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Identification and characterization of vancomycin-resistant *Staphylococcus aureus* in hospital wastewaters: evidence of horizontal spread of antimicrobial resistance

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ABSTRACT

Antibiotic resistance has become a major threat to human health around the world, but its spread through the aquatic environment has been often overlooked. This study aimed to determine the occurrence of vancomycin-resistant *Staphylococcus aureus* in hospital wastewaters and their transmission into public water bodies in Kerala, India. A total of 113 *S. aureus* were isolated from three hospital effluents in Kerala, India. Standard disc diffusion and the strip method were used for antibiotic susceptibility testing and minimum inhibitory concentration detection. Plasmid-mediated vancomycin resistance was confirmed by plasmid curing and conjugation; resistant genes were detected by the polymerase chain reaction (PCR). Nearly 76% of *S. aureus* isolates were resistant to β-lactams, chloramphenicol, macrolides, aminoglycosides, and glycopeptide class of antibiotics. Among the vancomycin-resistant *Staphylococcus aureus* (VRSA) isolates, the prevalence rates of *van*A and *van*B resistance-encoding genes were 46.5 and 59.3%, respectively. Through the broth mating method, *vanA* gene was successfully transferred from VRSA donor to vancomycin-sensitive *S. aureus*. The study strongly indicates the contamination of water bodies with antibiotic-resistant bacteria from hospital discharges, their dissemination and possible transfer to microbes in the aquatic environment, posing a serious threat for public health.

Key words: hospital effluent, multidrug resistance, *van*A, *Staphylococcus aureus*, *van*B

HIGHLIGHTS

- High vancomycin resistance was observed in *S. aureus* isolates in the hospital effluent.
- Vancomycin-resistant *Staphylococcus aureus* (VRSA) isolates with *van*A and *van*B resistance-encoding genes have a higher chance of surviving in the sewage treatment plants.
- Horizontal transfer of the resistance gene was confirmed by conjugation.
- The VRSA isolates have a strong capacity to acquire or transfer antibiotic-resistant genes, posing a threat to public health.

INTRODUCTION

Staphylococcus aureus is one of the most common microorganisms frequently associated with various diseases, ranging from mild infections of the skin to life-threatening endocarditis, chronic osteomyelitis, pneumonia, and bacteraemia (Lowy 1998; Murray 2005). During the mid-20th century, the introduction and use of antibiotics such as penicillin and methicillin proved successful against *S. aureus* infections. However, the bacterium quickly acquired resistance to these antibiotics posing an enormous challenge to both veterinary and human health clinicians (Brouillette & Malouin 2005). Treatment for this bacterium is a concern with the emergence and spread of penicillin-resistant *S. aureus* and in turn methicillin-resistant *S. aureus* (MRSA). MRSA has become one of the most common causes of hospital-associated and community-acquired infections, and the global spread of MRSA is a matter of great concern (Grundmann *et al.* 2006). Besides, community-based MRSA has recently emerged as a potential threat, causing infections in healthy individuals with no risk factors associated with healthcare (Kluytmans-Vandenbergh & Kluytmans 2006). The antibiotic glycopeptide vancomycin has proven effective in the treatment of serious MRSA infections (McGuinness *et al.* 2017). Moreover, in the last 20 years, *S. aureus* clinical isolates with reduced sensitivity to vancomycin and less frequently, with maximum resistance to vancomycin, have emerged (Hidayat *et al.* 2006).

In 1958, vancomycin was clinically introduced to treat Gram-positive bacterial infections by inhibiting the incorporation of *N*-acetylglucosamine and *N*-acetylmuramic acid polypeptides into the growing chain of peptidoglycines. This process is interfered by D-Ala–D-Ala, which inhibits the release of terminal D-Ala and the formation of intrachain bonds. In *S. aureus*, two modes of resistance to vancomycin have been described. The first type observed in intermediate vancomycin resistance is the piling up of an additional layer of peptidoglycan where within the bacterial cell wall, many vancomycin molecules were trapped. The trapped molecules block the peptidoglycan lipid bilayer and eventually form a physical barrier to more incoming molecules of vancomycin (Chaudhari & Bajaj 2015). The second type identified in vancomycin resistance strains is the result of the acquisition of *van*A gene cluster from *Enterococcus* spp. (Saha *et al.* 2008). In Enterococci, six types of van operons that confer glycopeptide resistance were identified based on the gene sequence and organization (Reynolds & Courvalin 2005). The different operons are named by the gene, which encodes either a D-Ala:D-Lac (*van*A, *van*B, and *van*D) or a D-Ala:D-Ser (*van*C, *van*E, and *van*G) ligase for the synthesis of lowest affinity peptidoglycan precursors for glycopeptides. The D-Ala:D-Lac ligase-coding operons include genes for a two-component regulatory system (*van*R and *van*S), three resistance genes (*van*H, *van*A or *van*B, or *van*D, and *van*X), an additional gene (*van*Y), and other unknown functional genes (*van*W or *van*Z).

