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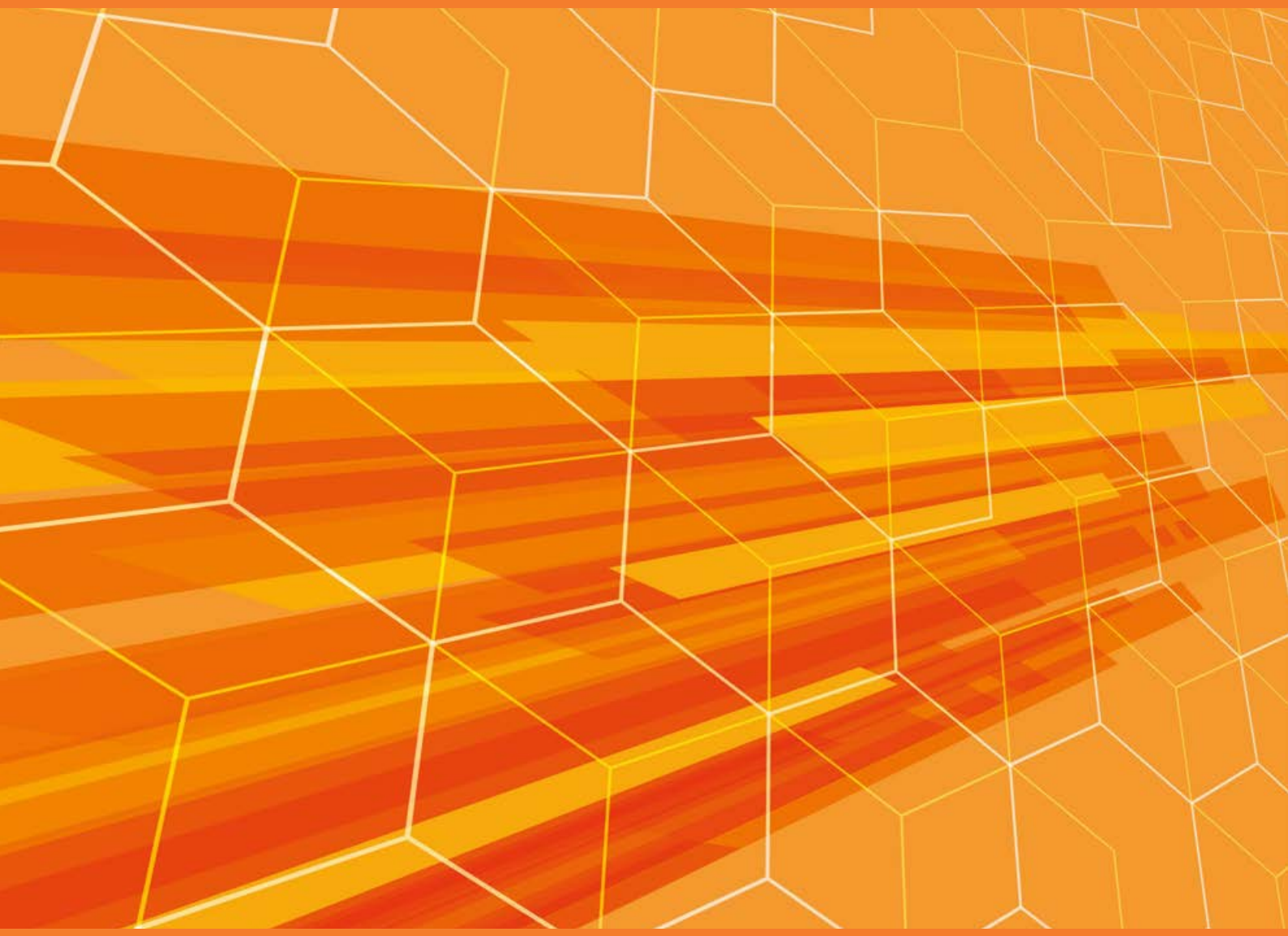

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6 Anatomical and functional responses to single brolucizumab injection in neovascular age-related macular degeneration patients not responding to antiangiogenics: a case series
Silvio Zuccarini, Fabrizio Puce, Alessandro Crisà

Focus on Antimicrobial Resistance (AMR)

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CASE REPORT

Erythrodermic psoriasis and palmoplantar hyperkeratosis successfully treated with secukinumab: a case report

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ABSTRACT

Introduction: Erythrodermic psoriasis (EP) is a rare and severe form of psoriasis that affects 1% to 2.25% of patients, increasing mortality risk. To date, very few therapies have been approved for the treatment of this condition. Recently, biological therapies that specifically target inflammatory cytokines have improved the management and treatment of EP. Secukinumab, a human monoclonal antibody that specifically targets interleukin-17A (IL-17A), has been shown to be beneficial in different psoriasis settings.

Methods: We report the case of a 72-year-old man affected by persistent EP and severe palmoplantar hyperkeratosis whose condition was not resolved after two rounds of treatment with prednisone and therapy with cyclosporine.

Results and conclusions: Treatment with secukinumab significantly improved the symptoms of palmoplantar hyperkeratosis as early as the first week, with a decrease of psoriasis area and severity index (PASI) score from 60 to 10, showing almost complete remission after 1 month. Consistent with the current literature, secukinumab treatment showed promising and encouraging clinical outcomes in the treatment of the patient's EP. However, more studies are needed to clarify the IL-17-dependent mechanism in the pathophysiology of EP.

Keywords: Biologic therapy, Erythrodermic psoriasis, Interleukin-17, Monoclonal antibody, Palmoplantar hyperkeratosis, Secukinumab

Introduction

Psoriasis is an immune-mediated inflammatory disease with unknown etiology, characterized by the presence of papules and plaques of various morphology and severity over the surface of skin (1). Psoriasis is classified as an inflammatory autoimmune disease in which an excessively aberrant hyperproliferation of keratinocytes is present, along with dilated, hyperplastic blood vessels and inflammatory infiltration of leukocytes predominantly into dermis (2). These features typically present as red skin patches that are itchy and scaly. As with other autoimmune diseases, psoriasis may have a systemic outcome beyond the skin, also affecting the joints, nails, and other organs (2). Currently, there is no cure available, and psoriasis imposes a substantial negative impact on the quality of life (QoL) of patients. Psoriasis can occur at any age and is most common in the age group 50-69 years. The reported country-specific prevalence of psoriasis ranges between 0.09% and 11.4%, making psoriasis a serious global problem (1), with approximately 3% of the US population and around 125 million people affected worldwide, ranging from 0.5% in Asian regions to 8% of the population in Norway. Male and female populations are equally affected in most regions (3).

Psoriasis may occur in different forms, such as plaque psoriasis (characterized by dry scaly patches), which represents 80%-90% of psoriasis cases; pustular psoriasis (contains pus-like fluid mainly infiltrated with white blood cells); erythrodermic psoriasis (EP, characterized by exfoliation of fine scaly skin with pain and itching); guttate psoriasis (characterized by drop-like dots); and inverse psoriasis (affects the flexure surfaces and characterized by smooth inflamed lesions) (1).

The pathogenesis of psoriasis is not completely understood, and the exact mechanism remains elusive, although the literature suggests that different genetic, epigenetic, and environmental factors may be responsible for the onset of psoriasis (2). Although not inherited in a Mendelian fashion, a familial predisposition may be present, which significantly

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increases the relative risk of psoriasis in families with a positive history among first-degree and second-degree relatives of patients, compared to the general population (2). Epigenetic factors including dysregulated deoxyribonucleic acid (DNA) methylation levels, abnormal histone modification, and microribonucleic acid (miRNA) expressions have been recognized as crucial players in the pathophysiology of psoriasis. Among other factors, excessive activation of the adaptive immune system and the interplay of immune cells and cytokines are thought to play a role in the onset of psoriasis (2,3). An initial secretion of cytokines that activate myeloid dendritic cells, from plasmacytoid dendritic cells, keratinocytes, natural killer T cells, and macrophages, is thought to initiate the first steps of the pathogenesis of psoriasis (3).

Erythrodermic psoriasis

Among the other forms of psoriasis, EP represents a rare and severe form that affects 1% to 2.25% of patients with psoriasis. The clinical characteristics of this form of psoriasis are the presence of erythematous, edematous, often exfoliative lesions that affect over 75% of the body surface area (4), and are often associated with numerous systemic symptoms such as fever, tachycardia, lymphadenopathy, arthralgia, and fatigue (5). There is a substantial risk of mortality due to transepidermal fluid and nutrient loss, and which in severe cases may lead to multiorgan failure and death (4).

EP generally develops in patients with poorly controlled psoriasis and as a result of the abrupt withdrawal of systemic medications such as corticosteroids, drug reactions to medications such as lithium, and underlying systemic infections (5). For a clear and correct diagnosis of EP, two general clinical subtypes that define the disease must be considered. The first subtype is characterized by psoriatic plaques that gradually differentiate and develop into generalized erythroderma, although, overall, the plaques remain differentiable from the erythroderma. This form of EP has a stable course and a good prognosis. In contrast, the second EP subtype is more common in a psoriatic arthritis setting and plaques usually cannot be distinguishable from whole-body erythema (6). As with psoriasis, the exact pathogenesis of EP is not fully clarified. The current literature suggests the involvement of an unbalanced T-helper (Th)1/Th2 differentiation in favor of the Th2 phenotype and its related cytokine secretion. Interleukin (IL)-4 and IL-13, in particular, have been shown to be elevated in EP relative to both psoriasis patients and healthy controls. Several studies have demonstrated that anti–tumor necrosis factor (TNF)-α agents improve EP outcomes, suggesting that TNF-α may play a role in the pathogenesis of EP (6).

Although difficult and challenging, the management of EP is possible through several treatment options. The US National Psoriasis Foundation had suggested, in 2010 in their consensus guidelines, the use of cyclosporine or infliximab as first-line therapy in unstable cases, with acitretin and methotrexate reserved for more stable cases. Since then, other therapies and treatment strategies have emerged, including topical treatment with steroids and vitamin D analogues, as well as phototherapy. Other studies have also demonstrated the efficacy of systemic agents, including second-generation retinoids (acitretin) and immunosuppressive drugs (methotrexate and cyclosporine), which showed complete remission and significant improvement in EP outcomes.

Recently, another class of drugs, called biological agents, has demonstrated high efficacy in EP management. Biological therapies represent an emerging class of immunosuppressive drugs, which, thanks to their enhanced selectivity to specific cytokines, may represent a valid alternative to the canonical treatments. Biological therapies include TNF-α inhibitors, IL-12/23 inhibitors, and IL-17 inhibitors (6).

Secukinumab: drug description and focus on the treatment of EP

As mentioned, an unbalanced proinflammatory response and massive cytokine secretion may promote and sustain worsening symptoms of EP. Besides Th1 and Th2, the recent discovery of the new class of T-helper cells, Th17, also highlighted the possible role of the proinflammatory cytokine IL-17 (secreted by Th17) in the pathogenesis of EP (7). Some environmental triggers, such as physical trauma, drugs, or infections, release proinflammatory cytokines, including IL-23 and TNF-α. Differentiation of T helper into Th17 cells and the release of cytokines, such as IL-17, promote keratinocyte proliferation, which, in the setting of EP, also release additional ILs and chemokines (8). The homodimeric glycoprotein IL-17A belongs to the IL-17 family and through its receptor complex IL-17RA/IL-17RC binds to keratinocytes, dendritic cells, dermal fibroblast, and endothelial cells.

Under physiological conditions, normal levels of IL-17A promote, upon binding, the proliferation of the keratinocytes necessary for healing, and protect against infectious agents. However, in psoriatic patients, levels of IL-17A are elevated, and correlate with the severity of the disease. Keratinocytes themselves, when stimulated, also synthesize many cytokines that can induce epidermal hyperplasia (autocrine growth factors) or neoangiogenesis (paracrine growth factors), resulting in worsening of EP and initiating a reverberating loop that perpetuates pro-proliferative and proinflammatory stimuli (9). Consistently, blocking IL-17A results in an improvement of psoriasis lesions, suggesting a key role in the pathogenesis of EP (8). Currently, one anti-IL-17A biological agent is approved for the treatment of plaque psoriasis, secukinumab, a monoclonal antibody that targets IL-17A (10).

Secukinumab is a novel biologic agent that specifically targets IL-17A. It is a fully human monoclonal antibody, and many clinical trials have demonstrated its efficacy in the treatment of plaque psoriasis, psoriatic arthritis, and ankylosing spondylitis (AS) (11-14). Indications approved by the European Medicines Agency (EMA) include adult and pediatric psoriasis, psoriatic arthritis, axial spondyloarthritis (axSpA), AS and non-radiographic axial spondyloarthritis (nr-axSpA) (15). The rationale behind the efficacy and mechanism of the action of secukinumab is a targeted approach to block the disease process at a very early stage along with a safe adverse event profile (16). By targeting IL-17A, secukinumab thereby blocks its binding with IL-17R and consequent expression of cytokines. Blocking the IL-17A pathway leads to a normalization of the inflammatory processes and thus combats epidermal

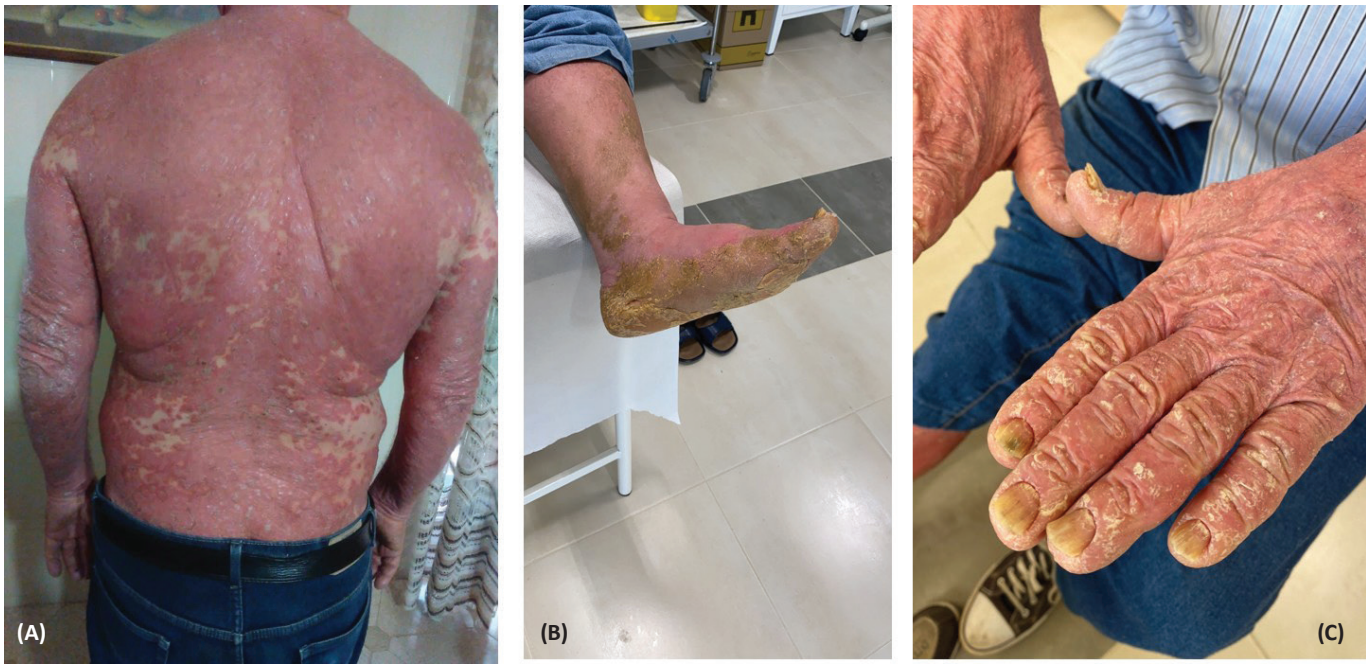

Fig. 1 - Widespread erythrodermic psoriasis (A) and palmoplantar hyperkeratosis (B, C) at initial presentation before secukinumab treatment.

hyperproliferation, T-cell infiltration, and excessive expression of pathogenic genes (16).

Several clinical trials have demonstrated the efficacy of secukinumab in the treatment of psoriasis (11-14). Two phase III, double-blind, 52-week trials (ERASURE and FIXTURE) (17) evaluated the efficacy of secukinumab in patients with moderate-to-severe plaque psoriasis. The ERASURE study enrolled 738 patients, while FIXTURE study included 1,306 patients. In both studies patients received either placebo or secukinumab subcutaneously once a week for 5 weeks, but in the FIXTURE study patients also received etanercept (50 mg twice a week for 12 weeks, then once a week). Results from the ERASURE study showed that a psoriasis area and severity index (PASI) 75 score at week 12 was achieved by 81.6% and 71.6% of patients administered with 300 mg and 150 mg of secukinumab, respectively, and by 4.5% of placebo recipients. Corresponding results from FIXTURE showed that 77.1% of patients administered 300 mg of secukinumab, 67% of those administered 150 mg of secukinumab, 44% of etanercept recipients, and only 4.9% of placebo patients achieved PASI 75 score at week 12 (17).

Another multicenter, international, retrospective, pilot study enrolled 13 EP patients who were treated with a loading dose of 300 mg of subcutaneous secukinumab at weeks 0, 1, 2, 3, and 4, followed by 300 mg every 4 weeks (18). Results from this study showed that the response rate to secukinumab was 10/13 patients (77%), with a median time to clearance of 3 weeks (1.5-3 weeks). No recurrences were registered in the 52-week follow-up and a PASI score of 90 was achieved by 10/13 patients. These results demonstrated that secukinumab remains a valid and effective therapeutic option for EP (18).

Case presentation

In this real-life clinical experience of the treatment of psoriasis with secukinumab drug, we report the case of a 72-year-old man, in general good health condition, who contacted the dermatologic outpatient clinic of the author on March 5, 2020, during the pandemic lockdown. The patient reported widespread EP with severe palmoplantar hyperkeratosis (shown in Fig. 1A-C), and he was not taking any other drugs or medications. He was started on prednisone (25 mg one tablet) treatment for 8 days and at half-dose for 8 more days. In addition, topical therapy with mometasone furoate cream was prescribed. On March 16, 2020, blood chemical tests were in the normal range, and the patient continued therapy with cyclosporine (100 mg twice a day) until July 2020. The patient's erythroderma significantly improved, but his palmoplantar hyperkeratosis persisted. Due to onset of hypertensive seizures and increased creatininemia, the patient discontinued the treatment. Then, after a pause of a few weeks, the patient resumed prednisone treatment in August 2020, due to the persistence of severe hyperkeratosis. However, there was no response or improvement.

On September 1, 2020, the patient started secukinumab treatment at the dose of 300 mg administered by two subcutaneous injections of 150 mg each (15); significant signs of clinical resolution were observed during the induction phase, for both erythrodermic psoriasis and palmoplantar

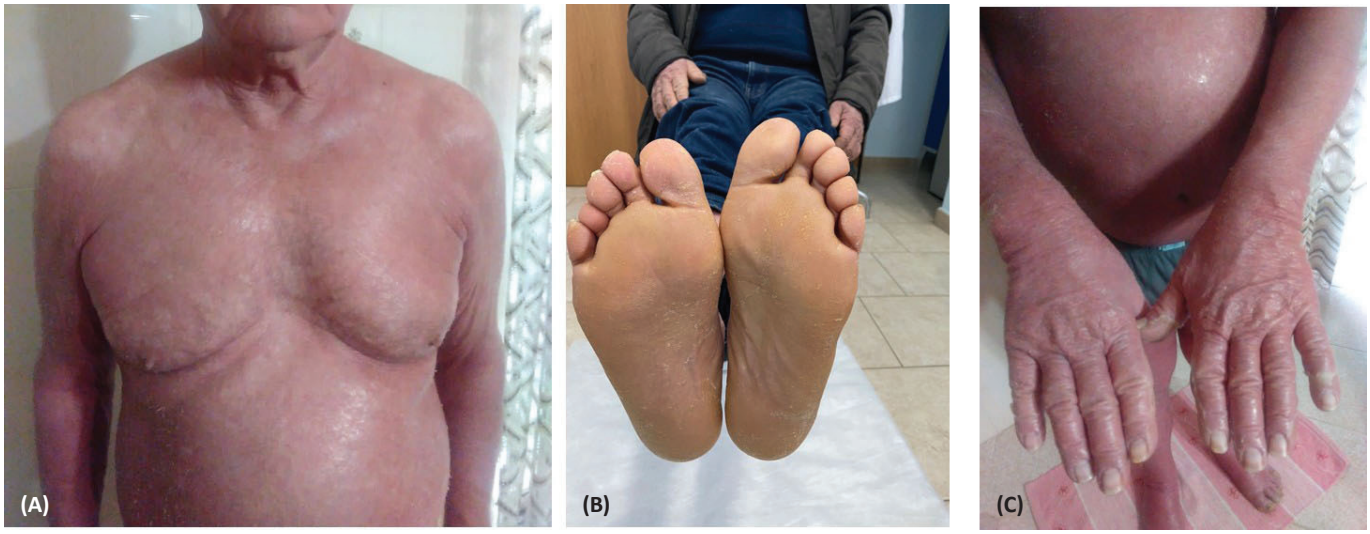

Fig. 2 - Improved erythrodermic (A) and palmoplantar hyperkeratosis (B, C) after secukinumab treatment.

hyperkeratosis. The patient is currently under maintenance treatment with secukinumab and his general condition is good. The PASI score at the first visit was 60, and decreased to 20 after induction (shown in Fig. 2A-C). At a follow-up visit, conducted at the end of February 2021, the patient showed an almost complete remission, with a PASI score of 10. As of January 2022 the patient, in perfect health conditions with normal hematochemical tests, presents a complete remission of the clinical picture.

Discussion and conclusions

Although the treatment options for EP have greatly expanded in recent years, psoriasis and its severe forms remain a major public health concern. Treatment of EP with secukinumab has shown promising and encouraging clinical outcomes, and the present case report showed data consistent with other case reports from the literature. Weng et al (19) reported a series of 10 cases in which secukinumab was prescribed for EP. Data from those case reports showed that at week 16, 4/10 patients and 7/10 patients, respectively, were able to achieve PASI 90 and PASI 75 scores (19). In this very first and unique case for our clinical practice, the patient, affected by palmoplantar psoriasis, but who also presented a very severe form of EP, showed significant signs of clinical resolution during the induction phase, with few side effects. The initial PASI score of the patient was 60, which significantly decreased to 20 after the induction and to 10 after the follow-up visit at 6 months, suggesting complete remission of EP. However, although treatment outcomes with secukinumab are promising, additional controlled trials with extended follow-up are needed to better understand the link between IL-17 inhibition and the resolution of EP.

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Author contributions

Dr. Carriero: conception and design of the work, acquisition and analysis of data, draft of the work; agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Statement of ethics

Written informed consent for publication (including images) has been obtained from the patient. The research was conducted ethically in accordance with the Declaration of Helsinki.

Disclosure

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ORIGINAL RESEARCH ARTICLE

Anatomical and functional responses to single brolucizumab injection in neovascular age-related macular degeneration patients not responding to antiangiogenics: a case series

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ABSTRACT

Introduction: Neovascular age-related macular degeneration (nAMD) is treated with antivascular endothelial growth factor (anti-VEGF) drugs. However, resistance to anti-VEGF therapy is observed in some patients. Brolucizumab is a new-generation anti-VEGF drug for the treatment of nAMD, with proven efficacy in fluid resolution and long-lasting effects.

Methods: We report here a case series of nAMD patients not responding to previous anti-VEGF therapy showing anatomical and functional response to a single intravitreal injection of brolucizumab.

Results: Nine patients with nAMD, undergoing treatment with anti-VEGF therapy (aflibercept, bevacizumab, or ranibizumab) but with either fluid persistence or frequent fluid recurrences in retinal compartments, were switched to intravitreal brolucizumab and examined 4 weeks postinjection. No signs of active disease were observed in all but one patient, with complete retinal fluid resolution in seven patients. Central macular thickness and visual acuity significantly improved, and changes were sustained for up to 12 weeks in a subset of three patients. No adverse reactions were observed.

Conclusions: This new anti-VEGF drug showed great efficacy since the first week from the injection with a significative reduction of subretinal fluid and rapid improvement of visual acuity. In conclusion, brolucizumab administered intravitreally appears to be an effective treatment in nAMD patients, leading to both early anatomical and functional improvements.

Keywords: Anti-VEGF, Brolucizumab, Case series, Neovascular age-related macular degeneration (nAMD)

Introduction

Neovascular age-related macular degeneration (nAMD) is a chronic degenerative disease characterized by the pathological growth of vessels, which leak blood and fluids in the various retinal compartments, leading to loss of visual acuity (VA) (1,2). Fluids accumulating in retinal compartments (i.e., intraretinal fluid [IRF], subretinal fluid [SRF], or fluid accumulating in the subretinal pigment epithelium [sub-RPE] space) are used as biomarkers for disease activity. Treatment of nAMD is based on the use of antivascular endothelial growth factor (anti-VEGF) drugs, with the objective of controlling the exudation from the vessels to minimize disease activity and consequently avoid VA loss (3,4). Appropriate treatment intervals maintain and sometimes improve patients' vision in the long term (3,5-9).

Macular neovascularization secondary to nAMD requires continuous treatment because it remains active for years (10). Randomized controlled trials showed that SRF and/or IRF is still present after two years of treatment in around 40%-50% of patients (3,11,12). Long-term retrospective observational studies have also reported around 40% of patients with detectable active disease at the end of the study, after 3 and 10 years, respectively (9,13). The activity pattern of nAMD is often unpredictable and patients must be continuously monitored and treated to avoid permanent VA loss

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(14-16), thus representing an important burden for both patients and clinicians (4,17).

In clinical practice, patients are treated in a personalized way with the aim of controlling recurrences, but over time anti-VEGF therapies may experience reduced effectiveness: after 5 and 10 years of treatment, 41% and 42% of patients, respectively, made at least one switch to other anti-VEGFs (9,18). The poor response (with either fluid persistence or recurrences) may be due to tachyphylaxis, changes in the neovascular membrane characteristics (e.g., increased fibrosis acting like a resorption barrier), chronic changes in vessel walls, changes in the type of lesion evolving over time, or an inadequate treatment frequency (19,20). As a consequence, switching treatment becomes necessary and it is important to re-establish control of the disease. While VA is not usually regained, treatment switch is consistently associated with anatomical improvement (20,21). In patients showing fluid persistence despite at least three-monthly injections with bevacizumab, switching to either aflibercept or ranibizumab showed comparable efficacy, with anatomical improvement noticeable after a single injection (22).

Brolucizumab 6 mg (Beovu®, Novartis AG, Basel, Switzerland), a monoclonal single-chain variable domain antibody fragment, is a new-generation anti-VEGF drug approved for the treatment of nAMD, with proven efficacy in fluid resolution and long-lasting effects (23). Brolucizumab shows great promise in reducing the risk of undertreatment and permanent VA loss. Results from the HAWK and HARRIER studies (24) showed that patients treated with brolucizumab (administered at 8- and 12-week intervals after three-monthly loading doses) had a significantly higher reduction in retinal central subfield thickness (CST) and absence of SRF/IRF at 48 and 95 weeks postinjection, compared with those treated with aflibercept (23). These data also showed that disease activity was controlled in a shorter timeframe after diagnosis when using brolucizumab compared with aflibercept (24). The use of brolucizumab in patients showing no response to other anti-VEGFs offers a valuable therapeutic option to maintain disease control and VA while reducing treatment burden.

Preliminary observations on patients switched to brolucizumab have recently been published (25,26), confirming its efficacy in controlling fluid accumulation. However, more data are needed to further characterize brolucizumab efficacy and rapidity in obtaining complete fluid resolution and the impact on functional outcomes.

We report here the anatomical and functional responses observed in a group of nAMD patients with persistent or recurrent fluids 1 month after a single injection of brolucizumab, as assessed retrospectively in a center in Italy.

Materials and methods

Study design

We retrospectively analyzed data from nine patients with nAMD who were switched to brolucizumab 6 mg because they were not responsive to other anti-VEGFs. All recruited patients were followed and treated at Villa Donatello Hospital, Florence, Italy. The use of brolucizumab for treating nAMD was officially introduced in October 2020. Patient demographic and clinical information collected at baseline (i.e., before the brolucizumab injection) included: age; gender; treatment prior to brolucizumab injection (anti-VEGF used, treatment duration, number of injections, last interval between injections, time from last injection to the first brolucizumab injection); VA; CST; type of lesion; and the presence of IRF, SRF, or sub-RPE fluid, fibrosis, subretinal fibrosis, and subretinal hyperreflective material (SHRM).

Following the first brolucizumab injection, patients were examined at two time points ($T1 = 1 \pm 1$ weeks and $T2 = 4 \pm 1$ weeks) to determine the presence of anatomical and functional changes using slit-lamp ophthalmoscopy, spectral domain optical coherence tomography (SD-OCT), fundus examination, and VA assessment (ETDRS scoring). Three patients had further assessments at a longer follow-up.

Due to the possibility of intraocular inflammation (IOI), vasculitis, or retinal vascular occlusion, all patients were informed and instructed to report, within 24 hours of the injection, any of the following symptoms: redness, reduction of viscous, floaters, eye pain or pressure, scotoma, or blurred vision. A notification letter was sent to the local Ethical Committee, according to Italian regulations. All patients provided written informed consent prior to receiving the brolucizumab injection.

Patient inclusion and exclusion criteria

The study only included patients with nAMD not responsive anymore to other anti-VEGFs (i.e., showing either fluid persistence or frequent fluid recurrences), who were first injected with brolucizumab by December 2020, and had a visit 1 month ($T2$ range 4±1 weeks) after the injection. Patients with a maculopathy other than nAMD or who received brolucizumab injection after December 2020 were excluded.

Study outcomes

The main outcome measured was the proportion of patients without SRF and IRF 1 month after brolucizumab injection. Secondary outcomes were the proportion of patients with reduced (or absent) SRF/IRF 1 month after injection, the proportion of sub-RPE fluid resolution 1 month after injection in patients with sub-RPE fluid at baseline, the proportion of patients without active disease 1 month after injection, the mean time to first disease inactivation, and the mean difference of CST and VA between baseline and 1-month postinjection.

Statistical analysis

Descriptive statistics were used to summarize demographic and clinical data at baseline and successive time points, as appropriate. Continuous data were described using the mean, standard deviation (SD), and range, while categorical data were expressed as percentages. The difference in CST and VA between baseline and the assessment at 1 month was tested using the nonparametric Wilcoxon

signed-rank test, with statistical significance set at $p < 0.05$. The time to first disease inactivation was determined using Kaplan-Meier survival analysis using the two available observation time points, *T*1 and *T*2. Analyses were performed using IBM® SPSS® version 26.

Results

Patient characteristics at baseline

A total of nine patients were eligible and included in the study. None of the patients had signs of IOI at baseline or study end. Patient demographics and clinical and treatment history at baseline are summarized in Table I.

Before switching to brolucizumab, six patients were treated with a single anti-VEGF: aflibercept (n = 2) or bevacizumab (n = 2) or ranibizumab (n = 2). The remaining patients switched from aflibercept to bevacizumab (n = 1), from bevacizumab to aflibercept (n = 1), and from bevacizumab to ranibizumab to aflibercept (n = 1).

TABLE I - Baseline characteristics

Characteristic	Data
Age (years), mean (SD; range)	77.2 (11.6; 58-90)
Female gender, no. (%)	7 (77.8)
Clinical profile, mean (SD)	
VA (letters)	54.4 (20.1)
CST (µm)	386.4 (97.5)
Angiographic lesion type, no. (%)	
1	4 (44.4)
2	5 (55.6)
With IRF	6 (66.7)
With SRF	7 (77.8)
With sub-RPE fluid	3 (33.3)
With fibrosis	3 (33.3)
With subretinal fibrosis	1 (11.1)
With SHRM	1 (11.1)
Anti-VEGF treatment prior to brolucizumab injection, mean (SD; min-max)	
Treatment length (months)	9.9 (5.8; 3-18)
No. of injections	5.7 (3.5; 2-12)
Last administration interval (months)	2 (0.7; 1-3)
Months from last injection to brolucizumab switch	3.1 (1.2; 2-6)
Reason for switching to brolucizumab, no. (%)	
Fluid persistence	4 (44.4)
Frequent fluid recurrences	5 (55.6)

µm = micrometers; CST = retinal central subfield thickness; IRF = intraretinal fluid; max = maximum; min = minimum; no. = number; RPE = retinal pigment epithelium; SD = standard deviation; SHRM = subretinal hyperreflective material; SRF = subretinal fluid; VA = visual acuity; VEGF = vascular endothelial growth factor.

Anatomical and functional outcomes at 1-month postinjection

Patients were assessed within 1 week and 1 month (mean 29.1 ± 4.6 days, range 24-35) after the first brolucizumab injection. No signs of active disease were observed in eight (88.9%) patients: in seven patients (77.8%) SRF and IRF were absent, while in one patient (11.1%) fluid was only partially reabsorbed. All three patients presenting with sub-RPE fluid at baseline showed complete resolution.

The CST showed a significant reduction from 398.4 (97.5 SD) µm at baseline to 258.3 (32.4 SD) µm at 1 month postinjection (mean difference 131.1 µm, 95% confidence interval [CI] 46.4-215.8; related-samples Wilcoxon signed-rank test $p = 0.007$).

In parallel, VA improved significantly from 54.4 (20.1 SD) letters at baseline to 72.8 (16 SD) letters at 1 month postinjection (mean difference 18.3 letters, 95% CI 6.64-30.02; related-samples Wilcoxon signed-rank test $p = 0.011$).

The estimated mean and median time to disease inactivation were 14.2 (range 5-23.4) days and 3 (range 2.03-3.97) days, respectively (shown in Fig. 1).

In the three patients who were further examined at 60, 62, and 92 days, respectively, from the injection, no sign of disease reactivation or fluid recurrence was observed, and the VA remained stable; thus, there was no need for additional injections. As a case example, the retinal SD-OCT B-scans of one of the patients at different time points following brolucizumab injection are shown in Figure 2.

IOI, vasculitis, or retinal vascular occlusion was not reported for any patient in the follow-up period under consideration.

Discussion

Significant anatomical improvements were observed in patients switching to brolucizumab, as expected according to the available data (25,27,28). These included a significant reduction in CST and almost complete resolution of fluids in most patients.

Although the most frequent timepoint used to evaluate the postinjection anatomical response is approximately 1 month, in the case of no response it would be useful to have an earlier assessment since there is a correlation between CST reduction and the time passed from the last injection (29,30). In the group of patients we described, the response to brolucizumab treatment was rapid according to anatomical improvement, which occurred on average 2 weeks after the injection.

Furthermore, in the subgroup of patients followed for more than 4 weeks, the improvements were stable for at least 8 to 12 weeks after injection. Despite these results being obtained in non-treatment-naïve patients, they seem to be in line with those from the HAWK and HARRIER studies, which showed that nAMD was controlled for at least 12 weeks in more than 50% of naïve patients injected with brolucizumab (24). Such long-term effects suggest patients may be stabilized with an interval of 12 weeks between injections, thus

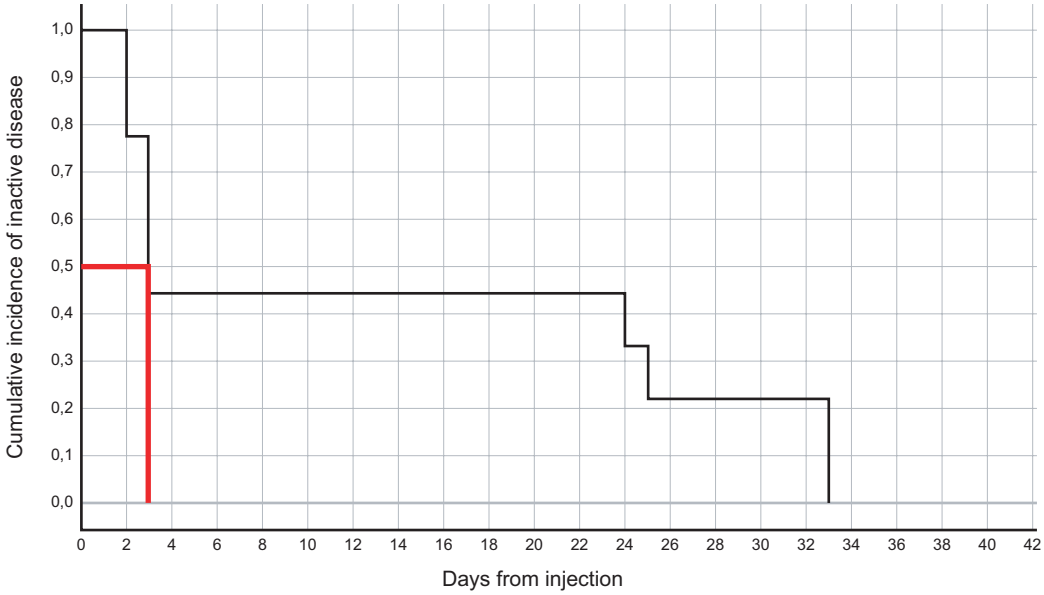

Fig. 1 - Kaplan-Meier plot for time to disease inactivation in the nine patients. The median (50th percentile) is indicated in red.

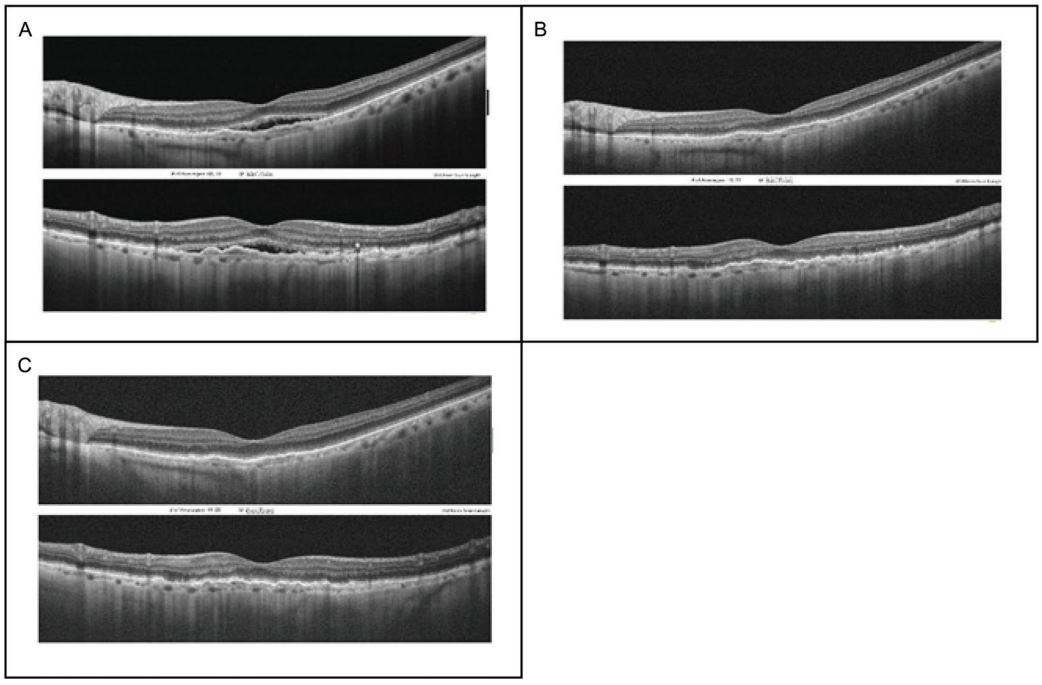

Fig. 2 - Representative case of a woman, 90 years old, treated with three intravitreal injections of aflibercept during the previous 9 months. Spectral domain optical coherence tomography (SD-OCT) B-scans at different time points: A) Baseline (before brolucizumab injection). B) Follow-up 1 month after injection. C) Follow-up 2 months after injection.

constituting a significant improvement of treatment burden. This is also clearer considering that retinal fluid was not successfully controlled with injections every 8 weeks before the switch to brolucizumab.

In our group of patients, we also observed a significant improvement in terms of VA. This appears to contradict a recent case series of six patients who did not show any increase in VA (26), and a retrospective analysis of 172 eyes, in which there was no improvement in VA after one to three brolucizumab injections (28). There are two possible explanations for our observation: the low number of cases in this case series, and the fact that our patients were treated for a shorter follow-up compared with the other studies (9.9 months and at least 2 years, respectively).

No inflammatory adverse events were reported in our patients, which may also be due to the small sample size and short follow-up. An independent Safety Review Committee, analyzing inflammatory ocular adverse events in the HAWK and HARRIER trials, observed an incidence of retinal vasculitis and retinal vascular occlusion of 3% and 2%, respectively, with a risk of severe visual loss in approximately 1 in every 200 patients (31).

In this context, it is important to consider the balance between the risk of adverse events and the benefits. In our case

series, the decision to initiate brolucizumab treatment was made for patients who were not responding anymore to other anti-VEGFs despite regular treatment. Notwithstanding, close contact with the patient is needed during follow-up, as it is expected that adverse inflammatory events can be successfully managed if they are recognized and treated promptly.

This retrospective case series has some limitations. Despite the important improvements observed, the sample size was small, and the measured changes had wide ranges. To better characterize the long-term efficacy and safety profile of brolucizumab in clinical practice, patients undergoing a treatment switch to brolucizumab should be followed for longer. Furthermore, naïve patients not undergoing treatment with other anti-VEGFs should be recruited to verify the anatomical superiority of brolucizumab and to optimize the treatment of nAMD by reducing the treatment burden for both patients and clinicians (24,25).

Conclusion

In conclusion, a single injection of brolucizumab was shown to be rapid and effective in fluid resolution, thus representing a valuable therapeutic option for controlling disease activity in nAMD patients. Indeed, the first month after the injection was shown to be very important to predict the response to the anti-VEGF drug of the single patient and brolucizumab showed a rapid and intense response.

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Disclosures

Statement of ethics: This retrospective study was conducted in accordance with the ethical standards set forth in the 1964 Declaration of Helsinki and its subsequent amendments. A notification letter was sent to the local Ethical Committee (Comitato Etico Area Vasta Centro at the Careggi University Hospital, Florence, Italy), as required by Italian regulations. Written informed consent for both medical treatment and publications of their data was obtained from all participants.
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ORIGINAL RESEARCH ARTICLE

The association of ESBL *Escherichia coli* with mortality in patients with *Escherichia coli* bacteremia at the emergency department

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ABSTRACT

Background: *Escherichia coli* is a common bloodstream infection pathogen in the emergency department (ED). Patients with extended-spectrum beta-lactamase (ESBL) *E. coli* have a higher risk of morbidity. However, there is still debate surrounding ESBL *E. coli*-associated mortality in community, intensive care unit, and tertiary care settings. In addition, there have been few studies regarding mortality in ESBL *E. coli* in ED settings, and results have been contradictory.

Methods: This was a retrospective cohort study conducted at the Department of Emergency Medicine, Faculty of Medicine, Khon Kaen University in Thailand aimed at evaluating the possible association between ESBL *E. coli* bacteremia and mortality in the ED. The inclusion criteria were age 18 years or over, clinical presentation suspicious of infection, and positive blood culture for *E. coli*. Predictors for mortality were analyzed by logistic regression analysis.

Results: During the study period, 273 patients presented at the ED with hemoculture positive for *E. coli*. Of those, 27 (9.89%) died. Five factors remained in the final model, of which plasma glucose levels, serum lactate levels, and ESBL *E. coli* were significantly associated with 28-day mortality in the ED with adjusted odds ratios of 0.970, 1.258, and 12.885, respectively. Plasma glucose of less than 113 mg/dL yielded a sensitivity of 80.95% and specificity of 64.29%, while serum lactate over 2.4 mmol/L had a sensitivity of 81.48% and specificity of 45.50%.

