribute to the high mortality of this study.

The study revealed that the independent factors associated with 30-day mortality were sepsis at the time of CRE infection diagnosis, increasing SAP II score, pneumonia, monotherapy, and improper empiric antibiotic. Similar to this study, de Maio Carrilho et al reported pneumonia and urinary tract infection were the most frequent source of CRE infection. The mortality rate was 34.6% and higher in pneumonia patients. This study demonstrated shock was the independent factor associated with mortality (12). A study from China by Li et al evaluated the mortality rate in bloodstream infections of CRE. This study demonstrated mortality rate was 53.1% and sepsis was the independent factor for mortality (13). Lim et al reported a high disease severity index defined as an APACHE score ≥15 had a higher mortality risk (14). Seo et al also demonstrated higher APACHE II scores were independent risk factors of mortality of CRE bacteremia (15). Papadimitriou-Olivgeris et al reported that a SAP II score upon infection onset was associated with mortality of carbapenemase-producing *K. pneumoniae* bacteremia (28). These reports suggested that a high disease severity index is associated with mortality of CRE infection, like the current study.

Daikos et al revealed that monotherapy for CRE infection was associated with mortality (16). Likewise, Lim et al revealed that a combination antibiotic therapy had lower mortality risk (14). Furthermore, several studies demonstrated combination antibiotic therapy had a good outcome for CRE infection (17,28-31). Similar to this current study, a combination antibiotic therapy was associated with lower mortality. This finding was unable to be applied to new BLBIs and cefiderocol because the aforementioned studies did not include new BLBIs and cefiderocol in the studies. This current study endorsed the ESMID guidelines that are recommended for CRE infection treatment; in case new BLBIs are not available, the combination antibiotic therapy of drugs active in vitro should be considered (8).

Tumbarello et al revealed that inadequate empiric antibiotic therapy was associated with mortality of carbapenemase-producing *Klebsiella pneumoniae* bacteremia (17). Another study by Zilberberg et al revealed that CRE infection was threefold more likely of receiving inappropriate empiric antibiotic (46.5% vs. 11.8%, $p < 0.001$), and receiving inappropriate empiric antibiotic was also associated with rising mortality (32). This result is similar to this current study; improper empiric antibiotic therapy had a high occurrence (55.2%) and was associated with mortality. Active surveillance, local data, and an antibiogram may guide a physician to decide on the proper empiric antibiotic (33-35). This might reduce the mortality of CRE infection.

This study emphasized the mortality and factors associated with mortality of CRE infection. The study had some limitations. First, this was a retrospective study, some data were missing, and the selection bias was unable to be avoided. Second, some factors were found significantly associated with mortality of CRE infection in previous studies but could not be identified in this study, this might be because this study had a relatively small sample size. Third, the temporal relationship could not be determined according to the study design.

Conclusion

The overall 30-day mortality of CRE infection was high. The factors associated with mortality were an increasing SAP II score, sepsis at time of CRE infection diagnosis, pneumonia, monotherapy, and improper empiric antibiotic. The study suggested that proper empiric antibiotic and combination antibiotics might reduce mortality from CRE infection.

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REVIEW

Management of urinary tract infections in the era of antimicrobial resistance

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ABSTRACT

Urinary tract infections (UTIs) are among the most common infections globally, imposing a substantial personal and economic burden on individuals and health resources. Despite international health concerns and sustained public awareness campaigns about the emergence of resistant microorganisms through the inappropriate therapeutic use of antimicrobial agents, the problem of antimicrobial resistance (AMR) is worsening, and AMR in UTIs represents a critical global healthcare issue. This narrative review summarizes evidence-based scientific material, recommendations from the current medical literature, and the latest clinical guidelines on antibiotic and antibiotic-sparing strategies for managing urological infections, including practical approaches to improve the management of patients with acute and recurrent UTIs (rUTIs) in routine clinical practice. Novel emerging therapies and prophylaxis options are described as potential alternatives to overcome the abuse and overuse of antibiotics and the practical application of the guideline recommendations and issues relating to best practice in managing UTIs.

Keywords: Antibiotic, Antibiotic sparing, Antimicrobial resistance, Prophylaxis, Recurrence, Urinary tract infections

Introduction

Urinary tract infections (UTIs) are among the most common infections globally, imposing a substantial personal and economic burden on individuals and health resources (1,2). Data from the Global Burden of Disease Study 2019 (3,4) have been used to investigate the incidence, mortality, and disability-adjusted life years (DALYs) related to UTIs globally, by region, and by country from 1990 to 2019. Globally, there were an estimated 405 million cases, 237,000 deaths, and 5.2 million DALYs associated with UTIs in 2019, with a 2.4 times growth in deaths from 1990 to 2019, accompanied by an increasing age-standardized mortality rate over time (1,2). Specifically, the age-standardized incidence rate of UTIs increased from 4,715 individuals per 100,00 population in 1990 to 5,229 per 100,000 in 2019, and the number of deaths related to UTIs increased from approximately 99,000 in 1990 to 237,000 in 2019 (1,2).

The incidence of UTIs was more prominent in higher sociodemographic regions, together with an increasing mortality rate, whereas countries with lower sociodemographic development or a higher baseline disease burden showed notable rates of decline over the three decades. The burden of UTIs was higher in females and tended to increase with age, particularly in regions with a higher sociodemographic status (1).

UTIs present a critical global healthcare issue, particularly as, along with other infectious diseases, antimicrobial resistance (AMR) in UTIs is a continuing challenge. Despite global health concerns and sustained public awareness campaigns about the emergence of resistant microorganisms through the inappropriate therapeutic use of antimicrobial agents, the AMR problem is worsening (5-7).

In this narrative review we summarize the evidence from the scientific literature and recommendations from the latest clinical guidelines on the management of urological infections and suggest practical approaches to improve the management of patients with acute and recurrent UTIs (rUTIs). Novel emerging therapies and prophylaxis options are described as potential alternatives to overcome the abuse and overuse of antibiotics, including new clinical outcomes on the use of glycosaminoglycan (GAG) therapy in the management of rUTIs

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and the practical application of the guideline recommendations and issues relating to best practice in the management of UTIs.

Methods

The present review is based on relevant articles retrieved from PubMed/MEDLINE and Google Scholar guided by material presented at an interactive scientific workshop sponsored by IBSA Institut Biochimique SA (IBSA) during the 38th Annual European Association of Urology (EAU) Congress (EAU23) that was held from March 10 to 13, 2023, in Milan, Italy. The workshop addressed the management of UTIs against the background of increasing AMR, focusing on the widespread problem due to the over- or inappropriate use of antibiotics and the related threat of AMR in patients with UTIs.