The use of antibiotics and the spread of antibiotic resistance in clinical settings are well-recognized issues but its environmental importance has been largely overlooked. It is the case in many nations, including those with large populations such as China and India, as well as in many countries in Africa and South America, where sales of antibiotics tend to have risen, in line with the rise of an affluent middle class (Taneja & Sharma 2019). Antibiotic resistance can emerge from mutations or the acquisition of resistance-encoding genes via horizontal gene transfer (HGT), with the latter being the most important factor in the current AMR pandemic.

Long-term exposure of microorganisms to low antibiotic concentration contributes to antibiotic resistance in pathogenic organisms. In addition, antibiotic residues existing in the hospital wastewater treatment plants and discharged through the hospital effluents increase the selection pressure; therefore, the normal microorganisms in the aquatic environment acquire resistance through different types of transfer mechanisms. These resistant bacteria spread not only in hospital wastewater, but also in municipal wastewater, urban water, and agricultural and aquaculture systems (Allen *et al.* 2010). The spread of antibiotic resistance plasmids in human pathogens is especially well studied and shows that once resistance genes have become established on successful plasmids, they may rapidly spread across different strains, species, or even genera. These genes are currently found in humans, animals, and the environment (Hartmann *et al.* 2012; Woerther *et al.* 2013). The transfer of plasmids in pathogens has been attributed to the worldwide dissemination of multiple ARGs encoding resistance to β-lactams, quinolones, aminoglycosides, tetracyclines, sulfonamides, and other drug classes, leading to the development of multidrug resistance.

In India, the hospital effluent is released into the municipal sewer system without any proper treatment (Mubedi *et al.* 2013). It is reported that India has a yearly revenue of USD45 billion from the pharmaceutical sector and ranks among the top five countries of the world with 25–300 pharmaceutical companies. India is also one of the greatest consumers of antibiotics. Antibiotic use has increased significantly in India over the last decade, with a 30% increase in per capita consumption. According to the Center for Disease Dynamics, Economics, and Policy (CDEEP) in Washington, the percentage change in total consumption between 2010 and 2020 has also been about 48% (Hindustan Times 2021). Recognizing this high rate of production and utilization, some studies suggest that 10–90% of the drug consumed is eliminated in its original form, while the rest is metabolized and/or conjugated (Kumari *et al.* 2020). The Central Pollution Control Board (CPCB), India, reported that out of 18.6% of total treatment capacity, only 13.5% of sewage is properly treated (CPCB 2017). According to Khan *et al.* (2019), the main methods of wastewater treatment used by Indian hospitals are conventional activated sludge and sand filtration. In a study conducted based on the efficiency of sewage treatment plants in South India, Prabhasankar *et al.* (2016) point out that, due to the improper treatment process, the treated hospital wastewater showed higher outlet concentrations of pharmaceutical compounds when compared with the domestic treatment plants.

Taking all these facts into consideration, we conducted a study of three direct hospital effluents to public water bodies in Kerala, India, which revealed the presence of vancomycin-resistant *S. aureus* in the samples. In particular, the study aimed to find out the sensitivity of these isolates to different antibiotics, the molecular characterization of vancomycin resistance-encoding genes, and the mode of transfer of these resistance genes by the conjugation method.

METHODS

Study area

Three prominent hospitals (H1, H2, and H3) located near water bodies in three districts of Kerala, India (Ernakulam, Kollam, and Kannur) were selected for the study. The study was conducted during August–December 2018–2019. All the hospitals mentioned in the study have a moving bed biofilm reactor (MBBR) system for sewage treatment. The hospitals released their sewage effluents into water bodies that were used for various purposes including inland fishing activities. There were no pharmaceutical industries adjacent to the water bodies.