Conclusion: ESBL *E. coli*, plasma glucose, and serum lactate levels were associated with 28-day mortality in patients with *E. coli* bacteremia presenting at the ED.

Keywords: Extended-spectrum beta-lactamase-producing *Escherichia coli*, Glucose, Lactate

Introduction

Bloodstream infection (BSI) with Gram-negative bacteria is common in the emergency department (ED), accounting for 39.4% of ED patients with suspected infection (1). A study from China found that *Escherichia coli* was the most common Gram-negative BSI in 3,199 patients and accounted for 34.3% of cases (2). One population-based study found the mortality rate of *E. coli* BSI to be 9.6%. Male patients aged 70 years or older are at higher risk of 30-day mortality with adjusted incidence rate ratios of 1.26 and 10.35 (3). Another study found a mortality rate of 30.6% in patients infected with extended-spectrum beta-lactamase (ESBL) *E. coli* vs 22.2% in those infected with non-ESBL strains or *Klebsiella pneumoniae* (4).

The prevalence of drug-resistant Gram-negative bacteria is increasing, particularly in in-hospital, intensive care unit (ICU), and tertiary care settings (5-8). A study from a multispecialty hospital in India found that rates of multidrug-resistant Gram-negative bacteria increased from 26.16% in 2012 to 33.33% in 2014 (6). Additionally, urinary tract infection patients with resistant Enterobacteriaceae have been shown to be 1.447 times more likely to have severe sepsis or septic shock at presentation than those with nonresistant strains (9). Data regarding the association of ESBL *E. coli* and mortality in community, ICU, and tertiary care settings have been inconclusive. Two studies conducted in community settings, for example, found differences in mortality between

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patients with ESBL and non-ESBL bacteremia (10,11), whereas a study from a tertiary care setting found comparable rates (9.7% vs 9.2%), as did a study in a teaching hospital in China (12,13). However, another study in a teaching hospital in Japan found higher rate of mortality in patients with ESBL strains (14), as did a study in an ICU (37.5% vs 15.6%; p = 0.04) (15). This study thus aimed to evaluate if ESBL *E. coli* bacteremia was associated with mortality in an ED setting.

Methods

This was a retrospective cohort study conducted at the Department of Emergency Medicine, Faculty of Medicine, Khon Kaen University in Thailand as part of an ED infection project. The inclusion criteria were age 18 years or over, clinical presentation suspicious of infection, and positive blood culture for *E. coli*. Patients who received prophylactic antibiotics, presented with cardiac arrest or symptoms related to trauma, were referred from other hospitals, or had missing clinical data were excluded. The study period was between 2016 and 2018.

Eligible patients were selected from the hospital database. We reviewed participants' clinical data at the time of presentation as well as mortality data over the following 28 days. Clinical data included baseline characteristics, laboratory results, and treatment. Baseline characteristics reviewed were age, sex, comorbid diseases, Charlson Comorbidity Index, physical signs, and quick Sepsis Related Organ Failure Assessment (qSOFA) score. Laboratory results included complete blood count, chemistry, arterial blood gas, serum lactate levels, and blood culture results (for ESBL *E. coli* positivity). The primary outcome was 28-day mortality.

Statistical analyses

Eligible patients were categorized into two groups by mortality. Descriptive statistics were used to calculate differences between the two groups. Predictors for mortality were analyzed using logistic regression analysis. Univariate logistic analysis was used to calculate the unadjusted odds ratio with 95% confidence interval and p value for each factor. Factors with a p value less than 0.05 by univariate logistic regression analysis or those that were clinically significant were subsequently subjected to stepwise, multivariate logistic regression analysis. The final model was tested for goodness of fit using the Hosmer-Lemeshow method. Results were reported as unadjusted/adjusted odds ratios with their 95% confidence intervals. A numerical predictor for mortality as an appropriate diagnostic cutoff point was computed with its sensitivity and specificity. All statistical analyses were performed using STATA version 10.1 (College Station, Texas, USA).

Results

During the study period, 273 patients presented at the ED with hemoculture positive for *E. coli*. Of those, 27 (9.89%) died. In terms of baseline characteristics and physical signs, there were 12 factors that differed significantly between those who survived and those who died (Tab. I). For example, nonsurvivors had a significantly higher Charlson Comorbidity Index (5 vs 4), respiratory rate (28 vs 24 breaths/min), and qSOFA score (2 vs 1), but oxygen saturation at presentation was lower (96% vs 97%; p 0.040). qSOFA scores were significantly higher in those who died than those who survived (2 vs 1; p < 0.001).

With regard to laboratory tests and treatment, seven factors differed significantly between groups (Tab. II). For example, the nonsurvival group had significantly lower levels of serum bicarbonate (17 vs 21 mEq/L) and plasma glucose (94 vs 131 mg/dL), higher serum lactate levels (4.5 vs 2.6 mmol/L), and a greater percentage of patients with ESBL

TABLE I - Baseline characteristics of patients with *Escherichia coli* bacteremia presenting at the emergency department categorized by mortality at 28 days

Factors	Survivors n = 246	Nonsurvivors n = 27	p-Value
Age, years	66 (18-100)	73 (19-93)	0.161
Male sex	125 (50.81)	10 (37.04)	0.224
Comorbid diseases			
Liver disease	50 (20.33)	10 (37.04)	0.053
Diabetes	57 (23.85)	2 (7.41)	0.053
CKD (moderate-severe)	24 (9.76)	4 (14.81)	0.499
Solid organ tumor	74 (30.08)	15 (55.56)	0.010
Palliative care	4 (1.63)	4 (14.81)	0.004
Leukemia	2 (0.81)	1 (3.70)	0.269
Lymphoma	2 (0.81)	2 (7.41)	0.050
Hypertension	90 (36.59)	7 (25.93)	0.299
HIV infection	2 (0.81)	0	0.999
Cholangiocarcinoma	34 (13.82)	8 (29.63)	0.045
Charlson Comorbidity Index	4 (0-12)	5 (1-12)	<0.001
Temperature, °C	38.6 (35.9-41.5)	38.2 (35.6-41.0)	0.184
Pulse rate, beats/min	96 (58-190)	96 (52-148)	0.898
Respiratory rate, breaths/min	24 (18-50)	28 (18-40)	0.008
SBP, mm Hg	126 (64-218)	112 (80-167)	0.003
DBP, mm Hg	70 (33-112)	67 (37-95)	0.071
MAP, mm Hg	91 (48-138)	80 (56-119)	0.009
Oxygen saturation, %	97 (60-100)	96 (65-100)	0.040
GCS	15 (4-15)	15 (7-15)	<0.001
Sepsis score			
qSOFA	1 (0-3)	2 (1-3)	<0.001

Data are presented as median (range) or number (percentage).
CKD = chronic kidney disease; DBP = diastolic blood pressure; GCS = Glasgow coma scale; MAP = mean arterial pressure; qSOFA = quick Sepsis Related Organ Failure Assessment; SBP = systolic blood pressure.
Data presented as number (percentage) unless indicated otherwise.

TABLE II - Laboratory results and treatment of patients with *Escherichia coli* bacteremia presenting at the emergency department categorized by mortality at 28 days

Factors	Survivors n = 246	Nonsurvivors n = 27	p-Value
Hb, g/dL	11.0 (4.6-16.0)	9.6 (4.8-13.8)	0.002
WBC, $\times 10^3/mm^3$	33.8 (13.0-51.7)	26.6 (14.9-41.9)	0.010
Platelet, $\times 10^6$	179 (4-584)	138 (13-451)	0.098
BUN, mg/dL	17.9 (3.7-144.8)	27.3 (6.7-153.2)	0.009
Creatinine, mg/dL	1.1 (0.4-10.4)	1.5 (0.5-11.1)	0.118
Bicarbonate, mEq/L	21 (7-30)	17 (7-27)	<0.001
Total bilirubin, mg/dL	1.4 (0.2-33.8)	2.1 (0.3-33.8)	0.251
Glucose, mg/dL	131 (53-548)	94 (35-172)	<0.001
PaO_2, mmHg	76 (23-512)	89 (33-253)	0.702
pH	7.44 (7.16-7.58)	7.40 (7.11-7.56)	0.194
Lactate level, mmol/L	2.6 (0.5-18.3)	4.5 (1.3-17.9)	0.003
ESBL *E. coli*	5 (2.03)	4 (14.81)	0.007
Treatment			
Mechanical ventilator	21 (8.54)	5 (18.52)	0.155
ICU admission	83 (3.74)	17 (62.96)	0.005
Vasopressor*	62 (25.20)	21 (77.78)	<0.001
LOS	11 (2-56)	8 (1-54)	0.013

Data are presented as median (range) or number (percentage).
BUN = blood urea nitrogen; ESBL ***E. coli*** = extended-spectrum beta-lactamase-producing *Escherichia coli*; Hb = hemoglobin; ICU = intensive care unit; LOS = length of stay; PaO_2 = partial pressure of oxygen; pH = power of hydrogen; WBC = white blood cell.
*indicates that the patient received norepinephrine, adrenaline, or dopamine.

TABLE III - Factors associated with a 28-day mortality in patients with *Escherichia coli* bacteremia presenting at the emergency department

Factors	Unadjusted odds ratio (95% confidence interval)	Adjusted odds ratio (95% confidence interval)
Oxygen saturation	0.933 (0.883, 0.985)	0.934 (0.860, 1.015)
Hemoglobin	0.732 (0.605, 0.885)	0.856 (0.662, 1.106)
Plasma glucose	0.971 (0.955, 0.987)	0.970 (0.954, 0.987)
Serum lactate level	1.185 (1.067, 1.317)	1.258 (1.090, 1.451)
ESBL *E. coli**	8.382 (2.103, 33.407)	12.885 (1.082, 153.338)
Age	1.008 (0.981, 1.036)	Not retained
Sex	0.569 (0.251, 1.293)	Not retained
Liver disease	2.306 (0.995, 5.344)	Not retained
Diabetes	0.255 (0.058, 1.111)	Not retained
Cholangiocarcinoma	2.625 (1.065, 6.469)	Not retained
Solid organ tumor	2.905 (1.297, 6.508)	Not retained
qSOFA	3.761 (1.974, 7.164)	Not retained
DBP	0.974 (0.947, 1.001)	Not retained
GCS	0.770 (0.630, 0.941)	Not retained
Hemoglobin	0.732 (0.605, 0.885)	Not retained
WBC	1.014 (0.995, 1.032)	Not retained
Serum bicarbonate	0.836 (0.762, 0.918)	Not retained

Factors in the model included age, sex, liver disease, diabetes, cholangiocarcinoma, solid organ tumor, qSOFA, DBP, GCS, WBC, and serum bicarbonate.
DBP = diastolic blood pressure; ESBL = extended-spectrum beta-lactamase; GCS = Glasgow coma scale; qSOFA = quick Sepsis Related Organ Failure Assessment; WBC = white blood cell.

E. coli (14.81% vs 2.03%) than the survival group. In addition, patients in the nonsurvival group underwent significantly more aggressive treatment (such as vasopressor treatment) and had a higher rate of ICU admission. However, duration of hospital stay in the nonsurvival group was shorter (8 vs 11 days; p 0.013).

Five factors remained in the final model for predicting death (Tab. III). Plasma glucose, serum lactate levels, and ESBL *E. coli* were significantly associated with mortality, with adjusted odds ratios of 0.970, 1.258, and 12.885, respectively. The final model had a Hosmer-Lemeshow Chi square of 6.73 (p = 0.565). Plasma glucose of 113 mg/dL or lower yielded a sensitivity of 80.95% and specificity of 64.29%, while serum lactate level of over 2.4 mmol/L had a sensitivity of 81.48% and specificity of 45.50%.

Discussion

The prevalence of ESBL *E. coli* bacteremia at the ED in this study was 3.29%, which is lower than previously reported in community settings (6.7%-9.5%) (10,11,16). In addition to the difference in setting, these results may indicate differing rates among countries, as higher rates have been found in developed countries (South Korea and Spain). A previous report found that frequent visits to the ED increased the risk of ESBL bacteremia by a factor of 9.98, including in those patients who had undergone previous antibiotic treatment. In Thailand, the rate of previous antibiotic use may be lower than in some other countries. Despite the inconsistency in the ESBL *E. coli* mortality rate in other settings, this study found that patients with ESBL *E. coli* had a 13 times higher risk of mortality than those with non-ESBL strains. Other factors associated with mortality in patients with *E. coli* infection may be personal characteristics and inappropriate antibiotic use. A report from Korea found that presenting with septic shock or malignancy increased mortality risk by 26.6 and 11.9 times, respectively, while another study found that mortality rates were comparable in patients with ESBL and non-ESBL *E. coli* if antibiotics were administered appropriately (p = 0.23) (11,15).

Hypoglycemia has been shown to be related with higher mortality in sepsis patients and critically ill patients (17-19). Although the causal relationship between hypoglycemia and mortality is not well understood, several mechanisms have been proposed including the inhibition of the physiological responses of hormones such as insulin and epinephrine,

increased inflammatory response, and cellular damage from glucose administration (19). Previous studies have also found low plasma glucose to be associated with mortality in patients with sepsis (11,20). This study found that glucose of 113 mg/dL or lower yielded a sensitivity of 80.95% compared to a previous study, in which plasma glucose of 40-69 mg/dL resulted in an adjusted odds ratio of 3.43 (95% confidence interval of 1.51, 7.82) for mortality (19,20). These results may imply that patients with *E. coli* bacteremia and hypoglycemia may have as high of a risk of mortality as other patients with sepsis. The different plasma glucose cutoff points in the two studies may be due to differences in study population. This study enrolled only patients with *E. coli* bacteremia at the ED, while the previous study included patients with sepsis, which may have been caused by various pathogens. The plasma glucose cutoff point in this study may be more specific to patients with *E. coli* bacteremia at the ED.

As previously reported, serum lactate is an indicator for mortality in patients with infection at the ED (21-23). A previous study found that serum lactate greater than 4 mmol/L was associated with higher mortality than at 2 mmol/L (40.7% vs 2.7%) (24). In this study, we found that serum lactate over 2.4 mmol/L yielded sufficient sensitivity to predict fatality in patients with *E. coli* bacteremia at the ED. Another study found a serum lactate cutoff point of 5.80 mmol/L in patients with necrotizing fasciitis (25). This indicates that *E. coli* bacteremia may be severe and that the serum lactate cutoff point may vary depending on the causative agents.

Although oxygen saturation and hemoglobin were significantly associated with mortality by univariate logistic regression analysis (Tab. III), they were no longer significant in the final model. These results may indicate that neither factor was a strong predictor compared with the other three. Additionally, there might have been some related confounding factors. Other factors included in the model that had p values of less than 0.05 by univariate analysis were not retained in the final model for the same reasons. Some comorbid diseases, such as diabetes, were found to be significant predictors for mortality in a previous observational study (26). However, comorbid diseases were not significant in this study, as previously mentioned. Additionally, the model used in this study differed from that in the previous study. In this study, we included clinical factors such as ESBL *E. coli* in the model, while the previous study did not include ESBL *E. coli* and included treatment-related factors such as peak inspiratory pressure and positive end-expiratory pressure.

There were some limitations to this study. First, the ED at which it was conducted was a single site at a university hospital. Further prospective studies in other settings may be required to confirm the results. In addition, this was an exploratory study without validation. The final predictive model included more factors than event outcomes. There were five factors in the model with only 27 nonsurvivors, resulting in a ratio of more than 1:10. Moreover, the total number of patients in the final model was 130. These limitations could have caused the model to be unbalanced or biased. However, the final model had a high goodness of fit. Another limitation was that some factors were not studied such as previous antibiotic use, previous history of resistant pathogens, or special conditions (27-35). Finally, mortality was defined as 28-day mortality.

ESBL *E. coli*, plasma glucose, and serum lactate levels were associated with 28-day mortality in patients with *E. coli* bacteremia presenting at the ED.

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ORIGINAL RESEARCH ARTICLE
Focus on Antimicrobial Resistance (AMR)

Redefining genomic view of *Clostridioides difficile* through pangenome analysis and identification of drug targets from its core genome

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ABSTRACT

Introduction: *Clostridioides difficile* infection (CDI) is a leading cause of gastrointestinal infections and in the present day is a major concern for global health care system. The unavailability of specific antibiotics for CDI treatment and its emerging cases worldwide further broaden the challenge to control CDI.

Methods: The availability of a large number of genome sequences for *C. difficile* and many bioinformatics tools for genome analysis provides the opportunity for *in silico* pangenomic analysis. In the present study, 97 strains of *C. difficile* were used for pangenomic studies and characterized for their phylogenomic and functional analysis.

Results: Pangenome analysis reveals open pangenome of *C. difficile* and high genetic diversity. Sequence and interactome analysis of 1,481 core genes was done and eight potent drug targets are identified. Three drug targets, namely, aminodeoxychorismate synthase (PabB), D-alanyl-D-alanine carboxypeptidase (DD-CPase) and undecaprenyl diphospho-muramoyl pentapeptide beta-*N*-acetylglucosaminyl transferase (MurG transferase), have been reported as drug targets for other human pathogens, and five targets, namely, bifunctional diguanylate cyclase/phosphodiesterase (cyclic-diGMP), sporulation transcription factor (Spo0A), histidinol-phosphate transaminase (HisC), 3-deoxy-7-phosphoheptulonate synthase (DAHP synthase) and c-di-GMP phosphodiesterase (PdcA), are novel.

Conclusion: The suggested potent targets could act as broad-spectrum drug targets for *C. difficile*. However, further validation needs to be done before using them for lead compound discovery.

Keywords: *Clostridioides difficile*, Drug target, Genome, Inhibition, Phylogenomics

Introduction

Clostridioides difficile, earlier known as *Clostridium difficile*, is a toxin-producing Gram-positive, anaerobic bacteria (1). During infection, it releases toxins that disrupt the intestinal epithelia, resulting in a variety of diseases ranging from mild, self-limiting diarrhoea to the fatal pseudomembranous colitis (PMC) (2-5). During the past two decades, there has been dramatic increase in the incidence and severity of *C. difficile* infection (CDI) (6,7). CDI is usually followed by the antibiotic treatment that impairs the protective gut microflora (8).

C. difficile was first identified from microbial flora of faeces of healthy newborn infants and was considered that it has no deleterious effects in human (9). But later on, it was identified as the cause of antibiotic-associated PMC (10). *C. difficile* has been reported to have genetic heterogeneity because of its wide ecological adaptability. Hence, in the past 20 years, significant changes in CDI epidemiology have been reported (11). The differences in the severity of the infection, presence of pathogen at multiple sites (human, animal and environment) and their genetic differences have revived interest in the genomic comparison of *C. difficile*.

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Several strains of *C. difficile* have been isolated and sequenced from different ecological niches; these sequences were procured for genomic comparison. The availability of genome sequence and further development in genomics and related sciences has provided a platform to understand the functions of various proteins encoded by its genome. The concept of pangenome can be applied to identify different genomes: core genome, that is, genes present in all strains of the dataset; dispensable or accessory genome, which are genes present in few strains of the dataset; and strain specific or unique genome, which are genes present in only one strain and absent in others (12). Core gene(s) can be utilized to identify the drug targets and design broad-spectrum antibiotics for pathogenic species, whereas accessory and unique genes are supposed to give them advantage in survival, pathogenicity or habitat adaptation (13). The analysis of gene functions reveals their incorporation in different genes and their proteins, which are functional in various metabolic processes that help pathogens to survive in the different ecological niches (14).

There are various bioinformatics approaches to investigate the drug target from the genome such as ligand-based interaction fingerprint, proteochemometrics modelling, linear interaction energy modelling and many more (15). Here we have used the core genome of *C. difficile* to identify the drug target by an integrative approach using sequence and interactome analysis. Conventional methods for drug discovery are very costly and time consuming; however, using computer analysis at initial stages can reduce the cost and time. In the present study, pangenome analysis was done and its core genome has been used to identify the drug targets. This method has been developed for the first time to identify the drug targets from the core genome of *C. difficile*, which can be used in the future for other pathogens too.

Methodology

Collection of genomic data

The strains of *C. difficile* isolated from almost all the geographical regions of the world were chosen for the present study. The complete genome sequences of these 97 *C. difficile* strains and their associated proteomes were retrieved from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) available at the National Center for Biotechnology Information (NCBI) (16). The assembly levels of all these genomes were complete, that is, all the expected necessary chromosomes are present with no gaps.

Pangenome analysis

Pangenome analysis was conducted on these 97 strains of *C. difficile* using the Bacterial Pan Genome Analysis (BPGA) tool (17). For this, we have used USEARCH algorithm to generate orthologous protein clusters with the default threshold of 50% identity (18). By examining 20 permutations at random and giving median values after each genome is added, the pan and core genome size is determined. By comparing the common gene and unique gene families to the entire genome, core and pan genome curves, respectively, are created. In addition, it also generates the pan phylogeny using the pan matrix data. Using neighbour-joining method, a pangenome tree was constructed with a default combination value of 20 iterations.

Functional analysis

All the accessory and unique genes were subjected to functional analysis using protein BLAST against COG (Clusters of Orthologous Genes) and KEGG (Kyoto Encyclopedia of Genes and Genomes) databases (19,20). The percentage frequencies of these COG and KEGG categories are calculated for each gene and their outputs are generated in the form of charts.

Identification of drug targets

Core genome obtained from pangenome analysis was used to identify the potent drug targets. Initially, all the core genes were subjected to BLAST search against human (21). Genes with the E-value greater than 1×10^{-3} were considered as non-homologous. This is done to reduce the cross-reactivity with the human genome and to decrease drug toxicity. The resultant non-homologous genes (to human) were subjected to BLAST against DEG (Database of Essential Genes) to identify the genes that were essential for bacterial sustainability. DEG contains experimentally validated genes of many genera that are essential for survival (22). To shortlist essential genes, E-value <0.0001 and bit score >100 was used. The essential genes involved in vital function are targeted, such that the pathogen is affected and killed. All non-homologous and essential genes were subjected to virulence study. VFDB (Virulence Factor Database) is a comprehensive database that provides information about virulence factors, which are the gene products that help the pathogen to grow inside host and increase its ability to cause disease (23).

All the selected proteins were filtered on the basis of their physicochemical properties such as number of amino acids, molecular weight, isoelectric point (pI), GRAVY (grand average of hydropathicity) value, aliphatic index and subcellular localization. Except subcellular localization, all the parameters are calculated using Protparam tool and subcellular localization is predicted using CELLO (24,25). Sequences with less than 100 amino acids called peptides are excluded from the present study. Similarly, drugs are more accessible to low molecular weight targets, therefore sequences with more than 75 kDa are also excluded (26). The drug targets having low pI have been included in this study, which is in accordance with the study of Bakheet and Doig (27). In addition, negative GRAVY value indicated the hydrophilic nature of drug target and higher value of aliphatic index indicates thermostability (28,29). All extracellularly localized proteins were also excluded from the study, these being secreted outside the cell (30).

Interactome analysis

To search the key proteins (from selected proteins), the choke point analysis using pathway tool is performed to find

out the proteins which were specific in the metabolic network and whose function cannot be replaced by any other protein (31). A choke point protein is compulsory for any pathway and is essential for pathogen survival. Targeting choke point protein affects the metabolic pathway, which results in death of the pathogen. Further analysis was done using interactome studies, in which the input of all choke point proteins was given to the STRING database and network was created at high confidence level (32). The metabolic functional interaction was created using various methods such as gene fusion, neighbourhood, co-occurrence, co-expression and text mining. The interactome was downloaded from STRING in xml format and its analysis was done using cytoscape (33).

In cytoscape, various critical network parameters such as clustering coefficient, characteristic path length and network centralization were calculated for each node of the network. Clustering coefficient C_n for node n was calculated using:

$$C_n = 2e_n/k(k_n - 1)$$

where e_n is the number of connected pairs between all neighbours of n and k_n is the number of neighbours. Characteristic path length is the distance between nodes. Network centralization is the measure of network association around the central node; node having value close to 1 is central to network and value near to 0 shows decentralization. The values for clustering coefficient and characteristic path length are calculated for a node as well as after deleting the node. The difference in these two values shows the impact of node in the network (33). The complete methodology is shown in Figure 1.

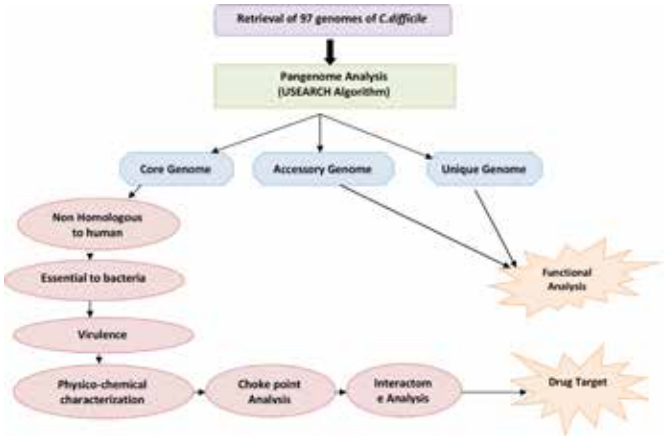

Fig. 1 - Flowchart depicting the workflow of the methodology adopted.

Results and discussion

The 97 complete genomes and associated proteomes of *C. difficile* available till the present study were downloaded from the NCBI. Their information such as accession number, name of the strain, country from where isolated and genome statistics is provided in Supplementary File 1. The pangenome analysis of these 97 strains reveals 6,286 gene families (pangenome), out of which 1,481 are core genes (present in all species). Thus the core genes form 23.5% of the total genome, which signifies a high genetic diversity among different strains. The same feature is represented in core-pan genome plot (Fig. 2). As the genomes are added, the size of pangenome increases, whereas the size of core genome declines. The curve of pangenome (yellow colour) is still progressing, indicating the likelihood of addition of more genes, that is, global gene repertoire is likely to change in the near future and its pangenome is almost open.

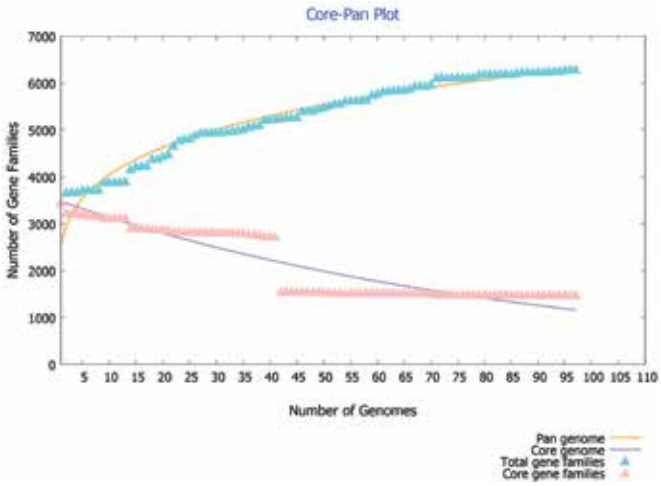

Fig. 2 - Core-pan plot of 97 strains of *C. difficile* genome.

The power law regression model equation,

$$f(x) = a.X^b$$

where $f(x)$ is the pangenome size, X is the number of genomes used, and a and b are fitting parameters used to find the openness and closeness of pangenome, has been used in the present study (17). In our study with *C. difficile* genes, the values of $f(x)$ = 6,286, X = 97, a = 2687.17 and b = 0.185459 indicated that the pangenome is open at $b > 0$; otherwise, the pangenome would be considered to be closed. The value of b = 0.185459 indicates that the pangenome is open but soon may be closed with increase in genome data. *C. difficile* has 186,308 accessory genes which are present in a few strains and has 976 unique genes which are present in specific strains of *C. difficile*. The conservation level of *C. difficile* does not seem to be very high. The genome level analysis reveals high genetic variability; this may be due to its existence in different niches.

On functional analysis, it is observed that unique genes (shown in blue) and accessory genes (shown in red) are mostly responsible for metabolism and transporter function (Fig. 3). This shows that both of them are more diversified in *C. difficile*. Recently in 2021, Kulecka et al. also reported the variability in the metabolism genes in recurrent CDI cases (34). In addition, the core genes like PolC-type deoxyribonucleic acid (DNA) polymerase III, exonuclease subunit C, cell wall–binding protein Cwp20, sensor histidine kinase KdpD and alanine-tRNA ligase (shown in green) are the genes

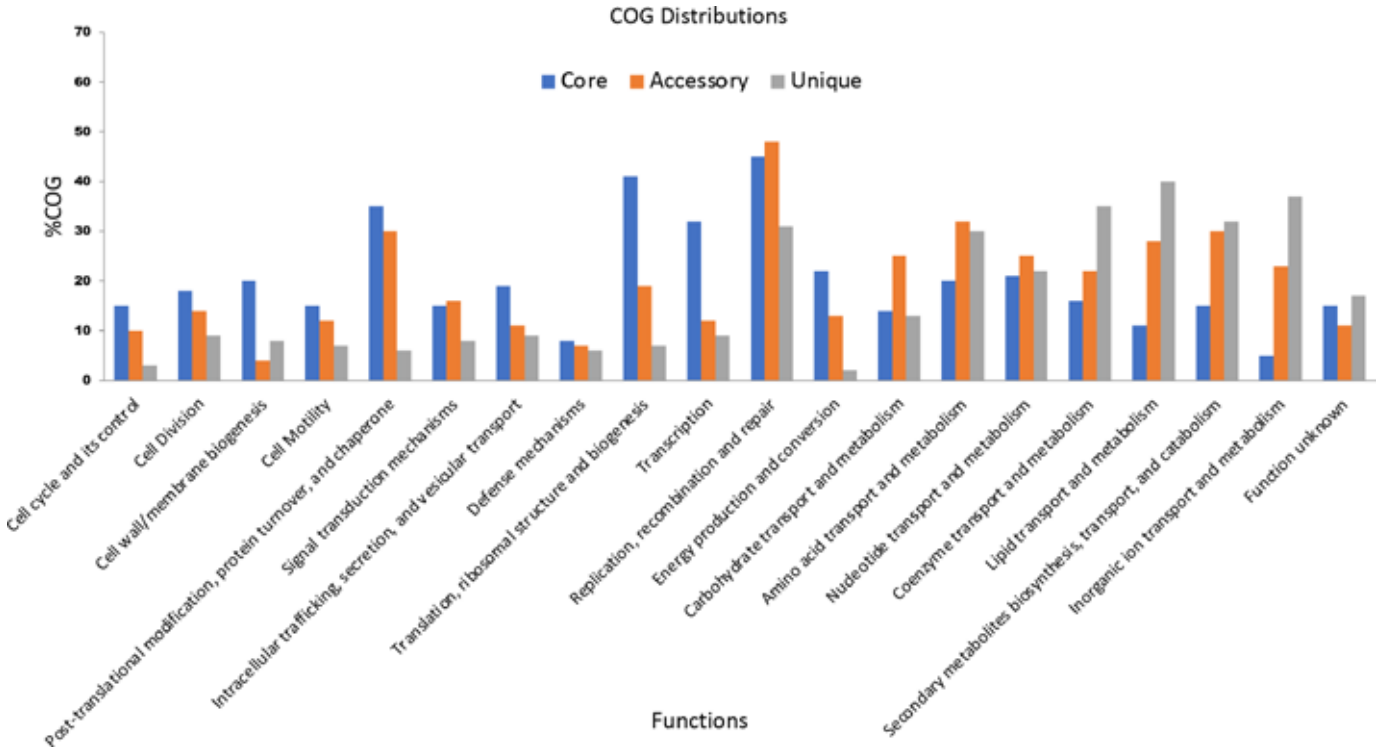

Fig. 3 - Functional analysis of various core, accessory and unique genes using COG and KEGG distributions.

for cellular processes such as cell division, cell cycle and its control, cell motility, cell wall/membrane biogenesis, transcription, translation, ribosomal structure and biogenesis, and are mostly conserved. This signifies that genes involved in important cellular mechanism are conserved, while genes required to adapt to the new ecological niches are variable.

The pan-phylogeny-based phylogenetic tree is shown in Figure 4. It is observed that strains are divided into different clades based on genome similarity. On observing each clade, it is noticed that similarity is mainly based on the country from where the samples are isolated. It suggests that *C. difficile* adapts to different environmental conditions by expression of relative proportions of the different gene products. For example, strains Cd9, Cd12, MT5121, Cd23 and W0023a are all assembled in one clade and are isolated from the USA. Similarly strains CD-10-00484, 10-00078, DSM 102860, DSM 102978 and DSM 29745 isolated from Germany show genome similarity and are assembled in one clade.

With high genetic variability and drug resistance for CDI, it is very necessary to design a drug that targets the core genes of the pathogen, as core genes are present in all the strains of the pathogen and are essential for the survival of the pathogen. Therefore, targeting core gene will surely help to overcome CDI. We have used an integrative approach based on sequence and interactome analysis to find the drug target against *C. difficile*.

From the core genome, genes that are homologous to humans are excluded in the first step, as it may adversely affect the host metabolism. A total of 1,130 proteins are found to be non-homologous to human (Supplementary File 2). On further screening, essential proteins that are vital for the survival of pathogens are searched using DEG. Among them, 370 proteins were found to be essential and crucial for *C. difficile* survival (Supplementary File 2). Essential proteins were further screened for their virulence, as these factors are responsible for pathogenesis. From 370 essential proteins, 130 proteins were found to be virulence-associated factors (Supplementary File 2).

All 130 proteins were checked for their physicochemical properties. Proteins having more than 100 amino acids, less molecular weight, low pI, negative GRAVY value, high aliphatic index and membrane or cytoplasmic localization were further considered (26-30). All these are the physicochemical properties required for the potent drug target. A total of 94 proteins were obtained after all physicochemical checks (Supplementary File 2); they are further used for choke point analysis.

On choke point analysis, only 39 proteins involved in the unique metabolic pathways were identified (Supplementary File 2). For these 39 proteins, interactome is created using STRING as shown in Figure 5.

On interactome analysis with cytoscape, eight potent drug targets were found. Their interactome analysis

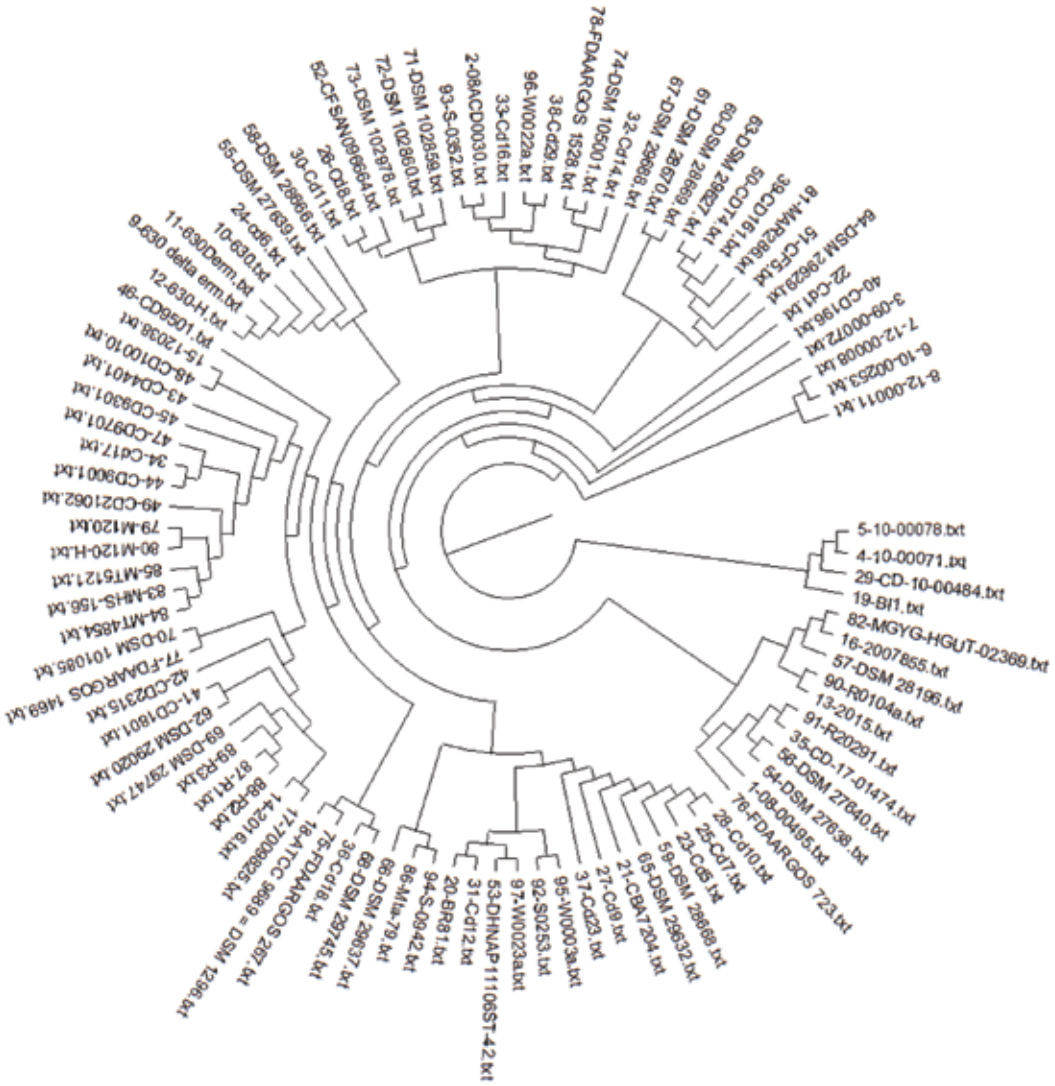

Fig. 4 - Pan phylogeny–based phylogenetic tree of 97 strains of *C. difficile.*

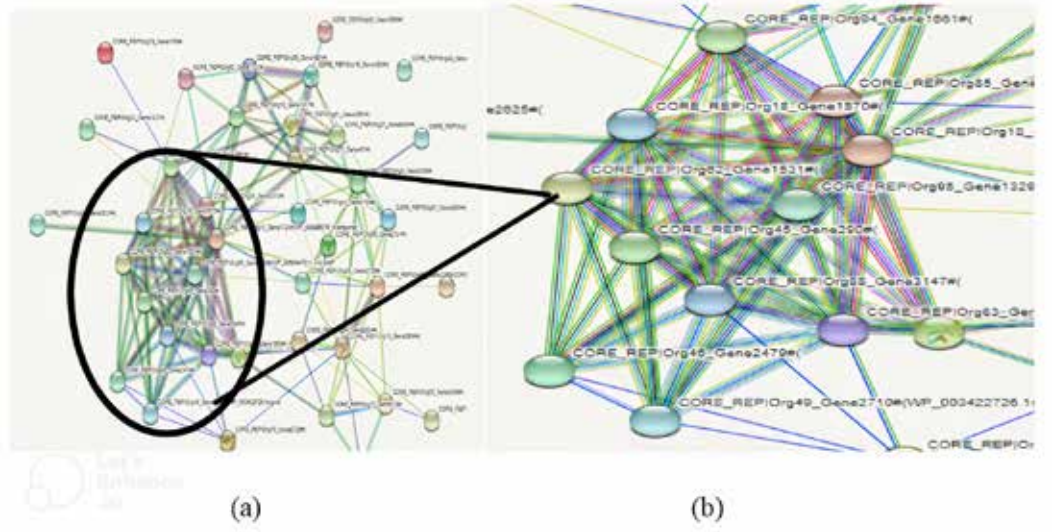

Fig. 5 - A) Interactome created using STRING. **B**) Zoomed view of interactome.

results are shown in Table I. Out of the eight drug targets, three targets, namely, aminodeoxychorismate synthase (PabB), D-alanyl-D-alanine carboxypeptidase (DD-CPase) and undecaprenyl diphospho-muramoyl pentapeptide beta-*N*-acetylglucosaminyl transferase (MurG transferase) were identified which have been previously reported (35).

PabB is involved in folate synthesis; its inhibition affects DNA and protein synthesis adversely. It is reported that it is targeted by the antibiotics 6-fluoroshikimic acid and atropabyssomycin C (35). Bifunctional diguanylate cyclase/phosphodiesterase (cyclic-diGMP) is a messenger protein that regulates motility, virulence and biofilm formation attributed to pathogenicity (36,37). Inhibition of cyclic-diGMP affects many processes of the pathogen that result in the death of the pathogen. Another identified target is sporulation transcription factor Spo0A, which is a key factor for entry into sporulation in stress conditions and biofilm formation (38).

Histidinol-phosphate transaminase (HisC) is a transferase that is mainly involved in transferring nitrogenous group. It is involved in synthesis and metabolism of many amino acids (39). 3-deoxy-7 phosphoheptulonate synthase (DAHP synthase) is involved in the shikimate pathway and is also responsible for the synthesis of aromatic amino acids, such as tyrosine, phenylalanine and tryptophan which are essential for bacterial metabolism (40). Another identified drug target, MurG transferase, is involved in the peptidoglycan biosynthesis and is reported to be the drug target for many pathogens such as *Neisseria meningitidis* (41), *Acinetobacter baumannii* (42) and *Mycobacterium tuberculosis* (43).

Another identified potent target, DD-CPase is a reported drug target and is involved in peptidoglycan biosynthesis and remodelling and is inhibited by β-lactam antibiotics (44). C-di-GMP phosphodiesterase (PdcA) is a potent drug target that regulates bacterial pathogenesis as well as is involved in surface adherence and biofilm development (45).

Conclusion

CDI is a challenging situation worldwide. The unavailability of specific antibiotic and emergence of antibiotic resistance against *C. difficile* is a matter of concern. Diversity in *C. difficile* genome drives the genomic comparison of the pathogen. Pangenome analysis reveals the open pangenome of *C. difficile* that may be soon closed. The diversity is due to its adaptability in different niches and different hosts. Due to such genomic diversity, a drug target can be designed only from its core gene, whose inhibition affects all strains of *C. difficile.* From sequence and interactome analysis of core genes, eight potent drug targets are reported. Out of these, three of the targets – PabB, DD-CPase and MurG transferase – are also reported as drug target for other pathogens, whereas bifunctional cyclic-diGMP, Spo0A, HisC, DAHP synthase and PdcA are newly reported targets. This indicates that the method originated in the present study has a high rate of success and saves considerable time and money. The same method can be used for other pathogens also.

Limitations of the study

This computational method uses multiple genomes with complete genome assembly, therefore this method cannot be employed for pathogens whose majority of the strains have not been sequenced. Pangenome analysis can only be performed if multiple sequenced strains are available for the organism. Pangenomics can easily be done only when proper software or tool is available; it is very complex to handle the genome manually. Another limitation with this study is the use of *in silico* method for drug target identification that saves considerable time and money, but its accuracy is still questionable. We have used the core genes which are conserved and involved in essential processes for drug target

TABLE I - Interactome analysis of potent eight drug targets

Sl. no	Sequence no.	Target name	Clustering coefficient		Characteristic path length		Network centralization
			Before N.D.	After N.D.	Before N.D.	After N.D.	
1.	Org18_Gene1417	Aminodeoxychorismate synthase (PabB)	0.666	0.397	3.98	2.76	0.325
2.	Org18_Gene1870	Bifunctional diguanylate cyclase/ phosphodiesterase (cyclic-diGMP)	0.863	0.425	2.36	1.83	0.693
3.	Org11_Gene1255	Sporulation transcription factor (Spo0A)	0.99	0.725	3.26	2.98	0.523
4.	Org39_Gene1501	Histidinol-phosphate transaminase (HisC)	0.356	0.120	2.35	1.25	0.364
5.	Org82_Gene1721	3-Deoxy-7-phosphoheptulonate synthase (DAHP synthase)	0.70	0.530	3.29	2.98	0.452
6.	Org18_Gene2684	Undecaprenyl diphospho-muramoyl pentapeptide beta-N-acetyl glucosaminyltransferase (MurG transferase)	0.893	0.452	4.63	1.88	0.832
7.	Org50_Gene2566	D-alanyl-D-alanine carboxypeptidase (DD-CPase)	0.528	0.257	3.00	2.08	0.452
8.	Org95_Gene1329	c-di-GMP phosphodiesterase (PdcA)	0.731	0.458	4.11	3.37	0.673

identification, but the drug targets from novel genes which the bacteria inherits from its adaptation in new niches cannot be considered in this type of study.