Papers presented as part of the workshop were augmented by searching the databases for additional publications necessary to advance the discussion or provide additional evidence. Original research papers published in English in internationally recognized journals and online journals were selected preferentially, but review articles that added to the understanding of the management of UTIs in the era of AMR were also selected. Identified articles were reviewed for relevance, with preference given to recent papers.

The EAU presents programs of cutting-edge urological science at their annual Congress, including the presentation of the latest edition of the EAU Guidelines on Urological Infections. These widely respected clinical guidelines, which provide medical professionals with best evidence-based information and recommendations for preventing and treating UTIs and male accessory gland infections (8), reinforce the material presented in this review. The guidelines also address important public health aspects of infection control and antimicrobial stewardship, including the growing problem of resistance among uropathogenic bacteria, the overuse and misuse of antibiotics, and the lack of new antibiotics. The European Association of Urology Nurses (EAUN), which has been developing practice guidelines for European urology nurses since 2004, also hosted their annual meeting (EAUN23) in conjunction with EAU23, providing a forum for leading urology nursing and medical professionals to share evidence-based research of particular relevance to urological care from a nursing perspective.

A patient awareness campaign was also introduced at the Congress to inform and raise awareness of the correct recognition of symptoms and treatment of UTIs, involving patients, patient organizations, and physicians in managing bacterial infections according to the EAU guidelines. A video supporting the campaign, *UTI and the use of antibiotics*, clearly and directly explains several aspects of urological disorders and is available, along with other information, in the EAU Patient Information Portal at Online.

Management of UTIs in times of increasing AMR

There is increasing interest in infectious diseases, and the EAU Guidelines on Urological Infections have become the second most downloaded of all European clinical guidelines after Prostate Cancer guidelines. In parallel, the issue of AMR in urological infections, as in other infections, is increasingly recognized as an urgent public health priority (6,9).

An independent review on AMR commissioned in July 2014 by the UK Government to address the challenge of AMR estimated that failure to address the problem could lead to 10 million deaths globally per year by 2050, costing an estimated US$ 100 trillion if action is not taken (7). The latest predictive models of the World Health Organization (WHO) AMR Collaborators estimate that there were 4.95 million deaths associated with bacterial AMR globally in 2019 (6). This includes 1.27 million deaths directly attributable to bacterial AMR and 3.57 million associated with AMR. Just six pathogens were each responsible for over 250,000 deaths attributable to or associated with bacterial AMR: *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* (Fig. 1).

Together, they were responsible for 929,000 deaths (6). Of these, UTIs were the fourth most prevalent infectious syndrome associated with global deaths attributable to or associated with AMR, after lower respiratory infections and all related infections in the thorax, bloodstream infections, and peritoneal and intra-abdominal infections (6).

Classification and diagnosis

UTIs can be classified differently, but a system developed by the EAU Section of Infections in Urology and the European Society for Infections in Urology (ESIU) and adopted by the EAU provides a valuable classification tool. The EAU/ESIU system classifies UTIs according to the prevalence of risk factors (uncomplicated and complicated UTIs), localization (lower and upper UTIs), the frequency of occurrence (rare and recurrent), relapse or reinfection, and in women and men (8). That is, the system is based on the clinical presentation of the UTI, the anatomical level of the UTI, the grade of severity of the infection, the categorization of risk factors, the frequency of occurrence, and the availability of appropriate antimicrobial therapy (10).

For example, a young, premenopausal, nonpregnant woman with no known relevant anatomical and functional abnormalities within the urinary tract or comorbidities presenting with the typical symptoms of a UTI can be considered to have an uncomplicated UTI. By definition, all other UTIs are complicated UTIs, with a higher risk of developing a complicated course. This classification system for UTIs is summarized in Table I.

Even an uncomplicated UTI is not just a simple infection, as it involves symptoms and restricted activity and may necessitate sick leave or bed rest. A UTI occurring at least three times a year or twice in the previous 6 months is defined as an rUTI. However, contrary to historical practice, it is strongly recommended not to treat cases of asymptomatic bacteriuria, where the presence of bacteria is found in a urine culture taken as part of a routine clinical visit in a patient without symptoms (8). In fact, there is evidence that asymptomatic bacteriuria is a common commensal colonization that may have a protective function against superinfecting

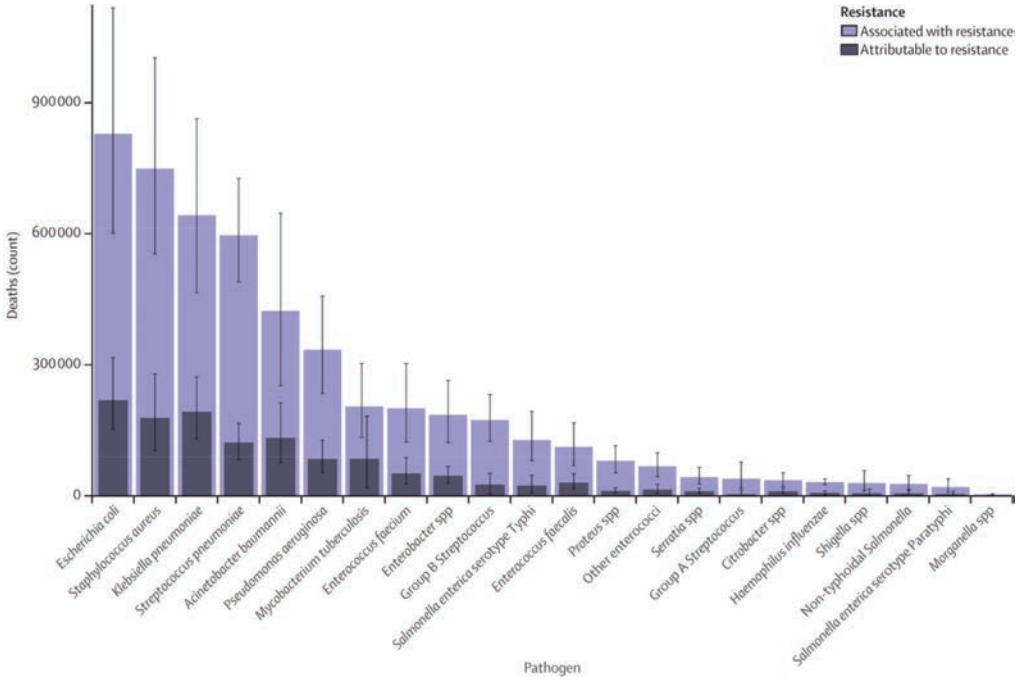

Fig. 1 - Global deaths attributable to or associated with antimicrobial resistance by pathogen in 2019 (6).
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TABLE I - The classification of urinary tract infections according to the European Association of Urology (EAU) Urological Infections Guidelines (8)