Sample collection and processing

Sediment and water samples were collected from the sampling points, following standard procedures. Water samples were taken from the outlet pipes of the hospitals in sterilized amber-coloured 500 ml glass bottles labelled as H1, H2, and H3 and transported to the laboratory on ice within 2 h of collection. Using an Ekman Dredge sediment sampler, sediment samples were also collected from the same three sites. From each site, approximately 100–200 g of the sediment sample was collected in 50 ml sterilized tubes. Ten-fold serial dilutions of surface water in 0.9% sterile saline (NaCl) solution were made. The entire sediment sample was dissolved in 100 ml of 0.9% normal saline, followed by subsequent 10-fold dilutions. The diluted samples were spread plated in duplicate plates of Trypticase soy agar (Himedia, India) and incubated at 37 °C for 24 h. Colonies were enumerated after incubation, and colonies with various morphologies were randomly selected and transferred at least three times in order to ensure their purity. Traditional biochemical tests were carried out to identify *S. aureus* isolates, including Gram staining, catalase, oxidase, coagulase, and mannitol utilization tests (Holt *et al.* 1994).

Antimicrobial susceptibility testing and determination of minimum inhibitory concentration

The antibiotic resistance profile was determined by the agar diffusion method using 14 antibiotic discs (Bauer *et al.* 1966). Antibiotic resistance phenotypes were determined according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2017). Overnight cultures of the bacterial isolates were plated on Mueller–Hinton agar. The following antibiotic discs were used for antimicrobial susceptibility testing: azithromycin (15 μg), ampicillin (10 μg), clindamycin (2 μg), clarithromycin (15 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), moxifloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), methicillin (5 μg), oxacillin (1 μg), streptomycin (10 μg), trimethoprim (5 μg), and vancomycin (30 μg). The microdilution tube method using Mueller–Hinton broth (Himedia, India) was used for determining the minimum inhibitory concentration (MIC) of vancomycin with a concentration ranging from 2 to 1,064 μg/ml as recommended by the CLSI.

Multiple antibiotic resistance index study

The multiple antibiotic resistance (MAR) index for each isolate was determined by dividing the number of antibiotics to which the organism was resistant by the total number of antibiotics tested (Kaplan *et al.* 2005). A general indication of the possible source of an organism is provided by the MAR index.

Plasmid curing and isolation of plasmid

Plasmid curing was performed by growing the isolates that showed high resistance to vancomycin in nutrient broth (Himedia, India) containing 0.1 mg/ml acridine orange for 24 h (Chaudhari & Bajaj 2015). A loopful of broth was inoculated in vancomycin screening agar along with nutrient agar. Growth in both screening agar and nutrient agar indicates chromosomal resistance which is not cured, whereas growth only in nutrient agar shows plasmid-mediated resistance. For the complete confirmation of plasmid curing, 3–6 days of daily sub-culturing in both the agar is preferred. The bacterial plasmid was extracted in accordance with the manufacturer's instructions using a plasmid extraction kit (GeNei, Bangalore). The purified plasmid DNA was dissolved and stored at 4 °C in TE buffer.

Transfer of vancomycin resistance by the broth mating procedure

Broth mating was performed as follows (Saha *et al.* 2008). In Luria–Bertani (LB) broth (Himedia, India), pure colonies of donor and recipient cells were inoculated separately and cultivated overnight at 37 °C with shaking. These overnight cultures were diluted in a fresh medium at 1:100 and each was grown to the early exponential phase. The mating combination was prepared by adding 0.1 ml of donor cells to 0.9 ml of recipient cells. The mixture was gently whirled for a few minutes, then incubated at 37 °C for 6 h (without shaking). This was followed by plating on LB agar medium (Himedia, India)

containing 16 μg/ml vancomycin and 2.5 μg/ml ciprofloxacin. After 48 and 72 h of incubation, colonies were counted. In addition, in the presence of vancomycin plus ciprofloxacin combination, donor and recipient cells were separately plated to assess their inability to grow because the donor was sensitive to ciprofloxacin and the recipient was susceptible to vancomycin.