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REVIEW

Understanding the environmental drivers of clinical azole resistance in *Aspergillus* species

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ABSTRACT

Aspergilli are ubiquitous fungal pathogens associated with severe life-threatening infections, especially in immunocompromised patients. Azoles are the first line of defence in the fight against most *Aspergillus*-related infections. However, resistance to these therapeutic compounds has developed, which is mainly due to the existence of mutations in lanosterol 14 alpha-demethylase (Cyp51A), a crucial enzyme in the pathway that produces ergosterol and is the target of azole antifungals. Azole-based antifungal medications are ineffective because of infections brought on by azole-resistant *Aspergillus* species, leading to a high fatality rate. However, resistant *Aspergillus* isolates have also been isolated from azole-naïve patients. Global agricultural practices promote the use of azole fungicides to protect crops from phytopathogens. Usage of azole fungicides on a large scale has been linked to the development of resistance among *Aspergillus* species prevalent in the environment. The infections caused by these azole-resistant *Aspergillus* species cannot be treated by the available azole drugs, in turn leading to high morbidity and mortality rates. Thus, knowledge of the environmental drivers and comprehending the genetic basis of fungal drug resistance evolution is pertinent, considering increasing numbers of patients with COVID-19 infections who are sensitive to opportunistic fungal infections. This article emphasises the prevalence and underlying mechanisms of azole resistance in *Aspergillus* species, with a focus on environmental triggers and resistance development. It also highlights the need for regular surveillance of pesticide use in agriculture, detection of triazole-resistant *Aspergillus* species in environmental and clinical settings and development of new antifungal drugs.

Keywords: *Aspergillus*, Azole resistance, Biofilm, *cyp51A* gene, Environmental origin, Triazoles

Introduction

The saprophytic genus *Aspergillus* is universal in the environment that releases large numbers of conidiospores. After inhalation, they either reach the terminal airways or settle in large groups in the upper ventilatory system and cause sensitisation. The small size of the spores, thermotolerance, resistance to oxidative damage and the capacity to create proteolytic or even immunosuppressive enzymes are among *Aspergillus*' biological characteristics which permits growth at body temperature (1,2). *Aspergillus* species reported as human pathogens are *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus nidulans*. Bronchitis, invasive aspergillosis (IA), and chronic pulmonary aspergillosis (CPA) are all signs of *Aspergillus* infection. In addition, severe asthma with fungal hypersensitivity and allergic bronchopulmonary aspergillosis (ABPA) are allergic symptoms of inhaled *Aspergillus* (3).

Current antifungal therapy for aspergillosis falls into three main categories: polyenes, echinocandins, and azoles. Of these, azoles are the drug of choice for treating *Aspergillus* infection. In addition, they are the only orally available antifungal agents for therapy and are essential for long-term treatment (3). *A. fumigatus* has developed azole resistance over the past few decades because of long-term azole therapy for aspergillosis in the clinical environment (4). Furthermore, azole-resistant isolates of *Aspergillus* detected in azole-naïve (5) individuals indicate that there may be a second route for the establishment of resistance through *A. fumigatus* azole fungicide exposure in agro ecosystems.

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Global prevalence of Aspergillosis

Aspergillosis can be either chronic or acute. Most commonly, aspergillosis diseases are CPA, IA and bronchitis (6). *Aspergillus* species are responsible for >2,00,000 cases of IA annually, as shown in Table I (7).

TABLE I - Global burden of aspergillosis (8,9)

Fungal disease	Global burden	Comments
Asthmatic allergic bronchopulmonary aspergillosis	~4,800,000	Adults only, rare in children
Cystic fibrosis–related allergic bronchopulmonary aspergillosis	~6675	Adults only, starts from age 4
Invasive aspergillosis	~3,00,000	About 10 million at risk annually
Chronic pulmonary aspergillosis	~3,000,000	

In immunocompromised individuals, such as those with severe neutropenia, individuals who had bone marrow transplant or solid organ transplants, those with advanced acquired immunodeficiency syndrome or with chronic granulomatous illness, IA can develop (10). Invasion of the lungs by *Aspergillus* leads to tissue damage leading to sepsis and sometimes haemoptysis in later stages (11). In addition to these classic risk factors for IA, liver cirrhosis, tuberculosis, diabetes mellitus and persistent lung disease can also develop (12-15). CPA is an infectious disease that progressively damages lung tissue. This is especially true for immunocompromised people with a previous or underlying lung disease, such as tuberculosis or chronic obstructive pulmonary disease (COPD). Awareness of this debilitating and ultimately deadly infection is growing. It is tentatively estimated that there are 3 million CPA (Chronic pulmonary aspergillosis) patients worldwide (6). Among Asian countries, the highest CPA burden was recorded in India (209,147) (15), followed by Pakistan (55,509) (16), Bangladesh (20,720) (17), Nepal (6,611) (18) and Sri Lanka (2,886) (19). Patients with ABPA frequently experience uncontrolled asthma or repeated infections brought on by bronchiectasis, which progresses to lung damage, respiratory failure, and ultimately death. ABPA contamination in the Indian subcontinent is summarised in Table II (9,21).

Antifungal agents to treat aspergillosis

In general, there are three different types of antifungal medications used to treat *Aspergillus*-related illnesses: polyenes (amphotericin B), azoles (itraconazole, voriconazole and posaconazole) and echinocandins (caspofungin).

Polyenes

Polyenes are large macrolide structures with amphipathic nature (Fig. 1). The oldest class of antifungal drugs are the polyenes, which include nystatin, amphotericin B and pimaricin. Of these, amphotericin B is the only drug used to treat systemic infections. Polyenes promote channelling in fungal membranes by interacting with sterols in cell membranes (ergosterol in fungal cells), which leads to changing the permeability of the membrane leading to the leaking of intracellular components. The major antifungal medication used to treat severe *Aspergillus* infections is amphotericin B. However, it has detrimental side effects including fever, chills, hypotension, tachypnoea, as well as renal toxicity such as renal ischaemia, hypokalaemia, tubular acidosis and reduced erythropoiesis in the kidney (10).

TABLE II - Burden of aspergillosis in the Indian subcontinent

Country	Chronic pulmonary aspergillosis		Invasive aspergillosis		Allergic bronchopulmonary aspergillosis	
	Burden	Rate/100,000	Burden	Rate/100,000	Burden	Rate/100,000
India (15)	209,147	24	–	–	1,380,000	114
Pakistan (16)	55,509	70	10,949	5.9	94,358	51
Bangladesh (17)	20,720	41	5166	5.1	90,262	56
Nepal (18)	6,611	24.2	1119	4.0	9546	35
Sri Lanka (19)	2,886	14.4	229	1.1	10,344	49

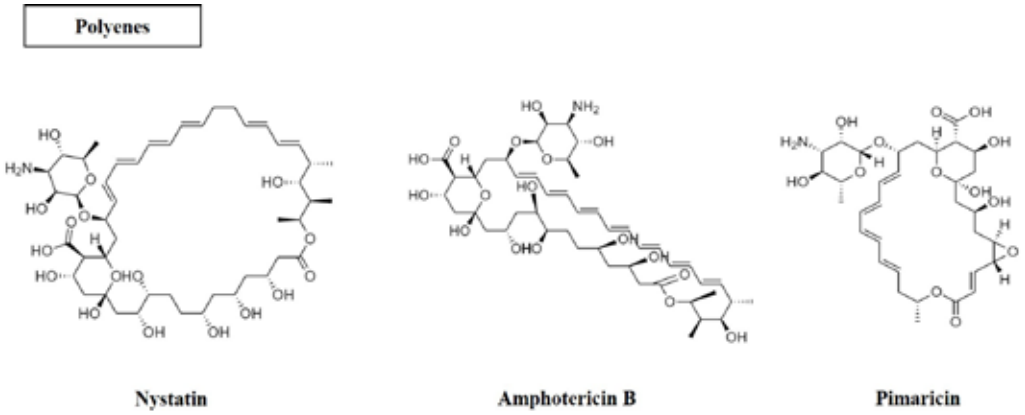

Fig. 1 - Structure of polyene antifungal compounds.

Azoles

The primary treatment includes azoles used to treat aspergillosis (20). This class of antifungals includes medicines with an azole ring (Fig. 2) that stops a variety of fungi from growing (22). Clotrimazole, econazole, ketoconazole, miconazole, and tioconazole are examples of two-nitrogen-atom imidazole. Triazoles, in contrast, have three nitrogen atoms in the azole ring (fluconazole, itraconazole, posaconazole and voriconazole). The antifungal effects of azole compounds were first described in 1944 (23). The first compound was imidazole, followed by triazole. The first azole antifungal that could be applied topically in a therapeutic setting was chlormidazole in 1958. Other imidazole-azole antifungals such as clotrimazole and miconazole were thereafter made available for topical application, and econazole was released in 1974 (24). The first drug was ketoconazole, an oral medication for systemic fungal infections, albeit its usage was constrained by its toxicity (25,26). Fluconazole and itraconazole, the two most used systemic triazoles, were first made available in the United States in 1990. Fluconazole and itraconazole have sufficient antifungal activity and are far less harmful than ketoconazole (27). Itraconazole and fluconazole are less toxic than ketoconazole and have adequate antifungal action (27). Voriconazole and posaconazole were the second-generation triazoles to be made available in the late 1990s and early 2000s. They have proven to be incredibly powerful against *A. fumigatus* (28) and are being utilised to manage fluconazole-resistant strains (29). Triazoles under investigation include albaconazole (30), isavuconazole and pramiconazole (31).

Azoles work by blocking the cytochrome P-450-dependent enzyme lanosterol demethylase, also known as 14α-sterol demethylase or P-450$_{DM}$, which is necessary for the formation of ergosterol, a crucial component of fungal plasma membranes (32). Exposure to azoles in *A. fumigatus* decreases ergosterol levels and accumulates 14α-methylated sterols (33). This leads to a change in the structure and shape of the membrane, affects nutrient uptake and chitin synthesis, and inhibits fungal growth (34,35). On fungal cells, ergosterol also exhibits hormone-like characteristics that promote growth and reproduction (34). This function can also be impaired if the breakdown of ergosterol is more than 99% complete (34). Triazole is indispensable for long-term treatment, because of the fact that it is the only anti-*Aspergillus* drug that can be taken orally (36). Although itraconazole is still frequently used to treat the long-established non-invasive allergic type of aspergillosis (38), voriconazole is still advised as the first-line treatment for AI (34,35,37). Amphotericin B, a more lethal medication than triazoles, is the only available alternative.

Echinocandins

The most recent addition to the family of antifungal drugs are the echinocandins, with the first example, caspofungin, entering clinical use a decade ago (39). They are lipopeptide compounds that inhibit the enzyme (1,3) beta-D-glucan synthase, which produces glucan, the primary building block of fungal cell walls. Caspofungin (Fig. 3) is non-competitively effective against *A. fumigatus*, *A. flavus* and *A. terreus.* It is indicated for *Aspergillus* infections among those patients unresponsive or intolerant to other treatments. However, it has not been considered for first-line treatment (39).

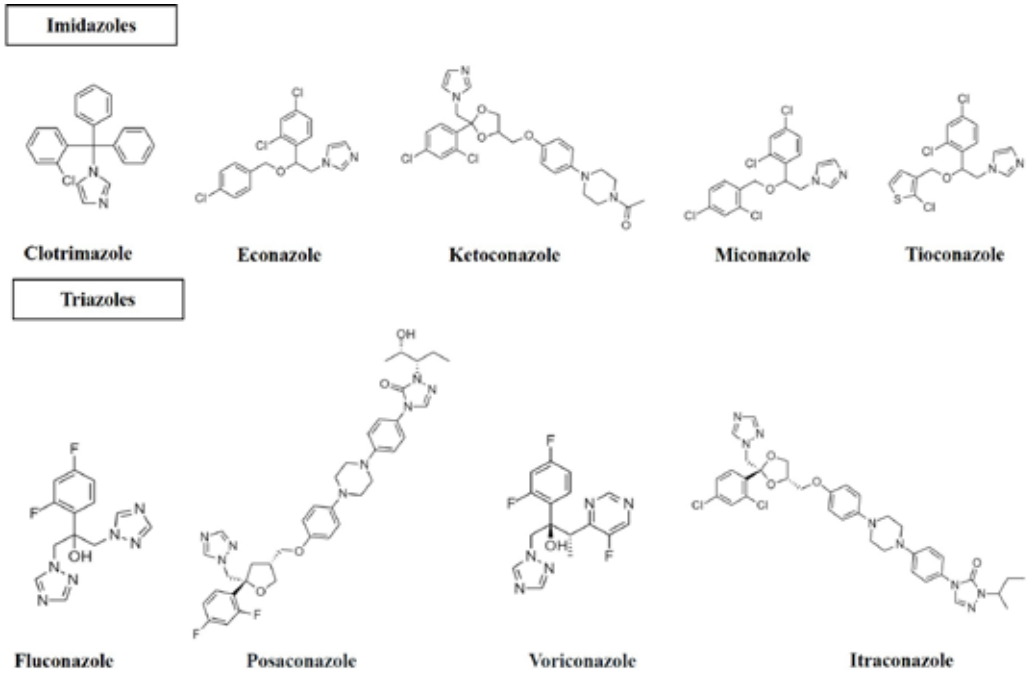

Fig. 2 - Structure of azole antifungals (imidazoles; two nitrogen in the azole ring and triazoles; three nitrogen in the azole ring).

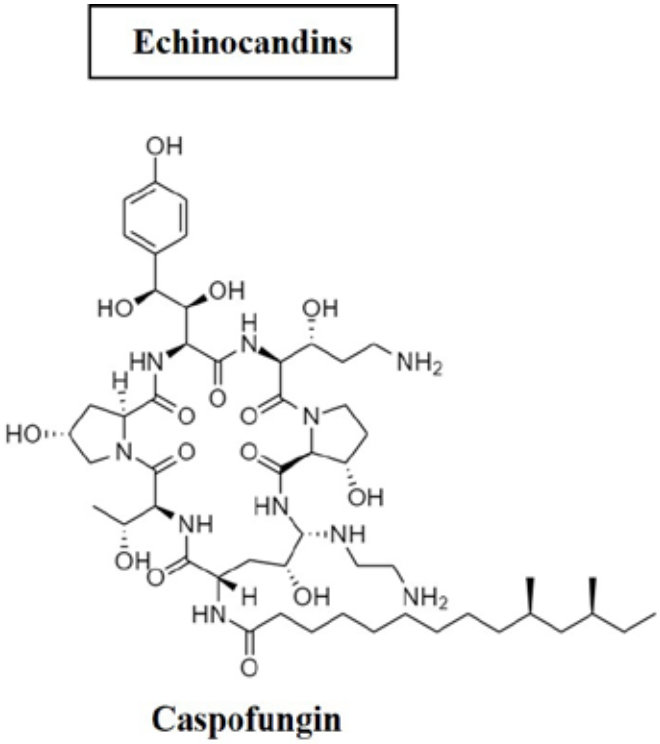

Fig. 3 - Structure of caspofungin antifungal.

Triazoles in the agricultural ecosystem

Plants are attacked by various pathogenic fungi that cause a variety of disease such as late blight, powdery mildew, leaf spot, blast, downy mildew, fruit rot, rust, etc. Throughout the world, the usage of synthetic insecticides and pesticides is quite common to overcome crop diseases and failure. However, these can have a significant negative impact on the environment (40). Thousands of tonnes of azoles are sold every year for crop protection. The advantages of azole pesticides include low cost and a diverse range of antifungal activity (41). Hexaconazole, propiconazole, triadimefon and tricyclazole are some of the prominent triazole fungicides available in the international market (42). The long-term stability of azoles is a crucial characteristic. With very slight modifications to their chemical composition, several azoles can continue to function for months in agricultural habitats (soil and water). Singh and Dureja (43) reported that hexaconazole can remain in soil for a long time due to its hydrophobic properties. Azoles are effective against several plant fungal diseases listed in Table II. The major metabolite of triadimefon, triadimenol, has a half-life in soil that varies from 110 to 375 days. The plants are sprayed several times per growing season at a dose of 100 g/ha, which is recommended to combat fungal diseases (41). There are currently 32 commercially available azole fungicides for crop protection (44). Several foods have been found to contain azole residues, for example, in samples of commercially available strawberries, grapes or mint. Therefore, there is evidence that large amounts of antifungal residues, especially azoles, can remain in the environment.

TABLE III - Azole fungicides used in market (42-44)

Azole fungicides	Crops	Diseases caused by *Aspergillus*
Triadimefon	Wheat, pea, grapes, coffee, mango, chilies, soybean	Bunt of wheat, powdery mildew, rust, powdery mildew, coffee rust, rust
Bitertanol	Apple, groundnut, tea, wheat, groundnut	Scab, rust, tikka, blister blight, Karnal bunt
Flusilazole	Grapes, apple, rice, chilies	Powdery mildew, scab, sheath blight
Hexaconazole	Apple, rice, groundnut, mango, soybean, tea	Blister blight, powdery mildew rust, scab, blast, sheath blight, tikka leaf spot
Tebuconazole	Wheat, groundnut, chili, rice	Blast sheath blight, loose smut, flag smut, collar rot, root rot, stem rot, fruit rot, powdery mildew
Difenoconazole	Apple, groundnut, rice, chili, cumin, onion	Fruit rot, blight powdery mildew, sheath blight, scab, leaf spot rust, purple blotch
Tricyclazole	Paddy	Blast

Resistance to azoles developing in Aspergillus *clinical isolates*

Recent years have seen a rise in the concern of azole therapy resistance in patients with *Aspergillus* infections. *A. fumigatus*, which causes around 80% of invasive infections, has been found to possess the highest azole resistance (45); Azole resistance has also been demonstrated in other species including *A. niger*, *A. terreus* and *A. flavus* (46). Infections with resistant *Aspergillus* strains lead to the effectiveness of azole antifungals, resulting in high mortality rates. Azole-resistant strains of *Aspergillus* were reported in the United States in the late 1990s (47). There have been numerous reports of infections with resistant strains in Europe, particularly in the UK and the Netherlands. Since that time, practically every European nation has reported cases of azole resistance, including Germany, Ireland, Italy, Austria, Denmark, France, Sweden, Portugal, Spain and Turkey (25,48-64). A surveillance study from the UK reported that the prevalence of azole resistance in *A. fumigatus* increased from 0.43% in 1998-2011 to 2.2% in 2015-2017 (65). A multicentre study conducted in Taiwan found a 4% prevalence rate for *A. fumigatus* resistant to azoles (66). Among Asian countries, Taiwan and China were the first to report resistance to azoles (67,68). Furthermore, this study has sparked significant worries about the use of azole antifungal medications to treat IA in the future.

The increase in resistance to triazoles in the clinical setting can be explained by two main phenomena: (a) an azole-resistant *Aspergillus* strain was found after long-term treatment with azoles in patients with cavernous lung disease and aspergilloma. When susceptible *Aspergillus* strains

acquire resistance to the pharmacological stress response of prolonged azole therapy, they develop resistance. Numerous point mutations were found in the azole-resistant *A. fumigatus* isolate, especially at codons 54 and 220 of *cyp51A* (69). Several resistance mechanisms associated with *cyp51A* have been identified in patients specifically associated with azole therapy (70). Human-to-human transmission is therefore highly unlikely and spread of resistance is very rare.

(b) Azole fungicides are frequently employed to protect crops in agricultural settings and are structurally like medicinal triazoles. *Aspergillus* species are found in soil together with other plant pathogens. Azoles that attack plant pathogens can also affect *Aspergillus* species found in the same ecosystem (3). Fungicides used repeatedly over a long period of time can create persistent selection pressure and lead to the development of resistant *Aspergillus* species. As a result, the environment contains *Aspergillus* species that are azole-resistant. When these conidia are inhaled by susceptible individuals, *Aspergillus* species become resistant to triazoles used for treatment. Several cases of triazole-resistant aspergillosis in humans and animals without prior triazole treatment have been reported worldwide (71,72).

Resistance to triazoles in Aspergillus *clinical isolates is associated with the use of azole fungicides in agriculture*

Many major agricultural fungi have developed resistance because of the site-specific mode of action and widespread application of 14α-demethylase inhibitor (DMI) fungicides to prevent post-harvest spoiling by plant-pathogenic fungus. Azole overuse in agriculture may have an impact on saprophytic microbiota species as well as plant-pathogenic fungi (73). The soil provides a natural habitat for several fungi that could be harmful, including *Aspergillus*, *Coccidioides*, *Histoplasma* and *Cryptococcus*. Recently, the use of azole pesticides has been identified as a significant contributing cause in the increasing prevalence of *A. fumigatus* isolates with a particular mechanism of resistance comprising the TR34/L98H mutation in the *cyp51A* gene.

Snelders et al.'s (74) findings that were obtained from both clinical and environmental sources exhibited cross-resistance to five triazole-DMI fungicides, notably bromuconazole, propiconazole, epoxiconazole, tebuconazole and difenoconazole, support the notion that *Aspergillus* species become resistant to triazoles as a result of environmental use of azole fungicide (74). Additionally, these researchers noted that all these five DMI-triazoles have efficacy against wild-type *A. fumigatus* but not against the resistant TR34/L98H *A. fumigatus* because of their molecular structure, which is similar to drug triazoles and when attached to the target enzyme, acquire a same conformation (74). In a related study from India, four of the five triazole DMIs – bromuconazole, tebuconazole, epoxiconazole and difenoconazole – showed substantially higher MIC (Minimum inhibitory concentration) values with TR34/L98H-resistant *A. fumigatus* from environmental and clinical samples than with wild-type non-resistant isolates. These drugs are known to have performance comparable molecular structures to drug triazoles (75).

In 2020, a study from India found that patients who had never taken triazole therapy had developed resistance, raising the possibility that environmental transmission may contribute to the emergence of resistance (5). Most of the resistance mechanisms found in patients without prior treatment with azole are resistance mechanisms associated with TR (tandem repeats). Most *Aspergillus* isolates from the environment also possessed this resistance mechanism (TR34/L98H or TR46/Y121F/T289A). Norway, the Netherlands, Denmark, the United Kingdom and India are some of the countries where all these resistant environmental isolates have been found (76). Thus, retrieval of a similar resistance mechanism from environmental isolates as patient isolates supported an environmental pathway for resistance development.

Environmental-induced mutations in azole-resistant Aspergillus *isolates*

Ergosterol is a crucial and distinct element of the fungal plasma membrane that gives the cell membrane stability and permeability. The enzyme cytochrome P450, also called sterol-14α-demethylase, converts lanosterol into ergosterol. *cyp51A* is a gene that codes for the cytochrome P450 enzyme. The ergosterol biosynthetic pathway is the general target of azole antifungals. Triazoles hinder the cytochrome P450 enzyme from performing its lanosterol-converting role in the ergosterol biosynthetic pathway, and causes the depletion of ergosterol and the deleterious build-up of lanosterol (74). Azole resistance is brought on by mutations in the *cyp51A* gene that change the *cyp51A* protein's structure and reduce the enzymes' affinity for azole therapies.

Various changes in *cyp51A* resulting in a pan-azole-resistant phenotype have been reported in *A. fumigatus* isolates from environment and clinical sources worldwide. The most frequent mechanisms of resistance reported in environmental and clinical strains of *A. fumigatus* are mutations in the TR34/L98H and TR46/Y121F/T289A genes. These changes in the *cyp51A* gene comprise TRs within the gene's promoter region (77). The *cyp51A* gene is overexpressed because of the insertion of 34 base pair (bp) TRs (TR34) into the *cyp51A* gene's promoter region and the substitution of the leucine 98 amino acid coding for histidine (TR34/L98H) (78). According to Table IV (77), TR34/L98H is the most typical resistance mechanism found in environmental and clinical strains of *A. fumigatus* in many different nations. Another resistance mechanism which has been proven is TR46/Y121F/T289A (Fig. 4) where a 46 bp TR promoter region of the *cyp51A* gene has substitutions of threonine 289 for alanine and tyrosine 121 for phenylalanine (TR46/Y121F/T289A), resulting in an elevated resistance to voriconazole in *A. fumigatus* (79). One of the most frequently detected mechanisms of resistance in environmental isolates from Europe are TR34/L98H and TR46/Y121F/T289A, and their emergence has already been connected to the widespread use of azole-based agricultural fungicides (tebuconazole, hexaconazole and epoxiconazole). Fungicide use is rising in India, where it already accounts for 19% of all pesticide use (80). Even though Europe is the worldwide leader in agricultural fungicide use (40%), it is followed by Japan and Latin America. In contrast to Europe, the United

States uses very less azoles in agriculture (http://ec.europa.eu/food/fs/se/ssc/out278_en.pdf). Therefore, no TR34/L98H mutation has been reported in environmental clinical isolates in the United States, but this mutation has been found in the European setting and now also in India (77).

An environmental study conducted examined a variety of soil and air samples from different regions of India. In their study, soil samples were taken from natural soils (where no azole fungicides were used). The samples tested positive for *Aspergillus* strains, but no resistant isolates were detected. Their findings were supported with those of the Dutch environmental study, which discovered that none of the *A. fumigatus* isolates cultured in natural soil exhibited azole resistance (81). Therefore, commercialised compost and samples taken from fields where fungicides are consistently treated can be the focus of environmental research to find TR34/L98H mutations in *A. fumigatus* isolates.

In environmental samples from China (82), certain novel mutations (G448S, TR46/Y121F/T289A) with 46 bp triple TRs in the promoter region have been discovered. Other Asian nations like India, Iran and Kuwait have demonstrated azole resistance in environmental *Aspergillus* strains (83,84). About 2% of *Aspergillus* species in Kuwait were found to be azole resistant, according to another study on environmental resistance (84). Indian researchers have reported that environmental studies on azole resistance describe the TR46/Y121F/T289A mechanism (85), and the findings indicated that 44 out of 630 *A. fumigatus* isolates from soil of indoor air, paddy fields, tea gardens, cotton groves, flowerpots and hospitals were resistant and managed to retain TR34/L98H resistance (75). An environmental mechanism of resistance (TR46/Y121F/1289A) in strains of *A. fumigatus* was also first reported in this study. In 2009, a similar mutation was discovered in a Dutch patient and has also been reported in several patients from the Netherlands (86). This survey, which took place between 2012 and 2013, studied 105 environmental samples taken from North Indian agricultural fields. The study concluded that azole fungicide-treated agricultural soils in northern India co-occurred with TR34/L98H and TR46/Y121F/T289A. The identified Indian *Aspergillus* strains were likely to be highly adaptable recombinant descendants of a cross between a native azole-sensitive strain from within India and azole-resistant strain that migrated from outside India, followed by a mutation, according to genomic analysis of the Indian resistance mechanism TR34/L98H (75). Reports indicated a rapid spread of this mutation in Asia (83).

TABLE IV - Common resistance mechanisms reported in the *cyp51A* gene of environmental *A. fumigatus*

Geographic region/ references	Sample	*cyp51A* resistance mechanisms
The Netherlands (81)	Soil	Unknown, F46Y/ M172V/E427K
France (87)	Dust from patients' home	H285Y
Germany (88)	Soil	G54A, M220I
India (89)	Soil	G54E
Taiwan (90)	Soil, air	Wild-type *cyp51A* or SNPs
France (91)	Soil	Unknown, P216L
Colombia (92)	Soil	TR46/Y121F/T289A, TR34/L98H and TR53
India (77)	Environment	TR34/L98H
India (93)	Azole-naïve patient	G54E
India (5)	Azole-naïve patient	G54R, P216L and Y431C

SNP = single nucleotide polymorphism.

Multiple triazole-resistant *A. fumigatus* isolates with the TR/L98H genotype were found in patients with chronic respiratory disease, according to a different study that was carried out for the first time in India (75). Only Europe and

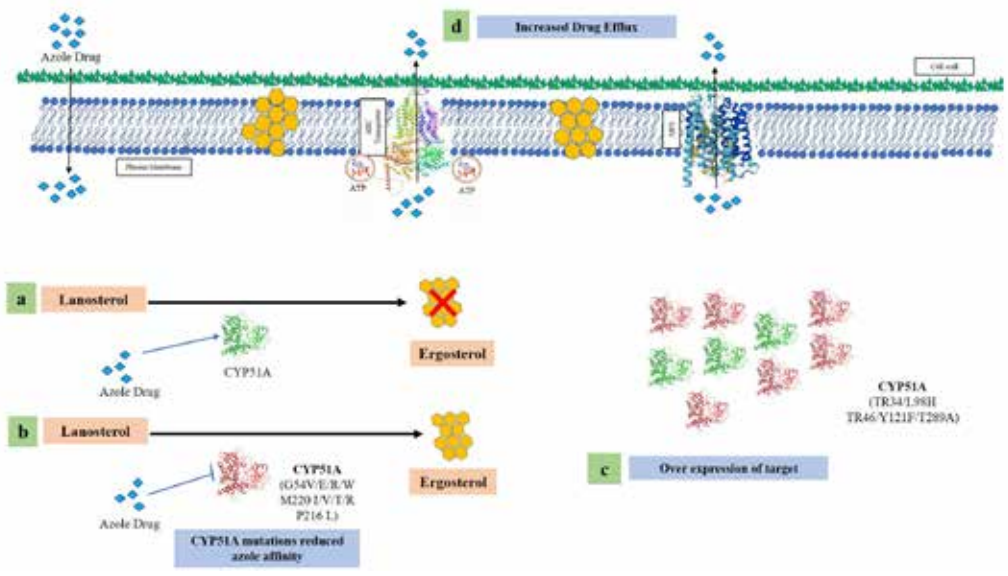

Fig. 4 - Various azole resistance mechanisms in *Aspergillus fumigatus* (a) Wild-type fungi in the presence of azole drug unable to make ergosterol. (b) Mutations in the *cyp51A* region alter the structural modifications of the enzyme leading to reduce azole affinity. (c) Insertion of 34 and 46 base pair in the promoter region along with point mutation in the *cyp51A* region causes overexpression of the gene. (d) Overexpression of efflux pump genes causing a reduced intracellular accumulation of azole drug.

China have been described as having the TR/L98H mutation linked to pan-azole resistance in *A. fumigatus* (70,81). The two triazole-resistant *A. fumigatus* isolates are epidemiologically unrelated, share the same TR genotypes, and come from patients with no prior history of exposure to azoles or travel to Europe, suggesting that they most likely mutated and developed resistance as a result of exposure to the environment in India. The two isolates were phylogenetically distinct from TR/L98H, which contained the 25 *A. fumigatus* isolates from Dutch. The usage of azole fungicides in the environment may be a contributing factor in the propagation of this resistance mechanism (TR/L98H) in *A. fumigatus* isolates. A total of 43.7% of *Aspergillus* isolates found in 25 agricultural soil samples were found to be resistant to azoles, according to a recent environmental study in India (94).

Other non-synonymous hotspot mutations in the *cyp51A* gene have also been discovered in azole-resistant *A. fumigatus* strains, in addition to the TR34/L98H and TR46/Y121F/T289A alterations. While resistance to ITC (Itraconazole) and POS (Posaconazole) was provided by the glycine modification mutations 54 (G54) and 138 (G138), lower susceptibility to ITC and POS related to the glycine 448 (G448S) (95) mutation-related resistance to VRC. Methionine 220 (M220) amino acid substitution was also linked to a pronounced pattern of decreased sensitivity to triazoles (70). There have also been sporadic reports of other point mutations, including P216L, F219C, F219I, A284T, Y431C, G432S and G434C (86). Patients who received around 4 months (range 3 weeks to 23 months) of long-term azole treatment for persistent aspergillosis have been discovered to have the point mutations G54E/R/V and M220I/V/T/K (96). It is important to mention here that studies conducted in India, Tanzania, Romania and Germany found G54 mutations in environmental isolates of *A. fumigatus* (97). An environmental study in India found an azole-resistant *Aspergillus* species with a G54E mutation (89). This point mutation in the *cyp51A* gene is commonly seen in patients undergoing long-term azole therapy (89). In another study in India, *A. fumigatus* isolates with G54R, P216L and Y431C mutations were obtained from azole corpus patients (4). The MICs of many additional point mutations, including F46Y, M172V, N248T, D255E and E427K, have been discovered in azole-susceptible and azole-resistant *Aspergillus* isolates. However, this is not always restricted to clinical breaking point (75). The non-*cyp51A* pathway has also been linked to azole resistance in *A. fumigatus*. In isolates of *A. fumigatus* (98), voriconazole was also used to treat the link between biofilm growth and efflux pump activity to regulate homeostasis in azole resistance. Additionally, *Aspergillus* species can effectively invade and colonise the host by activating efflux pumps, specifically adenosine triphosphate (ATP)-binding cassette transporters and carriers of the major facilitator superfamily, to overcome the build-up of intercellular toxins (78).

Spread of Aspergillus *from the environment to hospitals*

Aspergillus spp. is ubiquitous in the environment and cosmopolitan in nature. The main habitat of *Aspergillus* spp. is the soil, and this saprophytic fungus has a vegetative mycelial life that develops on the decomposing matter, whether organic or vegetable, found in the soil (99). Previous studies showed that the metabolic machinery in *Aspergillus* spp. contains certain enzymes such as endo-β-glucanase, acetylxylan esterase, polygalacturonase, tannase, etc., which can easily degrade components of the plant cell wall. On the other hand, it does not contain any enzymes that can decompose plant wood (100). Spread of conidia occurs by asexual sporulation, and there is airborne spread of asexual reproductive organs, or conidia. Conidia mainly predominate in the air and are inhaled by individuals. It is estimated that 200 conidia are inhaled per person. However, they are stripped of pulmonary macrophages and neutrophils present in the lungs of immunocompetent humans. The clinical manifestations of *Aspergillus* depend on the host's immune status. They cause severe infections in immunocompromised patients with other predisposing factors and develop life-threatening aspergillosis (101). A case study examined fatal IA and found that the source of *Aspergillus* infection was the patient's home, which was in an agricultural area with potentially high pressure of fungicides used to protect crops. Even after the patient died, household samples showed the persistence of azole-resistant strains of *Aspergillus* spp. (63). Another study tested the source of azole-resistant *Aspergillus* spp. in a hospital environment. Their samples were taken from different environments in the hospital. The assessment showed the main source of *Aspergillus* spp. in the hospital and in the corridors (102), where the floor was decorated with tulip pots. This indicates the easy transmission of environmental *Aspergillus* strains to hospitals and infecting patients who were mainly in immunocompromised states or in persistent drug states. Therefore, it is important to identify sources of infection, whether the patient is hospitalised or a source of in-hospital contamination, due to the potential for aerosol transmission from patient to patient (103).

Azole-resistant Aspergillus *biofilms*

Aspergillus is an opportunistic airborne pathogen capable of forming biofilms in clinical settings or in immunocompromised patients with underlying conditions leading to allergic aspergillosis or IA (104). Biofilms are a community of cells strongly adherent to abiotic and biotic surfaces and surrounded by an extracellular matrix (ECM) composed of polysaccharides. The ECM acts as a protective sheet and external scaffold for adhesion and integration with the surface, and cell spreading for subsequent invasion. This protective layer becomes more sensitive to antifungal drug treatments and attacks immune cells, making them harder to fight (105). Possible factors contributing to drug resistance in *Aspergillus* spp. biofilms are: upregulation of efflux pump genes such as *AfuMDR4, MDR1, MDR2, MDR4*; induction of the HSP90 stress response pathway, which increases resistance to the antifungal drugs amphotericin B and caspofungin; by extracellular DNA which reduces drug sensitivity by preventing the drug from reaching its cellular target through ECM and sister cell formation while acting as drug-tolerant cells to form new biofilms (106).

The most important factor in IPA (Invasive pulmonary aspergillosis) and aspergilloma is biofilm formation. Fungal components such as drug transporters, secondary metabolites and cell wall components promote biofilm formation in host cells and are resistant to antifungal drug treatment (107). Biofilm formation, which helps to penetrate the host immune system and reduce the patient's immune competence, also contributes to increased resistance to triazoles (108). A study showed that under in vitro conditions, *A. fumigatus* formed multicellular biofilms of polystyrene sheets that could resist the effects of antifungal drugs (109). Another study also showed an effect of itraconazole on hyphal germination and biofilm formation at an early stage, but no effect on mature biofilms, suggesting a predominance of resistant biofilms (110). Biofilms in the lungs are difficult to diagnose because they occur after mature biofilms form. In the adult stage, this tissue in the lungs develops into a more complex tissue with dense ECM and limited oxygen, which encourages further growth. This makes it increasingly difficult for immune cells to recognise and influence them. It also worsens when other microbial biofilms persist and are difficult to remove with antifungal drugs, particularly in cystic fibrosis (111). Therefore, a comprehensive analysis and understanding of *Aspergillus* biofilms is required to develop new and improved antifungal targets for the treatment of complex biofilm-related diseases (107).

Future directions

Azole resistance in environmental *Aspergillus* spp. is a matter of grave clinical concern as transfer of resistance from environment to clinical settings is inevitable. India needs to impose strict regulatory compliance to ensure regulated usage of pesticides in agricultural fields. Further studies are warranted to understand the level of transfer of resistance from the field to clinic. This can be undertaken in a state-wise study by mapping the mutations that are unique to the region. Additional studies focusing on the usage of azole fungicides and the presence of azole residues in developed environments are needed, because the amounts used or quantities present are not often measured or reported.

More surveillance, accurate data collection and comprehensive resistance surveillance programmes in agricultural ecosystems are needed to study the magnitude of the emerging problem of azole resistance. It will be very important to identify tipping points to ensure the agronomic use of fungicides without jeopardising their treatment goals. Research at the epidemiological level can uncover geographic differences in the emergence of resistance and help identify areas with high levels of resistance. Further, to overcome antifungal resistance in clinical settings, development of new antifungals with new drug target site is needed.

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ORIGINAL RESEARCH ARTICLE
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Antimicrobial resistance surveillance system mapping in different countries

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ABSTRACT

Objectives: Excessive use of antibiotics has increased antimicrobial resistance (AMR) worldwide, which is a major public concern among the countries. To control this threat proper monitoring of the antimicrobial usage with increasing rate of AMR is required. Moreover, alternatives for antibiotics are surveyed and are being researched for quick use in the future. Thus, multisector intervention is highly encouraged for better outcomes. In this research article, six different European countries are discussed in terms of antimicrobial usage and AMR in human and livestock sectors with the help of literature study and various reports published by different organizations.

Methods: Data study has been conducted to collect data for comparison study. Data sources of AMR and antimicrobial usage are analyzed and both antimicrobial use and AMR are compared.

Results: This article provides surveillance systems that are formed to keep a track on the upcoming situation of AMR and the consumption of antimicrobials by humans as well as animals. The article firmly allows the readers to get broad information about the AMR across six countries of Europe. These annual reports have hugely helped the government to decide for alternatives and have focused in many training activities to combat the AMR situation globally.

Conclusion: As antibiotic resistance genes persist on an interface between environment and animal and animal health, an approach is required in all three areas that stress the concept of "One Approach to Health."

Keywords: Alternative antibiotics, AMR, Comparative medicine, One Health Approach, Phage therapy, Surveillance

Introduction

In the last few decades extensive use of antibiotics has resulted in rising cases of antimicrobial resistance (AMR) against various organisms. From narrow-spectrum antibiotics, people shifted to broad-spectrum antibiotics, which eventually increased the high resistance rates. Multidrug-resistant (MDR) bacterial infections are rapidly emerging and spreading over the world, posing a severe threat to global healthcare. Carbapenem-resistant Enterobacteriaceae (CRE), a type of gram-negative bacteria that has resisted all or virtually all current antibiotics, is one cause for concern. Likewise, various other antibiotics are also becoming resistant against the microorganisms causing immense threat among the population. This global threat comprises of both commensal and pathogenic bacteria. The similarities between human and animal diseases, as well as the interactions between animals and humans who come into contact with them, have long been recognized. Human and veterinary medicine diverged in the twentieth century. During the same time span, our understanding of infectious diseases and antibiotics grew dramatically. The necessity for partnerships between human health and veterinary sectors to prevent and control zoonotic illnesses and antibiotic resistance grew in the second half of the twentieth century. The notion of ecosystem health developed toward the end of the twentieth century, extending the integration and collaboration of human and animal medicine to the environment. Later on, the phrase "One Health" was coined to describe a holistic approach to improving human, animal, and environmental health through multidisciplinary cooperation and communication. Several global plans have been established to combat the AMR epidemic, including the World Health Organization's (WHO) Global Action Plan (GAP), the new European One Health Action Plan against AMR, and the Central Asian and Eastern European Surveillance of

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Antimicrobial Resistance (CAESAR) network (1). Surveillance and monitoring systems for Antimicrobial Usage (AMU) and AMR in humans and animals are critical for assessing and controlling global trends in antimicrobial use and antimicrobial susceptibility patterns of bacteria in various populations. In the context of a One Health strategy, zoonotic and indicator microorganisms are especially important. A strategic framework for reducing infectious disease risks at the animal-human ecosystem interface was published in 2008, adopting and promoting the One Health concept. The One Health Approach has been supported and implemented by a wide number of national and international institutes since 2008. Research on the human-animal environment interaction is critical to supporting the call for a One Health Approach to AMR and infectious illnesses. Furthermore, training and extension initiatives are critical for promoting the One Health idea and facilitating its application among various stakeholders (2,3). Several governments and international organizations have now included a One Health Approach in the AMR action plans. Improvements in antimicrobial use, better regulation and policy, improved surveillance, stewardship, infection control, sanitation, animal husbandry, and identifying antimicrobial alternatives are all necessary efforts. This report summarizes research and educational activity in the field of One Health in Western Europe, with an emphasis on infectious diseases. It might act as a springboard for future collaborations and projects.

Materials and methods

Data sources

We conducted a database study for collecting major characteristics of surveillance and monitoring systems on antimicrobial use and AMR in cattle and people, as well as AMR systems in food, in this publication. Countries such as Spain, Germany, France, the Netherlands, Norway, and the United Kingdom were considered for this project. The literature searches in recent times have been carried out to understand and collect the data from different gray reports and other AMR databases. The database study has been conducted by searching the terms "Antimicrobial Resistance," "Antibiotic Usage," "One Health Approach" on PubMed and desired research papers or data sheets annually published by different agencies are studied for collective data required for this research article. Additionally, information about One Health Approach in these countries was also investigated for obtaining the results. One Health policy publications issued by international organizations and countries also provided background information on the One Health program and European One Health projects. Moreover, Google research on One Health with its associated activities and trainings among these countries was conducted for acquiring more relevant outcomes. Alternative antibiotics for resolving the issue of AMR were also researched which focuses on better solution for AMR and antimicrobial usage. One of the renowned projects also known as ARDIG (Antimicrobial Resistance Dynamics the Influence of Geographic origin) together collects and gathers data related to AMR and usage of antimicrobials from both human and veterinary sectors. The stipulated graph depicts the predicted deaths that will be increasing for the usage of antibiotics globally by the year 2050 (Fig. 1) (4). This helps us to portray the comparison study of all the data collected for different countries along with the Asian countries. In addition, all the surveillance systems for monitoring AMR in different countries of Europe have been explained thoroughly in this article. The primary reason for tracking the reports of European countries is because One Health has gained a lot of traction throughout Europe. The One Health strategy is currently being promoted in Europe mostly in regard to AMR. Many nations have adopted the One Health concept in their anti-AMR policies, and funding opportunities for AMR research have considerably increased. In the areas of zoonotic diseases and One Health, the number of national and international multidisciplinary research networks is growing (1,5,6).