Classification	
Uncomplicated UTIs	Acute, sporadic, or recurrent lower (uncomplicated cystitis) and/or upper (uncomplicated pyelonephritis) UTI, limited to nonpregnant women with no known relevant anatomical and functional abnormalities within the urinary tract or comorbidities.
Complicated UTIs	All UTIs that are not defined as uncomplicated, meaning in a narrower sense UTIs in a patient with an increased chance of a complicated course, that is, all men, pregnant women, patients with relevant anatomical or functional abnormalities of the urinary tract, indwelling urinary catheters, renal diseases, and/or with other concomitant immunocompromising diseases, for example, diabetes.
Recurrent UTIs	Recurrences of uncomplicated and/or complicated UTIs, with a frequency of at least three UTIs/year or two UTIs in the last 6 months.
Catheter-associated UTIs	Catheter-associated UTI (CA-UTI) refers to UTIs occurring in a person whose urinary tract is currently catheterized or has had a catheter in place within the past 48 hours.
Urosepsis	Urosepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection originating from the urinary tract and/or male genital organs (54).

UTI = urinary tract infection.

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symptomatic UTI, and treating it may be harmful in some cases (8,11).

UTI is strongly gender biased, exhibiting one of the most prominent sex disparities among infectious diseases. Premenopausal women are 20-40 times more likely to have a UTI than men of the same age (12-15). Anatomical differences may account for some of this disparity. For instance, men have a longer urethra, and the distance between the anus and urethral opening is shorter in females. However, anatomical differences do not explain why young males and females have similar rates of rUTIs, and the rates of UTIs in males over 65 years increase substantially until they are nearly equivalent to those in older women (12,16). Changes in the incidence of UTIs in older men may, instead, be due to urodynamic changes such as prostatic hypertrophy or other factors, for example, biological or immunological changes occurring over time (16-19). Indeed, the differences in UTI susceptibility by gender are most evident in postpubescent adults younger than 50 years, a time when estrogen and testosterone are at their highest levels in females and males, respectively (12,14).

Zychlinsky Scharff and colleagues hypothesized that sex-based differences in immunity influence response to UTI, which they explored in an animal model designed to bypass anatomical differences, enabling innate and adaptive immune responses in infected female and male mice to be directly compared (19). They identified a cytokine pathway essential for bacterial clearance after infection with uropathogenic *E. coli* (UPEC), demonstrating that interleukin 17 (IL-17) specifically influenced the innate immune response to bacterial infection in a sex-dependent manner (19). Whether this finding is directly relatable to human infection outcomes remains to be demonstrated. However, it provides a possible explanation for the similarly high frequency of infection in older men and women and the increased severity associated with UTI in men, suggesting hormone-mediated suppression of the innate immune response. This is supported by significant sex differences in the expression of immune-mediating genes induced during UTI, including in the IL-17/IL-33 axis (19). The authors concluded that a better understanding of the immune system's role in UTIs might contribute to developing nonantibiotic-based stratified therapies targeting sex-specific immune pathways in managing women and men (19).

In conclusion, while multiple factors, including genetics, age, and access to care, contribute to the gender differences in the incidence of UTIs, sex hormones have a powerful influence on the onset, severity, and patient outcomes (12,14).

UTI is a syndrome. The symptoms of urgency, frequency, fever, suprapubic pain, or dysuria, combined with the presence of bacteria in the urine, constitute a UTI. When a patient has symptoms without bacteriuria, other syndromes should be considered, such as overactive bladder (OAB) syndrome or interstitial cystitis/bladder pain syndrome (IC/BPS), which should not routinely be treated with antibiotics (20,21). Point-of-care testing by dipstick may help to clarify a diagnosis, but such analysis has drawbacks, and the characteristic clinical presentation is reliable for the diagnosis. A complete medical history and a short physical examination should still be taken. However, the diagnosis of a first episode of uncomplicated UTI can be symptom based, and there is usually no requirement for further diagnostic workup in women under 40 without other risk factors. Taking a urine culture is not recommended for the first episode but should be considered if presenting symptoms are not characteristic or when there is a failure to respond to antibiotics or the infection recurs within 1 month of antimicrobial therapy (8). A voided urine specimen, collected using a method to minimize contamination, is usually appropriate. An in-and-out catheter specimen is recommended if a voided specimen cannot be obtained. Any gram-negative organism isolated in counts $\geq 10^2$ colony-forming units (CFU)/mL is considered relevant for this presentation in these patients. Of note, true bacteriuria is considered to be a count $>10^5$ CFU/mL, both for pyelonephritis and cystitis, and the cut-off of 10^5 bacteria/mL remains the benchmark in many laboratories.

Data from international surveillance studies suggest that the most common pathogens associated with uncomplicated UTI are UPEC, which accounted for 76.7% of uncomplicated cystitis, *Enterococcus faecalis* (4.0%), *Staphylococcus saprophyticus* (3.6%), *Klebsiella pneumoniae* (3.5%), *Proteus mirabilis* (3.5%), and other bacteria (8.7%) (22,23). UPEC is also the most prevalent pathogen identified in complicated cystitis (43%), but to a much smaller extent than in uncomplicated UTI, and many other pathogens, including *Klebsiella* spp. (13%), *Enterococcus* spp. (10%), *Pseudomonas aeruginosa* (9%), *Enterobacter* spp. (7%), *Proteus* spp. (6%), *S. aureus* (3%), fungi (1%), and other bacteria, become important, as does the issue of AMR (22,24).

Treatment strategies

There are a variety of antibiotic strategies and antibiotic-sparing approaches to treating an episode of uncomplicated UTI (Fig. 2A).

A recent systematic review of randomized controlled trials of analgesics (nonsteroidal anti-inflammatory drugs [NSAIDs]/nonsteroidal antirheumatic drugs [NSARs]), herbal formulations, delayed prescription of antibiotics, and placebo therapy to prevent the overuse of antibiotics in women with uncomplicated UTIs found that antibiotic-sparing strategies can reduce antibiotic use by 60-70% (25). However, they may result in higher rates of incomplete recovery or therapy failure compared with immediate antibiotic use, and a higher incidence of secondary outcomes, such as pyelonephritis or febrile UTI (25-27). The use of antibiotic-sparing and other strategies in the treatment and prevention of rUTIs will be developed in the following sections.