Molecular characterization of vancomycin resistance-encoding genes

The isolates that phenotypically showed resistance to vancomycin were screened to detect the presence of resistance-encoding genes using appropriate primers (Clark *et al.* 1993; Saha *et al.* 2008). Table 1 shows the details of the primers used and the polymerase chain reaction (PCR) conditions. The PCR was carried out in 25 μl reaction mixture containing 15.75 μl of nuclease-free water, 10× Taq buffer (2.5 μl) (100 mM Tris–HCl, pH 8.3, 20 mM $MgCl_2$, 500 mM KCl, and 0.1% gelatin), 200 mM dNTPs (0.5 μl) (dATP, dTTP, dGTP, and dCTP), 10 pmol each of forward and reverse primers (2 μl), 1.0 unit of Taq DNA polymerase (0.25 μl), and 2 μl of the template. All the reagents were purchased from Origin Diagnostics and Research, Bangalore. The PCR was carried out in a My Cycler thermal cycler (Bio-Rad, USA). The PCR products were purified and sequencing was performed with an automated ABI 3100 Genetic analyzer using the ABI BigDYE terminator method (M/s

Table 1 | List of primers used for PCR amplification

Specific gene for amplification	Primer	Primer sequence (5′–3′)	Amplicon size (bp)	PCR conditions	References
*van*A	Forward Reverse	ATGAATAGAATAAAAGTTGC TCACCCCTTTAACGCTAATA	1,032 bp	98 °C/2 min – ID 98 °C/10 s – D 50 °C/1 min – A 72 °C/1 min – PE 72 °C/5 min – FE Cycles – 35	Saha *et al.* (2008)
*van*B	Forward Reverse	GTGACAAACCGGAGGCGAGGA CCGCCATCCTCCTGCAAAAAA	433 bp	95 °C/10 min 94 °C/30 s 58 °C/30 s 72 °C/30 s 72 °C/10 min Cycles – 30	Clark *et al.* (1993)
*van*C	Forward Reverse	GAAAGACAACAGGAAGACCGC ATCGCATCACAAGCACCAATC	796 bp	95 °C/10 min 94 °C/30 s 58 °C/30 s 72 °C/30 s 72 °C/10 min Cycles – 30	Clark *et al.* (1993)
*van*HAX	Forward Reverse	ATGAATAACATCGGCATTAC TTATTTAACGGGGAAATC	2.6 kb	98 °C/2 min 98 °C/10 s 50 °C/1 min 72 °C/1 min 30 s 72 °C/5 min Cycles – 35	Saha *et al.* (2008)
*van*H	Forward Reverse	ATGAATAACATCGGCATTAC CTATTCATGCTCCTGTCTCC	969 bp	98 °C/2 min 98 °C/10 s 50 °C/1 min 72 °C/1 min 72 °C/5 min Cycles – 35	Saha *et al.* (2008)
*van*X	Forward Reverse	ATGGAAATAGGATTTACTTT TTATTTAACGGGGAAATC	609 bp	98 °C/2 min 98 °C/10 s 50 °C/1 min 72 °C/30 s 72 °C/5 min Cycles – 35	Saha *et al.* (2008)

ID, initial denaturation; D, denaturation; A, annealing; PE, primer extension; FE, final.

Agrigenome Pvt Ltd, Kochi). A BLAST algorithm was used to analyse the nucleotide sequences (https://www.ncbi.nlm.nih.gov/BLAST).

RESULTS

Identification and characterization

Samples were collected from three direct hospital effluent discharge sites located in the north, central, and south districts of the state and screened for the presence of drug-resistant bacterial pathogens. From the collected hospital effluent samples, a total of 113 *S. aureus* (69.75%) isolates were identified from the sediment, water and fish/shellfish samples screened (H1 = 59, H2 = 37, H3 = 17). The number of cultivable bacteria in the H1 samples was much higher than that in the H2 and H3 samples. The results of culture on mannitol salt agar showed that all the *S. aureus* isolates produce positive results for fermentation of mannitol, changing the colour from red to yellow. The occurrence and distribution of multidrug-resistant *S. aureus* from different locations is shown in Table 2.

Antibiotic susceptibility testing, determination of MIC, and MAR index analysis of selected isolates

The pattern of antibiotic resistance among *S. aureus* strains varied in samples collected from different discharge points. On initial testing, the growth of *S. aureus* isolates on the MHA screen agar plate with 32–64 μg/ml of vancomycin indicated possible resistance to vancomycin since no inhibition zone was noted around the vancomycin disc. The highest number of isolates was obtained from the H1 site ($n = 59$). Among them, 48 isolates were found to be resistant to several antibiotics, such as amoxicillin, ampicillin, azithromycin, clindamycin, clarithromycin, chloramphenicol, ciprofloxacin, erythromycin, methicillin, oxacillin, streptomycin, and vancomycin. However, all the isolates were susceptible to gentamicin and ciprofloxacin as determined by the disc diffusion test. Twenty-eight *S. aureus* from H2 discharge points ($n = 37$) and 10 isolates from H3 ($n = 17$) were resistant to almost all classes of antibiotics similar to H1 isolates. The antimicrobial susceptibility pattern of multidrug-resistant *S. aureus* isolated from direct hospital effluents is illustrated in Table 3.