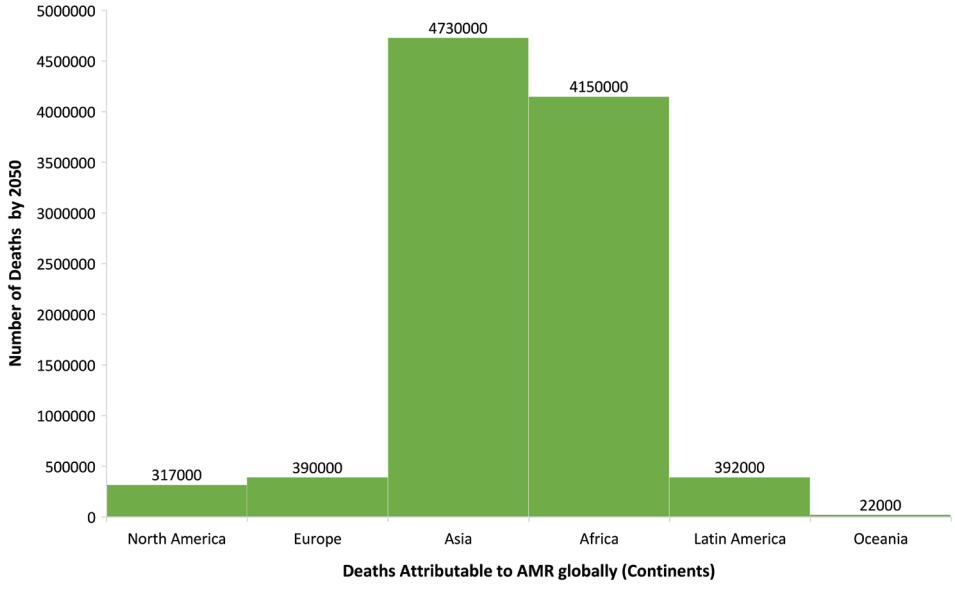

Fig. 1 - The stipulated graph depicts the predicted increase in the number of deaths for using antibiotics globally by the year 2050. X-axis denotes the continents and Y-axis denotes the number of deaths by the year 2050. (from: https://www.publichealthpost.org/databyte/antibiotic-resistant-bacteria/)

Surveillance strategies of AMR and monitoring system

Data collection and data analysis

Different European countries have various surveillance strategies and monitoring systems for controlling the rising threat of AMR. Additionally, multiple organizations are coming together for joint efforts that require combating this situation. A complete summary of the data collected is provided in a tabular form for better understanding of the data gathered.

France

AMR data related to agriculture, food, and the environment are monitored by the French Agency for Food, Environmental, and Occupational Health and Safety (ANSES). The French monitoring network for antibiotic resistance in pathogenic bacteria of animal origin (RESAPATH) and the Salmonella network are coordinated by this agency. The Salmonella network is a surveillance system designed to keep nonhuman Salmonella under control throughout the food chain. The Investigation and Surveillance of Nosocomial Infection Network (RAISIN) coordinates the nosocomial infection surveillance coordination centers across the country. BMR-RAISIN, a private RAISIN module for multidrug-resistant bacteria, reports on AMR data in the community. Healthy animals, food, and the environment are all sampled. The RESAPATH voluntary surveillance system compiles AMR data for primary bacterial species and general isolates from sick animals from each animal sector in the annual RESAPATH report (7).

Germany

Clinical AMR data from companion and food-producing animals is collected in Germany through the German veterinary monitoring system (GERM-VET). AMR testing in the Zoonosis-Monitoring System (ZOMO) report includes data on zoonotic and commensal bacteria in various food chains, as well as AMR data on Salmonella from national control programs, which are also reported to the European Food Safety Authority (EFSA). Antimicrobial Resistance Surveillance (ARS) is the human national AMR surveillance system. It gathers routine susceptibility data for all bacterial species from any sample site, including hospital and outpatient care facilities. The Hospital Infection Surveillance System (KISS) is a nosocomial infection surveillance system made up of multiple sub-systems that collect AMU and AMR data in hospitals. Surveillance of Antibiotic Use and Resistance in Intensive Care Units (SARI) gathered data on antimicrobial sensitivity for selected pathogenic microorganisms and the creation of AMU-AMR on a volunteer basis (1).

Spain

To keep track of AMR, the Spanish Veterinary Antimicrobial Resistance Surveillance Network (VAV) was formed. VAV provides nonclinical data to the EFSA, which is included in the agency's annual reports. According to EU legislation, this report contains information on zoonotic infections and diseases in animals, humans, and food, as well as data on AMR in select zoonotic bacteria and indicator bacteria (1,6).

Norway

The three AMR surveillance programs in Norway are the Norwegian Surveillance System for Antimicrobial Drug Resistance (NORM), Norwegian Veterinary Antimicrobial Resistance Monitoring (NORM-VET), and the Norwegian Surveillance System for Communicable Diseases (MSIS). This annual report contains updated information on AMU and AMR prevalence and distribution in the human, animal, and food sectors (8,9).

The Netherlands

The "Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands" (MARAN), which brings together the Food and Consumer Product Safety Authority's AMR food database, is the Netherlands' AMR monitoring system for animals and food. It disseminates information on foodborne pathogen resistance as well as commensal indicators from animals and food. The Infectious Disease Surveillance Information System on Antibiotic Resistance (ISIS-AR) monitors AMR in key pathogens in the human sector (10). These surveillance systems are extremely helpful in tracking down the situation caused by antimicrobial use and AMR. The various features of different organizations built by the agencies have successfully helped the researchers in providing the necessary data for handling the threat worldwide. In addition to strengthening the AMR surveillance, numerous policies have been prepared by WHO and other agencies that apparently help in working with the solution of either decreasing or avoiding the AMR situation. For teaching and training, surveillance and risk assessment, and research, the AMR Coordinating Office emphasizes a One Health Approach. Political commitment, policy formation, sustainable finance, program creation, knowledge sharing, institutional collaboration, capacity enhancement, civil society involvement, and active community participation are all part of the framework for effective One Health implementation. One health is a straightforward and strong idea with complex processes. The national response to zoonoses must be revised, food safety improved, and environmental integrity guaranteed. The transformation must be driven by the senior leadership. Strong, ongoing lobbying by international development partners, in particular: the FAO, the OIE and the WHO, should be shared with the leading national leadership, disseminating the evidentiary results, predicted economic benefits, and best practice globally. The interconnected sustainable development goals offer a unique opportunity for advocacy and an integrated approach to development. The effectiveness of One Health implementation depends on the extent to which institutional cooperation, common planning and coordination thorough monitoring for early detection and prevention of zoonoses are achieved. The key planning, implementation and surveillance are data and science. Initial efforts for rapid tracking should be performed quickly in order to create multisectoral capacity across various organizations. The theory and practice of One Health should be fully integrated and visible in the educational curriculum as well as in the constant upgrading of skills for all subjects for long-term implementation.

United Kingdom

In the United Kingdom, the EU-Harmonized Surveillance System (a native UK system) collects mandatory AMR data on indicator commensal *Escherichia coli* and/or *Campylobacter* spp. from meat and fecal content of healthy animals (chicken, beef, turkey, and pigs). There are also Salmonella National Control Programs in the United Kingdom that are hosted in the EU-Harmonized Surveillance System. In Scotland, the Scotland's Rural College Veterinary Services and Capital Diagnostics (SRUC) surveillance system collects clinical isolates from animals. In England, monitoring surveillance system Vet Pathogens APHA collects AMR data from infected animals that veterinarians proactively offer for diagnostic services, covering all relevant bacteria and animal species. On the human aspect, the British Society for Antimicrobial Chemotherapy's (BSAC) Resistance Surveillance Program provides antibiotic resistance data from cooperating labs in the UK and Ireland for a variety of clinically relevant bacteria from community-acquired respiratory illnesses. AMR data are collected through the Electronic Communication of Surveillance in Scotland (ECOSS) network from participating National Health Service (NHS) and reference laboratories in Scotland (1,11).

Results

The accomplished research revealed that various surveillance systems are actively working to follow a trail of the upcoming situation of AMR and antimicrobial consumption by humans as well as animals. These surveillance systems of European countries are jointly contributing in statistically analyzing the rising situation of AMR and the prominent measures taken by different organizations for implementing One Health Approach. Moreover, various training institutes and alternative measures for preventing AMR are firmly encouraged in these six European countries along with Taiwan and India. Data of AMR solely do not arise from consuming antibiotics. There are multiple more aspects such as food habits of the humans, food chains maintained by the healthy animals, and the environment that together exhibit the importance of surveillance systems for AMR as these features put up the AMR issue topmost. The EFSA is in charge of communication on food chain concerns. Annually, the EFSA and the European Centre for Disease Prevention and Control (ECDC) collect AMR data on humans, food, and healthy animals from EU States and some affiliated countries (5). The European Union summary report on AMR in zoonotic and indicator bacteria from humans, animals, and food is prepared and published by the EFSA. Also, some nongovernmental organizations such as European Animal Health Study Centre (CEESA) are also contributing by researching about AMR and forming relevant systems to perform the activities efficiently (6). Precisely, the organization is working in monitoring the antimicrobial susceptibility of the bacterial pathogens that have the potential of causing diseases among the animals along with the foodborne pathogens in animal's food. These organizations are not the only aide for this surveillance system; a prime system also called as the European Antimicrobial Resistance Surveillance Network (EARS-Net) mainly helps in the surveillance of AMR data. This is an AMR surveillance network established in compliance with European Union and European Economic Area legislation. The ECDC collects AMR data from EU States through EARS-Net and publishes the annual EARS-Net report. On that account it is extremely crucial to compare the percentages of antibiotic usage and AMR, which will help in displaying the numbers accurately acquired from different organizations and relevant measures will be implemented for better solution. Similarly, acknowledging this, two joint interagency reports for antibiotic consumption and the analysis of AMR were published that clearly demonstrate the effects of using extensive antibiotics on humans and animals and the data were compared to AMR reports for better understanding. This report is jointly published by the European Medicines Agency (EMA), the EFSA, and the ECDC. The ECDC and EARS-Net require other platforms to jointly work for this (Fig. 2). Every country from Europe has

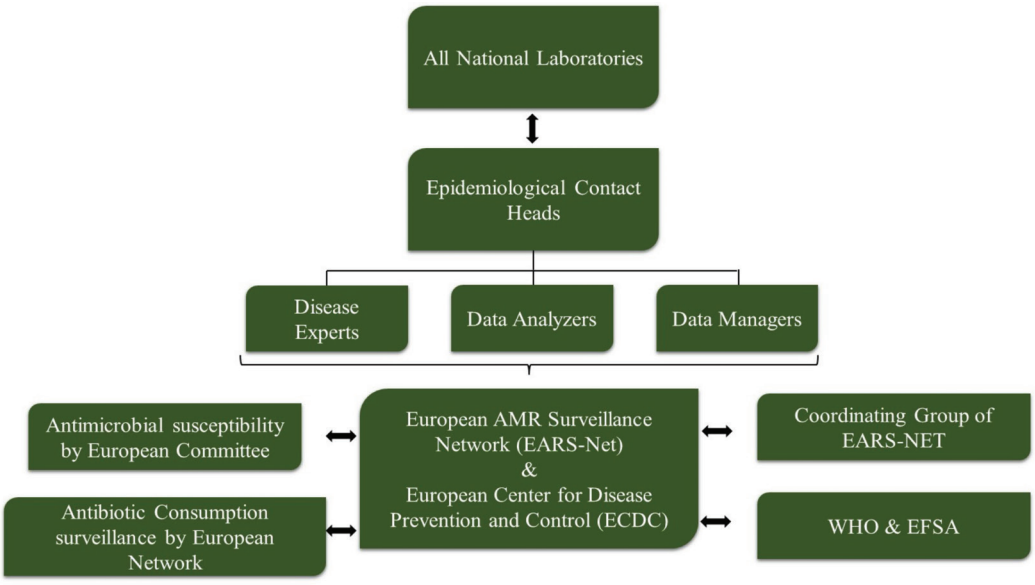

Fig. 2 - Different organizations jointly working together to provide data to EARS-Net and ECDC.

its own surveillance system for AMR that they follow, and prepared reports are further provided to EFSA. The AMR surveillance system is developed distinctly for humans and livestock (1).

Different systems of the country contribute in forming the reports, which are eventually published by EFSA or ECDC (Fig. 3).

Similarly, AMR surveillance system for humans is also analyzed by different organizations formed in these six European countries. Figure 4 depicts the organizations that are being established for keeping the record of the AMR surveillance (6).

Tables I and II give details about all the aforementioned surveillance system followed by the distinct countries along with the features and roles they perform (1,7).

Fig. 3 - AMR surveillance systems for livestock of different countries reporting the data to EFSA. ANSES = The French Agency for Food, Environmental and Occupational Health & Safety; EU-Harmonized = The EU-harmonized Surveillance System; MARAN = Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands; NORM-VET = Norwegian Veterinary Antimicrobial Resistance Monitoring system; VAV = The Spanish Veterinary Antimicrobial Resistance Surveillance Network (VAV); ZOMO = Zoonosis-Monitoring System.

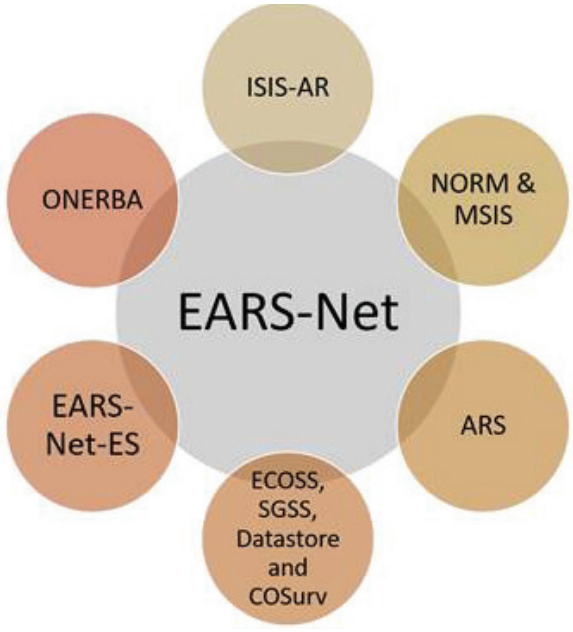

Fig. 4 - AMR surveillance systems for humans of different countries reporting the data to EFSA. ARS = antimicrobial resistance surveillance; EARS-Net-ES = European Antimicrobial Resistance Surveillance Network; ECOSS, SGSS, Datastore and COSurv = The Electronic Communication of Surveillance in Scotland, Second Generation Surveillance System; ISIS-AR = Infectious Disease Surveillance Information System on Antibiotic Resistance; NORM & MSIS = Norwegian Veterinary Antimicrobial Resistance Monitoring; ONERBA = National Observatory of the Epidemiology of Bacterial Antibiotic Resistance.

TABLE I - AMR surveillance system conducted in different countries for humans

Surveillance system	Country	Roles of surveillance system
ISIS-AR	The Netherlands	This aims at monitoring AMR in major pathogens.
NORM and MSIS	Norway	It is an AMR surveillance program in Norway. This annual report provides updated information on AMU and AMR occurrence and distribution in human beings.
ARS	Germany	It is the national human medicine AMR surveillance system. Established by the Robert Koch Institute, it collects routine sensitivity data from any sample site in the hospital and from ambulatory care institutions for all bacterial species.
EARS-Net-ES	Spain	Maintains the records of AMR surveillance across Spain.
ONERBA	France	AMU and AMR as well as a leading AMR network that collects data from a complex subsystem network is an annual French report, ONERBA.
ECOSS, SGSS, Datastore, and COSurv	United Kingdom	The ECOSS database gathers AMR data from participating NHS laboratories and reference laboratories in Scotland. Electronic Communication of Surveillance in Scotland (ECOSS) (SGSS) captures 98% of the National Health Service (NHS) laboratories across England, from routine laboratory surveillance data on infectious diseases and antimicrobial resistance.

AMR = antimicrobial resistance.

TABLE II - AMR surveillance system conducted in different countries for livestock

Surveillance system	Country	Roles of surveillance system
VAV	Spain	VAV monitors the AMR status throughout the country and is also responsible for monitoring animals and food. In addition, VAV supplies EFSA with nonclinical data.
ANSES	France	ANSES generally monitors AMR data related to food and livestock.
ZOMO	Germany	This report also provides data on zoonotic and commensal bacteria of the different food chains reported to EFSA.
MARAN	The Netherlands	Data on foodborne pathogens and commensal indicators from cattle and food are published in the annual report of the Netherlands.
NORM-VET	Norway	Facilitate updated incidence and distribution information on animal AMU and AMR.
EU-Harmonized	United Kingdom	Mandatory AMR data for meat and feces in healthy animals, using the appropriate indicator *Escherichia coli* and/or *Campylobacter* spp. are collected under the European harmonized supervisory system.

AMR = antimicrobial resistance; EFSA = European Food Safety Authority.

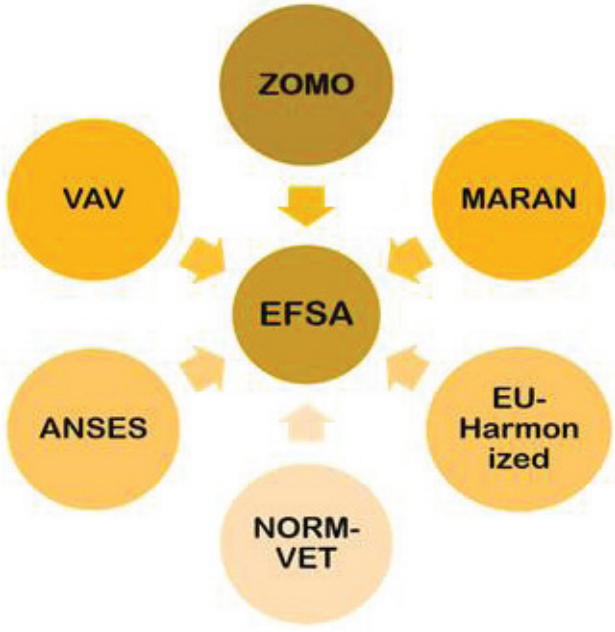

Fig. 5 - AMR surveillance systems for foods of different countries reporting the data to EFSA. ANSES = The French Agency for Food, Environmental and Occupational Health & Safety; EU-Harmonized = The EU-harmonized Surveillance System; MARAN = Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands; NORM-VET = Norwegian Veterinary Antimicrobial Resistance Monitoring system; VAV = The Spanish Veterinary Antimicrobial Resistance Surveillance Network (VAV); ZOMO = Zoonosis-Monitoring system.

TABLE III - Country-wise AMR surveillance systems not reporting data to EFSA

Surveillance system	Country	Hosts
GERM-VET	Germany	Livestock
RESAPATH	France	Animals
APHA-VET PATHOGENS	United Kingdom	Diseased animals
SRUC	United Kingdom	Animals
PEG	Germany	Human pathogens
ARMIN	Germany	Humans
BARDa	Germany	Humans
ICU-KISS, OP-KISS, SARI-KISS, MRSA-KISS	Germany	Human pathogens
BSAC	United Kingdom	Humans
BMR-RAISINS	France	Human pathogens

Humans and animals are exposed to AMR from their food habits. Thus, a surveillance system was set up especially for the food that is being consumed by both animals and humans. A thorough monitoring of the food consumed has the possibility of getting exposed to new pathogenic organisms, which could be a probable reason for pandemic, endemic, and epidemic. Again, some of the organizations similar to humans and livestock are formed for keeping the track of rising AMR cases from food habits (Fig. 5) (1).

All the aforementioned the organizations report their AMR data to EFSA but on the other hand, there are some organizations that do not report their AMR data to EFSA (Tab. III). The details of all these organizations contributing to different countries are described further (1).

Reported microorganisms accountable for AMR in Europe

Discussing about the surveillance systems available to control the AMR and antimicrobial usage will not help the population be aware about the pathogenic disease-causing microorganisms accurately. Therefore, it is very important to understand the pathogens responsible for causing AMR also with the antimicrobials that are extensively used. EARS-Net received data from 29 countries for all eight bacterial species under observation (*E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter* species, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Enterococcus faecium*). *E. coli* was the most commonly reported bacterial species (44.2%), followed by *S. aureus* (20.6%), *K. pneumoniae* (11.3%), *E. faecalis* (6.8%), *P. aeruginosa* (5.6%), *S. pneumoniae* (5.3%), *E. faecium* (4.5%), and

Acinetobacter species (4.5%) (Fig. 6) (12). In 2019, more than half of *E. coli* isolates reported to EARS-Net were resistant to at least one antimicrobial group under surveillance, and more than a third of *K. pneumoniae* isolates were resistant to multiple antimicrobial groups. In general, resistance percentages in *K. pneumoniae* were higher than in *E. coli*. While carbapenem resistance was uncommon in *E. coli*, carbapenem resistance rates in *K. pneumoniae* were reported to be more than 10% in numerous countries. Carbapenem resistance was also found in larger percentages in *P. aeruginosa* and *Acinetobacter* species than in *K. pneumoniae.* The increase in the percentage of vancomycin-resistant *E. faecium* isolates in the EU/EEA from 10.5% in 2015 to 18.3% in 2019 is a cause for concern. The results of antimicrobial susceptibility testing (AST) from invasive (blood or cerebrospinal fluid) isolates of eight bacterial species are provided in this article. *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *Acinetobacter* species, *S. pneumoniae*, *S. aureus*, *E. faecalis*, and *E. faecium* are all important bacteria for public health in Europe. In 2019, the estimated national population coverage of data provided to EARS-Net ranged from 11% to 100%, with more than a third of the nations reporting a population coverage of 80% or above (12).

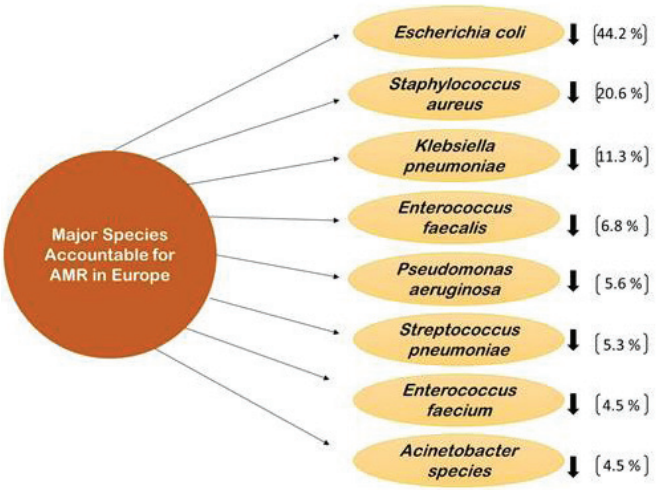

Fig. 6 - Major species responsible for AMR in Europe.

Escherichia coli

In Europe, *E. coli* is a common cause of bloodstream infection. Infections caused by antimicrobial-resistant *E. coli* account for the majority of AMR cases in the EU. The percentages of AMR reported in 2019 were substantially higher than in 2002, underlining the need for more antimicrobial stewardship and infection prevention and control activities. According to the latest data from the European Surveillance of Antimicrobial Consumption Network (ESAC-Net), there are large inter-country variations in the use of broad-spectrum antimicrobials, indicating a need for increased antimicrobial stewardship and the potential for further antimicrobial consumption reductions (13).

Pseudomonas aeruginosa

Although *P. aeruginosa* is naturally resistant to a wide range of antimicrobials, acquired resistance complicates the treatment of *P. aeruginosa* infections. Because *P. aeruginosa* is still one of the most common causes of healthcare-associated illness in Europe, the public health consequences of AMR in *P. aeruginosa* should not be overlooked (12).

Klebsiella pneumoniae

Due to K. pneumoniae's great resistance, the European Union is currently dealing with a significant issue. Although carbapenem resistance has increased more than sevenfold since 2006, it has been more moderate in the last 5 years than in earlier eras. The WHO believes that novel drugs targeting third-generation cephalosporin- and carbapenem-resistant Enterobacterales, such as *K. pneumoniae* and *E. coli*, are urgently needed.

Staphylococcus aureus

Many nations have created and implemented national methicillin-resistant *Staphylococcus aureus* (MRSA) prevention recommendations and guidance documents, emphasizing on enhanced infection prevention and control as well as sensible antibiotic usage. Despite this progress, MRSA remains a significant pathogen in Europe. *S. aureus* is one of the most frequent bacteria that causes bloodstream infections, with a significant morbidity and fatality rate. MRSA surveillance in animals and food is currently voluntary and only carried out in a few countries. This monitoring, however, reveals an ever-changing situation, including the detection of livestock-associated MRSA (LA-MRSA), healthcare-associated MRSA, and community-associated MRSA from companion animals and/or livestock. LA-MRSA has recently received increased attention as a zoonotic risk, particularly for those who work in close proximity to livestock.

Acinetobacter species

Acinetobacter species have the widest inter-country range in resistance percentages of any bacterial species under EARS-Net surveillance. Depending on the reporting country, the percentage of isolates resistant to at least one of the antimicrobial groups under surveillance (fluoroquinolones, aminoglycosides, or carbapenems) ranged from 0% to 95.8% in 2019. Because *Acinetobacter* species is naturally resistant to many antimicrobial agents, acquired resistance complicates treatment of *Acinetobacter* species infections. MDR *Acinetobacter* species are a problem in the healthcare environment because they can survive for long periods of time in the environment and are notoriously difficult to eradicate once established.

Streptococcus pneumoniae

In addition to EARS-Net, the enhanced surveillance program for invasive pneumococcal disease (IPD), which is also supervised by ECDC, collects additional data on IPD cases from reference laboratories across the EU/EEA. The

frequency of resistance to penicillin and erythromycin grew somewhat in all countries that consistently supplied antimicrobial susceptibility data, according to data from this surveillance project (13).

Enterococcus faecalis and Enterococcus faecium

There are grounds for concern that *E. faecium is* fast and constantly increasing in the percentage of vancomycin resistance in the EU. The ECDC study on AMR's health burden estimated that vancomycin-resistant enterococci (VRE) infections and fatalities virtually doubled. A large issue for infection prevention and an important cause for dietary-related illnesses remain high levels of antimicrobial-resistant enterococci. In addition to being difficult to cure infections caused by resistant strains, enterococci are easily spread in medical settings (12).

Overview of the reported microorganisms resistant against the antimicrobials

The above-mentioned subsequent organisms have been tried to be treated with multiple antimicrobials, which has not benefited healthcare. The initial treatment method implemented against these species was applying a single antimicrobial. Later on due to nonobservance of the former antimicrobials, the healthcare sector switched to provide double antimicrobial treatment to the patients for more efficient results but to our surprise, the species were found to be successfully resistant against them. Recently, a combination of antimicrobials is being applied to fight against the resistance that is acquired by the organisms but eventually extensive use of multiple antimicrobials has not only triggered AMR globally but has also shown significant amounts of increase in MDR cases worldwide (Tab. IV) (12-14). The number of deaths attributed to bacterial AMR in 2019 has been estimated at 4.95 million based on previous research and several statistical methods. *E. coli*, *S. aureus*, *K. pneumoniae*, *S. pneumoniae*, *Acinetobacter baumannii*, and *P. aeruginosa* are the top infections for mortality associated to resistance in 2019 (15). Understanding the exact cost of resistance is a difficult task when trying to combat AMR, especially in areas with little surveillance and scant data. High percentages of third-generation cephalosporin and carbapenem resistance in *K. pneumoniae*, as well as high percentages of carbapenem-resistant *Acinetobacter* in various countries are of concern, according to a WHO/ECDC report from the year 2022. Resistance to last-resort antibiotics like vancomycin and members of the carbapenem family is also strongly triggered. There are very few treatment choices available if these antibiotics stop working, and some of them may even be lethal if they don't. The effectiveness of life-saving medical measures like cancer treatment and organ transplantation is likewise threatened by resistance to last-line antibiotics (16). The prevalence of MDR and XDR tuberculosis as well as resistance in gram-negative bacteria are India's biggest worries. The community's Enterobacterales are producing extended-spectrum beta-lactamases at an alarming rate (17).

TABLE IV - Bacterial species and the antimicrobial groups to which they are resistant (18,19)

Bacterial species	Resistant against antimicrobial groups	Geographical location
Escherichia coli	Resistant to beta-lactam antibiotics	India, Europe, USA, and Taiwan
Staphylococcus aureus	MRSA (methicillin-resistant *Staphylococcus aureus*)	India, Europe, and USA
Klebsiella pneumoniae	Third-generation cephalosporin resistance, carbapenem resistance, aminoglycoside resistance, fluoroquinolone resistance	India, Europe
Pseudomonas aeruginosa	Carbapenem resistance, fluoroquinolone resistance, aminoglycoside resistance	India, Europe
Streptococcus pneumoniae	Resistant to macrolides	India, Europe
Acinetobacter species	Carbapenem resistance, aminoglycoside resistance, fluoroquinolone	India, Europe

One Health Approach and training programs regulating AMR

One Health largely emphasizes the collaboration between human and animal health issues today, but also other disciplines should be merged, such as the environmental and social sciences. These One Health training agreements are notably integrated more into veterinary schools than into medical training, as the review of One University Training projects in Western Europe shows. Moreover, multidisciplinary and global health research and training activities must be undertaken, as zoonotic illnesses and AMR do not stop at national borders. Increasing emergent human infectious diseases of zoonotic origin and microorganism resistance to antimicrobial medicinal products have demonstrated that there is a need for cooperation between the human, animal, and environmental sectors. Increasingly, the One Health concept is recognized by politicians and scientists all across the world. In this overview, research and training efforts have been assembled with the aim of focusing on infectious diseases in One Health in Western Europe, particularly in France, Spain, the Netherlands, UK, Germany, and Norway. It can serve as a basis for future projects and partnerships. This summary indicates that One Health in Europe is widely recognized, as most recent educational activities are. In Europe, the One Health strategy in respect to AMR is now being pushed. Many nations have included the One Health strategy in their anti-AMR policy and there have been considerable increases in funding options for AMR research. The number of multidisciplinary national and international research networks on zoonotic diseases and One Health has grown. European institutes have researched on the topic of One Health Approach and many minor projects and training activities are being conducted in the countries of Europe for spreading the awareness of the importance of One Health Approach to fight against AMR and figure out a solution for it. Tables 5 and 6 depict the information related to One Health Approach conducted or training activities performed in European countries (3,20).

TABLE V - European institutes which researched on One Health Approach and also published article on this topic (3)

Country	Research institute	Topic
France	OIE	Advocating the One Health Approach in general and in relation to rabies and Rift Valley fever
Germany	Freie Universitate Berlin	Publication on AMR and zoonoses in the food chain such as *Vibrio* and *Campylobacter*
United Kingdom	The Royal Veterinary College London School of Hygiene and Tropical Medicine University of Cambridge University of Liverpool University of Edinburgh	Research of AMR advocating the One Health concept and research of zoonoses and AMR research on zoonotic diseases such as emerging zoonosis and neglected research on zoonosis such as Japanese encephalitis virus and rabies
Norway	Norwegian Veterinary Institute	EU's Horizon 2020 One Health Project
The Netherlands	Netherland Centre for One Health	Netherland Centre for One Health Project
Spain	Center for Veterinary Health Surveillance (VISAVET)	Project on One Health

TABLE VI - Training activities conducted in the European counties on One Health

Country	Institute	Type of training
Spain	Veterinary School of the Universitat Autonoma de Barcelona	Masters on zoonoses and One Health
The Netherlands	Utrecht University	Honours Program One Health, One Health Track
United Kingdom	Royal Veterinary College (RVC)/ London School of Hygiene & Tropical Medicine (LSHTM), London Royal (Dick) School of Veterinary Sciences, Edinburgh, University of Bristol	Masters in One Health Honours Program population medicine and One Health
France	Nantes-Atlantic National College of Veterinary Medicine, Food Science and Engineering, in partnership with the University of Nantes' Department of Medicine and the University of Angers' Department of Medicine	Master in Animals in training

Various training activities for One Health are conducted in the universities of Europe. Students have also paticipated n One Health in recent years. Some countries have One Health student associations or public health veterinary organizations and networks, such as Holland and the United Kingdom. Extension activities are part of several European research projects. An annual One Health Workshop and One Health for Next Generation project is organized for instance in Anticipating a Global Onset of Novous Epidemics (ANTIGONE) (3,21).

One Health Approach regulating AMR in India

A national plan for controlling AMR has been formed in India. The plan suggests targeting a number of critical components of AMR in both the human sector and the nonhuman one, including agriculture, fishing, animal husbandry, and environment. The strategy addresses all the five main GAP goals and provides a further goal of boosting India's AMR leadership. There are certain priorities that are being maintained to address all the issues. Below are the main objectives of the plan:

enhance awareness of AMR through effective communication, training and education;
enhance surveillance knowledge and evidence;
reduce infection incidence by efficient infection, prevention, and control;
optimize the use of antibiotics in all industries;
promote AMR investment, research, and innovation activities;
enhance India's AMR leadership through international, national and sub-national collaborations on AMR.

The Indian NAP for AMR is a well-designed global plan that incorporates all of the key GAP goals and pledges to address critical antibiotic policy and regulatory problems within the "One Health Approach." India's National Action Plan (NAP) for AMR was released in April 2017 by the Union Ministry of Health and Family Welfare. Implementation was delayed but all parties needed a major push. Failure to achieve separate funding remains the major hurdle to implement NAPs and/or state action plans, not just in India (21,22). The mapping of the surveillance system set up for AMR in India is described in Figure 7.

One Health Approach regulating AMR in Taiwan

Taiwan's Centers for Disease Control (CDC) implemented the National Antimicrobial Stewardship Program; established multi-channel monitoring of MDR organisms, hospital accreditation, and hospital infection control inspections related to antimicrobial stewardship; coordinated infection control interventions; and carried out antimicrobial control interventions in response to the growing threat posed by AMR. Taiwan CDC also proactively establishes relevant guidelines, e-learning materials, manual hygiene, and antimicrobial awareness campaigns to encourage everyone to reduce this condition. Main objectives are:

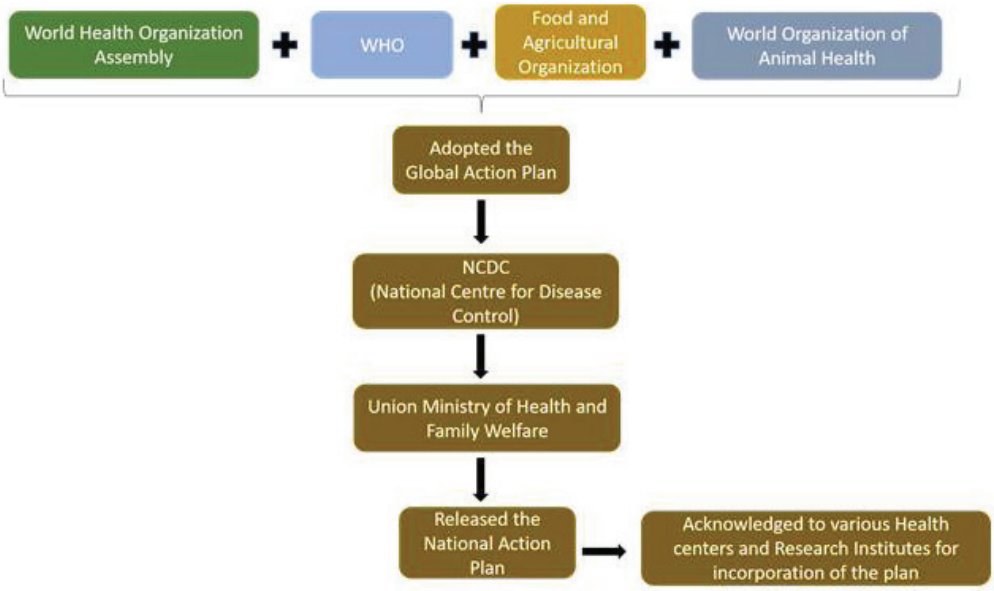

Fig. 7 - Mapping of the surveillance system set up in India for controlling antimicrobial resistance (21,22)

Enhanced surveillance and control of carbapenem-resistant *Enterobacteriaceae* of antimicrobial-resistant pathogens;
Accredit and control hospital infections;
Hospital inspections, the antimicrobial stewardship of all hospitals, are necessary or encouraged;
Offer a number of e-learning courses to improve the understanding and consciousness of health workers on antimicrobial stewardship;
Conduct national public and health awareness-raising campaigns;
Cooperate on the fight against AMR with human and animal health sectors (23).

Thus, a comparative study has highlighted the fact that European countries as well as Asian countries such as India and Taiwan are equally contributing in building various agencies and organizations for combating AMR by implementing various policies and many other surveillance systems, which has actively increased the implementation of One Health Approach. In addition, after European countries, Taiwan has successfully accomplished many of their objectives which have helped the country in fighting against the AMR. Some of the above-mentioned strategies to prevent AMR are broadly explained (Figs. 8 and 9) (24). The mapping of the surveillance system set up for AMR in Taiwan is described further (Fig. 8) (25).

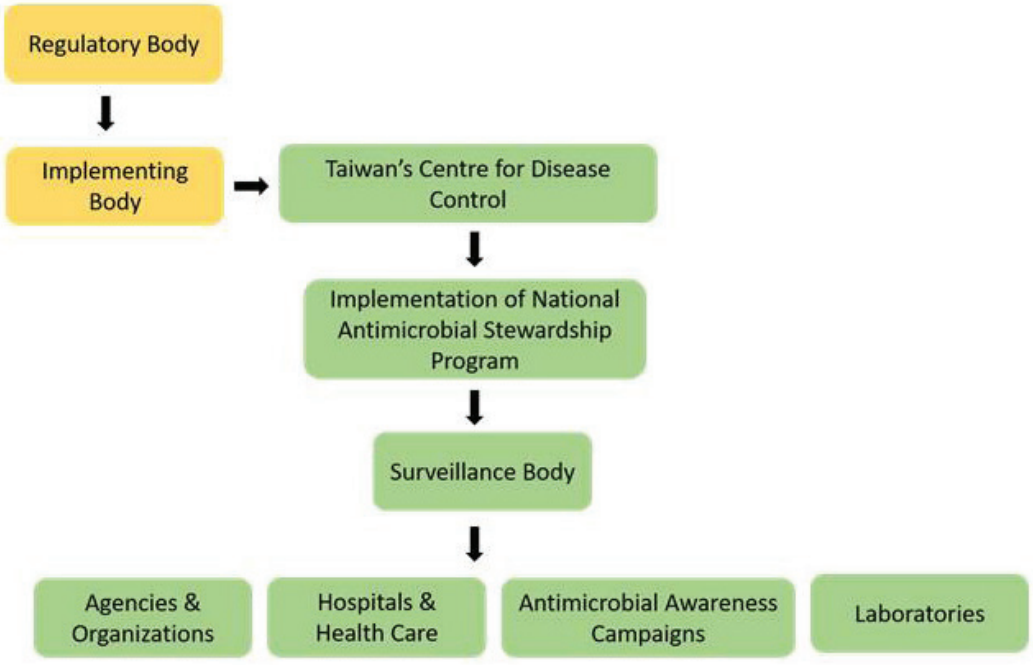

Fig. 8 - Mapping of the surveillance system set up in Taiwan for controlling antimicrobial resistance.

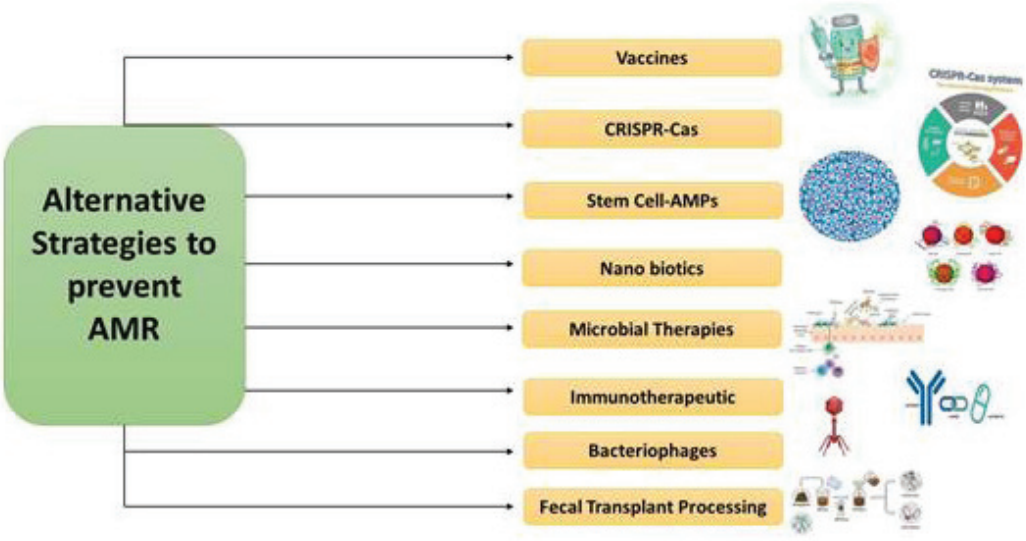

Fig. 9 - Possible alternative strategies to prevent AMR.