The EAU Guidelines for antimicrobial therapy in uncomplicated UTI recommend first-line therapy with fosfomycin trometamol, different formulations of nitrofurantoin, or pivmecillinam. Table II summarizes the regimens and details the doses and duration of therapy. Of note, fluoroquinolone and quinolone antibiotics are no longer included in the treatment of uncomplicated UTIs, having been banned from use by the European Commission from 2019 because of serious disabling and potentially permanent side effects (28).

To guide the choice of antibiotic for uncomplicated UTI, it is important to be aware of the local resistance data. For example, surveillance study conducted in Europe and Brazil in the Antimicrobial Resistance Epidemiology in Females with Cystitis (ARESC) study identified *E. coli* as by far the most frequent uropathogen identified, present in 74.6% of urine cultures, followed by *E. faecalis*, *S. saprophyticus*, *K. pneumoniae*, and *P. mirabilis*, all at less than 5% and found that, although susceptibility rates varied considerably from country to country, rates for fosfomycin, pivmecillinam, and nitrofurantoin approached 100% overall (98.1%, 95.8%, and 95.2%, respectively) (23). Susceptibility to cotrimoxazole was lower (70.5%), which is why cotrimoxazole is not recommended as a first-line treatment in uncomplicated UTI.

In contrast, a recent study of the prevalence and resistance patterns of uropathogens in different regions of India found that, while *E. coli* was still the most prevalent pathogen identified (68.3% overall), there was a much higher prevalence of *K. pneumoniae* (17.7%) than in Europe, and resistance to nitrofurantoin was higher for both pathogens (5.8% and 45.4%, respectively) (29). Of note, none of the UPEC were resistant to fosfomycin. These differences emphasize

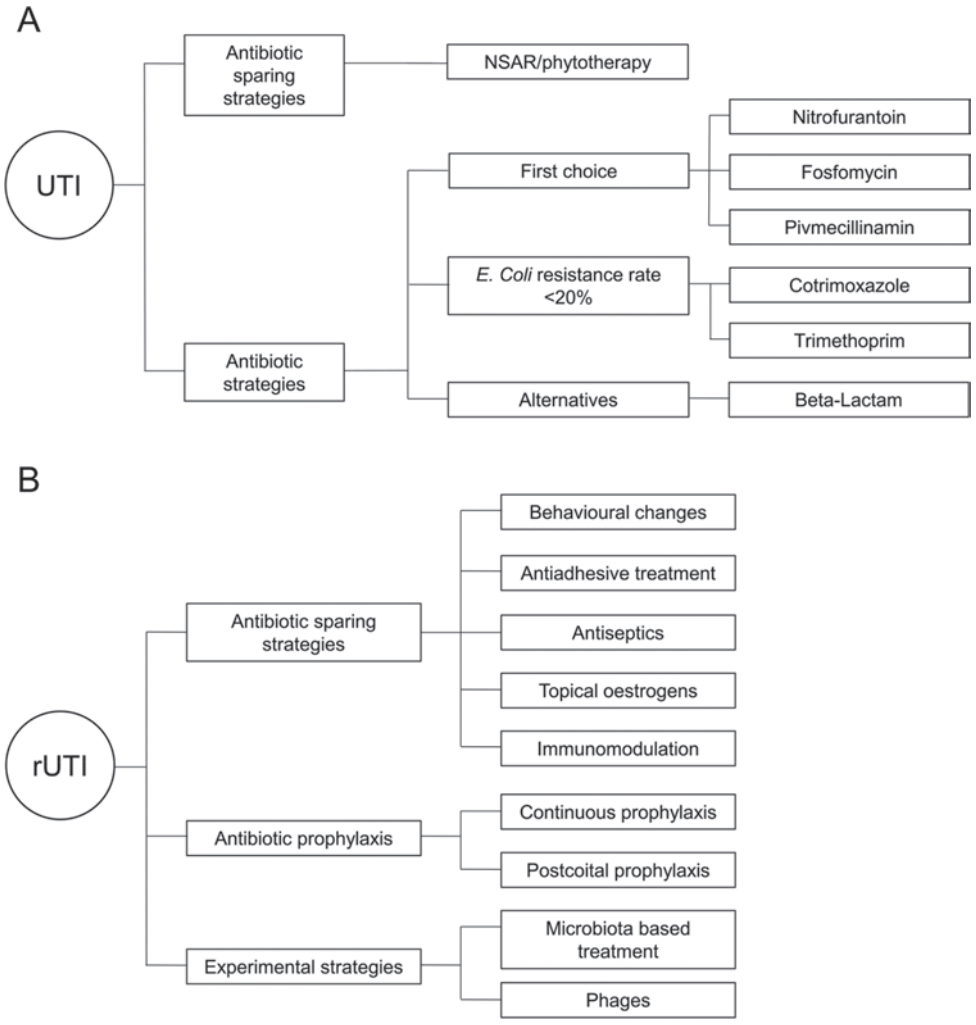

Fig. 2 - Treatment options for **A)** uncomplicated urinary tract infections (UTIs) and **B)** recurrent urinary tract infections (rUTIs).

TABLE II - Suggested regimens for antimicrobial therapy in uncomplicated cystitis. Recommendations of the European Association of Urology (EAU) (8)

Antimicrobial	Daily dose	Duration of therapy	Comments
First-line women			
Fosfomycin trometamol	3 g SD	1 day	Recommended only in women with uncomplicated cystitis.
Nitrofurantoin macrocrystal	50-100 mg four times a day	5 days	
Nitrofurantoin monohydrate/macrocrystals	100 mg b.i.d.	5 days	
Nitrofurantoin macrocrystal prolonged release	100 mg b.i.d.	5 days	
Pivmecillinam	400 mg t.i.d.	3-5 days	
Alternatives			
Cephalosporins (e.g., cefadroxil)	500 mg b.i.d.	3 days	Or comparable
If the local resistance pattern for *Escherichia coli* is <20%			
Trimethoprim	200 mg b.i.d.	5 days	Not in the first trimester of pregnancy
Trimethoprim-sulfamethoxazole	160/800 mg b.i.d.	3 days	Not in the last trimester of pregnancy
Treatment in men			
Trimethoprim-sulfamethoxazole	160/800 mg b.i.d.	7 days	Restricted to men. Fluoroquinolones can also be prescribed in accordance with local susceptibility testing.

b.i.d. = twice daily; SD = single dose; t.i.d. = three times daily.

the importance of local prevalence and resistance patterns to guide the choice of antibiotic.