The MICs of vancomycin for *S. aureus* isolates from different hospital discharge points were found to be 64–128 μg/ml, confirmed as vancomycin-resistant *Staphylococcus aureus* (VRSA) according to CLSI criteria. However, the MIC value increased to 1,024 μg/ml after sub-culturing the isolates in the presence of vancomycin. The 23 *S. aureus* isolates from the H1 discharge point were highly resistant to vancomycin and teicoplanin with MIC values ranging from 32 to 128 μg/ml. The 11 VRSA isolates from H2 and two isolates from H3 showed MIC values for vancomycin ranging from 32 to 64 μg/ml; among them, three isolates from H2 were resistant to teicoplanin also. The remaining isolates from H3 showed intermediate resistance to vancomycin with the MIC value of 16 μg/ml. An MAR index revealed that all the multidrug-resistant isolates had a high MAR index value of >0.9. These results confirmed that the selected isolates have originated from a clinically high risk source of contamination.

Molecular characterization of vancomycin resistance-encoding genes

A total of 86 (H1 = 48, H2 = 28, H3 = 10) VRSA isolates from the selected sites were screened for vancomycin resistance-encoding genes. The extracted plasmid was used as a template for PCR amplification of *van*HAX, *van*H, *van*A, *van*B, *van*C, *van*X, and *mecA* with appropriate primers. Amplicons of 1,032 bp for *van*A (Figure 1) and 433 bp for *van*B (Figure 2) were obtained by the PCR. Among the 86 VRSA isolates, 27 isolates from H1 and 13 isolates from H2 showed the existence of

Table 2 | Occurrence of VRSA from direct hospital effluent samples

Source	Total number of isolates	Types of samples	Total number of VRSA isolates	*vanA*-positive isolates	*vanB*-positive isolates
Ernakulam (H1)	59	Hospital effluents ($n = 42$) Sediment ($n = 17$)	48	27	32
Kollam (H2)	37	Hospital effluent ($n = 28$) Sediment ($n = 9$)	28	13	19
Kannur (H3)	17	Hospital effluent ($n = 11$) Sediment ($n = 6$)	10	Nil	Nil

Table 3 | Antimicrobial susceptibility pattern of multidrug-resistant *S. aureus* isolated from direct hospital effluents

	Number of isolates showed resistance towards antibiotics		
Antibiotics	**H1 samples (*n* = 59)**	**H2 samples (*n* = 37)**	**H3 samples (*n* = 17)**
Azithromycin	48	28	10
Ampicillin	48	28	10
Clindamycin	48	20	8
Clarithromycin	40	18	7
Chloramphenicol	39	21	10
Ciprofloxacin	0	0	0
Moxifloxacin	48	23	5
Erythromycin	36	28	6
Gentamicin	0	0	0
Methicillin	48	28	10
Oxacillin	48	28	10
Streptomycin	40	21	9
Trimethoprim	41	26	8
Vancomycin	48	28	10