Discussion

This article provides an overview of various surveillance systems that are formed only to keep a track on the upcoming situation of AMR and the consumption of antimicrobials by humans as well as animals. The article does not provide all the details required to monitor the AMR issue but firmly allows the readers to get acknowledged with the broad information about the AMR across the six countries of Europe along with a comparative study between Taiwan and India. There are also a lot of debates on the themes of research covered by the term "One Health." Any field of research, including anthropology, sociology, pedagogies, or comparative medicine, that may contribute to human, animal, or ecosystem health can be "One Health." Failure to treat certain infections with currently available antibiotics is a concern for biomedicine. Phage therapy as an alternative therapy against bacterial infections has been extensively investigated. Although various challenges exist, bacteriophages treatment could be used in the future to replace antimicrobial agents with pathogens that are drug-resistant. The technique is now becoming popular as photographs are omnipresent, host-specific and harmless and can be administered with food orally. Antibiotic protein in target bacteria is developed for the delivery of recombinant phages. Topical treatment for open wounds or systemic infections may be performed intravenously. However, phage therapy gives rise to some serious concerns. The main thing about the host bacterium is its fine specificity. This prevents their use for acute infections as empirical therapy. The basis for their investigation was bacteriophageal lysins, the extremely specific peptidoglycan hydrolases, and was also referred to as enzybiotics. Incorporated lysins represent a new therapy form that is powerful and readily available to fight AMR as MDR diseases are becoming increasingly common threats (26,27). With the emerging crisis of AMR, vaccine treatments are seen as a possible solution by health authorities, healthcare providers, and drug developers. The biomolecules that boost the host immune system and give immunity against infectious agents are immunotherapeutic. Developments in the new technology of recombinant vaccines have been essential for reducing the use of different antibiotics for primary and secondary bacterial infection. One of the most important ways to prevent infections continues to be vaccines. Increasing the internal immune system is the advantage of immunotherapeutic agents (24). The CRISPR case is a distinguishing adaptive immune feature in archaea and bacteria, which offers protection against invasively invading bacteriophages and provides a regularly cross-sectional breast repeat. Short bacteriophages or plasmids known as spacers are inserted as a CRISPR array into the bacterial genome; the Cas proteins use guide RNAs from spacers to target the invading nucleic acid with the same sequence. Phagemids from CRISPR-Cas9 could kill certain in vivo bacteria. CRISPR Nanosized Compounds can target the Mec-A gene that is involved in the MRSA effectively (28). Mesenchymal stem cells (MSCs) have been intensively investigated for a variety of chronic diseases over several decades in order to develop a safe and promising therapeutical product. MSCs show promising skills in promoting immunomodulation, tissue cure and excessive inflammation control. Recently, human MSCs have been shown to synthesize antimicrobial peptide (AMP) factors that eradicate bacteria through several mechanisms including an inhibition of bacterial cell wall synthesis. Nonbacterial effects of MSCs (HUCMSCs) on drug-resistant clinical pathogens like *E. coli*, *S. aureus*, and *K. pneumoniae* have been detected (29). A number of names known as fecal microbiota transplantation are known as fecal bacteriotherapy. The Fecal Microbiota Transplantation (FMT) process involves the transplantation, using various routes, including enema, nasogastric, nasoduodenal and colonoscopy, of a fecal suspension of commensal bacteria by a healthy individual donor into the intestinal lumen of the recipients. Clinical trials have found an automotive FMT (aFMT) in antibiotic-disrupted human patients that is better than probiotic therapy and that has induced a fast and almost complete recovery of gastrointestinal microbiota

(30). Nanoparticulate materials may be used for the supply or may contain antimicrobial materials. The nanoparticles and antibiotics based on metal and metal oxides are seen as promising therapeutic candidates for future applications of biomedical science, because they have lower toxicity and improved antibacterial, antiviral, and cancer efficacy. They are of unique size, such as an increased volume-to-surface ratio, making them efficient medicine carriers and improving their solubility, compatibility, and ease of delivery (31).

Advancing genetic engineering and next-generation sequence have enabled scientists to develop future strategies, such as bioengineered probiotics or pharmabiotics, that can become a bacterial infection biotherapy or prophylaxis. An option against antibiotics may be bioengineered probiotics with diverse immunogenic properties. Recombinant probiotics with high competence could provide a greater degree of site specificity than common drug administration regimes to produce drugs, therapeutic proteins, and gene therapy vectors (24).

Conclusion

The regular data collected by different organizations play a vital role in monitoring the status of AMR and antimicrobial usage by humans and livestock. These annual reports have highly helped the government to decide for alternatives and have focused in many training activities to combat the AMR situation globally. AMR prevention is linked to the One Health concept. As antibiotic resistance genes persist on an interface between environment and animal health, an approach is required in all three areas that stresses the concept of "One Approach to Health." Finally, at any stage of life, antibiotic resistance can affect humans or animals. Alternative therapies should be developed to reduce dependency on chemical therapy. As antibiotics become part of modern medicine before many decades, antibiotic effectiveness is decreasing. Clinical research, microbiology, genetics and computer engineering, imaging and modeling experts should work together to develop strategies to deal with this problem and to develop new therapies. Patients with normal infections should avoid unnecessary prescription and over-prescription of antibiotics and patients should be advised to follow good hygiene such as hand washing and adequate infection management measures.

For the purpose of addressing the global epidemic of drug-resistant infections, an accurate evaluation of the existing and future burden of diseases caused by AMR is crucial. To effectively combat an apparent rise in resistance infections, detailed and dynamic information is required. This information enables policymakers and healthcare professionals to put global AMR action plans into place and allocate resources in an effective manner (32). The standard and accessibility of the supplied data affect how accurate AMR results will be. The current state of the global surveillance system is unconnected and unsatisfactory (33). Only 70 nations have reportedly signed up for the WHO's Global Antimicrobial Resistance Surveillance System. The percentage is fewer than half of the AMR rates reported (34). Numerous restrictions, such as a lack of adequate global data, make it difficult to quantify AMR reports. Many countries lack the lab and data management capabilities needed to conduct efficient surveillance. Most significantly, surveillance data or analysis cannot remedy the problem right away; they can only estimate the burden that will arise. Only the creation of brand-new anti-pathogenic chemicals and herbal formulations can fix the problem. The exchange of data is also further constrained by many necessary and strict privacy concerns. More difficulties for the researchers are brought about by the lack of extensive and diverse datasets from various places, which is especially troubling in low-income countries where monitoring is essentially nonexistent.

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ORIGINAL RESEARCH ARTICLE
Focus on Antimicrobial Resistance (AMR)
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Success of 14-day triple and quadruple therapy for the control of *Helicobacter pylori* infections in Kohat district

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ABSTRACT

Introduction: *Helicobacter pylori* is an important medical pathogen present in more than half of the world's population. Various treatment regimen are in use for the eradication of *H. pylori*, but due to the emergence of antibiotic resistance, its management is a big issue for clinicians.

Methods: In this study all suspected cases that had visited District Headquarters Hospital Kohat were considered for screening of *H. pylori* infections. Preliminary information about their age, gender, general health conditions, occupation, etc. was taken for consideration. After recording initial signs and symptoms, samples were considered for *H. pylori* detection using stool antigen test and endoscopy. Fourteen-day proton pump inhibitor base triple and quadruple therapy were administered to each patient.

Results: In total (n = 178), there were high numbers of positivity in patients aged below 30 years (82; 46.06%), most of whom belonged to rural areas.

Conclusion: This study concludes that there were high numbers of positive patients aged below 30 years, and according to this study MEL (Metronidazole + Esomeprazole + Levofloxacin) is the most effective treatment regimen for the eradication of *H. pylori*.

Keywords: Gastric pathology, *Helicobacter pylori*, Intestinal metaplasia, Peptic ulcer

Introduction

Helicobacter pylori is a Gram negative, microaerophilic bacteria that is very common, infecting more than half of the world's population (1). *H. pylori* infection can cause gastric inflammation, peptic ulcer, intestinal metaplasia and can lead to gastric cancer (2,3). Its urease activity, flagella mobility, adhesive proteins and S-shape help to colonize the human stomach and initiate infection (4). Besides these, the *cagA* and *vacA* genes are the major virulence factors in *H. pylori*, responsible for the gastric pathology. The *cagA* gene is responsible for peptic ulcer disease and adenocarcinoma, while the *vagA* gene causes injury to gastric epithelium (5,6). Its invasiveness in the human stomach instigates mucosal and systemic immune responses in the infected host but it has acquired some mechanism that can evade host responses (7).

H. pylori infection rates vary by geographic location, age, ethnicity and socioeconomic status of population (8). It has been documented that infection rates are higher in poor socioeconomic conditions, particularly in developing countries (9). Its transmission takes place in different ways. Most common routes of transmission are iatrogenic, feco-oral and person-to-person contact (10,11). Along with this, contaminated food and water may be a source of infection (12).

Different strategies have been adopted for its treatment. Commonly and most acceptable treatment therapies are triple and quadruple therapy (13,14). In recent era due to the emergence of antibiotic resistance, its success has declined. Antibiotic resistance to *H. pylori* is considered the major cause of the eradication failure (15,16). One of the most enduring debates in the world is the optimal duration of therapy for its eradication (17).

The incidence of *H. pylori* eradication failure and antibiotic resistance has been documented worldwide. In Pakistan, the rate of *H. pylori* is very high due to lack of proper diagnosis of dyspepsia and the over-the-counter use of inappropriate

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doses of proton pump inhibitors (PPIs). In our population of the District of Kohat, the rate of *H. pylori* infection is increasing day by day, and it is yet to find factors that lead to eruption of resistance. Therefore, keeping in view the existence data regarding its resistance and treatment failure of *H. pylori* infection, the study was conducted to determine the incidence of *H. pylori* infection and its response to different regimen of eradication in Kohat district. The aim of the study was to recognize the best treatment regimen for the eradication of *H. pylori.*

Methods

This study was conducted at the District Headquarters Hospital Kohat and Kohat University of Science and Technology, from April 2021 to December 2021. It was approved by the University Ethical Committee and written consent was taken from all patients. Only positive cases were considered in this study for further analysis.

Patients' data collection and risk factors

All the patients were asked about the issues related to gastrointestinal problems and their socioeconomic status, and all data were recorded accordingly. A questionnaire was used as the data collection tool, and it was given after obtaining written informed consent. All the patients were also questioned about their gastric information or complaints like nausea, vomiting, epigastric pain and ballottement.

Sample collection

From all suspected cases stool samples (10-20 g) were collected from all the patients and placed in a clean container. Stool antigen test was used for the detection of *H. pylori* infection. Blood sera were also collected from each patient. Three to 5 mL of blood was taken from each patient and analyzed through an automated hematology analyzer for the complete blood count (18).

Stool antigen test

Stool antigen test was performed for the detection of *H. pylori* infection. About 1 g of collected sample was diluted with the buffer present in the specimen collection tube. Suspending diluted sample for 2 min, 2-3 drops of the diluted specimen were added to the well and then waited for the appearance of faint line to read the result (19).

Treatment regimen for H. pylori eradication

Three different treatment regimen were used for control of *H. pylori* positive cases. These were **MEL** (Metronidazole + Esomeprazole + Levofloxacin), **MRL** (Metronidazole + Rabeprazole + Levofloxacin) and **MELB** (Metronidazole + Esomeprazole + Levofloxacin + Bismuth subcitrate) treatment strategies. In the designated study each patient received PPI base triple and bismuth quadruple treatment. All the patients were randomly assigned to the 14-day treatment comprising of Esomeprazole 40 mg, Rabeprazole 20 mg, Metronidazole 500 mg, Levofloxacin 500 mg and bismuth subcitrate. PPI and bismuth were recommended to be taken before meals while all the antibiotics were taken after meals. Successful eradication was defined as negative result after reconfirmation through the stool antigen test after successful 14-day therapy (20).

Statistical analysis

Qualitative and quantitative variables are shown as percentages. The relationship between hematological parameters of *H. pylori* positive patients *and H. pylori* negative control group was evaluated using confidential interval method by which the values are calculated for each parameter that will fall between intervals.

Results

In total there were 178 patients positive for *H. pylori* infection; there were 38.76% (n = 69) female and 61.23% (n = 109) male positive cases. Among the total, there were high numbers of positivity (n = 82; 46.06%) in patients aged below 30 years, while the number of positive patients in group aged 30-50 years is 38.76% (n = 69) and in that of above 50 years is 15.16% (n = 27). Of the 178 patients, 71.91% (n = 128) were living in the rural area while 28.08% (n = 50) were living in the urban area. According to the above results, high numbers of the patients were living in the rural area and only a small number of patients were living in the urban areas. The demographic information and characteristic of the suspected patients are shown in Table I.

TABLE I - Demographic characteristic of positive patients

Demographic factors	Numbers	Percentage (%)
Age		
Below 30 years	82	46.06
30-50 years	69	38.76
Above 50 years	27	15.16
Gender		
Male	109	61.23
Female	69	38.76
Literate	40	22.47
Illiterate	138	77.52
Place of living		
Rural	128	71.91
Urban	50	28.08

Gastrointestinal symptoms were almost similar in all the patients, but there were high numbers of patients who complained of epigastric pain and recurrent abdominal pain; all the patients showed more than one symptom as mentioned in Table II.

TABLE II - Gastrointestinal symptom of patients

Symptom	Numbers	Percentage (%)
Epigastric pain	153/178	85.95
Recurrent abdominal pain	138/178	77.52
Nausea	141/178	79.21
Vomiting	70/178	39.32
Ballottement	30/178	16.85
Water brush	65/178	36.51

The hematological parameters of *H. pylori* patients included (n = 52) *H. pylori* positive patients whose hematological values were compared with *H. pylori* negative control group (n = 52). It showed that the hemoglobin level was low in the positive patients. Comparing platelets and neutrophile, it was increased in the infected patients as shown in Table III.

TABLE III - Hematological parameter

Parameter	Patients (N = 52)	Negative control (N = 52)
Hemoglobin, g/dL	12.82 ± 1.79	14.37 ± 1.06
Platelet count, %	304,043 ± 8311.8	223,360 ± 3498
Neutrophile	61.32 ± 5.57	59.34 ± 6.91
Eosinophile	2.85 ± 0.89	3.22 ± 1.12
Monocytes	2.41 ± 0.53	4.29 ± 1.16
Lymphocytes	30.36 ± 7.80	27.87 ± 5.97

The above results show the eradication percentage of each given regimen in which 100 patients were given MEL, out of whom 88 (88%) patients showed successful eradication (Fig. 1). MRL was given to 53 patients, of whom 40 patients recovered, exhibiting 75.47% eradication percentage. The bismuth-based quadruple therapy (MELB) was given to 25 patients, of whom 21 (84%) patients showed successful eradication after 14 days of therapy, as shown in Table IV.

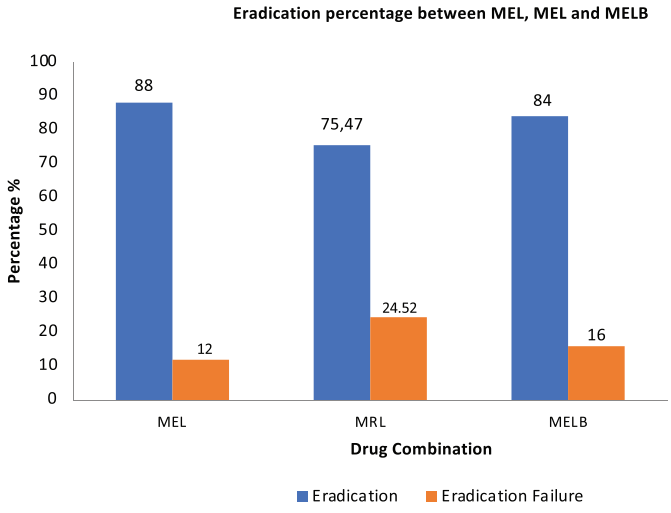

Fig. 1 - Successful eradication and failure percentage between MEL, MRL and MELB drug combinations. MEL = Metronidazole + Esomeprazole + Levofloxacin; MRL = Metronidazole + Rabeprazole + Levofloxacin; MELB = Metronidazole + Esomeprazole + Levofloxacin + Bismuth subcitrate.

TABLE IV - *Helicobacter pylori* eradication percentage between MEL, MRL and MELB regimen

Regimen	Eradication (%)	Eradication failure
MEL	88/100 (88%)	12/100 (12%)
MRL	40/53 (75.47%)	13/53 (24.52%)
MELB	21/25 (84%)	4/25 (16%)

MEL = Metronidazole + Esomeprazole + Levofloxacin; MRL = Metronidazole + Rabeprazole + Levofloxacin; MELB = Metronidazole + Esomeprazole + Levofloxacin + Bismuth subcitrate

Discussion

H. pylori infection is the most common bacterial infection in the world, infecting about half of the world's population. This infection is more common in areas where there are poor hygienic conditions such as use of contaminated food and water. This bacterium is mainly transmitted by feco-oral route from the fecal contaminated water. The oral-oral route is also the leading cause of the infection, as few authentic studies have cultured *H. pylori* from the oral cavity (21); the oral-oral transmission has been examined in the eating of premasticated food, the use of the same spoon by mother and children (22). Various diagnostic methods have been identified for the detection of *H. pylori* but the choice usually depends on the sampling and condition of the patient. In this study, the stool antigen test has been used for the diagnosis of *H. pylori* as stool antigen test is noninvasive and rapid for the detection of *H. pylori* infection (23).

H. pylori analysis included 178 positive patients in this study in which most of the patients were less than 30 years of age. One of the studies in Egypt included 89 asymptomatic young patients, out of whom 78 were positive for *H. pylori* antigen, all aged below 30 years (24). A total of 128 patients out of 178 belonged to the rural areas, exhibiting high percentage because of the poor hygienic conditions of the people living in the rural areas. Most authentic studies have also shown that most of the *H. pylori* positive patients were from rural areas, where the environment was not hygienic. Similarly, one of the studies in Venezuela revealed that *H. pylori* in the rural population was found in 87.2% (34/39) of the patients (25). *H. pylori* is associated with a number of symptoms that are still in debate. In this study, all the patients complained of gastrointestinal symptoms, but there were high number of patients who complained about epigastric pain and recurrent abdominal pain. The main reason behind this is the hyperacidity during peptic ulcer. Primarily, gastrin and oxyntic gland are responsible for the production of more acid during *H. pylori* infection that can lead to epigastric pain (26).

In this study, we found abnormalities in some of the hematological parameters of *H. pylori* positive patients when compared to the control group. The hemoglobin level is quite lower than the control group, while the platelets and neutrophil level

TABLE V - Comparison of *Helicobacter pylori* eradication regimen (MEL, MRL and MELB)

Patient details	Treatment 1 (MEL)		Treatment 2 (MRL)		Treatment 3 (MELB)	
	Success %	Failure %	Success %	Failure%	Success %	Failure%
1. Male (n = 109)	57 (90.47%)	6 (9.53 = 2%)	17 (70.83%)	7 (30.43%)	20 (90.90%)	2 (9.09%)
2. Female (n = 69)	31 (83.78%)	6 (16.21%)	23 (79.31%)	6 (20.68%)	1 (33.3%)	2 (66.6%)
Success of treatment regimen according to age						
3. Below 30 years (n = 82)	45 (84.90%)	8 (15.09%)	12 (63.15%)	7 (36.84%)	8 (80%)	2 (20%)
4. 30-50 years (n = 69)	31 (91.17%)	3 (8.82%)	20 (83.33%)	4 (16.66%)	9 (81.81%)	2 (18.18%)
5. After 50 years (n = 27)	12 (92.30%)	1 (7.69%)	8 (80%)	2 (20%)	4 (100%)	0 (0%)
Resident wise						
6. Rural (n = 128)	60 (86.95%)	9 (13.04%)	32 (76.19%)	10 (23.80%)	14 (82.35%)	3 (17.64%)
7. Urban (n = 50)	28 (90.32%)	3 (9.67%)	8 (72.72%)	3 (27.27%)	7 (87.5%)	1 (12.5%)
Total 178	88/100 (88%)	12/100 (13.72%)	40/53 (75.47%)	13/53 (24.52%)	21/25 (84%)	4/25 (16%)

MEL = Metronidazole + Esomeprazole + Levofloxacin; MRL = Metronidazole + Rabeprazole + Levofloxacin; MELB = Metronidazole + Esomeprazole + Levofloxacin + Bismuth subcitrate

remarkably increased. This may be due to the inflammatory conditions and immune response to *H. pylori.*

Eradication of *H. pylori* needs combinations of drug treatment with adjuvant regimen that increase antibiotic activity and host responses. The duration of therapy also strongly affects the eradication of *H. pylori*. One of the studies in the United States was based on the duration of therapy of *H. pylori*, which shows that RAC (Rabeprazole, Amoxicillin, and Clarithromycin) treatment of 7 days and 10 days had a higher percentage of eradication than the 3-day treatment (27). Similarly, a study in Turkey based on levofloxacin triple therapy in which MEL was given to 92 patients showed 95.5% positive response toward MEL combination. In this study, each patient was given 14 days treatment of PPI base triple and quadruple therapy, which show that MEL had a high percentage of eradication (88/100; 88%) followed by MELB (21/25; 84%). MEL and MRL are the same triple therapy with two different PPIs (Tab. V). The reason for changing one PPI to another was that some of the patients in either group were already using that drug with the same name, so for patient satisfaction, psychologically, we changed the drug. Another reason is some of the studies showed better results with rabeprazole than esomeprazole (28).

Conclusion

This study concludes that there were high numbers of positive patients aged below 30 years in which many patients were from rural area, and according to this study MEL is the most effective treatment regimen for the eradication of *H. pylori.* This study recommends that clinicians may suggest MEL treatment for *H. pylori* positive patients for complete eradication of *H. pylori*.

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Authors' contribution: Syed Fahim Shah and Sohail Aziz Paracha conducted the experiments; Somaid iqbal helped in editing the manuscript and in conducting experimentations; Sadar Zaman, Mudassir Hussain, Hafeez Ullah and Iqbal Muhammad helped in sample collection; Aisha Gul helped in experimentations; and Waheed Ullah designed the project and wrote the manuscript.

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ORIGINAL RESEARCH ARTICLE

Focus on Antimicrobial Resistance (AMR)
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Exploring the inhibitory mechanisms of indazole compounds against SAH/MTAN-mediated quorum sensing utilizing QSAR and docking

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ABSTRACT

The world is under the great threat of antimicrobial resistance (AMR) leading to premature deaths. Microorganisms can produce AMR via quorum sensing mechanisms utilizing *S*-adenosyl homocysteine/methylthioadenosine nucleosidase (SAH/MTAN) biosynthesis. But there is no specific drug developed to date to stop SAH/MTAN, which is a crucial target for the discovery of anti-quorum sensing compound. It has been shown that indazole compounds cause inhibition of SAH/MTAN-mediated quorum sensing, but the biochemical mechanisms have not yet been explored. Therefore, in this original research, an attempt has been made to explore essential structural features of these compounds by quantitative structure-activity relationship (QSAR) and molecular docking of indazole compounds having inhibition of SAH/MTAN-mediated quorum sensing. The validated QSAR predicted five essential descriptors and molecular docking helps to identify the active binding amino acid residues involved in ligand-receptor interactions that are responsible for producing the quorum sensing inhibitory mechanisms of indazole compounds against SAH/MTAN-mediated AMR.

Keywords: Antimicrobial resistance, Indazole compounds, Molecular docking, QSAR, Quorum sensing, SAH/MTAN

Introduction

Microbial invasion and its virulence can cause damage to the host cells and antimicrobial resistance (AMR) via quorum sensing (QS) mechanism, which is responsible for the intercellular communication among microbes. As per British government statistical record, it was recently estimated that by 2050 AMR can engulf 10 million lives each year and cause cumulative losses of US$ 100 trillion to world GDP (1). QS represents microbiome population density and utilizes signal molecules responsible for producing drug resistance (2). These signals are chemical autoinducers (AIs) (3).

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There are two types of AIs such as AI-1 and AI-2. AI-1 is *N*-acyl homoserine lactone (AHL) whereas AI-2 is furanosyl borate diester (4). The *S*-adenosyl methionine (SAM) and *S*-ribosylhomocysteine (SRH) are key components that can be catalyzed by *S*-adenosyl homocysteine/methylthioadenosine nucleosidase (SAH/MTAN) to produce AI-1 and AI-2 signal molecules. SAH/MTAN is an important enzyme and essential for bacterial metabolism (5). Our lab recently reviewed QS biosynthetic pathway-mediated enzymes responsible for antimicrobial drug resistance. SAM is utilized to synthesize SAH, which is being catalyzed by MTAN to produce SRH. SAH/MTAN is responsible for the recycling of adenine and methionine necessary for bacterial DNA and protein synthesis, respectively (6). SAH/MTAN, a very essential component for creating bacterial virulence, could be an attractive target for the disruption of SAM biosynthesis. Schramm developed some MTAN transition state analogues such as methyl (MT), ethyl (EtT), and butyl (But)-substituted immucillin A and DADMe-immucillin A derivatives such as methyl (MT), ethyl (EtT), butyl (BuT), and PhT (Phenyl) substituted having structural resemblance with MTAN. MT-immucillin A and MT-DADMe-immucillin A were found to be slow-onset tight-binding inhibitors of cellular MTAN activity in *Vibrio cholerae* and wild-type *Escherichia coli* (7).

Tedder et al designed and synthesized many SAH/MTAN inhibitors using 6-substituted purine and deaza purines as the core scaffolds. Some of them produced low nM inhibitors with broad-spectrum antimicrobial activity (8). Li et al designed 5-aminoindazole derivatives using structure-guided methods that screened several low-nanomolar inhibitors with broad-spectrum antimicrobial activity to combat SAH/MTAN-mediated QS. X-ray crystal structure of lead compounds cocrystallized with SAH/MTAN obtained from *E. coli* and other pathogenic bacteria revealed the mode of binding of the inhibitor toward the target site. These cocrystal structures could provide structural information for the design of more active congeneric compounds in the series (9).

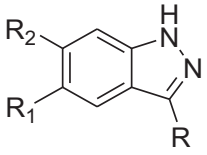
But there is hardly any QSAR utilizing theoretical molecular descriptors and docking studies carried out toward these potential congeners. Therefore, an attempt has been made in the present study to explore the biochemical mechanisms of indazole compounds against SAH/MTAN utilizing QSAR and docking tools.

Experimental methods

Activity data

The biological activity data consist of 40 indazole compounds (Tab. I) designed, synthesized, and tested by Li et al (9). These compounds evaluated the biological inhibitory effect of taking SAH/MTAN enzyme expressed on the full-length *E. coli pfs* gene having high conservation on the bacterial species. The SAH/MTAN is the key target for the production of AHL-mediated AI-1 and SAH-mediated AI-2, which are the building blocks for the synthesis of the QS AIs (9). The enzyme inhibitory activities have been measured in terms of *K*i measuring the affinity of the compound to bind the active cavity of SAH/MTAN. A negative logarithm of these *K*i values (p*K*i) has been done for data reduction and taken as a dependent variable whereas molecular structural descriptor has been computed as independent variables for these compounds.

TABLE I - Biological activity data



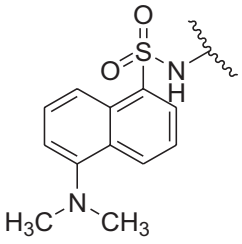
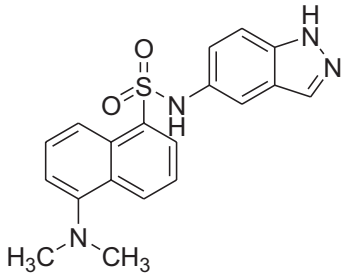
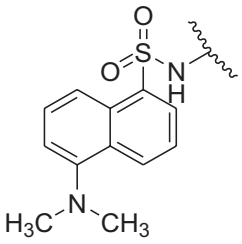
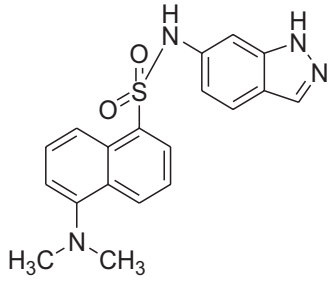
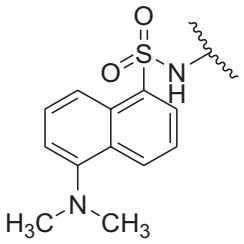
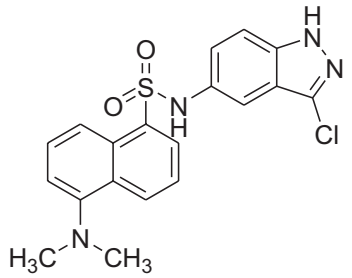
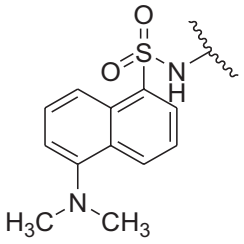
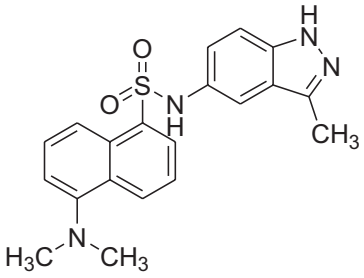
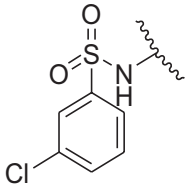
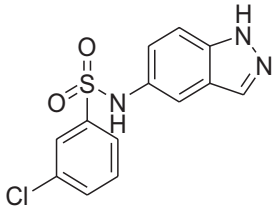
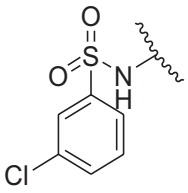
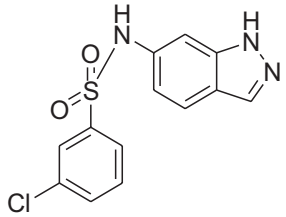
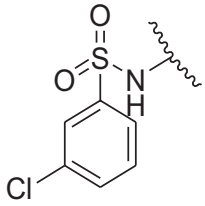
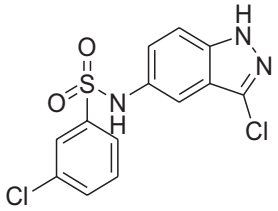
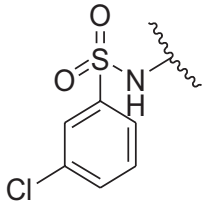
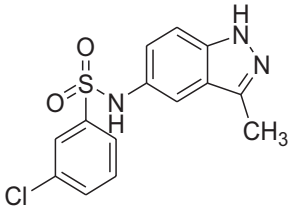
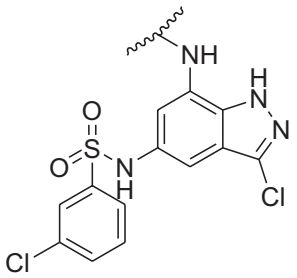
Sl. no.	Substitution points			Structure	pK_i (μm)
	R	R_1	R_2		
*1.	H		H		–0.447
2.	H	H			0

TABLE I - (*Continued*)

*3.	Cl		H		–1.34
4.	CH_3		H		–1.80
5.	H		H		–1.43
6.	H	H			–1.23
7.	Cl		H		–0.71
8.	CH_3		H		–0.602



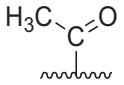
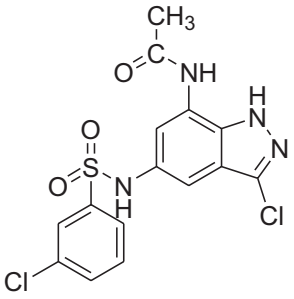
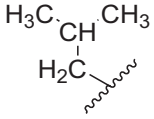
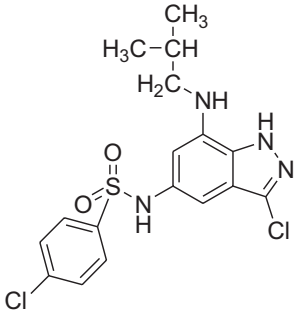
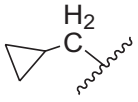
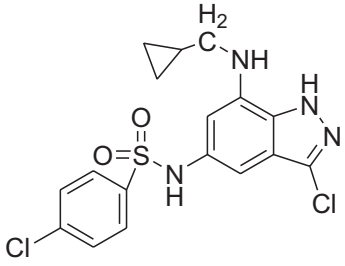
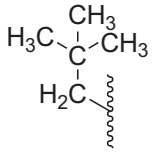
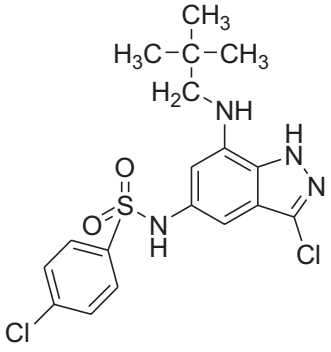
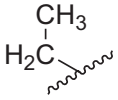
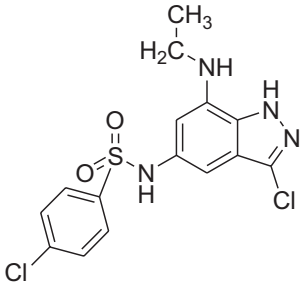
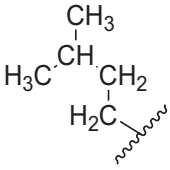
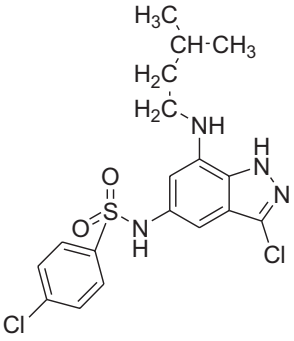
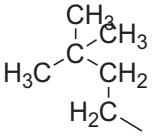
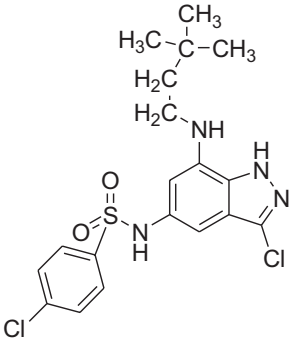
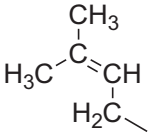
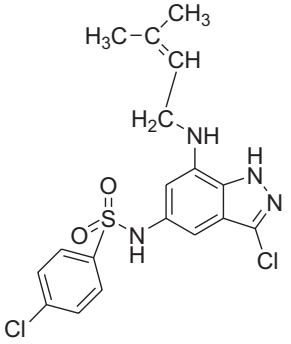
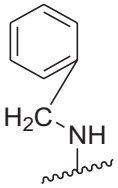
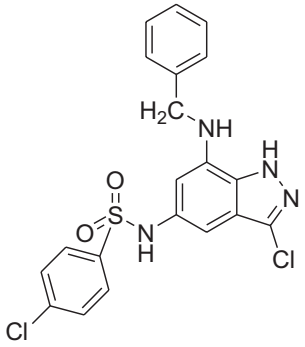
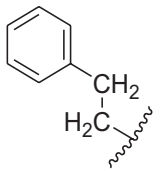
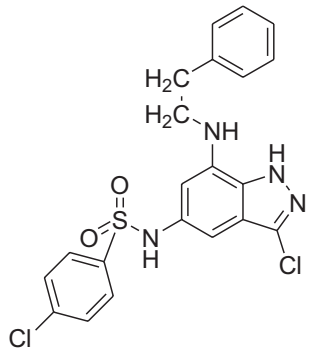
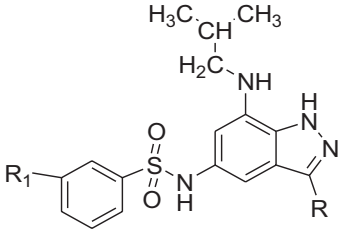
Sl. No.	R1	Structure	p*Ki*
*9.			–1.041
*10.			0.119
11.			0.301
12.			–0.079

TABLE I - (*Continued*)

13.			–0.431
14.			–0.69
15.			–0.812
16.			–0.342
17.			–0.255

18.			–0.361



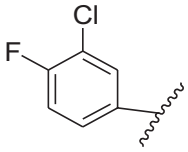
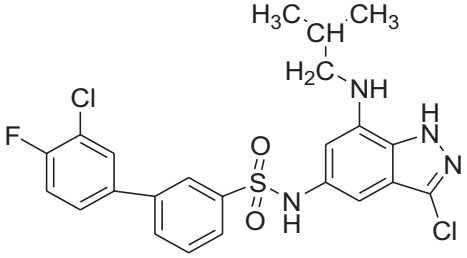
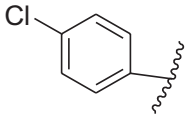
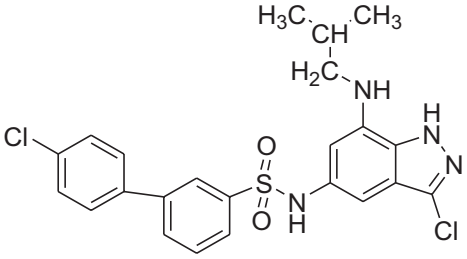
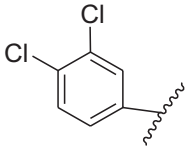
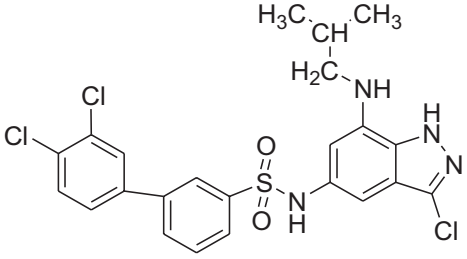
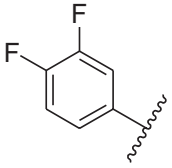
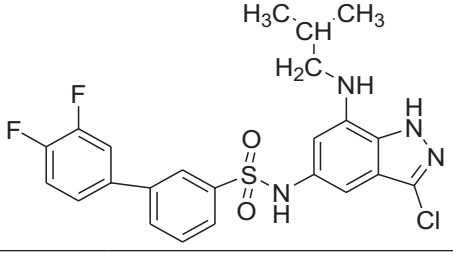
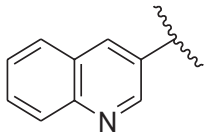
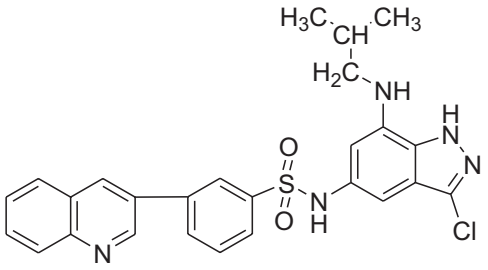
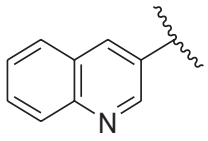
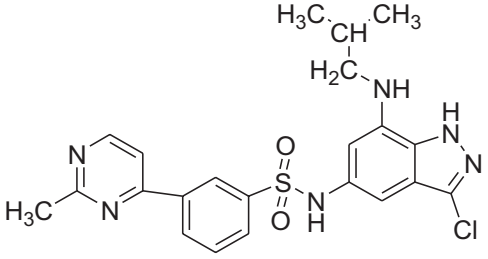
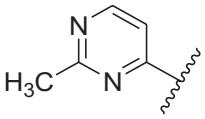
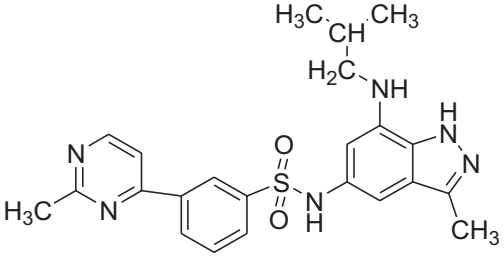
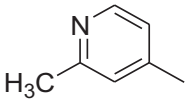
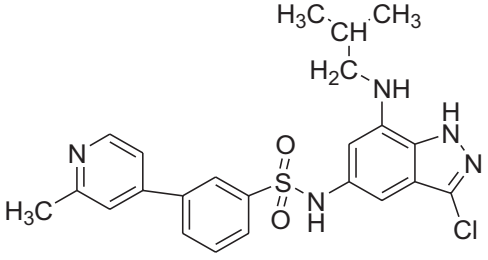
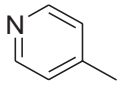
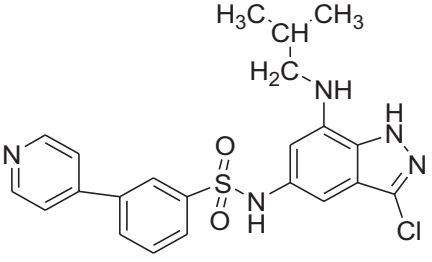
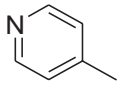
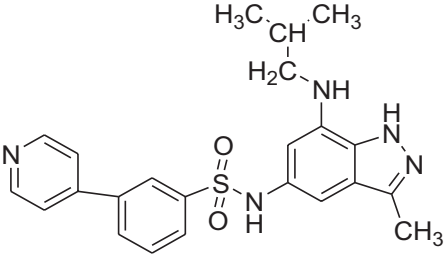
Sl. No.	R	R1	Structure	p*Ki*
*19.	Cl			2.468
20.	Cl			1.903
*21.	Cl			2.795
22.	Cl			2.091

TABLE I - (*Continued*)

*23.	Cl			1.966
*24.	Cl			1.443
25.	CH3			0.718
26.	Cl			1.545
27.	Cl			1.283
28.	CH3			0.619

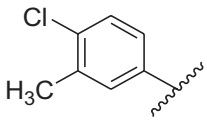
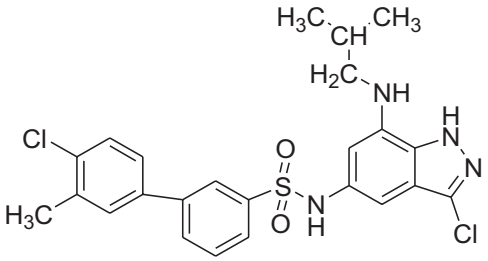
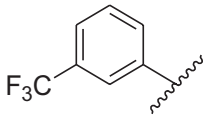
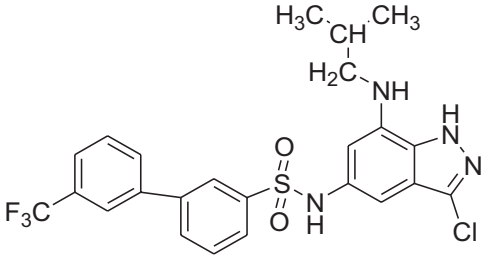
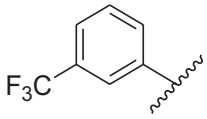
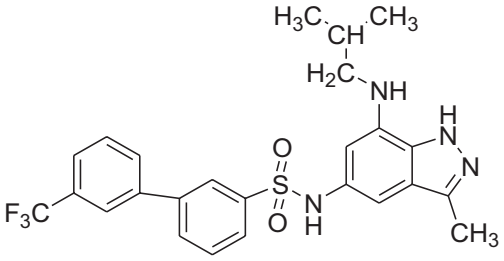
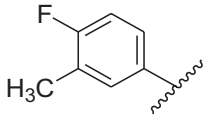
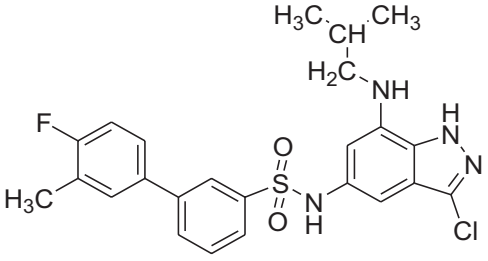
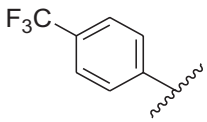
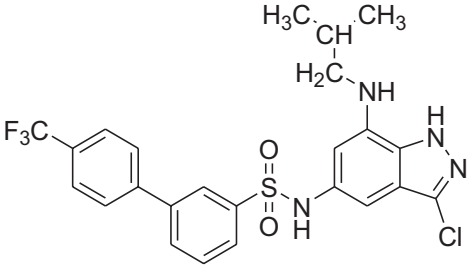
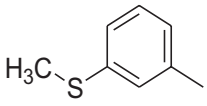
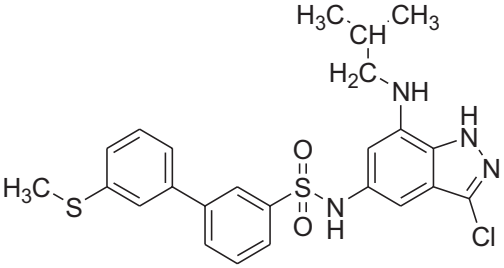
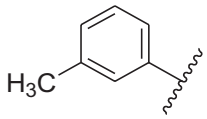
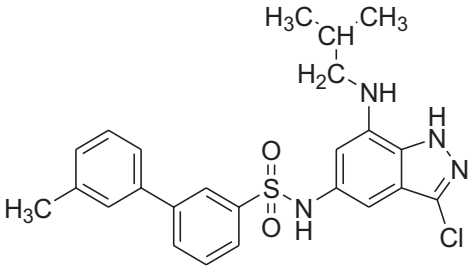
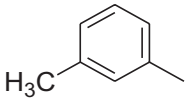
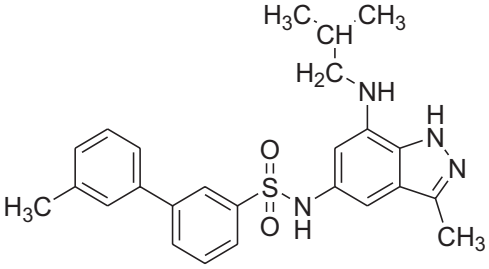
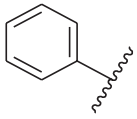
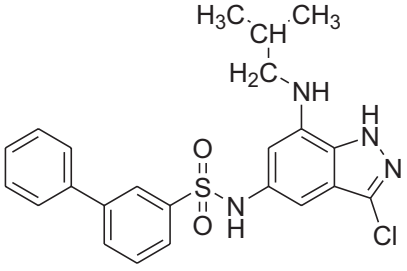
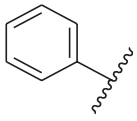
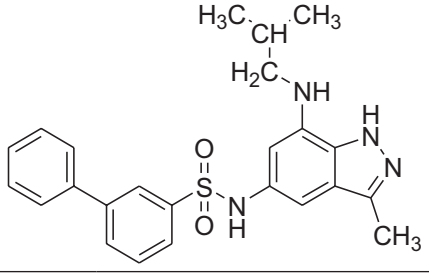
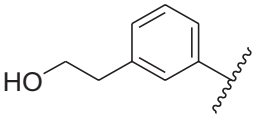
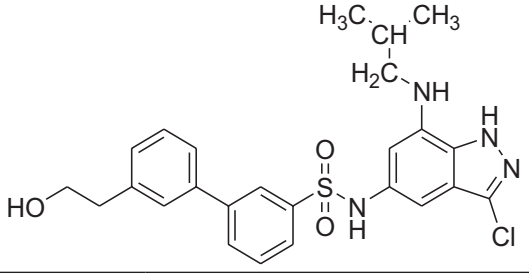
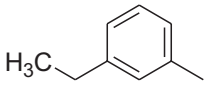
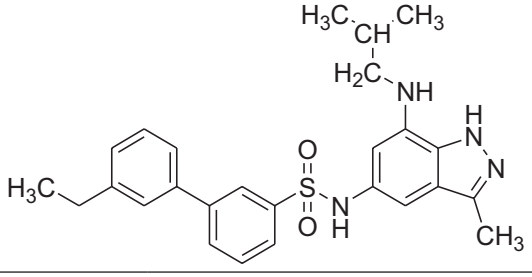
29.	Cl			2.408
*30.	Cl			1.958
*31.	CH3			1.698
*32.	Cl			1.835
33.	Cl			1.886
34.	Cl			1.769

TABLE I - (*Continued*)

35.	Cl			0.443
36.	CH3			1
37.	Cl			0.026
38.	CH3			0.302
39.	Cl			1.602
40.	CH3			0.903

*Test compounds.