Prevention of recurrent cystitis: current state of the art

As noted, an rUTI is defined as an UTI occurring at least three times a year or twice in the previous 6 months. However, in real-world practice, only about 20% of episodes occur twice in 6 months or three times a year; in a recent European study (GESPRIT), 47.4% of women had at least six UTIs per year, and 14.4% had over 12 infections per year (30). In fact, before preventive measures were started, almost a third of women experienced at least ten episodes per year. The health and economic burden caused by rUTIs is significant; rUTIs impact patients' daily activities and mental health, leading to high levels of frustration, anger, and dissatisfaction (30). Women may need bed rest, be absent from work, and require doctor visits and prescriptions for antibiotics.

There are many strategies for preventing the recurrence of UTIs, which can be summarized as antibiotic-sparing strategies, antibiotic prophylaxis, and experimental strategies (Fig. 2B).

Non-antimicrobial prophylaxis

In following a step-by-step approach to prophylaxis for rUTIs, avoidance of risk factors and behavioral modifications (increasing fluid intake, pre- or postcoital urination, and hygiene procedures) can be considered the first step. However, evidence for their benefits in reducing episodes of rUTI is limited. A recent study showed that increasing the daily water intake by about 1.5 L reduced cystitis episodes and antibiotic usage in premenopausal women with rUTIs (31). During 12 months of follow-up, the mean number of cystitis episodes in the 70 women randomized to the increased water intake group was 1.7 (95% confidence interval [CI], 1.5 to 1.8) vs 3.2 (95% CI, 3.0 to 3.4) in the 70 women in the control group who did not increase their usual water intake, a difference in means of 1.5 (95% CI, 1.2 to 1.8; $p < 0.001$). Over that time, the mean number of antibiotic regimens required to treat cystitis episodes was 1.9 vs 3.6, respectively ($p < 0.001$) (31).

Non-antimicrobial strategies avoid the risk of AMR and are the next step to consider before antimicrobial therapy is used. These strategies include hormonal replacement therapy, immunoactive prophylaxis, probiotics, cranberry, D-mannose, methenamine hippurate, and intravesical instillations of GAGs (8,32-34).

Hormonal replacement using topical (but not oral) estrogen therapy in postmenopausal women, intended to enhance vaginal and urethral flora, has been shown to have some beneficial effects in reducing rUTIs without systematic side effects, although local irritation and minor bleeding can occur (8,34-36). Topical estrogen administration by vaginal cream or pessary is more effective than placebo but less effective than antimicrobial prophylaxis.

A number of different immune cells are present in the bladder, including macrophages, dendritic cells, lymphocytes, monocytes, neutrophils, and eosinophils, suggesting a role for prophylaxis with immunoactive agents in UTIs (8,34). Immunoactive agents are understood to stimulate both the innate and adaptive immune systems to increase the production of bacteria-specific antibodies. OM-89 (Uro-Vaxom) is perhaps the most widely studied immunostimulant, consisting of an extract for oral administration of 18 strains of heat-killed UPEC. OM-89-S is a newer formulation prepared by a different lysis process. OM-89 (but not OM-89S) has shown significant efficacy in the prophylaxis of rUTIs and is included in the EAU guidelines as an option with a high level of evidence for preventing rUTI in women (8,33,34,37,38). A systematic review of the role of vaccines in the treatment of rUTIs concluded that OM-89 (usually at a dose of one oral tablet once daily for 3 months, and/or a booster of one tablet daily for the first 10 days of months 6-9) had a short-term role in preventing rUTIs, significantly reducing the risk of recurrence compared with placebo, with only mild side effects (33). However, vaccination therapies remain under-reviewed, and further research is needed.

When the balanced ecosystem of normal flora in the vagina is disturbed through repeated antibiotic use, spermicide use, or in postmenopausal women, the vagina may become a reservoir for uropathogenic bacteria (12,34,39). Probiotics may help to prevent rUTIs through many mechanisms, including restoration of the natural vaginal microbiota by increasing the level of protective *Lactobacillus* species, altering the pH of the vagina, inhibiting bacterial biofilm formation, downregulating inflammatory cytokines, and inhibiting the uptake of *E. coli* by vaginal epithelial receptors. While there are some contradictory findings, oral or vaginal administration of the lactobacillus strains *L. rhamnosus* GR-1, *L. reuteri* B-54 and RC-14, *L. casei shirota*, or *L. crispatus* CTV-05 have been shown to be effective for vaginal flora restoration and a beneficial effect in preventing rUTIs (8,12,34,36,39,40). However, the level of evidence is insufficient to allow definitive recommendations on the route of admission, optimal dosage, or treatment duration (8,35).

A number of meta-analyses and systematic reviews that have investigated the efficacy of cranberry-containing products have found a favorable benefit-to-harm ratio but limited evidence for their efficacy in protection against UTIs. However, a recent Cochrane Database systematic review of 50 randomized or quasi-randomized controlled trials (8,857 patients) of cranberry products vs placebo, no specific treatment, or other interventions for the prevention of UTIs supports their use to reduce the risk of symptomatic, culture-verified UTIs in women with rUTIs, children, and people susceptible to UTIs following interventions (41). Currently, the level of evidence for cranberry products compared to antibiotics or probiotics alone is very low, and the available evidence does not support their use in older patients, patients with bladder emptying problems, or pregnant women.

The monosaccharide isomer of glucose, D-mannose, is thought to act in the bladder by inhibiting the adhesion of UPEC to uroepithelial cells (34,36). Again, the overall level of evidence for efficacy in the prevention of rUTI is weak and contradictory, and, as with cranberry products, a good patient history to identify what has already been tried and to determine what triggers the UTI should first be taken. The patient should be informed that further studies are

needed before D-mannose can be fully recommended (8). A recent Cochrane systematic review of randomized controlled trials also concluded that there is currently little to no evidence for or against D-mannose use in preventing rUTIs (42).