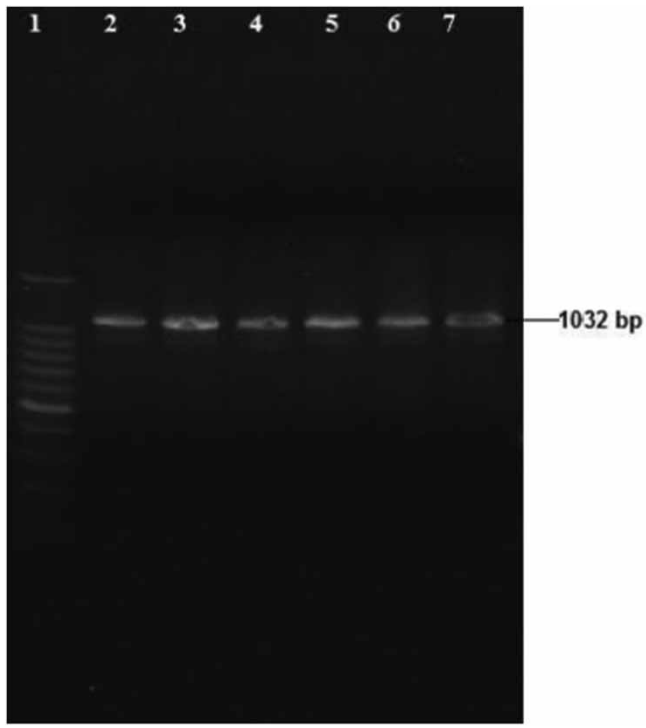

Figure 1 | Amplification of *van*A gene with 1,032 bp amplicon size product; lane 1: 100 bp molecular weight marker, lanes 2–5, samples from Ernakulam (H1); lanes 6–7: samples from Kollam (H2).

*van*A gene. The number of *van*B-positive isolates was 32 in H1 and 19 in H2, whereas none of the phenotypically resistant VRSA isolates from H3 harboured either *van*A or *van*B genes. Most of the *vanB*-positive isolates from the direct hospital effluent co-harboured *vanA* gene also.

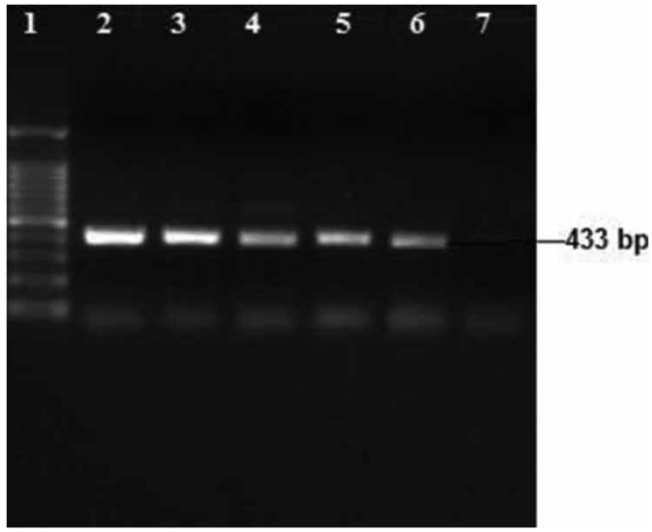

Figure 2 | Amplification of *van*B gene with 433 bp amplicon size product; lane 1: 100 bp molecular weight marker, lanes 2–4: samples from Ernakulam (H1); lanes 5–6: samples from Kollam (H2).

Plasmid analysis and transfer of vancomycin resistance-encoding genes

The plasmid profile analysis of VRSA isolates revealed that most of them carried three to four plasmids with different molecular weights ranging from 51.2, 5.5, 5.1 and 1.5 kb. The plasmid-mediated *vanA* gene was successfully transferred from donor to recipient *S. aureus* isolate by conjugation. Twenty-eight transconjugant colonies were found on LB agar with appropriate selective antibiotics, and no growth was observed in the selective medium of recipient and donor *S. aureus* when inoculated separately. The MIC of transconjugant was found to be 32 μg/ml.

Plasmid curing

Plasmid curing was performed with nine VRSA isolates that showed the presence of *van*A gene. Following 3–6 days of daily sub-culturing, an examination of the phenotypically resistance status showed that seven plasmids cured VRSA isolates lost one large plasmid of 50 kb size and these isolates became sensitive to glycopeptide as well as macrolide classes of antibiotics.

DISCUSSION

In this study, 113 multidrug-resistant *S. aureus* isolates were recovered from different hospital discharge points, clearly pointing to the improper treatment of the effluent in these hospitals. Even though all the three hospitals had an MBBR system for effluent treatment in place, it was either dysfunctional or not being used. Several studies have shown that hospitals are one of the major contributors to the emergence and dissemination of multidrug-resistant bacteria into surrounding aquatic environments (ARB) (Kalasseril *et al.* 2020) and the dissemination of these isolates is greatly influenced by the antibiotic policies of a particular country such as the manufacture of antibiotics, the dispensation of antibiotics, and inappropriate and incorrect dosing of antibiotics (Manyi-Loh *et al.* 2018). Analysing the findings of previous studies showed that one of the key routes leading to the dissemination of ARB into the environment might be hospital wastewater treatment plants (WWTPs) as suggested by Szczepanowski *et al.* (2009).