Methods of computation

Structure computation and molecular optimization

The two-dimensional structures of 40 indazole compounds were drawn using Chemdraw 8.0 software. These structures were transformed into 3D files, which are minimized using the MM2 force field. The molecular energy minimization is carried out taking the convergence criterion and dielectric constant of 0.01 Kcal/mol and 1.0 respectively (10) utilizing the Chem3D Ultra window (11).

Molecular descriptors computation

All the three-dimensional mol files were incorporated into PaDEL Descriptor Computation software for the computation of structural descriptors. PaDEL is a freeware for the calculation of molecular properties (12). A total number of 1,875 2D and 3D descriptors were calculated and before the model generation, this descriptor data had been reduced to 1,055 (Tab. II). Perfectly constant and highly intercorrelated descriptors were removed taking variance and correlation coefficient cut-off values of 0.0001 and 0.9 using the V-WSP algorithm of NanoBridges software (13). The reduced descriptor data set has been used for the development of QSAR models.

TABLE II - Descriptors used in the current study

ALogP, ALogp2, AMR, apol, naAromAtom, nAtom, nHeavyAtom, nH, nC, nN, nO, nS, nF, nCl, nX, ATS0m, ATS1m, ATS2m, ATS3m, ATS4m, ATS5m, ATS6m, ATS7m, ATS8m, ATS2v, ATS4v, ATS6v, ATS7v, ATS8v, ATS0e, ATS3e, ATS4e, ATS5e, ATS6e, ATS7e, ATS8e, ATS0p, ATS3p, ATS5p, ATS0s, ATS1s, ATS2s, ATS3s, ATS4s, ATS5s, ATS6s, ATS7s, ATS8s, AATS0m, AATS1m, AATS2m, AATS3m, AATS4m, AATS5m, AATS6m, AATS7m, AATS8m, AATS0v, AATS1v, AATS2v, AATS3v, AATS4v, AATS5v, AATS6v, AATS7v, AATS8v, AATS0e, AATS1e, AATS2e, AATS3e, AATS4e, AATS5e, AATS6e, AATS7e, AATS8e, AATS0p, AATS1p, AATS2p, AATS3p, AATS4p, AATS5p, AATS6p, AATS7p, AATS8p, AATS0i, AATS1i, AATS2i, AATS3i, AATS4i, AATS5i, AATS6i, AATS7i, AATS8i, AATS0s, AATS1s, AATS2s, AATS3s, AATS4s, AATS5s, AATS6s, AATS7s, AATS8s, ATSC0c, ATSC1c, ATSC2c, ATSC3c, ATSC4c, ATSC5c, ATSC6c, ATSC7c, ATSC8c, ATSC0m, ATSC1m, ATSC2m, ATSC3m, ATSC4m, ATSC5m, ATSC6m, ATSC7m, ATSC8m, ATSC0v, ATSC1v, ATSC2v, ATSC3v, ATSC4v, ATSC5v, ATSC6v, ATSC7v, ATSC8v, ATSC0e, ATSC1e, ATSC2e, ATSC3e, ATSC4e, ATSC5e, ATSC6e, ATSC7e, ATSC8e, ATSC0p, ATSC1p, ATSC2p, ATSC3p, ATSC4p, ATSC5p, ATSC6p, ATSC7p, ATSC8p, ATSC0i, ATSC1i, ATSC2i, ATSC3i, ATSC4i, ATSC5i, ATSC6i, ATSC7i, ATSC8i, ATSC0s, ATSC1s, ATSC2s, ATSC3s, ATSC4s, ATSC5s, ATSC6s, ATSC7s, ATSC8s, AATSC0m, AATSC1m, AATSC2m, AATSC3m, AATSC4m, AATSC5m, AATSC6m, AATSC7m, AATSC8m, AATSC0v, AATSC1v, AATSC2v, AATSC3v, AATSC4v, AATSC5v, AATSC6v, AATSC7v, AATSC8v, AATSC0e, AATSC2e, AATSC6e, AATSC7e, AATSC0p, AATSC2p, AATSC3p, AATSC4p, AATSC5p, AATSC6p, AATSC7p, AATSC8p, AATSC0i, AATSC1i, AATSC2i, AATSC3i, AATSC4i, AATSC5i, AATSC6i, AATSC7i, AATSC8i, AATSC0s, AATSC1s, AATSC2s, AATSC3s, AATSC4s, AATSC5s, AATSC6s, AATSC7s, AATSC8s, MATS1c, MATS2c, MATS3c, MATS4c, MATS5c, MATS6c, MATS7c, MATS8c, MATS1m, MATS2m, MATS3m, MATS4m, MATS5m, MATS6m, MATS7m, MATS8m, MATS1e, MATS2e, MATS3e, MATS4e, MATS5e, MATS6e, MATS7e, MATS8e, MATS1p, MATS2i, MATS3i, MATS5i, MATS6i, MATS7i, MATS8i, MATS1s, MATS2s, MATS3s, MATS4s, MATS5s, MATS6s, MATS7s, MATS8s, GATS1c, GATS2c, GATS3c, GATS4c, GATS5c, GATS6c, GATS7c, GATS8c, GATS1m, GATS2m, GATS3m, GATS4m, GATS5m, GATS6m, GATS7m, GATS8m, GATS1v, GATS2v, GATS3v, GATS4v, GATS5v, GATS6v, GATS7v, GATS8v, GATS1e, GATS2e, GATS3e, GATS4e , GATS5e, GATS6e, GATS7e, GATS8e, GATS1p, GATS2p, GATS3p, GATS4p, GATS5p, GATS6p, GATS7p, GATS8p, GATS1i, GATS2i, GATS3i, GATS4i, GATS5i, GATS6i, GATS7i, GATS8i, GATS1s, GATS2s, GATS3s, GATS4s, GATS5s, GATS6s, GATS7s, GATS8s, SpAbs_DzZ, SpMAD_DzZ, SM1_DzZ, VE1_DzZ, VE3_DzZ, VR1_DzZ, VR3_DzZ, VR1_Dzm, VR2_Dzm, VR3_Dzm, SM1_Dzv, VE1_Dzv, VE3_Dzv, VR1_Dzv, VR2_Dzv, VR3_Dzv, SM1_Dze, VE1_Dze, VE3_Dze, VR1_Dze, VR3_Dze, SpAbs_Dzp, SpMAD_Dzp, SM1_Dzp, VE1_Dzp, VE3_Dzp, VR1_Dzp, VR3_Dzp, SM1_Dzi, VE1_Dzi, VE3_Dzi, VR1_Dzi, VR3_Dzi, SpAbs_Dzs, SpMAD_Dzs, SM1_Dzs, VE1_Dzs, VE3_Dzs, VR1_Dzs, VR2_Dzs, VR3_Dzs, BCUTw-1l, BCUTw-1h, BCUTc-1l, BCUTc-1h, BCUTp-1l, BCUTp-1h, nBondsS2, nBondsS3, nBondsD, nBondsD2, nBondsM bpol, SpMax2_Bhm, SpMax3_Bhm, SpMax4_Bhm, SpMax5_Bhm, SpMax6_Bhm, SpMax7_Bhm, SpMax8_Bhm, SpMin1_Bhm, SpMin2_Bhm, SpMin3_Bhm, SpMin4_Bhm, SpMin5_Bhm, SpMin6_Bhm, SpMin7_Bhm, SpMin8_Bhm, SpMax1_Bhv, SpMax2_Bhv, SpMax3_Bhv, SpMax4_Bhv, SpMax5_Bhv, SpMax6_Bhv, SpMax7_Bhv, SpMax8_Bhv, SpMin1_Bhv, SpMin2_Bhv, SpMin3_Bhv, SpMin4_Bhv, SpMin5_Bhv, SpMin6_Bhv, SpMin7_Bhv, SpMin8_Bhv, SpMax1_Bhe, SpMax2_Bhe, SpMax3_Bhe, SpMax4_Bhe, SpMax6_Bhe, SpMax7_Bhe, SpMax8_Bhe, SpMin1_Bhe, SpMin2_Bhe, SpMin3_Bhe, SpMin4_Bhe, SpMin5_Bhe, SpMin6_Bhe, SpMin7_Bhe, SpMin8_Bhe, SpMax1_Bhp, SpMax2_Bhp, SpMax3_Bhp, SpMax4_Bhp, SpMax7_Bhp, SpMin1_Bhp, SpMin2_Bhp, SpMin3_Bhp, SpMin4_Bhp, SpMin7_Bhp, SpMin8_Bhp, SpMax2_Bhi, SpMax3_Bhi, SpMax4_Bhi, SpMax5_Bhi, SpMax8_Bhi, SpMin2_Bhi, SpMin3_Bhi, SpMin4_Bhi, SpMin5_Bhi, SpMin7_Bhi, SpMax1_Bhs, SpMax2_Bhs, SpMax3_Bhs, SpMax4_Bhs, SpMax5_Bhs, SpMax6_Bhs, SpMax7_Bhs, SpMax8_Bhs, SpMin1_Bhs, SpMin2_Bhs, SpMin3_Bhs, SpMin4_Bhs, SpMin5_Bhs, SpMin6_Bhs, SpMin7_Bhs, SpMin8_Bhs, C1SP2, C2SP2, C3SP2, C1SP3, C2SP3, C3SP3, C4SP3, SCH-3, SCH-6, SCH-7, VCH-6, VCH-7, SC-3, SC-4, SC-5, SC-6, VC-3, VC-5, SPC-4,SPC-5, SPC-6, VPC-4, VPC-5, VPC-6, SP-2, SP-3, SP-4, SP-6, SP-7, VP-0, VP-2, VP-3, VP-4, VP-5, VP-6, VP-7, AVP-0, AVP-1, AVP-2, Mare, Mi, CrippenLogP, SpMax_Dt, SpMAD_Dt, VE1_Dt, VE3_Dt, VR1_Dt, VR2_Dt, VR3_Dt, ECCEN, nHBd, nHBa, nwHBa, nHBint2, nHBint3, nHBint4, nHBint5, nHBint6, nHBint7, nHBint8, nHBint9, nHBint10, nHsOH, nHssNH, nHdsCH, nHaaCH, nHCsats, nHCsatu, nsCH3, nssCH2, naasC, naaaC, nssssC, naaN, nsssN, SHBd, SHBa, SwHBa, SHBint2, SHBint3, SHBint4, SHBint5, SHBint6, SHBint7, SHBint8, SHBint9, SHBint10, SHssNH, SHaaNH, SHaaCH, SHCsats, SssCH2, SaaCH, SsssCH, SdssC, SaasC, SaaaC, SssNH, SaaNH, SdO, SddssS, SsCl, minHBd, minHBa, minwHBa, minHBint2, minHBint3, minHBint5, minHBint6, minHBint9, minHBint10, minHaaCH, minHCsats, minHCsatu, minHother, minsCH3, minssCH2, minaaCH, minaasC, minaaaC, minssNH, minaaN, mindO, minsF, minsCl, maxHBd, maxHBa, maxwHBa, maxHBint2, maxHBint3, maxHBint5, maxHBint6, maxHBint10, maxHssNH, maxHaaCH, maxHCsats, maxsCH3, maxssCH2, maxaaCH, maxaasC, maxaaaC, maxssssC, maxssNH, maxaaN, maxdO, maxsCl, sumI, meanI, hmax, LipoaffinityIndex, DELS, MAXDN2, DELS2, ETA_Alpha, ETA_Epsilon_1, ETA_Epsilon_2, ETA_Epsilon_4, ETA_Epsilon_5, ETA_dEpsilon_B, ETA_Psi_1, ETA_Shape_P, ETA_Shape_Y, ETA_Shape_X, ETA_Beta, ETA_BetaP, ETA_Beta_s, ETA_BetaP_s, ETA_Beta_ns, ETA_BetaP_ns, ETA_dBeta, ETA_dBetaP, ETA_Beta_ns_d, ETA_BetaP_ns_d, ETA_Eta, ETA_EtaP, ETA_Eta_F, ETA_EtaP_F, ETA_Eta_L, ETA_EtaP_L, ETA_Eta_F_L, ETA_EtaP_F_L, ETA_Eta_B, ETA_Eta_B_RC, FMF, fragC, nHBAcc, nHBAcc2, nHBAcc3, HybRatio, IC0, IC1, IC2, IC3, IC4, IC5, TIC0, TIC1, TIC2, TIC3, SIC0, SIC1, SIC2, SIC3, SIC4, SIC5, CIC0, CIC1, CIC2, CIC3, CIC4, BIC1, BIC2, BIC3, BIC4, BIC5, MIC0, MIC1, MIC2, MIC3, MIC4, ZMIC0, ZMIC1, ZMIC2,

TABLE II - (*Continued*)

ZMIC3, ZMIC4, ZMIC5, Kier1, Kier2, Kier3, nAtomLC, nAtomP, nAtomLAC, MLogP, MDEC-11, MDEC-12, MDEC-13, MDEC-22, MDEC-23, MDEC-24, MDEC-33, MDEC-34, MDEO-11, MDEN-22, MLFER_A, MLFER_BH, MLFER_S, MLFER_E, MLFER_L, MPC2, MPC3, MPC8, MPC10, piPC1, piPC3, piPC5, piPC6, piPC10, R_TpiPCTPC, PetitjeanNumber, nRing, n6Ring, nTRing, nHeteroRing, nF10HeteroRing, nRotB, RotBFrac, nRotBt, RotBtFrac, LipinskiFailures, topoRadius, topoDiameter, GGI1, GGI2, GGI3, GGI4, GGI5, GGI6, GGI7, GGI8 , GGI9, GGI10, JGI1, JGT, VE1_D, VE3_D, VR1_D VR3_D, TopoPSA, MWC3, MWC6, MWC10, SRW7, SRW9, AMW, WTPT-2, WTPT-3, WPATH, XLogP, TDB1u, TDB2u, TDB3u, TDB4u, TDB5u, TDB6u, TDB7u, TDB8u, TDB9u, TDB10u, TDB6m, TDB7m, TDB8m, TDB9m, TDB10m, TDB1v, TDB3v, TDB4v, TDB5v, TDB6v, TDB7v, TDB8v, TDB9v, TDB10v, TDB1e, TDB2e, TDB3e, TDB4e, TDB5e, TDB6e, TDB7e, TDB8e, TDB9e, TDB10e, TDB1p, TDB3p, TDB4p, TDB5p, TDB6p, TDB7p, TDB8p, TDB9p, TDB10p, TDB1i, TDB2i, TDB3i, TDB4i, TDB5i, TDB6i, TDB7i, TDB8i, TDB9i, TDB10i, TDB1s, TDB3s, TDB5s, TDB6s, TDB7s, TDB8s, TDB9s, TDB10s, TDB1r, TDB2r, TDB3r, TDB4r, TDB5r, TDB6r, TDB7r, TDB8r ,TDB9r, TDB10r, PPSA-1, PPSA-2, PPSA-3, PNSA-1, PNSA-2, PNSA-3, DPSA-1, DPSA-2, DPSA-3, FPSA-1, FPSA-2, FNSA-2, FNSA-3, WPSA-1, WPSA-2, WPSA-3, WNSA-1, WNSA-2, WNSA-3, RPCG, RNCG, RPCS, RNCS, THSA, TPSA, RHSA, GRAV-1, GRAVH-3, GRAV-4, LOBMAX, LOBMIN, MOMI-X, MOMI-Y, MOMI-Z, MOMI-XY, MOMI-XZ, MOMI-R, geomRadius, geomDiameter, geomShape, RDF10u, RDF15u, RDF20u, RDF25u, RDF30u, RDF35u, RDF40u, RDF45u, RDF50u, RDF55u, RDF60u, RDF65u, RDF70u, RDF75u, RDF80u, RDF85u, RDF90u, RDF95u, RDF100u, RDF105u, RDF110u, RDF115u, RDF120u, RDF125u, RDF130u, RDF135u, RDF140u, RDF145u, RDF150u, RDF155u, RDF15m, RDF20m, RDF25m, RDF30m, RDF35m, RDF40m, RDF45m, RDF50m, RDF55m, RDF60m, RDF65m, RDF70m, RDF75m, RDF80m, RDF85m, RDF90m, RDF95m, RDF100m, RDF105m, RDF110m, RDF115m, RDF120m, RDF125m, RDF130m, RDF135m, RDF140m, RDF145m, RDF150m, RDF155m, RDF20v, RDF25v, RDF30v, RDF35v, RDF40v, RDF45v, RDF50v, RDF55v, RDF60v, RDF65v, RDF70v, RDF75v, RDF80v, RDF85v, RDF90v, RDF95v, RDF100v, RDF105v, RDF110v, RDF115v, RDF120v, RDF125v, RDF130v, RDF135v, RDF140v, RDF145v, RDF150v, RDF155v, RDF30e, RDF35e, RDF70e, RDF80e, RDF95e, RDF100e, RDF155e, RDF15p, RDF20p, RDF30p, RDF35p, RDF40p, RDF45p, RDF50p,RDF60p, RDF65p, RDF70p, RDF75p, RDF80p, RDF85p, RDF90p, RDF95p, RDF100p, RDF115p, RDF130p, RDF135p, RDF140p, RDF145p, RDF150p, RDF155p, RDF30i, RDF65i, RDF10s, RDF15s, RDF20s, RDF25s, RDF30s, RDF35s, RDF40s, RDF45s, RDF50s, RDF55s, RDF60s, RDF65s, RDF70s, RDF75s, RDF80s, RDF85s, RDF90s, RDF95s, RDF100s, RDF105s, RDF110s, RDF115s, RDF120s, RDF125s, RDF130s, RDF135s, RDF140s, RDF145s, RDF150s, RDF155s, L1u, L2u, L3u, P1u, P2u, E1u, E2u, E3u, Tu, Au, Vu, Du, L1m, L2m, L3m, P1m, P2m, E1m, E2m, E3m, Tm, Am, Vm, Dm, L1v, L2v, L3v, P1v, P2v, E1v, E2v, E3v, Tv, Av, Vv, Dv, P2e, E1e, E2e, E3e, De, L1p, P1p, P2p, E1p, E2p, E3p, Dp, E1i, E2i, Di, E1s, E2s.

QSAR model generation followed by validation

A number of QSAR models have been generated for the deliberated indazole compounds utilizing various sets of a combination of 2D and 3D descriptors using genetic algorithm coupled multiple linear regression (GA-MLR) methods (14) based on the theory of mutation and crossover of the parents' genes to generate the new solutions, taking the most appropriate transformations of the independent variables incorporated in the NanoBridges software (15,16). A population of 100 different random combinations of the structural descriptors is generated taking default parameters as set in the NanoBridges software (17). The impact of these indazole compounds' computed descriptors on SAH/MTAN inhibitory activities has been shown through QSAR model development by considering each parent combination of descriptors for the entire data set using MLR. The entire data set was divided randomly into test and training sets before QSAR modeling. The developed models were validated statistically. The validation parameters are denoted by R^2 (R is the square root of multiple R-square for regression), Q^2 (cross-validated r^2) values for the training set, whereas external validation was carried out by calculating predictive R^2 (R^2_{pred}) and the standard error of estimation (SEE) represents standard deviation measured by the error mean square, which expresses the variation of the residuals or the variation about the regression line (18). Further, the external predictability of the generated QSAR models was scrutinized by calculating modified r^2 (r^2_m), average modified r^2 ($r_m{}^2$), and delta modified r^2 (Δr^2_m) respectively (19). The best training model is composed of 72.5% and the test set consists of 27.5% of the total data. The test compounds have been marked in Table I with an asterisk.

Ligand docking

All optimized ligands were docked into the receptor active cavity using molecular docking, which is a powerful structure-based drug discovery simulation for the identification of ligand-receptor complexes having minimal interaction energy. The energy of interactions between ligand and protein was calculated in terms of the score, which can predict the affinity of the compound toward active binding (20,21). The crystal structure of *E. coli* SAH/MTAN (PDB ID: 1JYS) in complex with adenine cocrystal was selected as a receptor for in silico molecular docking studies (22).

The protein was downloaded and prepared by removing water molecules, and hydrogen atoms in the H-depleted target molecule were added. Grid points were generated surrounding the cocrystallized ligand bound with the active cavity of the target. This cocrystallized molecule is considered a reference to make the binding site for the ligand X-ray group. A flexible docking module was incorporated in ArgusLab 4.0.1, which is a very powerful docking simulation freeware (23-25). In the present docking simulation, the ligand is freely rotated inside the target cavity to generate multiple 150 conformers that can produce many docked complex poses considering grid resolution (angle) of 0.4 degrees as the default value. The term *pose* usually designates the specific set of coordinates of a docked ligand. The coordinates of conformation will change concerning this docked pose. The ligand is docked inside the target of the active site, which is well-kept within the grid box. The best complex pose with minimal interaction energy has been taken into consideration for a better explanation of the mode of interaction between the ligand and active amino acid residues of the receptor protein (26).

Results and discussion

QSAR modeling

In the present study, QSAR modeling of indazole compounds having inhibitory activities against SAH/MTAN-mediated QS has been carried out utilizing a different combination of 2D and 3D structural descriptors. The impact of the different classes of computed descriptors on SAH/MTAN inhibitory activities of these compounds has been discussed by the development of optimal training QSAR model formulated by 72.5% of total data and the remaining 27.5% of total data is used as a test set marked by an asterisk. These model parameters have been expressed as R^2 and Q^2 (cross-validated r^2) values for the training set while the external model validation significance is carried out by calculating predictive R^2 (R^2_{pred}), the SEE and modified r^2 (r^2_m) given in Table III.

It was shown that Equation [1] can produce an explained variance of 71.4% and an internal predicted variance of 55.6% of the observed data. For a predictive QSAR model, the value of R^2 should be more than 0.6 (27). The external model validation parameters such as R^2_{pred}, r^2_m, and SEE are given as 0.151, 0.112, and 0.660, which do not produce significant predictability because R^2_{pred} and r^2_m must be greater than 0.5, while the SEE values should be less than 0.5 to have a significant model (28).

Therefore, outlier analyses have been carried out by testing of applicability domain of the training QSAR model, which determines its acceptance as per the Organization for Economic Cooperation and Development (OECD) incorporated in NanoBridges software (29). The training molecules 14 and 18 were detected as an outlier and again best QSAR model (2) was modeled by deleting the outliers showing the best result on the SAH/MTAN inhibition. The developed QSAR model (2) can explain and predict 85.2% and 78.1% of variances of the SAH/MTAN inhibitory activity of the deliberated compounds. This model can also produce 68.5% external predictability and r^2_m (test) and SEE values of 0.636 and 0.490 respectively. This model is quite acceptable as per statistical validation. The square correlation coefficient between observed activities vs. predicted activities of the test compounds obtained from the correlation plot (Fig. 1) is calculated as 0.751, which suggests good model predictivity.

The model (2) parameters such as RDF55m, E1s, and AATSC7s have a positive impact on aromaticity toward SAH/MTAN inhibition, whereas the decrease in value of AATS1v and ATSC3s of the deliberated indazoles may increase the enzyme inhibition to stop the QS.

Indazole-SAH/MTAN docking

The resultant binding affinity along with details of amino acid residues bound with studied 40 indazole compounds toward inhibition of SAH/MTAN target is shown in Table IV. When ligands of interest are docked inside the defined target cavity of SAH/MTAN, both the conformational changes of ligand and receptor occur to make a number of ligand-receptor complexes. These ligand-receptor complexes represent the

TABLE III - QSAR models

QSAR model-1	
p*K*i = 2.73333 (±0.84046) –3.13346 (±0.84654) CIC2 –0.96779 (±0.23832) nHBint4 +0.00324 (±0.00408) Am +1.06367 (±0.15668) C3SP2 –0.22577 (±0.07554) RDF135m.	
N = 29, R^2 = 0.714, Q^2 = 0.556, R^2_{pred} = 0.151, r^2_m (test) = 0.112, SEE = 0.660	
Parameters	**Physical interpretation**
CIC2	Complementary Information Content index (neighborhood symmetry of 2-order)
nHBint4	Count of E-State descriptors of strength for potential Hydrogen Bonds of path length 4
Am	A total size index/weighted by mass
C3SP2	Doubly bound carbon bound to three other carbons
RDF135m	Radial Distribution Function-135/weighted by mass
QSAR model 2	
p*K*i = –2.66387 (±2.92509) –0.01691 (±0.00928) AATS1v +0.12995 (±0.01611) RDF55m + 9.69053 (±2.60499) E1s –0.0602 (±0.01124) ATSC3s +2.93253 (±0.59661) AATSC7s	
N = 27, R^2 = 0.852, Q^2 = 0.781, R^2_{pred} = 0.685, r^2_m (test) = 0.636, SEE = 0.490	
Parameters	**Physical interpretation**
AATS1v	Averaged Moreau Broto autocorrelation of lag 1 weighted by vdW volume
RDF55m	Radial Distribution Function – 055/weighted by mass
E1s	1st component accessibility directional WHIM index/weighted by I-state
ATSC3s	Centered Moreau Broto autocorrelation of lag 3 weighted by I-state
AATSC7s	Averaged and centered Moreau Broto autocorrelation of lag 7 weighted by intrinsic state

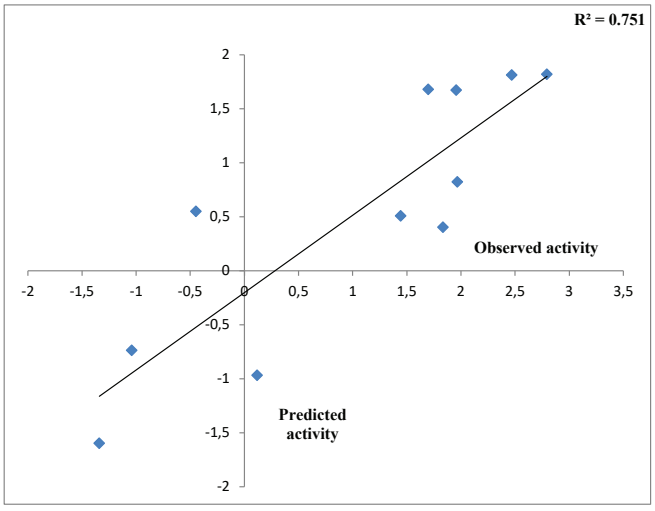


Fig. 1 - Observed versus predicted activity of test molecules after Outlier Analysis (model 2).

conformational rotation of the ligand inside the cavity. The ligand-receptor complex having a minimal dock score may produce maximum binding affinity toward the cavity of the target. The docking of indazole compounds having inhibition of SAH/MTAN resulted in common amino acid interaction inside the SAH/MTAN cavity for all compounds. These common amino acid residues such as ASP197, PHE151, GLU172, ILE152, and MET173 (highlighted in Tab. IV) are essential for ligand-protein interaction and help in unique conformational changing of ligand-receptor interaction complex, which is responsible for producing biological activity. If these amino acids are removed from the cavity then the binding affinity of a compound may be reduced. So these common amino acids play an important role in the suitable binding of the ligand toward the active site.

For a better interpretation of the binding mode of the indazole ligands, molecules are categorized into three highly active, intermediate active, and lower active by considering their biological activities (p*K*i) range as 1.50 to 2.79 µm, 0.44 to 1.4 µm, and <0.4 µm respectively. The highly active compounds such as 20, 22, 23, 26, 30-34, and 39 have more interactions with ALA150, PHE335, and VAL171 along with common amino acids bearing –14.477 to –11.191 kcal/mol dock score. The intermediate active compounds 24, 25, 27, 28, 35, 36, and 40 have –11.186 to –10.367 kcal/mol dock score with a lack of interactions with ALA150 except compound 28 which does not capture both ALA150 and PHE335. The remaining compounds are 1-18, 37, and 38 with lower active range having dock score greater than –10 kcal/mol, and these compounds lack either ALA150 and PHE335 or VAL171 amino acid interactions.

From Table IV, we can say that the binding affinity of compounds depends on a number of residues and minimal dock score involved in the ligand-receptor interactions. The highest active compound 21 shows the following pattern of interactions (Fig. 2).

TABLE IV - Detailed study of indazole analogs-receptor interactions

Comp. no.	Amino acid residues interacting with ligand	pK_i (µm)	Dock score
1.	**ASP197, PHE151, GLU172, ILE152, MET173**, ALA150, ALA199, GLY78, LEU158, VAL 171	–0.447	–10.2544
2.	**ASP197, PHE151, GLU172, ILE152, MET173**, ALA150, ALA77, GLY78, ASN153, SER196	0	–10.134
3.	**ASP197, PHE151, GLU172, ILE152, MET173**, ALA150, GLY78, GLU 174, VAL171, LEU158	–1.34	–10.12
4.	**ASP197, PHE151, GLU172, ILE152, MET173,** ALA150, GLY78, SER76, ALA77	–1.80	–9.4128
5.	**ASP197, PHE151, GLU172, ILE152, MET173**, VAL 332, ALA150	–1.43	–9.4128
6.	**ASP197, PHE151, GLU172, ILE152, MET173**, PRO343, PHE335, VAL332, ALA334, ALA77, GLY78	–1.23	–10.931
7.	**ASP197, PHE151, GLU172, ILE152, MET173**, SER196, SER76, PHE335, VAL332, ALA150	–0.71	–9.49573
8.	**ASP197, PHE151, GLU172, ILE152, MET173**, ASN153, ALA150, VAL171	–0.602	–9.46457
9.	**ASP197, PHE151, GLU172, ILE152, MET173**, GLY78, SER76, ALA77, PHE335, ASN153, ALA150	–1.041	–10.4928
10.	**ASP197, PHE151, GLU172, ILE152, MET173**, HIS98, PRO343, ALA150, VAL171, ALA77, VAL332, GLY78, PHE335	0.119	–9.88002
11.	**ASP197, PHE151, GLU172, ILE152, MET173**, ALA150, VAL332, ALA77, GLY78, ALA334, PHE335	0.301	–10.631
12.	**ASP197, PHE151, GLU172, ILE152, MET173**, ALA199, LEU158, LEU80, GLY78, VAL171, ALA150, PHE335, GLU174	–0.079	–9.89744
13.	**ASP197, PHE151, GLU172, ILE152, MET173**, ALA150, VAL332, GLU174	–0.431	–9.78021
14.	**ASP197, PHE151, GLU172, ILE152, MET173**, GLY78, ALA77, VAL332, PHE335, ALA150	–0.690	–10.6611
15.	**ASP197, PHE151, GLU172, ILE152, MET173**, ALA150, VAL332, SER76	–0.812	–9.69068
16.	**ASP197, PHE151, GLU172, ILE152, MET173**, ALA150, PHE335, VAL332, GLU174	–0.342	–10.2657
17.	**ASP197, PHE151, GLU172, ILE152, MET173**, ALA150, PHE335, GLY78, ALA77, VAL171	–0.255	–11.4409
18.	**ASP197, PHE151, GLU172, ILE152, MET173**, ALA150, SER76, VAL332	–0.361	–10.6145

Comp. no.	Amino acid residues interacting with ligand	pK_i (μm)	Dock score
19.	**ASP197, PHE151, GLU172, ILE152, MET173**, ALA150, PHE335, PHE210, SER196, ALA334, VAL171	2.468	–12.8469
20.	**ASP197, PHE151, GLU172, ILE152, MET173**, ALA150, PHE335, PHE210, SER196, ALA334, VAL171, SER76	1.903	–12.855
21.	**ASP197, PHE151, GLU172, ILE152, MET173**, ALA150, PHE335, VAL171, GLY78, ALA77, ALA199, VAL332, TYR337, PRO343, LEU158	2.795	–14.4772
22.	**ASP197, PHE151, GLU172, ILE152, MET173**, ALA150, PHE335, PHE210, SER196, VAL171, SER76	2.091	–12.2961
23.	**ASP197, PHE151, GLU172, ILE152, MET173**, ALA150, VAL171, GLY78, ALA77, VAL332, PRO343, SER76	1.966	–10.3176
24.	**ASP197, PHE151, GLU172, ILE152, MET173**, ALA150, PHE335, SER196, VAL171, GLY78, ALA77	1.443	–10.367
25.	**ASP197, PHE151, GLU172, ILE152, MET173**, ALA150, PHE335, VAL171, GLY78, ALA77, VAL332, PHE207, ASN153	0.718	–10.367
26.	**ASP197, PHE151, GLU172, ILE152, MET173**, ALA150, PHE335, SER196, VAL171, PHE201, SER76	1.545	–11.1919
27.	**ASP197, PHE151, GLU172, ILE152, MET173**, PHE335, VAL171, GLY78, ALA77, VAL332, PRO343, SER76	1.283	–11.0913
28.	**ASP197, PHE151, GLU172, ILE152, MET173**, VAL171, GLY78, ALA77	0.619	–11.1815
29.	**ASP197, PHE151, GLU172, ILE152, MET173**, PHE335, VAL171, SER76	2.408	–11.6766
30.	**ASP197, PHE151, GLU172, ILE152, MET173**, ALA150, PHE335, PHE210, SER196, VAL171, SER76	1.958	–11.6479
31.	**ASP197, PHE151, GLU172, ILE152, MET173**, ALA150, PHE335, PHE210, LEU211, PRO343, GLY78, VAL332, ILE50	1.698	–11.0191
32.	**ASP197, PHE151, GLU172, ILE152, MET173**, ALA150, PHE335, SER196, VAL171, VAL332	1.835	–12.8532
33.	**PHE151, GLU172, ILE152, MET173**, ALA150, PHE335, PHE210, SER196, LEU158, VAL332, ASN153, GLY154	1.886	–9.56766
34.	**ASP197, PHE151, GLU172, ILE152, MET173**, ALA150, SER196, VAL332, GLY78, SER76, ALA8, MET9, ALA77, GLU174, ILE50	1.769	–12.697
35.	**ASP197, PHE151, GLU172, ILE152, MET173**, ALA150, PHE335, SER196, VAL171, VAL332, SER76, PRO343, PHE207, LEU211	0.443	–12.8692
36.	**ASP197, PHE151, GLU172, ILE152, MET173**, PHE335, VAL171, VAL332, PHE210, GLY78	1	–12.9969
37.	**ASP197, PHE151, GLU172, ILE152, MET173**, PHE335, VAL171, VAL332, SER76, PRO343, ALA77	0.026	–14.2768
38.	**ASP197, PHE151, GLU172, ILE152, MET173**, ALA150, VAL171, VAL332, ASN153, GLY78, ALA77, PRO343, SER76	0.302	–14.2777
39.	**ASP197, PHE151, GLU172, ILE152, MET173**, PHE335, VAL171	1.602	–11.6493
40.	**ASP197, PHE151, GLU172, ILE152, MET173**, PHE335, PHE210, VAL332, GLY78	0.903	–13.6849

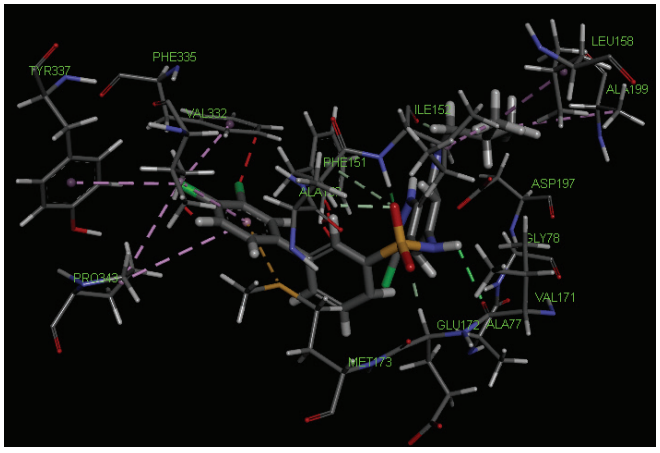


Fig. 2 - Best docking pose of highest active compound no. 21 docked in the cavity of *S*-adenosyl homocysteine/methylthioadenosine nucleosidase.

The amino group of sulfonyl amino linkage interacts with VAL171 and sulfonyl group interacts with GLU172, ILE152, and PHE151 by hydrogen bonding. ILE152 also interacts with isobutyl group by the same bonding. Chlorine atom at position-3 of 3,4-dichlorophenyl interacts with PHE335, TYR337, and PRO343, and VAL332 along with PRO343 interacts with 3,4-dichlorophenyl ring by hydrophobic bonding, whereas MET173 interacts with the sulfur bonding. Some amino acid residues such as ASP197, ALA150, ALA77, and GLY78 are also present at the binding site.

Conclusion

Parameters such as AATS1v, RDF55m, E1s, ATSC3s, and AATSC7s are crucially captured in the training QSAR model responsible for producing inhibition of SAH/MTAN. After molecular docking of indazole compounds, it was found that amino acids ASP197, PHE151, ILE152, GLU172, and MET173

are common for all compounds' modes of interaction and to produce biological activity. Apart from that, the higher active compounds capture ALA150, PHE335, and VAL171 amino acid residues, which are very crucial for the inhibition of the SAH/MTA-mediated QS mechanism. The intermediate and lower active compounds lack any of these interactions. The binding affinity of indazole compounds depends on the number of amino acid residues involved in ligand-receptor interaction, for example, the highest active compound number 21 has 15 amino acid residues, which is more than the other 40 indazole compounds. A higher pIC_{50} value and minimal dock score help to find the greater binding affinity of the compound. This utility helps in lead optimization.

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Authors contribution: SN supervised RK and MK, who equally did the present work. AS contributed to the quorum sensing-mediated antimicrobial resistance.

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EDITORIAL

Focus on Antimicrobial Resistance (AMR)
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AMR research: a perspective from personal experience

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Antimicrobial resistance (AMR) has been well recognized as a global health issue. It is a 'slow pandemic' with huge socioeconomic impact. With around 15 years of experience of working with antibiotic-resistant pathogenic bacteria and anti-pathogenic natural products, I believe I have developed some insight into the issue, and I consider it worth sharing with the readers the variety of experiences I had while working in the AMR field. The views expressed are not claimed to be free from personal beliefs and bias, and are likely to be more relevant to researchers in the Low- and Middle-Income Countries (LMIC). Some of the points discussed are not exclusively relevant to AMR, non-AMR researchers may also correlate their experience with them, and of course, many may disagree with my observations as this is a non-diplomatic personal account!

1. Finding a critical mass of people working on similar aspects of AMR can be a challenge! With AMR getting quite a bit of attention in scientific circles as well as the media, this statement may sound strange, but this is a reality at least for certain geographic area. When you do not have sufficient number of AMR labs in your city/state, it may be difficult to find people with whom you can exchange ideas, resistant strains, protocols, etc. Even finding people with most relevant expertise to act as members of Research Progress Committees/Thesis Evaluation Committees of your PhD students becomes difficult when you do not have many of them in your near vicinity. Though online meetings with experts anywhere in the world are possible, this in my opinion is never as effective as offline face-to-face interactions.
2. Oversimplified perception of AMR research in certain circles of scientific community: While you are presenting before grant review committees, often the committee will comprise a mix of expertise, with few of them not directly involved in wet-lab AMR work. They may perceive AMR research too simplistically as if it is all about determining the minimum inhibitory concentration (MIC)/minimum bactericidal concentration (MBC) of test compounds, that is, screening molecules/natural extracts for bactericidal activity through broth dilution assay. In my personal experience, many of these committee members are not updated with the most recent trends in AMR research, for example, use of alternative model organisms (*Caenorhabditis elegans* and Zebrafish) for the study of host-pathogen interactions, and for screening a library of natural/synthetic compounds for preliminary detection of *in vivo* anti-pathogenic activity. Such model systems also provide an excellent opportunity for detecting anti-virulence activity in test compounds and extracts (1). Recently while presenting a grant proposal involving use of *C. elegans* as a model host, and implementing whole-transcriptome analysis of bacterial pathogen treated with certain anti-pathogenic herbal formulation for novel target identification, I had to face these naughty comments from the grant-reviewing panel:
   A. "Instead of working with *C. elegans*, do experiments directly with higher animals": Despite arguing that use of simpler organisms like *C. elegans* at an early stage can reduce animal sacrifice at later stages, and informing the committee of few hundred papers citing *C. elegans* as a valid and useful model for AMR research, I failed to convince the committee (or the committee failed to understand the value of *C. elegans* in AMR research).
   B. "Since whole genome sequence of most of the pathogenic bacteria is available, we already have sufficient targets known"!!!: While dearth of validated novel antimicrobial targets is widely accepted as one of the major hurdles in discovering new antibiotics (2), one of the committee members *educated* me that full-genome sequencing of pathogens has already solved that problem, and he claimed that we need to focus more on antimicrobial surveillance. I again failed to make the committee understand that surveillance at best tells us which resistant phenotypes are more prevalent in the given geographic area, but it cannot solve the problem of finding novel targets and antibiotics.