The urinary antiseptic methenamine hippurate (hexamine hippurate) has bacteriostatic activity via the production of formaldehyde from hexamine in an acid environment (34,36). The most recent Cochrane review of methenamine hippurate for preventing UTIs dates back to 2012 when it was concluded that methenamine hippurate might be effective short-term prophylaxis in patients without renal tract abnormalities (43). There was a 76% reduction in the incidence of UTIs (risk ratio 0.24; 95% CI, 0.07 to 0.89), comparable to antibiotic prophylaxis, but the quality of studies included in the analysis was mixed, and the data were heterogeneous. More recent studies have shown that long-term (6-12 months) methenamine hippurate is not inferior to daily low-dose antibiotics to prevent rUTI (26,44). In a study conducted at eight centers in the United Kingdom, 240 women with rUTIs were randomized to methenamine hippurate twice daily (n = 120) or daily low-dose antibiotics (nitrofurantoin, trimethoprim, or cefalexin; n = 120) for 12 months (26). During the 12-month treatment period, the incidence of UTIs requiring antibiotic treatment was 1.38 (95% CI, 1.05 to 1.72) episodes per person-year in the methenamine hippurate group and 0.89 (95% CI, 0.65 to 1.12) in the antibiotics group. The absolute difference of 0.49 (90% CI, 0.15 to 0.84) indicated noninferiority between the two strategies. Therefore, the current recommendations support using methenamine hippurate to prevent rUTIs in women without urinary tract abnormalities (8).

As disruption of the bladder lining (urothelium) and resultant loss of the protective GAG layer is considered to be a key factor promoting rUTIs, GAG therapy with intravesical instillations of hyaluronic acid (HA) or HA in combination with chondroitin sulfate (CS) has been suggested as an option to prevent rUTIs, particularly in patients where less invasive preventive approaches have been unsuccessful (8). The quality of evidence for the benefits of combination therapy is highest, as randomized controlled trials of the instillation of single-agent HA or CS are lacking. In a randomized trial in 57 women with rUTI, intravesical administration of 50 mL of HA 1.6% plus CS 2.0% (Ialuril®, IBSA) for 6 months, the decrease in mean UTI rate per patient-year at the end of the 12-month study was 86.6% ± 47.6 compared with 9.6% ± 24.6 for placebo (intravesical saline), a mean difference of 77% (95% CI, 72.3 to 80.8, p = 0.0002) (45). The mean time to UTI recurrence was also significantly shorter in the placebo group. Given the issue of antibiotic resistance, the instillation of GAGs may become a recommended prophylactic option to antibiotic prophylaxis when more data are available.

Another, retrospective, case-control study in 276 women treated for rUTIs at seven European centers compared intravesical administration of combination HA+CS with EAU-recommended standard of care (continuous or postcoital antimicrobial prophylaxis, immunoactive prophylaxis, prophylaxis with probiotics or cranberry, or a combination of these) (46). In this real-world setting, bladder instillation of combined HA and CS reduced the risk of UTI recurrences, compared with standard management (55.7% vs 62.1%, respectively), although the difference did not reach statistical significance (p = 0.313). However, when the adjusted odds ratio (OR) for developing a bacteriologically confirmed rUTI within 12 months was calculated, there was a 49% reduced risk of developing a recurrence in women treated with HA+CS compared with standard care (OR 0.51, 95% CI, 0.27 to 0.96) (46). Treatment adherence (≥5 instillations) was associated with improved benefits of HA+CS therapy.

Data from a systematic review and meta-analysis of randomized and nonrandomized trials found that HA plus CS decreased the rate of rUTI per patient-year by a pooled mean difference of 2.56 compared with controls (95% CI, 3.86, −1.26, p < 0.001) (47). The time to a first recurrence of a UTI was also increased by a mean of 130.05 days. However, patients should be informed that further studies are needed to confirm the results of existing trials (8). An oral formulation of HA, CS, quercetin, and curcumin (Ialuril® Soft Gels, IBSA) is available and may be appropriate in patients with pelvic pain, although hard data on usefulness in the prevention of rUTIs are limited.

Many other non-antimicrobial options have been investigated or are already in use as strategies to prevent rUTIs. A detailed review of these approaches is beyond the scope of this article. However, a comprehensive review by Paul Loubet and colleagues provides a useful overview of alternative therapeutic options to antibiotics (40). Among the approaches are small compounds, often molecules that are by-products of or mimic bacterial substrates, designed to occupy binding sites to prevent adherence of pathogens to the uroepithelium, directly target the protective capsule of bacteria, inhibit enzymes essential to UPEC, or reduce biofilm formation (40). The formation of biofilms, either directly on the urothelial surface or formed around indwelling catheters, is a common strategy adopted by UPEC, providing a favorable environment for bacterial colonization and a persistent source for bacterial access to the urinary tract, thus facilitating rUTIs (16,22). Vitamin C, Chinese medical herbs, and various vaccine approaches that target microbial adhesion, the bacterial capsule, toxins secreted by UPEC that act as virulence factors, or iron metabolism processes essential for bacterial growth and colonization are also alternative nonantibiotic therapeutic options for UTIs (34,40). Finally, although not commonly utilized in the West, bacteriophages, viruses that cause lysis of bacterial cells, including antibiotic-resistant UPEC, have been used in eastern European countries for decades (36).

Antibiotic prophylaxis

When non-antimicrobial interventions have failed, continuous low-dose antimicrobials and postcoital antimicrobial prophylaxis have been shown to reduce the rate of rUTIs (8,36) and are recommended for use (8). There is no significant difference between the two strategies. In both approaches, patients should be counseled on possible side effects. The optimal duration of continuous antimicrobial prophylaxis is uncertain, with studies reporting durations of from 3 to as long as 12 months; between 3 and 6 months is typical (8).

Intermittent self-start treatment (self-diagnosis and self-treatment) with a short course of antibiotic therapy is also

effective and safe in women with rUTIs who have shown good compliance and motivation and can be an economical approach to preventing rUTIs (8,36).

The choice of antimicrobial agent is the same as that for acute uncomplicated UTI and should be based on local resistance patterns. Patients on antimicrobial prophylaxis may have been advised to increase the dose of the same antibiotic if they have a breakthrough infection. However, a more appropriate course of action would be for a culture to be taken to determine if switching to an antibiotic more helpful in the setting is indicated. If the patient has severe symptoms and wishes to start empirical treatment immediately, the recommended first-line antibiotics (i.e., fosfomycin trometamol, nitrofurantoin, or pivmecillinam) should be chosen (8).

Managing patients with UTIs in daily practice: the nurse's perspective

Managing UTIs is a major problem for patients, caregivers, and the physicians and specialist nurses involved in daily clinical practice. As well as the economic cost, UTIs impose a considerable loss of quality of life for the patient (1,2,30,48).

Increasingly, public health policy is to keep older people in their homes for as long as possible, promoting self-care for everyone and providing home care services where necessary. For patients with UTIs, there is likely to be a preference for self-instilling bladder compounds at home, and visiting specialist nurses will teach self-catheterization and self-instillation procedures. A specialized urological team that can provide almost all urological care in the home, including managing UTIs, is effective in freeing up hospital care. In the absence of specialized in-home care, solutions must be found in the outpatient setting.