The concern about environmental antibiotic pollution in general and that of aquatic ecosystems in particular is increasing worldwide. Effluents from hospitals and even wastewater treatment plants are an important source for the release of antibiotics and antibiotic-resistant bacteria into the environment. Kummerer (2009) concluded that globally 'antibiotics are released in hospital wastewater continuously, daily, and all year round'. The aquatic environment, in particular, can act as a natural reservoir of antibiotic resistance as well as a route for the dissemination of clinical resistance traits (Michael *et al.* 2013). Due to the improper waste management, large amounts of antibiotics may be released into hospital wastewater due to the excretion of antibiotics used and the incorrect disposal of unused compounds, which can later be released into the environment. The hospital effluents contain a wide range of compounds used for medical, laboratory, and research purposes, as well as human excreta (Biobooster 2016). These biological active compounds are insoluble/soluble organic/inorganic

pollutants that have a harmful effect on people and aquatic animals even at extremely low concentrations. Pathogenic microbes such as viruses, bacteria, fungi, protozoans, and helminths are also found in these effluents (Santoro *et al.* 2015). Studies have shown that once the antibiotics reach the aquatic environment, due to their slow biodegradation, some of these drugs may persist in the environment for longer time due to bioaccumulation and biomagnification. Antibiotics in the natural environment, even in low quantities, can affect the survival, reproduction, and metabolism of aquatic organisms, as well as alter the structure of communities and ecosystem functions (Pereira *et al.* 2015; Martin-Laurent *et al.* 2019).

Kerala, India's southernmost state, is bordered on the east by the Western Ghats and on the west by the Lakshadweep Sea. The state comprises of 14 districts. Kerala is considered a pharmaceutical consumer state, with a total drug consumption of over 20,000 crores per year, and antibiotics contributing for 20% of the total drugs taken annually in the state. Although there are regulations for effluent discharge in the state, there appears to be a lack of strict implementation of these regulations and thus, most WWTP units have not been fully functional (The Times of India Report 2018; The New Indian Express 2020), as also indicated by the results obtained in the present study. The wastewater from these systems can easily contaminate the environment, allowing multidrug-resistant bacteria to spread. Extreme temperatures and high relative humidity lead to the survival of such superbugs in hospitals and the emergence of new ones in the environment, which could pose an occupational and food threat not only to fishermen and farmers, but ultimately to the wider population. The Government of Kerala has initiated the Antibiotic Stewardship Program (ASP) to regulate and promote the judicial use of antibiotics in all sectors as part of the 'One-Health' Programme (KARSAP 2018).

The presence and survival of *S. aureus* in the hospital wastewater discharge, observed in the present study, is thus, not too surprising. Thompson *et al.* (2013) reported similar results, observing elevated levels of antibiotic-resistant *S. aureus* in untreated hospital wastewaters and receiving sewage treatment plant of a metropolitan hospital in Australia. Antibiotic resistance among pathogenic bacteria is a well-documented phenomenon which has significant consequences for the treatment of infections. *S. aureus* has a unique ability to respond rapidly to any new antibiotic by developing a resistance mechanism from penicillin and methicillin to the most recent linezolid and daptomycin (Kaur & Chate 2015). Our findings were comparable with other studies which also found high levels of antibiotic resistance among *S. aureus* isolated from hospital wastewaters in Australia, North Europe and clinical samples in Damascus (Ali *et al.* 2014). Interestingly, similar to the findings obtained in our study, Saha *et al.* (2008) had earlier reported that the *S. aureus* isolated from an Indian hospital showed 100% tolerance to ampicillin, chloramphenicol, and erythromycin, but was susceptible to ciprofloxacin and gentamicin.

According to the Clinical and Laboratory Standards Institute, *S. aureus* isolates for which vancomycin MIC is 4–8 μg/ml are classified as vancomycin-intermediate (VISA), and isolates for which vancomycin MICs are greater than 8 μg/ml are classified as vancomycin-resistant isolates (VRSA). The MIC of vancomycin for VRSA isolates from different hospital discharge points was found to be 64–128 μg/ml. However, the MIC value increased to 1,024 μg/ml after sub-culturing the isolates in the presence of vancomycin which indicate that *S. aureus* isolates were becoming increasingly resistant to vancomycin compared with earlier reports (Ramana & Chaudhury 2012). This is a matter of serious concern and may pose a major problem with its use as the main drug against MRSA infections. MAR index studies revealed that all the multidrug-resistant isolates had a high MAR index value of >0.9. These results indicated that the isolates have originated from a clinically high risk source of contamination where antibiotics are often used and possibly abused, which is similar to many findings from other parts of the world (Habibi *et al.* 2008).