The point is that oversimplification of the AMR research reducing it to simple antibacterial growth inhibition assay can do many harms. If people with such exaggerated simplistic perception of AMR research happen to head some academic institute, they may do even more harm by indirectly dissuading brilliant young minds to join AMR labs.

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3. AMR surveillance vs. antibiotic discovery: While many countries have floated their national action plans to combat AMR, most applicant labs are inclined towards AMR surveillance. While AMR surveillance is an important area of investigation, it contributes largely towards characterization of the problem and helps in identifying the priority pathogens, but the solution is arrived at only from discovery and development of novel antimicrobial compounds and formulations. Inherently AMR surveillance projects are guaranteed to generate some visible output because irrespective of where you source the sample from (soil, water, or clinical samples), almost all samples can be shown to contain AMR genes to a more or less extent through metagenomics. On the other hand, labs pursuing identification of novel targets and/or new antibiotics cannot be sure of a visible output as the probability of negative results is quite high. I personally feel that while AMR surveillance should actively be pursued by public health organizations, academic labs and university-industry partnerships should be funded more for antibiotic discovery programmes.
4. Exploring natural products for anti-pathogenic activity can be tricky: While traditional medicine (TM) can offer potent leads against various diseases including antibiotic-resistant infections, the wholistic philosophy of TM largely mismatches with the reductionist approach of modern drug discovery programmes (3). Concepts like hormesis (non-linear dose-response patterns) and 'multiplicity of targets' have to be understood by the researcher dealing with polyherbal formulations or multicomponent plant extracts. Unfortunately, not many people can claim familiarity with both modern science as well as TM. When you present your research to an audience largely comprising either TM practitioners or modern scientists trained in reductionist approach, it is difficult to be appreciated. Most TM formulations do not exert outright bactericidal effect at low concentrations, instead they may exert anti-virulence effect by simultaneously affecting multiple cellular and molecular targets in susceptible pathogens. To identify such polyphasic effect, simple growth inhibition assay can never be sufficient. Such widespread effects can only be grasped through 'omics' approach. Novel antimicrobial mechanisms can be identified through novel types of assays only. Training of the next generation of microbiologists needs to go beyond conventional MIC determination assays.
5. AMR among non-bacterial pathogens needs more attention: While resistant infections caused by bacterial pathogens are responsible for considerable morbidity and mortality, infection burden owing to fungal, protozoan, viral, and helminth infection is also heavy. For a variety of reasons, most AMR research has revolved around pathogenic bacteria, and AMR in non-bacterial pathogens could not get sufficient attention. Since meeting the criteria of 'selective toxicity' is even more difficult with potential new antimicrobials against eukaryotic and viral agent of diseases, building human resource skilled in investigating such non-bacterial pathogens is urgently required. Graduate courses in microbiology should be reframed to put more emphasis on eukaryotic microorganisms in theory as well as lab component of syllabus (4).

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Happy reading to all readers!

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ORIGINAL RESEARCH ARTICLE
Focus on Antimicrobial Resistance (AMR)
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MRSA carriage among healthcare workers in a Vietnamese intensive care unit: a prospective cohort study

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ABSTRACT

Background: Little is known about the magnitude and patterns of methicillin-resistant *Staphylococcus aureus* (MRSA) carriage among intensive care unit (ICU) healthcare workers (HCWs), especially in lower-middle-income countries like Vietnam.

Materials and methods: A prospective cohort study was conducted on HCWs working in the adult ICU of the Hospital for Tropical Diseases in Vietnam between October 28 and December 20, 2019. These HCWs included physicians, nurses, and nursing assistants who were responsible for all essential medical activities and basic patient care. A questionnaire was used to collect participants' information, including age, sex, profession, ICU working time, and underlying diseases. Hand and nasal swabs were collected weekly for 8 consecutive weeks for MRSA screening. Staphylococcal isolates were checked for catalase and coagulase and, for methicillin resistance using cefoxitin disk diffusion, then rechecked on the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Results: Among 55 HCWs, 16 (29.1%) carried MRSA in their noses or hands. MRSA intermittent hand carriage was documented in 2 (3.6%) HCWs. Among 53 HCWs undertaking nasal swabs, 13 (24.5%) were MRSA persistent and 3 (5.6%) were intermittent carriers. The MRSA carriage rate was highest among nursing assistants (50%, 4/8). More HCWs with underlying diseases were found to be MRSA carriers (31.8%, 7/22) compared with those without comorbidities (27.3%, 9/33).

Conclusion: MRSA carriage among HCWs is not rare. The findings highlight an urgent need to review and update the local infection prevention and control measures to prevent MRSA transmission from HCWs to patients.

Keywords: Healthcare workers, Intensive care unit, Methicillin-resistant *Staphylococcus aureus* carriage, Vietnam

Introduction

Colonization is the presence of an organism on or in a host with growth and multiplication to a sufficiently high concentration but does not invade the host's tissues or cause disease (1). The causal relationship between prior colonization and subsequent infections has been well-established in high-resource clinical settings (1). *Staphylococcus aureus* is a well-described organism of the normal human flora, frequently colonizing the nose, pharynx, and skin (1). Most *S. aureus* isolates are sensitive to currently used antibiotics; thus, infections caused by this agent can be effectively treated. However, the emerging methicillin-resistant *S. aureus* (MRSA) has resulted in significant morbidity and mortality in susceptible patients (2). Notably, patients colonized with MRSA are more likely to develop *S. aureus* infection compared to methicillin-sensitive *S. aureus* (MSSA)-colonized or MSSA-non-colonized patients (2). In hospital settings, MRSA colonization among healthcare workers (HCWs) is a huge challenge because they may spread MRSA to their patients as a result of poor infection control practices (3-5). MRSA outbreaks in hospitals are

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epidemiologically associated with MRSA-colonized or MRSA-infected HCWs, especially those who had exfoliative skin conditions, skin infections, or respiratory tract infections (3,5). According to the current clinical practice guidelines, routine screening of HCWs for MRSA is not recommended. However, it is suggested that screening for MRSA can be beneficial in circumstances including (i) if transmission continues in a ward, despite active control measures; or if epidemiological aspects of an outbreak are unusual; or if there is evidence suggesting persistent MRSA carriage among HCWs; and (ii) if new MRSA carriers have been found among patients in a ward, and thus, HCWs with skin lesions should be identified and screened (5).

Currently, little is known about the magnitude of MRSA among HCWs, especially in low-resource settings like Vietnam, likely due to the lack of a routine screening program that is costly. To the best of our knowledge, available data focus on the magnitude of MRSA in those who are not intensive care unit (ICU) staff such as healthy adults, medical conference attendees, and ICU patients (6-8). Despite this, in 2006, an outbreak of severe community-acquired MRSA infections following routine immunization was reported in Ho Chi Minh City (HCMC), Vietnam (9). The outbreak investigation found that HCWs' insufficient hand hygiene during routine injection led to the transmission of MRSA between children. A recent study conducted in the adult ICU at the Hospital for Tropical Diseases (HTD), HCMC, Vietnam, reported that 16.2% of patients acquired MRSA colonization during their ICU stay (10). This study also found that MRSA accounted for more than half (66.7%) of all *S. aureus* infections and suggested the role of HCWs in transmitting MRSA, leading to hospital-acquired infections (10). To strengthen MRSA prevention and control practices in Vietnam and other comparable settings, this study was conducted in the adult ICU of the HTD, which is among the largest local hospitals for infectious diseases in Vietnam, to examine the antimicrobial susceptibility profile of *S. aureus* isolates and the patterns of MRSA carriage among HCWs.

Materials and methods

Study design

A prospective cohort study was conducted in the 20-bed adult ICU of the HTD in Vietnam between October 28 and December 20, 2019. All ICU HCWs were invited to participate in the study. These HCWs included physicians, nurses, and nursing assistants who were responsible for all essential medical activities and basic patient care. A written informed consent was obtained, and the study was approved by HTD's ethics committee (approval number 24/HDDD) and the University of New South Wales (approval number HC190730).

The adult ICU includes four pods in which there are five to seven patients in each pod. The HCW roster is divided into four different staff shifts (i.e., eight nurses and nursing assistants and three doctors per shift). In a normal working day, three shifts are on duty by turns (i.e., 8 hours per shift), and one shift is off. Within a shift, all medical staff are further split into four small groups to care for patients in the four corresponding ICU pods. According to the local policy, staff within each shift are rotated every 8 weeks, so that all staff have an equal chance to work across the ICU and share the same responsibilities. According to previous studies conducted at the same ICU, weekly swabs are sufficient to detect potential bacterial colonization among study participants (6,10). Therefore, to ensure that all staff's potential bacterial colonizations were captured when working in different ICU pods, swabs were taken weekly during the 8-week study period. A questionnaire was used to collect participants' characteristics, including age, sex, profession (medical doctor, nurse, and nursing assistant), ICU working time, and underlying diseases (sinusitis, skin diseases, diabetes, and others). The questionnaire was developed based on the available literature regarding the sources and vectors of MRSA as well as risk factors for MRSA carriage in healthcare settings (3,11).

Swabbing procedure

Hand and nasal swab samples were taken weekly using the Sterile Transport Swab (Jiangsu Kangjian Medical Apparatus Co., Ltd., China) for *S. aureus* screening. The swabbing procedure was based on the HTD's infection control guidelines. A qualified study nurse performed hand and nasal swabs of participants at the start of each work shift. The study nurse put on gloves and a surgical mask to prevent contamination of the samples. For hand swabbing, HCWs washed their hands and let them dry according to the World Health Organization guidelines on hand hygiene in healthcare (12). Then, a moist and sterile swab was rotated across the palm and back of both hands as well as fingertips, fingernails, and between fingers. For nasal swabbing, another swab was inserted about 2 cm into the anterior nares of both nostrils of HCWs and rotated a few times against the nasal mucosa until it was covered in secretions.

Microbiological methods

Blood agar (bioMérieux) was used to isolate *S. aureus* from swabbing samples. *S. aureus* was confirmed based on its morphology and hemolytic activity. When grown in culture, several staphylococcal colonies could develop. However, due to resource constraints, a maximum of two staphylococcal colonies were included in this study in case several staphylococcal colonies were isolated. The selection of these two colonies was based on their levels of predominance (13). Staphylococcal colonies were checked for catalase and coagulase, and for methicillin resistance using cefoxitin disk diffusion (14), then rechecked on the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDITOF, Bruker Daltonics, United States). Catalase assay was used to detect the catalase enzyme that releases oxygen from hydrogen peroxide (H_2O_2), which helps differentiate between staphylococci (catalase positive) and streptococci (catalase negative). To detect the presence of catalase in bacteria in the culture, several drops of 3% H_2O_2 were added to the culture. The rapid formation of bubbles indicates catalase-positive culture. Coagulase assay was used to detect the coagulase enzyme that converts fibrinogen (soluble)

to fibrin (insoluble), which helps differentiate between *S. aureus* (coagulase positive) and other staphylococci (coagulase negative). The coagulase assay used in this study was the coagulase slide test to detect the bound coagulase of *S. aureus*. A suspension of the isolated colony is emulsified on a slide with a drop of rabbit plasma. Clumping of the organisms indicates the presence of bound coagulase. The principle of MALDITOF is that bacterial cells are ionized into charged molecules, then their mass-to-charge ratio is measured and analyzed by a mass spectrometer. Every bacterial genus/species has a distinctive protein spectrum that can be compared with a database software so that the nearest organism can be identified (15,16). The process is rapid, sensitive, and economical in terms of both labor and costs involved. No control MRSA strain was used in the study. Testing for susceptibility to eight most commonly used antibiotics including penicillin, oxacillin, vancomycin, erythromycin, ciprofloxacin, sulfamethoxazole-trimethoprim, rifampin, and clindamycin was performed using the Kirby/Bauer disk diffusion method and the 2015 Clinical and Laboratory Standards Institute (CLSI) guidelines. These microbiological methods have been validated elsewhere (6,10).

S. aureus carriage patterns

S. aureus carriage reported in our study included MSSA and MRSA carriage. *S. aureus* carriage was classified into three different categories: persistent carriage, intermittent carriage, and noncarriage. Given each study participant was swabbed weekly for 8 consecutive weeks, persistent carriage was defined as ≥2 positive consecutive cultures of either hand or nasal swabs with *S. aureus*. Intermittent carriage referred to the isolation of *S. aureus* in less than two positive consecutive cultures of either hand or nasal swabs. All negative cultures of hand and nasal swabs were categorized as noncarriage.

Statistical analysis

Descriptive analyses were performed and consisted of frequency and percentage (95% confidence interval [CI]) for categorical data, and median (interquartile range [IQR]) for continuous data using R statistical software. Chi-squared test was used to examine the significant relationship between categorical variables. The comparison of continuous variables was performed using Mann-Whitney U-test. Alpha was set at 5% level.

Results

Study participants' characteristics

Most HCWs working in the adult ICU (92%, 55/60) participated in the study, including all 11 doctors (100%), 36 nurses (87.8%, 36/41), and all 8 nursing assistants (100%) (Tab. I). Two-thirds of participants were female (67.3%, 37/55). Most of the participants (87.3%, 48/55) were younger than 41 years, and 45.5% (25/55) of them have been working for more than 5 years in the adult ICU. Gastritis and sinusitis (29.2%, 16/55) were the most common underlying diseases, but none of those having these diseases experienced any acute symptoms during the study period.

TABLE I - Baseline characteristics of 55 healthcare workers working in the adult intensive care unit (ICU)

Characteristics	Summary statistics*
Age (years)	32 (27-36)
Age groups	
≤30	23 (41.8)
31-40	25 (45.5)
≥41	7 (12.7)
Male	18 (32.7)
Profession	
Medical doctors	11 (20)
Nurses	36 (65.5)
Nursing assistants	8 (14.5)
Working time in ICU (years)	5 (1.2-11)
Period of time working in ICU	
<1	11 (20)
1-5	19 (34.6)
6-10	9 (16.4)
11-15	12 (21.8)
>15	4 (7.2)
Healthcare workers' underlying diseases	
Gastritis	11 (20)
Sinusitis	5 (9.2)
Diabetes mellitus	2 (3.6)
Gastroesophageal reflux disease	1 (1.8)
Chronic colitis	1 (1.8)
Rheumatoid arthritis	1 (1.8)
Thyroid cancer	1 (1.8)
No underlying diseases	33 (60)

*Median (interquartile range) for continuous variables and *n* (%) for categorical variables.

Antimicrobial susceptibility of S. aureus isolates

A total of 128 *S. aureus* isolates were cultured and included 123 (96.1%) from nasal swabs and 5 (3.9%) from hand swabs. MRSA accounted for 71.1% (91/128) of all *S. aureus* isolates (Fig. 1), with almost all (97.8%, 89/91) cultured from nasal samples. Almost three-quarters of 128 isolates were resistant to erythromycin (71.9%, 92/128) and clindamycin (70.3%, 90/128), while one-third (34.4%, 44/128) were resistant to ciprofloxacin. Vancomycin-resistant *S. aureus* was not detected. All *S. aureus* strains were fully sensitive to sulfamethoxazole-trimethoprim and rifampicin.

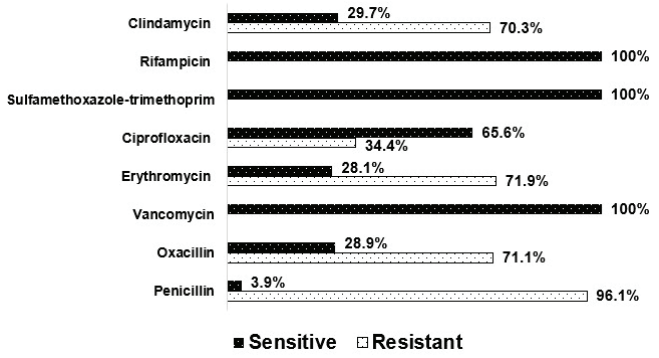

Fig. 1 - Antimicrobial susceptibility of *Staphylococcus aureus* isolates from 55 healthcare workers working in the adult intensive care unit.

MRSA carriage patterns

All 55 participants agreed to have hand swabs taken, while 2 refused to undertake nasal swabs. The proportion of MRSA hand carriers increased with hand swabbing frequency from 0% in the first 4 weeks to 3.6% (2/55) in the last 4 weeks of the study period. Similarly, MRSA nasal carriers were detected in 18.9% (10/53) of study participants in the first week and increased to 22.6% (12/53) at the end of the study period.

A total of 20 (36.4% of 55) HCWs carried *S. aureus* (MSSA and MRSA) in their noses or hands. MRSA carriage accounted for 29.1% (16/55). All participants who tested positive for *S. aureus* were asymptomatic. Persistent carriage was not detected from hand swabs. *S. aureus* intermittent carriers were documented in 7.2% (4/55) of participants, of whom 2 were MRSA intermittent carriers (Tab. II). For nasal swabbing, 34% (18/53) of participants were found to be *S. aureus* persistent carriers, of whom 13 (24.5% of 53) were MRSA persistent carriers. Five participants were *S. aureus* intermittent carriers, and 3 of them were MRSA intermittent carriers, all from nasal swabs. Additionally, of the 18 HCWs with *S. aureus* persistent nasal carriage, 3 were found to be *S. aureus* intermittent hand carriers, and *S. aureus* isolates recovered from their nasal and hand swab cultures shared the same antimicrobial susceptibility profile.

TABLE II - Patterns of *S. aureus* carriage among 55 healthcare workers working in the adult ICU

Swab taken	*S. aureus* carriage categories		n (%)	95% CI
Hand swab (n = 55)	Persistent carriage	MRSA or MSSA	0	
	Intermittent carriage	MRSA	2 (3.6)	1-12.3
		MSSA	2 (3.6)	1-12.3
	Noncarriage		51 (92.8)	82.7-97.1
Nasal swab (n = 53)	Persistent carriage	MRSA	13 (24.5)	14.9-37.6
		MSSA	5 (9.5)	4.1-20.3
	Intermittent carriage	MRSA	3 (5.6)	1.9-15.4
		MSSA	2 (3.8)	1-12.8
	Noncarriage		30 (56.6)	43.3-69.1
Total (n = 55)	MRSA carriage		16 (29.1)	18.8-42.1
	MSSA carriage		4 (7.3)	2.9-17.3
	Noncarriage		35 (63.6)	50.4-75.1

MRSA = methicillin-resistant *Staphylococcus aureus*; MSSA = methicillin-sensitive *Staphylococcus aureus*.

Risk factors of MRSA carriage

The proportion of nursing assistants (50%, 4/8) who were MRSA carriers was higher than that of doctors (36.4%, 4/11) and nurses (22.2%, 8/36), but these differences were not statistically significant ($p > 0.05$). Similarly, more HCWs with underlying diseases were found to be MRSA carriers compared with those without comorbidities (31.8%, 7/22 vs. 27.3%, 9/33), although this difference was not statistically significant ($p > 0.05$). There was no statistically significant association between MRSA hand and nasal carriage and age, sex, profession, and underlying diseases ($p > 0.05$) (Tab. III).

TABLE III - Risk factors of MRSA carriage among 55 healthcare workers working in the adult ICU

Characteristics†	MRSA (+) (n = 16)	MRSA (–) (n = 39)	p-Value*
Age (years)	30.5 (26-36)	32 (29.5-35.5)	0.41
Working time in ICU (years)	2.25 (0.67-7.13)	6 (2-11)	0.16
Male	3 (18.8)	15 (38.5)	0.16
Underlying diseases‡	7 (43.8)	15 (38.5)	0.72
Profession			
Medical doctors	4 (36.4)	7 (63.6)	0.25
Nurses	8 (22.2)	28 (77.8)	
Nursing assistants	4 (50)	4 (50)	

ICU = intensive care unit; IQR = interquartile range; MRSA = methicillin-resistant *Staphylococcus aureus*.
*Mann-Whitney U-test for continuous variables and chi-squared test for categorical variables.
†Median (IQR) for continuous variables and n (%) for categorical variables.
‡Gastritis, sinusitis, diabetes mellitus, gastroesophageal reflux disease, chronic colitis, rheumatoid arthritis, and thyroid cancer.

Discussion

The prevalence of MRSA carriage among our ICU HCWs was 29.1% (16/55, 95% CI: 18.8-42.1%). Specifically, 24.5% of HCWs were MRSA persistent nasal carriers. In Vietnam, previous studies found that the prevalence of MRSA colonization among healthy adults and ICU patients was 4.2% (28/662, 95% CI: 2.9-6.1%) (8) and 8.6% (72/838, 95% CI: 6.9-10.7%) (6), respectively, which is lower than the rate found in HCWs. Our findings are also in line with previous studies in

Europe and the United States, which documented a higher rate of MRSA nasal colonization in HCWs compared with non-healthcare professionals (17,18). This can be explained by the increased exposure of HCWs to patients, hospital environment, and potential MRSA-contaminated objects including medical devices compared to non-healthcare individuals. Indeed, a multicenter study conducted in Nepal, which is a comparable country, found that MRSA can be isolated from the commonly used medical devices in ICU settings such as stethoscopes, pulse oximeters, ventilators, and defibrillators (19). The biofilm-forming property enables *S. aureus* to survive longer on the surfaces of these instruments, which are potential sources of nosocomial infections (19). A global review found that the estimated average MRSA carriage rate in HCWs was 5% (3). In detail, the rate of MRSA carriage among HCWs in Europe was 3.4% (95% CI: 3.1%-3.7%), the United States 4.2% (95% CI: 3.8%-4.7%), Africa 15.5% (95% CI: 13%-18.4%), the Middle East 6.1% (95% CI: 5.2%-7.2%), Australia and New Zealand 9.7% (95% CI: 8.5%-11.1%), and Asia excluding Vietnam 9.8% (95% CI: 8.4%-11.4%) (3). The prevalence of MRSA carriage in our HCWs was substantially higher than these reports and data from MRSA-endemic settings, where the prevalence in HCWs was 8.1% (95% CI: 7.4%-8.9%) (3). Globally, information on the burden of MRSA among ICU HCWs is scarce. Only 4.7% (95% CI: 4%-5.4%) of ICU staff from other regions were found to be MRSA carriers (3), which was lower compared with our data. Our findings further confirm the burden of MRSA in ICU settings in Vietnam, supported by high rates of MRSA colonization and infection in Vietnamese ICU patients (6,10). In light of this, Vietnam should be listed as a country with hyperendemic MRSA. Our study also indicates that MRSA carriage in HCWs in Vietnam is an urgent health problem that needs to be addressed, though this warrants further large-scale studies.

Nurses are associated with a higher risk of MRSA colonization. A meta-analysis showed that the risk of MRSA colonization among nurses was 2.6 (95% CI: 1.8-3.7) times higher than other healthcare staff including doctors and nursing assistants (17). This is probably due to the more frequent and close contact of nurses with patients compared with other healthcare staff. However, we noticed a higher proportion of nursing assistants who were colonized with MRSA compared with doctors and nurses, although this difference was not statistically significant. Suboptimal infection control practices have been indicated as a risk factor for MRSA carriage in HCWs (20). Despite the availability of local infection control guidelines, recent studies found that infection control compliance among HCWs is suboptimal in Vietnam (20,21). Especially, nursing assistants are found to have a lower infection control knowledge compared with other healthcare staff (22). This may explain the high proportion of MRSA carriage among our nursing assistants. To address this, infection control education programs should be tailored to meet the nursing assistants' level of knowledge, and an audit program to measure infection control practice, especially hand hygiene, should be reinforced. Indeed, tailored infection control programs have been proven to be effective in comparable developing countries (23).

We also found that HCWs with gastritis or sinusitis had a higher prevalence of MRSA carriage compared with those without these comorbidities, although this association was not statistically significant. It is documented that HCWs with sinusitis are at an increased risk of transmitting MRSA in hospital settings and have been implicated in several MRSA outbreaks (3,24). Presently, the MRSA universal screening policy is controversial because of the lack of robust evidence for the effectiveness of such a costly measure. Recommendations for this infection control policy are suggested to be made by healthcare professionals based on their specific contexts (25). We believe that in low-resource settings with a high burden of MRSA like Vietnam, an infection prevention and control program needs to be designed to actively screen for MRSA among HCWs with these comorbidities for prompt interventions.

Among MRSA hand and nasal carriers, the distinction between persistent and intermittent carriage is important because persistent carriage is associated with a significantly higher bacterial load than intermittent carriage, resulting in an increased risk of transmitting MRSA to others (3,26). For MRSA hand carriage in our study, persistent carriage was not recorded, but 3.6% (95% CI: 1%-12.3%) of participants were found to be intermittent carriers. This prevalence was not different compared with the MRSA hand carriage rate in HCWs from other regions, including North America (8.3%, 95% CI: 3.5%-14.5%), Asia (4%, 95% CI: 2.1%-6.3%), and Europe (2.5%, 95% CI: 1%-4.5%) (27). Staphylococcal hand carriage in HCWs is usually transient, which means it is detectable after a working shift and gone before the next shift (3). The lack of persistent hand carriage in our study is a reassuring result. Moreover, intermittent carriage is often self-limiting and requires no treatment in healthy people (28). However, the contaminated hands of HCWs who are persistent or intermittent carriers are the main MRSA transmission route in hospitals, which can be prevented by effective hand hygiene (3,12). The overall prevalence of MRSA nasal carriage in our study was 30.1% (95% CI: 19.5%-43.5%), which was not different from the rate reported from Gaza Strip (25.5%, 95% CI: 20%-32%) (29). However, our rate was higher than that reported from Ethiopia (5.8%, 95% CI: 3.5%-9.5%) (30) and Nigeria (8%, 95% CI: 4.6%-13.5%) (31). Furthermore, the prevalence of persistent carriage of 24.5% was 4.5 times higher than the rate of intermittent carriage (5.6%) in our study. Unlike MRSA hand carriage, persistent nasal carriage cannot be managed by hand hygiene (32), but nasal mupirocin has been demonstrated to be efficacious in decolonizing MRSA in HCWs (3). Nasal mupirocin also provides a cost-effective adjunct to other infection control measures, including the screening and isolation strategies in controlling MRSA (33,34). However, nasal decolonization using mupirocin has not yet been implemented in Vietnam. A localized infection control guideline with detailed instructions on how to prevent and control MRSA nasal carriage with a focus on nasal decolonization will provide long-term benefits to both HCWs and patients.

In our study, *S. aureus* isolates were highly resistant to penicillin (96.1%), erythromycin (71.9%), and clindamycin (70.3%). We also found a moderate resistance rate with

ciprofloxacin (34.4%). All *S. aureus* strains were fully sensitive to sulfamethoxazole-trimethoprim and rifampicin. Our results are consistent with a study reported from Ethiopia, in which 93.1% (27/29) of *S. aureus* isolates colonizing in HCWs showed resistance to penicillin followed by erythromycin (62.1%) and ciprofloxacin (37.9%) (30). Although a lower resistance rate was documented with clindamycin (17.2%) in this report, a higher resistance rate of 51.7% was recorded for co-trimoxazole compared to our findings (30). The resistance rates with erythromycin (29.1%), clindamycin (11.2%), and ciprofloxacin (9.6%) of the 62 *S. aureus* isolates colonizing in HCWs in Gaza Strip were also lower than ours (29). Surprisingly, 14.5% of *S. aureus* isolates were found to be resistant to vancomycin in the Gaza Strip study, while this strain was not detected in our study (29). Higher resistance to the aforementioned antibiotics in our study could be due to excessive use, misuse, and irrational prescriptions of these medications in both hospitals and community in Vietnam (35,36). Therefore, there is an urgent need for robust antimicrobial stewardship programs in combination with adherence to infection control measures to tackle the growing threat of antibiotic resistance in *S. aureus.* The results of this antimicrobial stewardship would help tailor the MRSA infection prevention and control program to meet the local needs.

There are some limitations in our study. Our sample size was small, and thus may not be able to statistically detect the differences between study groups. However, our sample represented almost all staff (92%, 55/60) in the ICU of a leading tertiary hospital in Vietnam. In addition, it has been found that MRSA counts (colony-forming units/mL) may decrease over time among subjects exposed to a source of MRSA (37). Hence, MRSA counts can provide more insights into the clearance of MRSA among our participants. However, information on HCWs' MRSA counts was not available in our study. We also did not use genotyping methods to identify the resistance gene *mecA*, which is commonly used to examine MRSA due to limited financial resources. However, our study was set up to prospectively screen for MRSA carriage among HCWs for 8 consecutive weeks, and an antimicrobial susceptibility profile was performed for all cultured *S. aureus* isolates. The multiple testing times for antibiotic susceptibility of *S. aureus* in our study helped increase the possibility of detection of MRSA carriage and reduce the inconsistency of phenotyping resistance compared to the genotyping method. Given that there is no similar study in Vietnam, our study is the first attempt to examine the burden of MRSA among local HCWs. Therefore, we may have missed some possible risk factors of MRSA carriage in HCWs in Vietnam.

In conclusion, our data suggest that southern Vietnam may be an emerging MRSA hotspot, where the study was conducted . HCWs, especially nursing assistants, with comorbidities tended to be MRSA carriers. Infection control education programs should be tailored to meet the different knowledge levels of all HCWs. In addition to strengthening hand hygiene practice and antimicrobial stewardship, infection control guidelines need to be designed to actively screen for MRSA among HCWs with comorbidities for prompt interventions.

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SHORT COMMUNICATION

Focus on Antimicrobial Resistance (AMR)
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Unmet needs for management of drug-resistant infections: low- and middle-income countries' viewpoint

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ABSTRACT

Drug-resistant infections are a serious threat globally which demands cost-effective solutions to meet the unmet needs in their diagnosis and treatment. Gram-negative pathogens, drug-resistant tuberculosis, and multidrug-resistant *Salmonella typhi* have been reported as cause of resistant infections in developing countries. Here, we discuss the priority pathogens and conditions for which feasible solutions adaptable to low-resource settings are required to address the antimicrobial resistance in pathogens. These solutions will be helpful in containing the spread of antimicrobial resistance and better patient outcomes.

Keywords: Antibiotic, Antimicrobial resistance, Diagnostics, India, Infection, Low- and middle-income countries

Introduction

Drug-resistant infections are major public health concerns that are associated with high morbidity and mortality in patients, and result in exorbitant health and economic burden (1,2). In 2019, 4.95 million deaths were estimated due to resistant bacterial infections (3). The burden of drug-resistant infections remains high in low-resource healthcare settings, especially in low- and middle-income countries (LMICs) including India (3,4,1,5). Rampant misuse of antimicrobials (in humans and animal husbandry), unregulated over-the-counter availability, poor healthcare facilities and infection control practices, and lack of accurate diagnostics to support treatment decisions are few of the major drivers of antimicrobial resistance (AMR) in communities and hospitals (5,6).

In the past few years, reports on multidrug-resistant infections due to gram-negative bacteria have increased globally (3,4), which represent a critical unmet medical need, for which interventions are required. Gram-negative pathogens *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and Enterobacter spp. have become public health concerns (3,7,8) that are difficult to control due to acquisition of different resistance markers. *Streptococcus pneumoniae* infections have been associated with high mortality in children aged below 5 years, and in 2015, 50% of all pneumococcal deaths were reported in four countries of Africa and Asia (3,9). In neonates, antimicrobial-resistant infections have been associated with approximately 23% of deaths, with over 55% of AMR prevalence among sepsis-causing pathogens (3,10).

Published data from India have reported concerning rates of AMR among pathogens including *Enterococcus faecium*, *Staphylococcus aureus*, *Enterobacter* spp., *Salmonella Typhi*, etc. (7,11,12). An increase in infections due to carbapenem-resistant *K. pneumoniae* and *A. baumannii* has been reported from India and other countries (7,11,13). Hospital-acquired infections (HAIs) are another major concern especially in resource-constrained low-income settings where higher infection rates due to gram-negative pathogens have been reported than in developed countries (11,14). A recent HAI surveillance study from India reported high rates of carbapenem resistance in *Klebsiella* spp., *Acinetobacter* spp., and *Pseudomonas* spp. (15) and observed reduced susceptibility to extended-spectrum cephalosporins. This study also highlights *Candida auris* as an emerging multidrug-resistant threat in bloodstream infection (BSI) and urinary tract infection (UTI). Therefore, fungal pathogens such as *Candida* spp. require close monitoring for trends of infections, outbreaks, and susceptibility.

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Enteric fever and especially typhoidal *Salmonella* endemic to South and Southeast Asia and sub-Saharan Africa require continuous monitoring where changing resistance trends have been reported due to fluoroquinolones and cephalosporins (16, 17). Drug-resistant bacteria such as third-generation cephalosporin-resistant *E. coli*, carbapenem-resistant *A. baumannii*, fluoroquinolone-resistant *E. coli*, carbapenem-resistant *K. pneumoniae*, and third-generation cephalosporin-resistant *K. pneumoniae* represent serious challenges in patient management, especially in critically ill patient with comorbidities (3,4,7,11,14).

Treating these drug-resistant infections exerts high economic burden on healthcare and patients. Therefore, pathogens exhibiting resistance to antimicrobials for which there are limited treatment options represent a critical unmet medical need for diagnostics and therapeutic interventions. However, the priority pathogens or conditions as well as healthcare settings need to be prioritized as per local healthcare systems and burden of resistant infections.

Managing and containing drug-resistant infections in low-resource healthcare settings requires a multipronged approach consisting of the following steps:

- Effective surveillance systems are required to provide correct estimates of drug-resistant infections at national and regional levels.
- Strengthening of existing healthcare facilities by improving availability of laboratory services, diagnostics, and trained health personnel and, in parallel, enabling new health facilities at peripheral settings.
- Supporting research and innovations to develop rapid diagnostics and new treatment options such as drug molecules, targeted therapies, vaccines, and repurposing old antibiotics.
- Effective socio-behavioral tools and systems to create awareness among prescribers and general public to enable judicious utilization of existing antimicrobials.

Addressing the diagnostic gap

Improving diagnosis helps in generating evidence-based prescriptions to reduce empirical use of antimicrobials. There is a need for quality diagnostics with fast turnaround time for pathogen identification and susceptibility testing to address the problem of AMR (18). In LMICs, accurate and affordable diagnostic tests to differentiate viral and bacterial infections are a priority where resources and capacities are limited, and antibiotics are routinely misused for viral infections. Diagnostics for diagnosis of sepsis and typhoid fever are urgently required to contain spread of resistant infections and reduce morbidity and mortality, especially in neonatal sepsis. Typhoid and enteric fever are major concerns for LMICs with poor sanitation practices. Rapid diagnostic tests for UTIs, fever, and respiratory infections (lower and upper tract) are another unmet need, where empirical treatments result in heavy misuse of antibiotics. Global burden study in 2019 has also reported the dominance of lower respiratory infections, BSIs, and intra-abdominal infections as major infections attributable to AMR (3).

Over the last decade, several molecular diagnostics have been developed for pathogen identification and detection of genes(s) or gene mutations for presence or absence of resistance to antimicrobials (19). Despite the expedited diagnosis, these tests are expensive and need infrastructure and trained resources to provide results, thus limiting their use in resource-limiting settings. In LMICs, indigenously developed low-cost diagnostics have potential to fill this diagnostic gap and help in containing AMR (18). However, indigenous efforts in developing new diagnostics for AMR need to be supported and guided by delineating the local priorities and facilitators as per country's disease burden and healthcare requirements.

Need of new and reformulated antimicrobials and alternatives

The reliance on new drugs or combinations to fight AMR will only provide momentary relief as pathogens will evolve mechanisms to acquire resistance to antimicrobials. Analyzing and reducing antibiotic consumptions across human and animal husbandry sectors, research on intervention strategies and resistance rates can help in tailoring strategies and infection-specific treatment guidelines. The existing antibiotics also need to be utilized for developing new formulations to improve patient outcomes. There is a need to determine top combinations of antibiotics for BSI and study the efficacy of these combinations, preferably with contemporary isolates to provide effective combinations for the treatment of resistant infections. Pharmacokinetic/pharmacodynamic (PK/PD) and safety data on antibiotics and their combinations in different age groups are essential to effectively treat resistant infections.

It is also imperative to understand that the need for new antibiotics for LMIC is different from those of high-income countries as in countries like India, for example, predominant carbapenemase present in gram-negative isolates are metallo-β-lactamases (NDM, VIM) and not KPC (7). This also holds true for *P. aeruginosa* as the newer drugs for gram-negatives (ceftazidime-avibactam, imipenem-relebactam, and meropenem-vaborbactam) do not have activity against metallo-β-lactamases. This becomes an important aspect for drug developers for consideration with regard to LMICs. Low-cost solutions are therefore urgently needed to preserve the efficacy of available antibiotics/antibiotic alternatives such as virulence blockers, immune modulators, vaccines, etc. Research efforts need to be prioritized for new treatment strategies such as re-appropriation of old drugs, delivery strategies and for resistance mechanisms such as efflux pump inhibitors, and bacteriophage treatment.

Strengthening surveillance of drug-resistant infections

Tracking resistance trends is important to reduce the burden of infections in hospitals and community settings. Data generated through surveillance can be used in defining guidance for treatment in common syndromes and diagnosis of infections. It is important to implement infection prevention and control, antimicrobial stewardship practices, and

immunization program and reduce over- and misuse of antimicrobials. National estimates of resistant infections, etiology of infections, type of infections, and local resistance rates and patterns can be utilized to set research priorities regarding challenges and opportunities to tackle resistant pathogens in different healthcare and community settings.

In conclusion, evidence-based treatment strategies will be effective in preventing and managing resistant infections. Affordable and accessible quality diagnostics with fast turnaround time will help in enabling better patient care and survival outcomes, thus reducing economic burden on healthcare.

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REVIEW

Focus on Antimicrobial Resistance (AMR)
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Antibiotic-resistant bacteria originating from the gut may modulate the mucosal immune response during sepsis and septic shock

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ABSTRACT

The enrichment and diversity of gut microbiota play an important role in sepsis, but the role of gut microbiota composition and early-life colonization in sepsis and septic shock has not yet been characterized. The impact of gut microbiota diversity on host immunological disorders and future treatments of inflammatory diseases are not yet fully elucidated. Further, the association between the microbiota and immune development in sepsis remains unknown, and the underlying mechanisms are not well understood. The altered composition of gut microbiota during sepsis is profoundly associated with a loss of commensal bacteria and an overgrowth of potentially pathogenic bacteria, especially AMR bacteria. Disruptions of gut microbiota diversity are directly associated with susceptibility to sepsis and a higher risk of adverse outcomes. Several studies have confirmed that a mutual association between gut microbiota and the host is important for the metabolism of essential nutrients for the organism, for gut development, and for the maturation and development of a fully functional immune system. Therefore, understanding the gut microbiota diversity, composition, and function during various inflammatory conditions and sepsis may provide a comprehensive knowledge of the mechanisms behind the pathogenesis of gut-derived infection in diseases and the design of new treatment options (e.g., probiotics or fecal microbiota transplantation).

Emerging evidence displays an important role of gut microbiota and their derived metabolites in modulating the host mucosal immune response and determining the susceptibility to, as well as outcomes of sepsis.

Keywords: Immune response, Inflammation, Metabolites, Microbiota, Sepsis

Introduction

Antimicrobial resistance (AMR) also known as drug resistance, is a naturally occurring process that happens when germs like bacteria, fungi, viruses, and parasites develop the ability to defeat the drugs designed to kill them (1). Microbes change over time and no longer respond to antibiotics and other antimicrobial drugs, making infections harder to treat and increasing the risk of disease spread, severe illness, and death (2). Resistant infections can be difficult, and sometimes impossible, to treat. When the microorganisms become resistant to most of the antibiotics and other medications commonly used to treat the infections they cause, they are often referred to as "superbugs." AMR is considered one of the leading public health threats of the 21st century (3). About 700,000 deaths have been reported due to drug-resistant infections (4). About 2.8 million people suffer from acquired drug-resistant infections across the globe and 35,000 patients die annually in the United States due to infections alone (5). It is responsible for an estimated 33,000 deaths per year in the European Union (EU) (6). In 2019, the World Health Organization (WHO) reported that, if left unchecked, the anticipated deaths due to AMR would rise to 10 million by 2050 (5). These infections have significant economic and human costs. Economic projections suggest that by 2050, the economic costs of healthcare-associated infections (HAIs) to the US healthcare system will range from 28 to 45 billion dollars per year (7). Further, recent data released by the UK government argued that AMR could kill 10 million people per year by 2050 (8). The WHO and numerous other

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groups anticipated that AMR is a global issue that requires a coordinated action plan to address. The way AMR infections are spreading could make many bacterial pathogens resistant to emerging antibiotics and more lethal in the future than they are today (9).

India is considered the AMR capital of the world. India has one of the largest numbers of antibiotic-resistant pathogens worldwide (10). The highest burden of multidrug-resistant tuberculosis cases has been reported in different parts of India, as alarmingly high-resistance bacterial cases (11). India is one of the largest consumers of antibiotics worldwide and consumption and sales of antibiotics continue to rise rapidly. Despite the decline in the number of cases of communicable diseases, the consumption of antibiotics continues to increase (12). On the one hand, emerging new multidrug-resistant (MDR) organisms pose newer diagnostic and therapeutic challenges in front of policymakers and health care workers, while on the other hand India is still striving to battle old enemies such as malaria, cholera, and tuberculosis (13). Infectious disease remains a leading cause of mortality in India. About 50,000 newborns lose the battle to sepsis annually due to pathogens resistant to first-line antibiotics (14). Two million deaths are anticipated in India due to AMR by the year 2050 (13). There are several factors such as illiteracy, congestion, poverty, malnutrition, and excessive antibiotic use that contribute AMR situation being worse in India. The lack of awareness about the pathogenesis of infectious diseases and their spread among the public and inaccessibility to healthcare further compound the situation. Easy availability of over-the-counter (OTC) antimicrobial drugs and self-prescription of these drugs without any professional knowledge regarding the dose and duration of treatment significantly contribute to AMR. The lack of tertiary care hospital facilities to diagnose patients with MDR, a significant load of resistant infections, and unregulated sales of antibiotics have contributed to a speedy rise in resistant infections in India. This has an enormous socioeconomic impact due to a large number of deaths and increased costs due to protracted stay in the hospital. Despite the high burden of AMR cases and the continuous rise in resistance cases, India spends only 4.7% of its total Gross Domestic Product (GDP) on health. However, the government shared only one-fourth (1.15%) of its GDP, making the task massive (15). The contribution of the Government of India to health is very poor (16). In 2017 the Government of India adopted a National Action Plan (NAP) on AMR.

The gut microbiota is essentially required to maintain gut homeostasis by mutually interacting with intestinal epithelial cells and mucosal immune cells (17). During prolonged inflammation, this interaction could become pathological due to changes in the composition and diversity of gut microbiota (18). The loss of diversity and compositions of gut microbiota may lead to disruption of intestinal homeostasis and deleterious clinical manifestations (19). Recent studies reported the key role of microbiota and their metabolites in the development of gut-derived infection, sepsis, and multiple organ dysfunctions in sepsis (20). Therefore, it is important to understand the gut microbiota composition, diversity, and functions of their metabolites during sepsis and other inflammatory conditions. The present review article may provide a more inclusive understanding of the mechanisms of gut-derived infection in the pathogenesis of sepsis and the design of new treatment options. Here, we present current knowledge and key concepts linking gut microbiota to the development and function of the immune system. Through this article, we discuss how AMR causes the defective host immune system activation by modulating the gut microbiota, the current progress in the field, and identify the need for experimental studies investigating the use of these treatments in sepsis management. Finally, we highlighted the challenges and perspectives of microbiome-targeted approaches in studying disease pathogenesis and developing new microbiome-related treatments.