Ideally, seeing patients in their homes provides an insight into the most helpful approach to patient needs. UTIs often contribute to delirium, falls, and confusion in vulnerable older patients, even before a UTI is diagnosed (49,50). Therefore, prevention is the best solution; the challenge is achieving this. Close cooperation involving the nurse, physician, patient, caregivers, and even neighbors is fundamental. Knowledge and understanding of the relevant clinical guidelines for urological infections should be shared, and agreement on the relative roles and time points for interventions should be established. An open and proactive multidisciplinary approach should be adopted, sharing knowledge about patients and bringing the individual members' experience and expertise to bear on the issue.

The patient and caregivers will benefit from being educated by the urologic nurse on prevention strategies. These include drinking enough water, following an adequate diet (e.g., limiting the amount of sugar, a substrate for bacteria, in the diet for diabetic patients), good hygiene procedures daily and after sex, solving any issues of incontinence as best as possible, encouraging patients to keep active and moving, and, where appropriate, good catheter management (8,30,51,52). This can be a big challenge with elderly and vulnerable people, particularly where dementia, depression, refusal of care, or living alone without support are issues. The problem can be different in every situation, and if a meaningful difference is to be achieved, it is essential to know what is acceptable to the patient and their level of compliance. Searching to find the best solution should always be the goal.

In daily practice, a cautious approach to antibiotics is appropriate, and their use is reserved only in case of infection after performing the necessary analysis. Antibiotics may initially be used if cystoscopy imaging shows the bladder to be very infected, with alternative treatments to antibiotic prophylaxis considered at a later time where appropriate, perhaps 3-4 months after initial antibiotic therapy. Despite reservations about its effectiveness, D-mannose may be appropriate to help reduce rUTI episodes in some patients, topical estrogens can be beneficial in postmenopausal women, and probiotics or GAG therapy may also be considered to help prevent rUTIs (8). If intravesical instillation of GAGs is applicable, high-dose HA and CS are preferred (8), and training on self-instillation should be given. Seven instillations are typically used, the first four at weekly intervals, then two at 2 weeks apart, and one a month later. However, if patients find pain relief from the treatment, it may be extended empirically in consultation with their needs.

A number of issues should be addressed to improve catheter management in rUTIs. It should be considered if catheterization is necessary, the risk of potential infections, how accepting the patient is of the procedure, issues of pain or blocking of catheter insertion, and the choice of catheterization methods. In all instances, the aim should be to find the best solution for the individual patient. In seeking alternative methods to standard catheterization, the Ialuadapter® device has been developed for instillation into the bladder (Fig. 3). It can be attached to Luer Lock or Luer Slip syringes and offers an efficient method of administering medicine into the bladder without needing a catheter. The adapter tip can be guided into the urethral orifice of female and male patients with minimal risk of pain or other complications (53).

Fig. 3 - The Ialuadapter® device for use with a Luer Lock or Luer Slip syringe as an alternative to intravesical instillation by standard catheterization. Figure the property of Institut Biochimique SA (IBSA).

After an initial learning curve involving some practice with the Ialuadapter®, the use of the device for the instillation of HA and CS is a pain-free alternative to standard catheterization in both men and women, associated with fewer infections while providing simultaneous treatment of the bladder and the urethra. The method is also more effective and gives faster results in patients with bladder pain and urethritis. Although a residual urine volume of 50 mL is acceptable, the patient should ideally have an empty bladder before use.

TABLE III - Use of the Ialuadapter® in patients with urological conditions (n = 80)

Parameter	*N* (%)
Gender	
Female	78 (97.5)
Male	2 (2.5)
Age of participants	
≥50 years	50 (62.5)
<50 years	30 (37.5)
Urological indication	
BPS/IC	64 (80.2)
rUTIs	14 (17.4)
OAB syndrome	2 (2.3)

BPS/IC = bladder pain syndrome/interstitial cystitis; OAB = overactive bladder; rUTI = recurrent urinary tract infection.

In a 6-month study in the Andros Clinic in Rijswijk, around 170 instillations were performed with the device in 80 patients, predominantly in women with BPS/IC (80.2%), although 17.4% of patients had rUTIs and 2.3% OAB syndrome (Tab. III) (R Pothoven, data on file). The majority (62.5%) of patients were aged over 50, and only two men were included in the study.

The main reason patients tried the Ialuadapter® as an alternative to standard catheterization was an expectation of experiencing less pain during the procedure (40.8% of patients) (Fig. 4). Ease of use and anticipation of fewer infections were each given as reasons by around a quarter of patients, while 1.9% of patients had a fear of standard catheterization. Other reasons (fewer complications, less pain after instillation, more comfortable, lower volume of fluid to be given, and greater effect) were expressed by 5.8% of patients.

Eighty-two percent of patients over 50 and 63.3% of those under 50 wanted to continue using the Ialuadapter® (Fig. 5), mainly because of less pain (50.1% of patients overall). Other reasons were that the device was easier to use than a catheter (20.9%) and could be done at home (10.5%).

It was thought that a greater proportion of the younger patients were too tense and less able to relax than those over 50, leading to a higher rate of leakage after instillation, probably contributing to the lower percentage of patients under 50 wishing to continue to use the device. Of the 59 patients who continued using the Ialuadapter®, none experienced an rUTI. Research is ongoing to identify the patients best suited for instillation therapy.

Discussion and conclusions

UTIs impose a sizable personal and economic burden on individuals and health resources worldwide (1-4), and effectively managing UTIs is a major challenge faced not only by physicians and specialist nurses in their daily clinical practices but also by patients and their caregivers in the community.

While antibiotic treatment of symptoms suggestive of UTIs and rUTIs has clear benefits to patients, overuse and inappropriate use have led to a worldwide problem of AMR that has become an increasing threat to public health.