Of the six major phenotypes of vancomycin resistance, phenotypes of *van*A and *van*B are both common and transferable. The *van*A phenotype is highly resistant to vancomycin and teicoplanin, while *van*B phenotype has variable levels of vancomycin resistance, but not teicoplanin. We found both the vancomycin encoding genes, *van*A and *van*B, in our study among 86 VRSA isolates which showed complete resistance to vancomycin. Chang *et al.* (2003) reported a clinical isolate of vancomycin-resistant *S. aureus* with the presence of *van*A resistance-encoding gene that showed a high MIC value of 1,024 μg/ml for vancomycin isolated in United States. In India, Chakraborty *et al.* (2011) also found that VRSA strains which harboured both *van*A and *van*B genes showed complete resistance to vancomycin.

Besides, a small percentage (31.3%) of phenotypically resistant isolates did not have any common VAN resistance mechanisms. In the present study, some of the VRSA and VISA strains were negative for vancomycin resistance-encoding genes by the PCR. However, the absence of these genes in the present isolates does not rule out that these strains are not VRSA or VISA. There is another hypothesis that proposes that the thickening of cell walls is responsible for the development of vancomycin resistance. The vancomycin resistance mechanism has been studied extensively with the first clinical VRSA strain, Mu50 (Hanaki *et al.* 1998; Cui *et al.* 2000). Examination of the Mu50 cell by the biochemical test and transmission electron

microscopy indicated that it contains increased levels of peptidoglycan. Vancomycin-resistant staphylococci with thickening of cell walls were also demonstrated by Palazzo *et al.* (2005). The thickened cell wall of VRSA becomes thinner with the loss of vancomycin through sub-culturing in the absence of antibiotics. This may be the possible mechanism behind the vancomycin-resistant staphylococcal isolates that we identified, even though we were unable to perform the test for cell wall thickening demonstration in these isolates.

Vancomycin resistance was successfully transferred from *S. aureus* with the *van*A gene to vancomycin-sensitive *S. aureus* by the broth mating procedure. *van*A genes are located on mobile plasmids which can promote the transfer of this gene between different bacterial groups. The *van*A, pheromone-responsive conjugative plasmids, are anticipated to be critical for the occurrence of VRSA. The presence of sex pheromone in *S. aureus*, which promotes plasmid transfer in *Enterococcus* spp., was further demonstrated by Showsh *et al.* (2001). The *van*A phenotype can be transferred to other MRSA strains and through microbial species, with significantly greater potential for spread, even without vancomycin (Hageman *et al.* 2006). However, the detection of VRSA isolates with *van*A and *van*B genes in public water bodies in our study indicates that there is high possibility of the intrageneric transfer of vancomycin resistance from *S. aureus* strain to another.

Plasmid curing was performed with nine VRSA isolates that showed the presence of *van*A gene. Following 3–6 weeks of daily sub-culturing, an examination of the phenotypically resistance status showed that seven plasmids cured VRSA isolates lost one large plasmid of 50 kb size and these isolates became sensitive to glycopeptide as well as macrolide classes of antibiotics. It has been suggested that high levels of glycopeptides, vancomycin, and teicoplanin resistance are mediated by the *vanA* type that may be chromosomally or plasmid located (Perichon & Courvalin 2000). The results obtained agree with the study by Shriram *et al.* (2013) who proved that vancomycin resistance was mainly plasmid mediated as they became sensitive to low antibiotic concentrations after plasmid curing (Shriram *et al.* 2013).

CONCLUSION

The data obtained in this study showed the presence of multidrug-resistant *S. aureus* isolates with resistance-encoding genes in public water bodies illustrating the possible spread of bacterial pathogens from direct hospital wastewaters into natural environments. This study also points to a deeper systemic issue for a state like Kerala, where hospitals are main hotspots for resistant bacteria and its dissemination. A large number of strains was found in samples collected from the direct hospital effluent indicating their survival in the treatment plants. This study demonstrates the urgent need for the effective use of wastewater treatment plants in hospitals as part of ASPs to allow the constant monitoring of hospital wastewater discharges for the presence of antibiotic residues and antibiotic-resistant bacteria into natural aquatic environment.

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CONFLICT OF INTEREST

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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