Gut microbiota in health and disease

The mammalian gut contains highly diverse and wide varieties of the microbial community called the microbiome, which includes mostly bacteria, viruses, fungi, etc. Gut microbiota includes about 1,000 to 1,500 bacterial species (21). Gut microbiota is highly dynamic and varies from one individual to another individual. The diversity of gut microbiota can be imagined from the data obtained from an individual that contains only about 160 bacterial species (22). It indicates that the composition of gut microbiota is highly diverse among individuals and depends on nutrition, environmental changes, and genetic inheritance (21,22). Nutrition and environmental factors are very important in determining bacterial richness and diversity among individuals (23). A direct mutual association between gut microbiota and the host is reported in several studies. The gut provides a favorable condition for the growth and development of microbiota, and the gut microbiota supports the maturation of the mucosal immune system and metabolic system by providing beneficial nutrients such as vitamins and short-chain fatty acids (SCFAs) (24). Therefore, understanding the association between the gut microbiota and its metabolites with the intestinal immune system is vital for the development and maturation of the mucosal immune system.

A change in the richness and diversity of the microbiome profile in the gut is known as dysbiosis. The dysbiosis of gut microbiota is closely linked to several diseases, such as type 2 diabetes, obesity, hypertension, necrotizing enterocolitis (NEC), inflammatory bowel disease (IBD), etc. (18,25). Gut dysbiosis leads to the development of gut barrier dysfunction and bacterial translocation. It impairs the ability to maintain mucosal membrane function and contributes to systemic inflammation (26). When dysbiosis occurs, bacteria and bacterial endotoxins or toxins can leak from the gut, along with food particles. This systemic translocation of bacteria and bacterial products is responsible for other clinical manifestations in critically ill patients (27). The different bacterial genera present in the gut are likely to affect the intestinal environment of the hosts and alter the metabolic patterns and influence the occurrence of diseases. The altered gut microbiota is also associated with metabolic parameters, sex hormones, and the mediators of the gut-brain axis (22,28). Several studies have confirmed the role of gut microbiota in

sepsis, but the direct association of gut microbiota diversity with the pathogenesis of disease and outcomes has not yet been fully understood (20). Figure 1 shows the association of gut microbiota dysbiosis with changed metabolites and immune system dysregulation in critical illness and disease.

Role of gut microbiota in sepsis and septic shock

Sepsis, defined as life-threatening organ dysfunction caused by a dysregulated host response to infection, affects 1.7 million people annually in the United States (14). About 20%-30% of patients die annually across the globe due to sepsis (29). It is recognized as a global health emergency by WHO (30). The overwhelming inflammatory response is a hallmark property of sepsis. The exaggerated inflammatory response that occurs during sepsis may lead to immune suppression and dysregulated immune response (31). These dysregulated host immune responses during sepsis may lead to the dysfunction of multiple organ systems, which includes the cardiovascular, renal, pulmonary, hepatic, and gastrointestinal systems (31). Sepsis-induced hyperactivation of immune cells and immune suppression may be considered the main contributors to the pathophysiology of sepsis (32). Immune suppression often increases the individual's susceptibility to secondary infections, further increasing the risk of death (33). The recent development of molecular-based sequencing tools has exposed the importance of gut microbiota diversity in human health and disease (34). Several studies have reported gut dysbiosis with a sharp decrease in diversity, overgrowth of pathogenic bacteria, and loss of commensal bacteria (21). Recent studies have elucidated key immune pathways that are modulated by gut microbiota and their metabolites (24). The altered gut microbiota during sepsis may influence inflammatory responses and increase gut barrier permeability, which could enable the translocation of pathogenic bacteria to the systemic circulation and distant organs. The increased gut permeability during inflammatory conditions and sepsis may lead to the translocation of enteric bacteria from the gut to the systemic circulation served as the motor of multiple organ dysfunction syndromes (MODS) and, subsequently, cause acute septic responses (35). The altered gut microbiota composition and diversity during inflammatory conditions and subsequently enhanced translocation of gut microbiota may cause mucosal immune dysfunction (35).

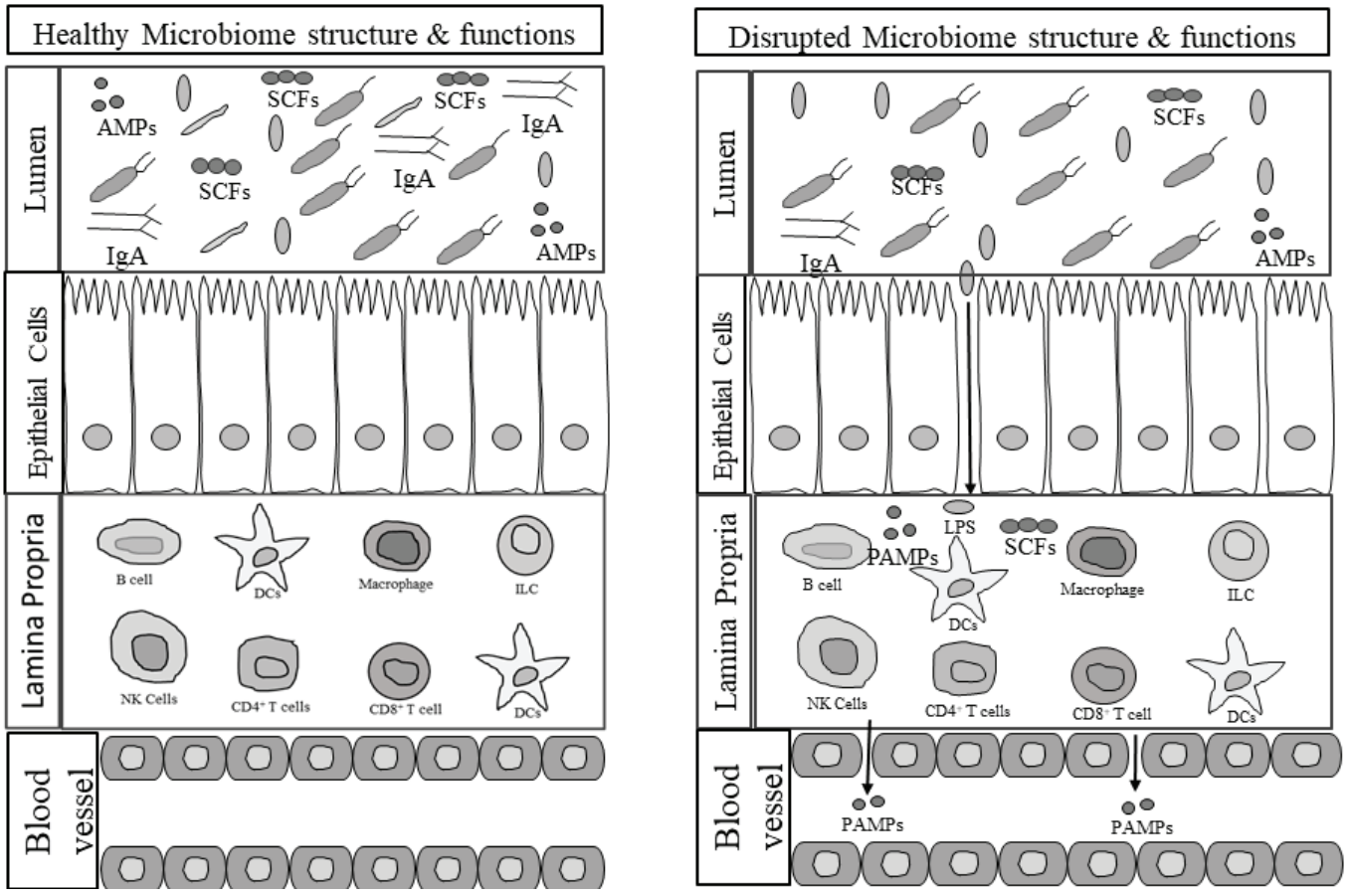

Fig. 1 - Gut microbiome dysbiosis predisposes to selection for pathogenic bacteria that leads to immune dysregulation, and decreased production of beneficial metabolites by the gut microbiome. AMPs = antimicrobial peptides; DCs = dendritic cells; ILC = innate lymphoid cell; LPS = lipopolysaccharides; PAMPs = pathogen associated molecular patterns; SCFAs = short-chain fatty acids

The loss of membrane integrity and translocation of enteric bacteria and its metabolites from the gut to the systemic circulation is a hallmark property of sepsis and other inflammatory diseases (17). Further, the antibiotics treatment may change the composition and diversity of gut microbiota that may lead to the translocation of enteric bacteria and their metabolites across the epithelium, which may provide vital information about the importance of the microbiota in host resistance against pathogens (36).

Despite the recent findings of gut dysbiosis to sepsis outcomes, the precise mechanisms underlying the protective effects of gut microbes in sepsis have not been well defined. Further, the role of gut microbiota and their metabolites in modulating the host mucosal immune response is not well known. More importantly, at present, we do not have diagnostic kits or therapies directed at the gut microbiome that could be implemented in the clinical management of sepsis. Patients in the early stages of sepsis manifest differences in their microbiome composition and diversity as compared to critically ill stages of sepsis (20). As compared to survivors, non-survivors with sepsis have pathogenic and antibiotic-resistant bacterial species such as *Clostridia* species and *Enterococcus* species (37). Use of excessive antibiotics for the treatment of sepsis patients inclines to gut dysbiosis and a state of immune suppression, with subsequent poor outcomes in the later course of hospitalization during sepsis (38). A study conducted in the murine model of sepsis confirmed the translocation of bacteria *Klebsiella pneumoniae* from the gut to the systemic circulation (35). A prospective cohort study conducted on 71 preterm infants with sepsis showed domination of the gut microbiota with bacilli and decreased abundance of anaerobic bacteria (39). A recent study highlighted that gut dysbiosis with an accumulation of bacilli (largely coagulase-negative staphylococci) and their fermentation metabolites could precede late-onset sepsis (40). A significant reduction of commensal bacteria and overgrowth of enteric bacteria may lead to overwhelming inflammation and inflammatory diseases. Several studies have highlighted the importance of diverse and balanced intestinal microbiota in enhancing the host's immunity to intestinal and systemic pathogens, and disturbing this balance is likely to increase the susceptibility to sepsis (14,17). However, the role of gut microbiota and its association with the pathogenesis of sepsis is not yet fully understood. Moreover, association studies of the gut microbiota with clinical parameters and the outcome of patients with sepsis are urgently needed.

Effects of gut microbiota in modulating the mucosal immune response

The major components of the immune system that are involved in protecting the host against diverse pathogens are immune cells, tissues, organs, soluble mediators such as cytokines, and cell receptors. The gastrointestinal tract is considered the most important immunological organ in the body because it harbors up to 70% of the body's lymphocyte population. The intestine mucosal immune system is an integral component of innate and adaptive immunity that includes three different mucosal lymphoid structures: Peyer's patches (PP), the lamina propria (LP), and the epithelia. Beneath the epithelium, the LP harbors dendritic cells (DCs), which are potent antigen-presenting cells (APCs), and the gut-associated lymphoid tissue (GALT), which includes PP, lymphocytes, and intraepithelial lymphocytes (IELs). IELs are the first immune cells that encounter invading pathogens through an epithelial surface of the intestinal tract, urinogenital tract, and respiratory tract. The mucus layer present on the surface of epithelial cells along with secreting antimicrobial peptides (AMPs) in response to bacteria or pathogens primarily contributes to the intestinal innate host defense system (1). The intestinal epithelial cells are directly involved in defense against invading pathogens and also send signals to the mucosal immune system by producing soluble mediators such as cytokines and chemokines (41). The innate lymphoid cells (ILCs) located in the epithelial cells also work as the first line of defense and get activated in response to stimuli. Once ILCs get activated it produces various soluble mediators such as cytokines and chemokines that are essentially required for the development and maturation of the mucosal immune system (42). The composition and diversity of gut microbiota are vital in maintaining intestinal homeostasis in mammals. Dysbiosis of gut microbiota occurs due to excessive use of antibiotics during inflammatory conditions and sepsis may lead to the uncontrolled production of inflammatory mediators and overwhelming activation of innate immune cells. In addition, the adaptive immune system of the gut contributes to intestinal barrier defense by secreting immunoglobulins (Ig). The secreted Ig by the activated B cells in response to invading pathogens into the intestinal lumen neutralize the pathogenic microorganism and protect the mucosal tissue (43). The role of gut microbiota in shaping the host mucosal immune response is confirmed by several studies (24). The various metabolites such as SCFAs and tryptophan decomposition metabolites produced by gut microbiota are required to stimulate ILCs and enhance gut integrity (44). IELs are considered an important player in the adaptive immune response against invading pathogens (45). They are rich in αβ+ and γδ+ T-cell populations that are required to protect against germs and pathogens during inflammation (46). IELs showed a diverse immune response when they get activated with stimuli. Once IELs are activated, they express cytokines such as interferon-γ and growth factor, to protect epithelial cells from injury.

Recently, the association of gut microbiota with the development of host immunity has been confirmed by several studies. Colonization of commensal bacteria in early life is important for the metabolism of essential nutrients required for the host, for gut development, and for the maturation of the innate and adaptive immune system (47). A study conducted in germ-free (GF) animals showed that colonization of gut microbiota in the early stages of life is crucial for the optimum development and maturation of the immune system (48). Early studies on GF animals showed that the lack of colonization of commensal microbes is associated with significant intestinal defects in immune cell development and functions (49). Intestinal microbial colonization during the early-life stage is critical for the development of αβ and γδ IELs, induction of mucosal IgA antibodies, and Th17 cells

(47). These immune dysfunctions are restored by microbial colonization, most notably with segmented filamentous bacteria and other commensal bacteria. Any abnormalities that lead to gut dysbiosis severely affected the development of intestinal mucosal immunity and make individuals more susceptible to secondary infections. A small animal study conducted in GF mice showed comparatively smaller mesenteric lymph nodes, PP, and reduced numbers of immune cells such as CD4+ T cells, CD8+ T cells, IgA-producing plasma cells, and intraepithelial T-cell receptors in mice having sepsis as compared to the control (14).

Challenges, pitfalls and future aspects in immune-microbiome research

Despite the impressive achievement that has greatly enhanced our understanding of gut microbiota diversity and its association with immune system development, many challenges remain in disentangling microbiome-immune system interactions in homeostasis and disease (50). Several mechanistic studies are required to explore the role of the commensal microbiome in modulating the host's innate and adaptive immunity in health and disease. Recent studies conducted in animals show a bidirectional relationship exists between microbiome perturbation and immune dysregulation (48). Early-life colonization of gut microbiota and metabolites causing immune development, activation, and chronic inflammation conversely may shape the dysbiotic configuration and functions of microbial communities. However, a direct causal association between the richness and diversity of gut microbiota with immune development before the onset or during the early stages of the disease has not been established in most medical conditions. In the context of septic patients, a large human study cohort is required to find microbiota composition, diversity, and dysbiotic changes before, during, and after the occurrence of sepsis to identify the protective commensals and microbiota potentially associated with susceptibility to sepsis and worse outcomes. In addition, a multidimensional approach, including metabolomics, proteomics, single-cell transcriptomics, epigenomics, and meta-genomics, is required to elucidate how the gut microbiome and immune system are cross-regulated during sepsis. Finally, the microbiome composition and immune responses are highly variables among human individuals and disease states. This inherent inter-individual variability of the gut microbiome and associated complexity constitutes a major experimental challenge. This increases the likelihood of precision medicine concerning microbiota. It intrigued us to predict the personalized, host immune responses based on gut microbiome profiles in terms of treatment and prognosis. Therefore, the microbiota is a next-generation medicine and may facilitate the development of personalized microbiome-targeted treatments for immunological disease.

Conclusions

In summary, the intestinal microbiota is essentially required for the development and maturation of host immunity and contributes to maintaining intestinal homeostasis. Recent studies have shown the pivotal role of intestinal microbiota in modulating the host cellular immune response to stimuli and enhancing mucosal immunity. The mutual association between the gut microbiota and the host is required for the maturation and development of host gut immunity. The host provides a suitable environment for the growth of the microbiome, and subsequently, the gut microbiota facilitates the development and maturation of the mucosal immune system. The altered composition and diversity of gut microbiota especially AMR bacteria due to antibiotics treatment can lead to the translocation of enteric bacteria from the gut to the systemic circulation and cause the pathogenesis of sepsis. The interaction between the gut microbiota and mucosal immune system is key for controlling normal homeostasis and inflammatory response. Impaired communication between these two is associated with the pathogenesis of several inflammatory diseases and sepsis, and it highlights the importance of exploring the function of microbiota in such diseases.

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REVIEW

Current molecular approach for diagnosis of MRSA: a meta-narrative review

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ABSTRACT

Introduction: Detection and diagnosis of methicillin-resistant *Staphylococcus aureus* (MRSA) are important in ensuring a correct and effective treatment, further reducing its spread. A wide range of molecular approaches has been used for the diagnosis of antimicrobial resistance (AMR) in MRSA. This review aims to study and appraise widely used molecular diagnostic methods for detecting MRSA.

Methods: This meta-narrative review was performed by searching PubMed using the following search terms: (molecular diagnosis) AND (antimicrobial resistance) AND (methicillin-resistant Staphylococcus aureus). Studies using molecular diagnostic techniques for the detection of MRSA were included, while non-English language, duplicates and non-article studies were excluded. After reviewing the libraries and a further manual search, 20 studies were included in this article. RAMESES publication standard for narrative reviews was used for this synthesis.

Results: A total of 20 full papers were reviewed and appraised in this synthesis, consisting of PCR technique (n = 7), deoxyribonucleic acid (DNA) Microarray (n = 1), DNA sequencing (n = 2), Xpert MRSA/SA BC assay (n = 2), matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) (n = 2), MLST (n = 4), SCC*mec* typing (n = 1) and GENECUBE (n = 1).

Discussion: Different diagnostic methods used to diagnose MRSA have been studied in this review. This study concludes that PCR has been extensively used due to its higher sensitivity and cost-effectiveness in the past five years

Keywords: Antimicrobial resistance, Molecular diagnosis, MRSA

Introduction

Antimicrobial resistance (AMR) is defined as changes in bacteria that result in the drug being used for its treatment becoming inefficacious (1). *Staphylococcus aureus* is an opportunistic pathogen with a tremendous capacity to adapt to human hosts and healthcare environments, causing detrimental effects to healthcare-associated infections such as bloodstream infections (2). AMR is reported as the world's biggest 21st-century health threat, and the World Health Organization (WHO) is calling for immediate action. As AMR spreads, common infections are becoming incurable. Reports state that over 700,000 die yearly due to drug-resistant illnesses; by 2050, the number is predicted to rise to 10 million (3).

A major issue pertaining to AMR is the excessive and injudicious use of antibiotics that have led to widespread resistant bacteria and dissemination of their antimicrobial resistant genes (ARGs) (4). It is concerning that the AMR rates are predicted to increase if measures are not taken. One way to overcome this is through early detection, which enables effective management, allowing efficient identification and detection of microbes such that the patient can be treated with the appropriate drug in time.

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Over the years, great leaps have been made in the diagnosis of AMR and diagnostic tests are reported to be an essential tool in early diagnosis, hence it is a robust strategy against AMR (4).

To enhance existing approaches, this review aims to summarize new and current molecular techniques and technologies used to identify AMR using a systematic meta-narrative approach, with a focus on the key benefits and drawbacks. Furthermore, a critical overview of recently developed molecular approaches and an informed assessment of future direction will also be discussed.

Methodology

Study design and inclusion criteria

This systematic review was carried out in a meta-narrative framework. This study qualitatively appraised different molecular methods used in the recent 5 years for the diagnosis of methicillin-resistant *Staphylococcus aureus* (MRSA). This study protocol was created according to the RAMESES (Realist And Meta-narrative Evidence Syntheses: Evolving Standards) meta-narrative review publication guidelines (5). Articles that satisfied the following requirements were considered for the review: (i) original articles written in English that were published between January 2017 and May 2022, (ii) cross-sectional or cohort studies that assessed the technical performance of molecular methods (sensitivity, specificity, accuracy or concordance) for diagnosing MRSA. Articles were excluded if they were: (i) case reports; (ii) review articles, commentary articles, and short communications.

Search strategies

Articles were searched using PubMed. Search keywords were (((((molecular diagnosis) AND (antimicrobial resistance)) NOT (review [publication type)) NOT (systematic review [publication type)) NOT (meta-analysis [publication type)) AND (methicillin-resistant staphylococcus aureus).

Selection and appraisal of articles

Two independent reviewers (Lee and Sim) screened the titles and abstracts. Articles with abstracts indicating the use of a molecular approach to diagnose MRSA were read in full. A final consensus was discussed between the two reviewers, and disagreements were resolved with discussion from the third reviewer (SM). EndNote Version 20 was used for article duplicate removal and archives. All the studies reviewed and appraised in this synthesis are summarized in Table I.

TABLE I - Summaries of studies appraised in this review

No	Author	Year	Country	Condition/patients	Sample	Study design	Molecular diagnosis methods	Reference
1	Moutaouakkil et al	2022	China	Children diagnosed with *Staphylococcus aureus* OAI	Blood cultures, articular fluids, synovial tissues and/or bone fragments	Prospective study	Multiplex polymerase chain reaction	(6)
2	Jin et al	2022	China	1,952 MSSA strains isolated from blood across 17 provinces	MSSA-PENS isolated from invasive BSIs	Retrospective study	Whole-genome sequencing	(2)
3	Senok et al	2021	United Arab Emirates	135 patients with a clinical diagnosis of severe skin and soft-tissue infections	*S. aureus* isolates associated with SSTI were tested for PVL detection	n/a	DNA microarray assays	(7)
4	Reddy and Whitelaw	2021	South Africa	231 samples	2,822 patients with positive blood cultures exclusively showing GPCC on Gram stain were included	Prospective study	Xpert MRSA/SA BC assay	(8)
5	Choi et al	2021	South Korea	26 children aged <15 years diagnosed with SSSS	Involved area of the skin, the presence of Nikolsky's sign, and the status of desquamation	n/a	PCR	(9)
6	Anafo et al	2021	Ghana	300 diabetes patients and 106 non-diabetic individuals	Anterior nasal swabs	Cross-sectional	PCR	(10)
7	Verdú-Expósito et al	2020	Ethiopia	80 *S. aureus* strains isolated from human patients with SSTIs	Human samples	n/a	MALDI-TOF and PCR	(11)
8	Tang et al	2020	China	MRSE strains from the dental plaque of a normal, healthy human population	Dental plaque specimens	n/a	PCR	(12)

No	Author	Year	Country	Condition/patients	Sample	Study design	Molecular diagnosis methods	Reference
9	Khawaja et al	2020	Pakistan	105 samples	Human samples	Descriptive cross-sectional study	PCR	(13)
10	Jin et al	2020	China	65-Year-old healthy man with a history of leprosy	Isolate was obtained from the patient's blood, and identified as an ST9-MRSA strain	n/a	Whole-genome sequencing	(14)
11	Geng et al	2020	China	536 neonates	Nasal swabs	Prospective surveillance study	Staphylococcal chromosomal cassette (and) type, *spa* type, MLST	(15)
12	Crandall et al	2020	USA	357 children with invasive *S. aureus* infections	Pleural fluid and/or blood	Prospective study	PCR, MLST, SCC*mec* typing	(16)
13	Bouza et al	2020	Spain	155 adult inpatients diagnosed with skin and soft-tissue infection	Microbiological samples	Prospective study	Gram stain plus GeneXpert® MSSA/MRSA SSTI	(17)
14	Yang et al	2019	China	269 nonduplicate *S. aureus* clinical isolates were isolated from children	Steril specimens and non-STERIL specimen using VITEK MS system	n/a	MALDI-TOF	(18)
15	Mutonga et al	2019	Kenya	83 adult patients diagnosed with diabetic foot ulcers	Wound swab cultures	Cross-sectional study	Real-time PCR	(19)
16	Latour et al	2019	Belgium	1,447 residents from nursing homes	Pooled sampling of nose, throat and perineum	Cross-sectional prevalence survey	Triplex PCR and MLST	(20)
17	Hida et al	2019	Japan	263 patients suspected of having staphylococcal bacteremia	Fresh and frozen blood culture samples	n/a	GENECUBE *mec*A	(21)
18	Luo et al	2018	China	275 isolates of *S. aureus*, including 148 isolates from patients, 127 from ready-to-eat food samples	Secretions, blood, phlegm, cerebrospinal fluid, transudation, urine, fresh meat, meat product, cereal products, fruits and vegetables	n/a	PCR, multiplex PCR	(22)
19	Lin et al	2018	Taiwan	106 hemodialysis patients diagnosed with MRSA	Blood cultures	Retrospective study	PCR and MLST	(23)
20	Yang et al	2017	China	104 children diagnosed with MRSA	Sputum, bronchioalveolar lavage fluid, skin and soft tissues, pus, secretions, secretions of omphalitis, blood, joint effusion, pleural effusion	n/a	MLST	(24)

BSI = bloodstream infection; DNA = deoxyribonucleic acid; MALDI-TOF = matrix-assisted laser desorption/ionization-time of flight; MLST = multilocus sequence typing; MRSA = methicillin-resistant *Staphylococcus aureus*; MRSE = methicillin-resistant *Staphylococcus epidermidis*; MSSA = methicillin-sensitive *Staphylococcus aureus*; n/a = not available; PCR = polymerase chain reaction; SCC*mec* = staphylococcal cassette chromosome *mec*; spa = staphylococcal protein A.

GPCC = Gram positive cocci in clusters; MSSA-PENS = methicillin-sensitive S. aureus – penicillin-susceptible; OAI = osteoarticular infections; SSSS = Staphylococcal scalded skin syndrome ; SSTI = skin and soft tissue infections; PVL = Panton Valentine leukocidin

Results

The dataset includes 20 different authors from Asia (n = 13), Africa (n = 5), Europe (n = 1) and America (n = 1). A total of 20 studies were included in this synthesis: seven studies employed polymerase chain reaction (PCR) for diagnosing MRSA (6,9,10,12,13,19,22), one study employed deoxyribonucleic acid (DNA) Microarray (7), two studies used DNA sequencing (2,14), Xpert MRSA/SA BC assay (n = 2) (8,17), matrix-assisted laser desorption/ionization-time

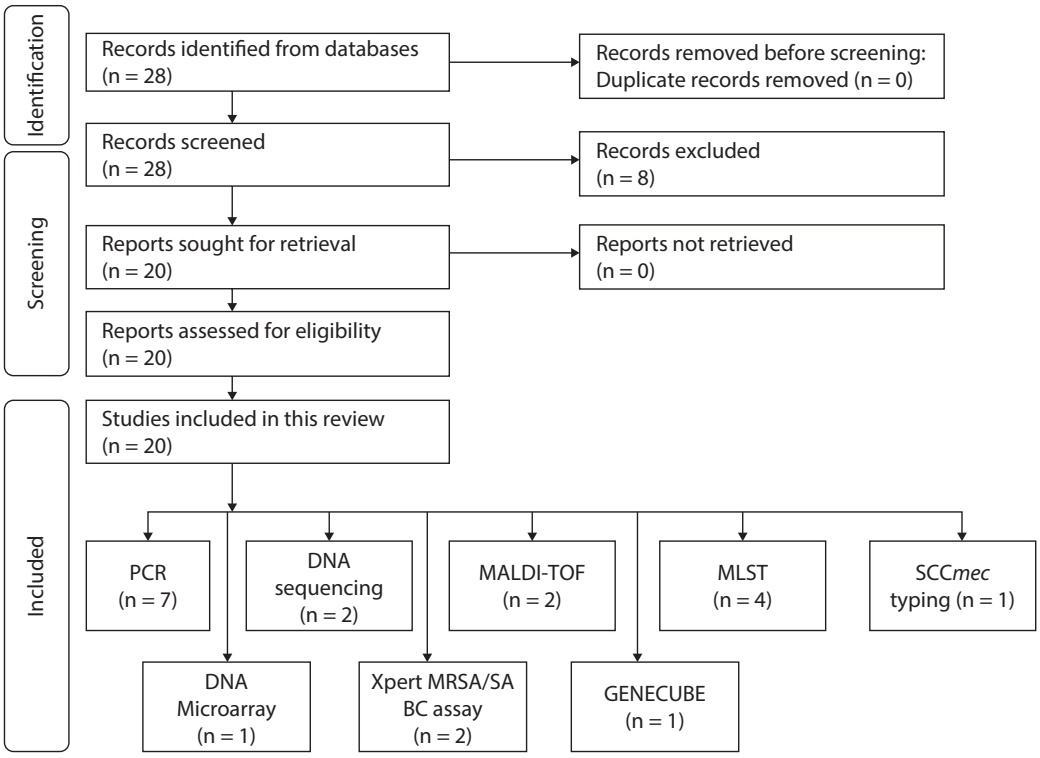

Fig. 1 - Diagrammatic flow of the study selection and list of techniques appraised in this review.

of flight (MALDI-TOF; n = 2) (11,18), multilocus sequence typing (MLST; n = 4) (15,20,23,24), GENECUBE (n = 1) (21) and staphylococcal cassette chromosome *mec* (SCC*mec*) typing (n = 1) (16). Figure 1 is the diagrammatic flow of the study selection and list of techniques appraised in this review.

Recent molecular methods for diagnosis of MRSA

Polymerase chain reaction

PCR approaches have been commonly used for the effective diagnosis of MRSA, and the rapid emergence of MRSA has led to a series of PCR approaches that have been developed for the identification of MRSA (25). PCR approach identifies *S. aureus* based on a single-base-pair mismatch in the staphylococcal 16S ribosomal RNA gene sequence (26). Recent researchers have also cited the use of the PCR approach for *mec*A gene detection as the gold standard method for the detection and identification of the prevalence of MRSA (27,28). In this synthesis, a total of seven studies have employed PCR for the detection and diagnosis of MRSA. A study conducted by Moutaouakkil and colleagues among patients suspected of *S. aureus* hospitalized in pediatric orthopedic clinic reported the detection of *mec*A using PCR (6). This study also utilized different biological samples such as blood cultures, articular fluids, synovial tissues and bone fragments for the detection of MRSA. Another study showed that the fluorescence signal of real-time (RT)-PCR could display the quantity of products formed and increases exponentially, enabling a user-friendly diagnostic (29). Furthermore, Mutonga and colleagues (2019) have demonstrated that the sensitivity of RT-PCR for MRSA is 100% (19).

Multiplex PCR amplifies multiple DNA sequences simultaneously, which gives an advantage over conventional PCR (30). The detection of target sequences, such as the *nuc* and *coa*A or elements necessary for methicillin resistance, such as *fem*A, or *fem*B, has provided the basis for PCR identification of *S. aureus*. It uses two pairs of primers specific to the staphylococcal *nuc* and *mec*A for PCR amplification of a 280-bp *nuc*-based fragment and a 533-bp *mec*A-based fragment (31). Tsai and colleagues (2019) reported *mec*A gene (*mec*A-F and *mec*A-R) is amplified and can be used to diagnose MRSA (32). Chikkala and colleagues showed that it exhibits 97% of specificity and 90% sensitivity (33).

DNA sequencing

DNA sequencing allows the detection of single-nucleotide polymorphisms (SNPs) and known resistance-associated genes and their variations (34). The availability of bacterial genomes in public databases facilitates the use of whole-genome sequencing for MRSA detection. It enables high-resolution characterization of antibiotic resistance (35). Whole-genome sequencing has a definite edge over conventional Sanger sequencing because it may produce millions of reads that are roughly 35 to 700 bp in length (36). There is growing evidence on the effectiveness of bacterial

whole-genome sequencing in controlling outbreaks. Whole-genome analysis, such as DNA microarray, simultaneously identifies relative concentration of different nucleic acid sequence (37). It allows a bulk number of nucleic acid sequences in a mixture to be tested and analyzed. The study by Jin and colleagues (2,14) used StaphyType DNA microarray (Abbott [Alere Technologies GmbH], Jena, Germany) and the INTER-ARRAY Genotyping Kit *S. aureus* (Inter-Array GmbH, Bad Langensalza, Germany) for the detection of MRSA. The study by Senok and colleagues (2021) also reported that DNA microarray exhibited 100% specificity and sensitivity (7). In a study done by Ma and fellow colleagues, Illumina's Nextera DNA library preparation kit was used to create whole-genome sequencing libraries, which were then sequenced on an Illumina MiSeq using the 500 cycle V2 protocol (38).

Xpert MRSA/SA BC assay

Xpert MRSA/SA Blood Culture is an in vitro diagnostic test for *S. aureus* and MRSA. The targeted DNA is amplified using automated RT-PCR and Fluorogenic target-specific hybridization, providing real-time detection of specific genes of MRSA and *S. aureus*. A study by Buchan and colleagues (39) reported the use of blood cultures for the detection of *Staphylococcus* protein A (spa) sequences, gene that encodes for methicillin resistance (*mec*A) and SCC*mec*. A study by Reddy and colleagues has shown the performance of the Xpert MRSA/SA BC assay to be 100% in specificity and sensitivity. It shows a failure rate for an interpretable result of just 1.7% (8). However, it is notable that the microbiological sampling should be of high quality to ensure rapid and accurate results, despite the significance of Xpert MRSA system.

MALDI-TOF

MALDI-TOF mass spectrometry (MS) has become a widely used technique for the rapid and accurate identification of bacteria (40). Despite the efficiency and sensitivity of MALDI-TOF, this method's limitation is that new isolates can only be detected if the spectral database contains peptide mass fingerprints (PMFs) of the type strains of specific genera/species/subspecies/strains. This method identifies microbes by comparing the PMF of unknown organisms with the PMFs deposited in the database or matching the masses of biomarkers with the proteome database. A recent study by Tang and colleagues (41) reported that MALDI-TOF MS on intact bacteria combined with a refined analysis framework allows accurate classification of methicillin-sensitive *Staphylococcus aureus* (MSSA) and MRSA. Esener and colleagues showed that MALDI-TOF has a sensitivity of 99.93% ± 0.25%, specificity of 95.04% ± 3.83%, and accuracy = 97.54% ± 1.91% (42). MALDI-TOF is low in cost, and analysis can be conducted within a short time, allowing rapid microbial resistance to be detected. Latour and colleagues employed MALDI BioTyper database for bacterial identification of suspected colonies (20). A study by Chen and colleagues has shown that MLST has been used for the past decades for MRSA epidemiological typing (43). However, it is only based on the sequences of seven house-keeping genes' internal fragments to identify individual isolate lineages.

MLST

MLST is a technique that distinguishes between isolates of bacteria species by utilizing sequences of internal fragment house-keeping genes (44). The strands are sequenced on both side by using an automated DNA sequencer. Different sequences of house-keeping genes found in bacterial species are characterized as distinct alleles. In contrast, seven loci alleles address each isolate's allelic profile or sequence type. Hence, species isolates are unambiguously characterized by a series of seven integers which label the alleles at the seven house-keeping genes. The seven house-keeping genes used in MLST for *S. aureus* are the Carbamate kinase (*arc*C), Shikimate dehydrogenase (*aro*E), glycerol kinase (*glp*F), Guanylate kinase (*gmk*), Phosphate acetyltransferase (*pta*), Triosephosphate isomerase (*tpi*), acetyl coenzyme A acetyltransferase (18,24,45).

SPA typing

Spa is an important gene virulence factor that allows *S. aureus* to avoid host immune responses (46). It codes for protein A, which is found in the cell wall of *S. aureus* (47). SPA genes were replicated using PCR followed by DNA sequencing (48). This method identifies the polymorphic X region of the protein A gene (spa). Based Upon Repeat Pattern (BURP) algorithm was used, and spa types with more than five repeats were clustered into different groups, with the calculated cost between group members being less than or equal to 6 (49). Spa typing is evidently reproducible and provides interchangeable information. However, a disadvantage of this method is that it requires additional targets such as SCC*mec*, lineage-specific virulence or resistance genes or alternative polymorphic regions of the *S. aureus* chromosome. Studies included in this synthesis employed Ridom Staph Database and SPA typer tool (http://spatyper.fortinbras.us/) (24,50,51). Reports cited that spa type of t437 was more prevalent in MRSA (24). A study by Luo and colleagues showed that the most prominent spa type was t030, reported to be 15.64% (43/275) (22).

GENECUBE assays

GENECUBE (TOYOBO Co., Ltd., Osaka, Japan) is a fully automated genetic analyzer that uses PCR to amplify a target gene (21). This tool can evaluate up to eight samples simultaneously. The target DNA is amplified, and fluorescently labeled oligonucleotides are used to hybridize targets based on fluorescence intensity changes (52). Data are automatically obtained on the GENECUBE monitor after completion of the assay. The advantage of this assay is that it is time efficient and easy to prepare. GENECUBE tests are anticipated to be clinically valuable for effectively identifying MRSA. Studies have reported the sensitivity and specificity

of the GENECUBE to be 100% (33). The system is accurate, rapid (52 minutes), and reliable; however, it does not detect the *mec*C gene (21).

SCCmec typing

SCC*mec* is a diagnostic method that divides SCC*mec* elements into groups based on their structural variations (53). The *mec* complex, which comprises the *mec* gene, its regulatory genes, the *mec*I and *mec*R1 genes, and several insertion sequences, confers methicillin resistance (54,55). The specific SCC*mec* type is determined by combining the *ccr* gene complex and the *mec* gene class. SCC*mec* typing provides valuable information about the resistance of genes to methicillin and identifies the origin of strains. A recent study by Chongtrakool et al (56) typed SCC*mec* of methicillin-resistant *S. aureus* strains isolated in 11 Asian countries. Another study showed that 610 of 615 (99.2%) MRSA strains could be classified into four SCC*mec* elements: type 3A, 370 strains; type 2A, 207; type 2B, 32; type 1B, 1 strain. This study on pandemic MRSA clones in Asia reported the ST59-SCCmecIVa as the most prevalent MRSA clone (15). A study by Chen and colleagues that used the web-based SCC*mec*Finder reported that this technique is efficient for detecting MRSA (43). SCC*mec*Finder is a web-based tool for SCC*mec* typing using whole-genome sequences (https://cge.cbs.dtu.dk/services/SCCmecFinder/, accessed on January 11, 2023). The SCC*mec*Finder website uses read data for whole-genome sequencing or preassembled genome/contigs to determine homology to the complete cassette in prediction of *SCCmec* types, *mec* complex and J regions (57).

Discussion

This meta-narrative review reports the commonly used molecular methods for the detection of MRSA in the past 5 years. This review has also summarized the advantages and disadvantages of each technique included in this synthesis.

S. aureus is a common cause of community and hospital-acquired infection (58,59). The WHO has regarded it as one of the primary clinical concerns, due to the global recognition of MRSA as a public health issue and the antibiotic resistance pattern of MRSA (60). The primary issue with MRSA is the incidence of multidrug resistance, which remains high (61).

The *mec*A encodes penicillin-binding protein 2a (PBP2a), which is an enzyme responsible for crosslinking peptidoglycans in the bacterial cell wall (62). The low affinity of PBP2a for β-lactams leads to resistance to β-lactam antibiotics, including penicillins, cephalosporins (except ceftaroline and ceftobiprole) and carbapenems (63). Recent reports have reported growing resistance to clindamycin and levofloxacin, necessitating an effective treatment.

The virulence factor of *S. aureus* is multifactorial and depends on a variety of toxins, adhesion, immune evasion and other virulence characteristics (64). Evaluation of the virulence factor is an effective method of predicting how these bacteria would behave in the host, enabling prediction of the onset and progression of an infection. The first stage of staphylococcal infection is when the bacterial cells connect to the host's tissues. The surface-exposed proteins, MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), are made by *S. aureus*, which functions to attach to one or more host extracellular matrix (ECM) components, such as laminin, elastin, fibrinogen, fibronectin and collagen (65,66). The extracellular adherence protein (Eap) produced by *S. aureus* is a member of the SERAMs (secretable expanded repertoire adhesive molecules) family, binds to ECM glycoproteins, including fibronectin, fibrinogen, sialoprotein and collagens (67). This protein is involved in the internalization of bacteria and the adherence of *S. aureus* to fibroblasts. Proteases are crucial virulence factors for *S. aureus* and can cleave host proteins to enable MRSA cells to change from an adhesive to an invasive phenotype.

Early diagnostic and therapeutic intervention in patients with MRSA infection risk factors is essential (68). Treatment with empiric antibiotics against MRSA should not be delayed in the event that MRSA infection is diagnosed. Molecular diagnostic tests can robustly identify staphylococcal species in clinical samples, thus improving antimicrobial stewardship (69).

In this review, multiple molecular methods such as PCR, DNA sequencing, Xpert MRSA/SA BC array, MALDI-TOF, MLST, SPA typing and SCC*mec* typing, have been appraised. This review summarizes that PCR technique has been widely used for the diagnosis of MRSA within the last 5 years (2017-2022).

PCR technique is frequently and commonly used to detect *S. aureus* and it identifies a single-base-pair mismatch in the staphylococcal 16S ribosomal RNA gene sequence for detection (26). PCR assay is cost and labor effective and can be conducted within a short period of time (70,71). However, studies have reported that different target genes may impact the specificity and sensitivity of PCR for diagnosis. The *nuc* gene has a 100% success rate (25,72). Several PCR techniques such as multiplex PCR, RT-PCR and isothermal identification have been developed to identify MRSA as a result of its rapid emergence. The *mec*A and *nuc* genes are being used due to their 100% sensitivity and 97% specificity respectively with a shorter turnaround time of 48 hours (73,74).

The second commonly used molecular techniques are SCC*mec* typing and MLST, respectively. Over the years, the structures of novel SCC*mec* have been identified and verified by molecular cloning and traditional sequencing (75). In a study by Singh-Moodley and colleagues (76), SCC*mec* typing method was used to replace multiplex PCR and was employed to classify additional un-typeable SCC*mec* elements based on *ccr* and *mec* gene complex combinations. However, this technique has been deemed highly complex because the SCC*mec* region is variable and newer types are permanently being developed. Another possible reason for using SCC*mec* typing could be its potential as a benchmark for testing for the *ccr* gene and *mec*A gene compared to other methods.

MLST is well-established and assigns alleles at multiple house-keeping loci directly by DNA sequencing. Sequence type is obtained based on the alleles identified at each of the seven loci using the SA MLST database. MLST detection of MRSA is based on the sequencing of the seven house-keeping conserved genes in the bacterial chromosome (77). MLST is also widely used due to its straightforward procedure for characterizing isolates of bacterial species (78). Due to

numerous alleles in each of the seven loci, it is unlikely that two isolates will have the same allelic profile. Instead, isolates with the same allelic profile can be identified as belonging to the exact clone. MLST has several advantages: (1) it uses sequence data to detect changes at the DNA level; (2) it is readily reproduced and does not require specialized reagents or training; (3) it does not require high-quality genomic DNA; and (4) the data generated are fully portable (79). The disadvantage of MLST is that it only uses seven genes, limiting its ability.

DNA microarray and Xpert MRSA/SA BC assay are the least used in the last 5 years. DNA microarray contains covalently immobilized probes specific for about 180 genes and 300 alleles of *S. aureus* (80). It allows simultaneous detection of the presence of numerous genomic loci. Studies have reported that DNA microarray may serve as an alternate molecular typing method, offering complementary characterization of the MRSA strains. However, this technique is labor and cost extensive and a single experiment could significantly increase the budget of the experiment. Subsequently, many probe designs are based on a sequence of relatively low specificity, sensitivity and accuracy (81).

Conclusion

This meta-narrative review has appraised and summarized molecular diagnostic methods frequently used to detect MRSA in the last 5 years (2017-2022), thus concluding that PCR technique is the most frequently used technique due to its high specificity, low cost and labor effectiveness.

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Disclosure

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