Programs of antimicrobial stewardship that aim to optimize clinical outcomes while reducing inappropriate antibiotic use have been developed to provide guidance encouraging prudent and appropriate antibiotic prescribing that minimizes the unintended consequences of antibiotic overuse, including antibiotic-related adverse events and the emergence of resistant bacterial strains (5,8). When antibiotic therapy is indicated for treating UTIs or rUTIs, choosing an appropriate antibiotic based on local resistance and susceptibility patterns is essential, guided by evidence-based clinical guidelines such as those compiled by the EAU, which

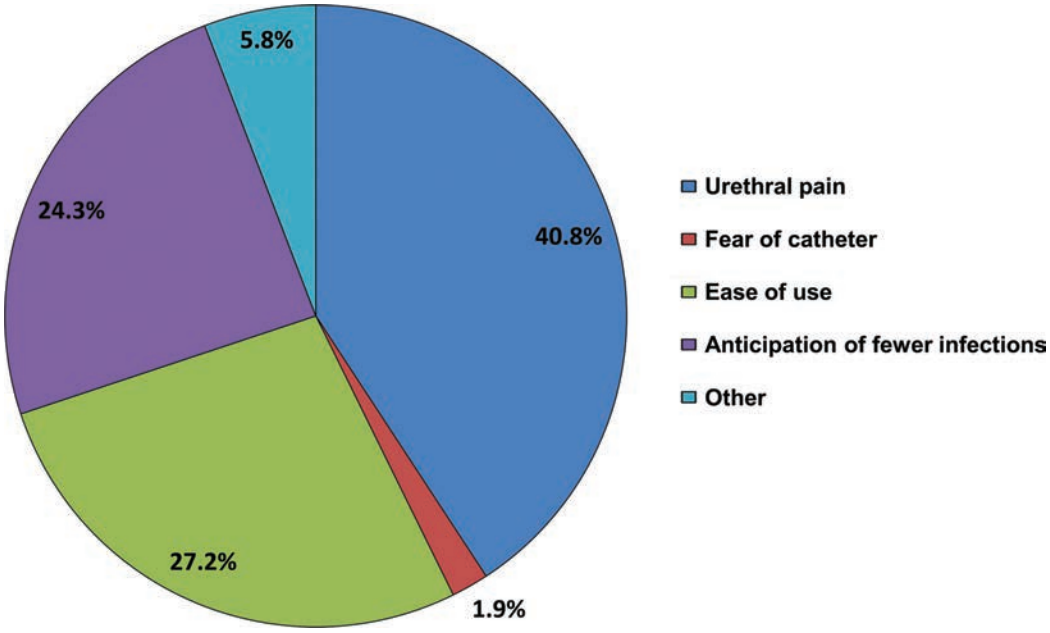

Fig. 4 - Reasons for trying the Ialuadapter® as an alternative to standard catheterization (n = 80). Data the property of the author.

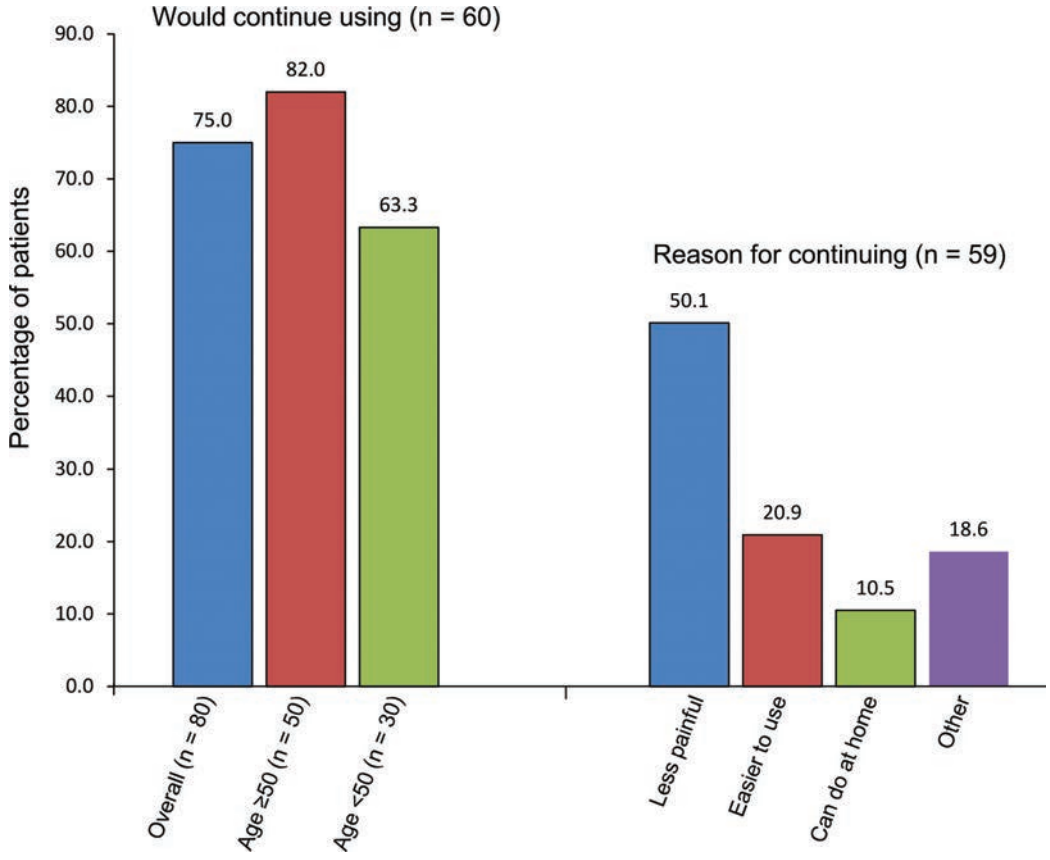

Fig. 5 - Patient feedback on using the Ialuadapter® device. Data the property of the author.

recommends first-line therapy with fosfomycin trometamol, nitrofurantoin, or pivmecillinam as first-line antimicrobial therapy for uncomplicated UTIs (8).

Appropriate non-antimicrobial strategies aimed at reducing the incidence of rUTIs should also be considered. Indeed, the increasing global burden of drug-resistant pathogens makes identifying effective non-antimicrobial strategies to reduce the burden of bacterial AMR an urgent priority (6). Strategies are available to reduce the dependence on antimicrobial use in uncomplicated UTIs, including methenamine hippurate, cranberry extract, D-mannose, probiotics, intravesical GAG therapy, and prophylactic vaccination.

Regardless of whether an antimicrobial or non-antimicrobial approach is taken, counseling patients about avoidance of risk factors and behavioral modifications can be a first step toward prophylaxis of rUTIs. Furthermore, the use of non-antimicrobial strategies should be based on physician expertise supported by clinical evidence, as reducing the overall use of antibiotics should not be at the expense of compromising clinical outcomes.

Further well-designed studies evaluating non-antimicrobial prophylaxis of rUTIs is needed to extend the level of evidence and fully define the place of non-antimicrobial strategies in routine clinical practice. Also important is the need to identify which patients might benefit most from non-antimicrobial treatment for acute UTIs or non-antimicrobial prophylaxis for rUTIs.

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