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Detection of vancomycin resistance in *Staphylococci* by antibiotic sensitivity test and PCR

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Abstract
Globally, infection cause by methicillin-resistant *Staphylococcus aureus* (MRSA) poses a severe threat to public health. One of the first-line medications for the treatment of MRSA and other resistant Gram-positive strains infections is still glycopeptide vancomycin. However, + isolates with complete resistance to vancomycin have emerged in recent years. In present study total 135 swab samples collected from clinically infected animals in Anand, Gujarat. Total 83 *Staphylococcus* isolates isolated from 135 samples and screened for vancomycin resistance genes and vancomycin resistance patterns by disc diffusion. Out of 83 staphylococci isolates 29 isolates showed resistance by disc diffusion method whereas all the isolates were found sensitive to vancomycin by E-test. During molecular detection of vancomycin resistance gene (*vanA* and *vanB*), only two isolates found positive for *vanB* gene. This isolate was found susceptible on disc diffusion as well as E-test suggest that this gene might not be expressed for the antagonistic effects against vancomycin.

Keywords: *Staphylococcus aureus*, MRSA, VRSA, Antimicrobial resistance gene, disc diffusion, epsilometer test

1. Introduction
Staphylococcus aureus (*S. aureus*) is one of the most medically important bacterial pathogens. It was first discovered as a cause of post-operative wound sepsis in the late 1870s by Professor Sir Alexander Ogston of Aberdeen. *Staphylococcus* is Gram-positive cocci that is recognized as globally important opportunistic pathogens that can cause serious infections in humans, especially in hospitalized patients (Otto, 2009; Gilmore *et al.*, 2013 and Lee *et al.*, 2018a) [15, 9, 11]. Over recent decades, there has been a significant increase in the rates of acquired antimicrobial resistance (AMR) in these species, either through the acquisition of resistance determinants by horizontal gene transfer of mobile genetic elements (MGEs), or through mutations that alter gene expression or binding sites in native genes. This has resulted in the emergence of polyclonal lineages that are resistant to front-line therapeutic agents. In staphylococci, this includes the development and global spread of methicillin-resistant *S. aureus* (MRSA) (Enright *et al.*, 2002) [8] and the recent emergence of multidrug-resistant *Staphylococcus epidermidis* (MDRSE) (Lee *et al*., 2018b) [12].
The glycopeptide vancomycin has been regarded as the last therapeutic agent for the treatment of infections due to severe MRSA and other resistant Gram-positive strains (McGuinness *et al.*, 2017) [14]. In 2002, the first case of vancomycin-resistant *S. aureus* (VRSA) was recovered in a 40-year-old Michigan woman with diabetes (CDC, 2002). Among the glycopeptides, vancomycin is the only one used in veterinary medicine. A recent published systematic review and meta-analysis (Shariati *et al.*, 2020) [17] analysed the prevalence VRSA, vancomycin intermediate *S. aureus* (VISA) and heterogeneous VISA (hVISA) variability depending on different years and locations. First clinical case of a vancomycin-resistant *S. aureus* isolates (MIC 1024 mg/L), carrying the *vanA* vancomycin resistance operon, was reported (Chang *et al.*, 2003) [4].
Bacterial resistance to antibiotics is a threat to public health and drug-resistant bacteria could cause 10 million deaths each year until 2050. This problem has become a concern in veterinary medicine; the concept of "One Health", where human health, animal health and the environment are fully integrated, is extremely important when considering bacterial resistance to antimicrobials. Looking to the paucity of information regarding sensitivity patterns of staphylococci against vancomycin this study was undertaken to determined antibiotic sensitivity pattern of staphylococcal isolates and molecular determination for presence of genes (*vanA* & *vanB*) related to vancomycin resistance by PCR.

2. Materials and Methods

2.1 Study area

The present study was conducted between January 2021 to May 2021 for the isolation and characterization of staphylococci from different infection in animals. Where total 135 samples were collected from various location of Anand district. The research was conducted in the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Anand.

2.2 Isolation and identification of the isolates

Total 83 staphylococcal isolates (Up to Genus) were isolated on 5% sheep blood agar (BA) and mannitol salt selective media. The plates were evaluated for bacterial growth and morphological characteristics of bacterial colonies after incubation. The pure culture of each isolate was further identified based upon morphological, cultural and biochemical characteristics (catalase test, oxidase test, potassium hydroxide (KOH) string test, pigment production on nutrient agar, hemolysin production) as per method described by Cowan and Steel (2004) [5] and Markey *et al.* (2013) [13].

2.3 *In-vitro* antibiotic profile of staphylococci against vancomycin

All the staphylococcal isolates were subjected to antibacterial susceptibility test. The *in vitro* antimicrobial susceptibility tests of the isolates were conducted with minor modifications as per the method of Bauer *et al.* (1966) [12].

2.4 *In-vitro* Minimum inhibitory concentration (MIC) test of staphylococci against vancomycin

All the staphylococcal isolates were subjected to epsilometer test (E-test, procured (Hi Media Ltd, Mumbai).) for the determination of their MIC. The *in vitro* E-tests of the isolates were conducted with minor modifications as per the method of Bauer *et al.* (1966) [12]. Interpretation of the MIC was carried out as per the Clinical and Laboratory Standards Institute (CLSI) in which the vancomycin MIC break point for *Staphylococcus* spp is categorize as ≤2 mg/mL for "susceptible," 4–8 mg/mL for "intermediate," and ≥16 mg/mL for "resistant".

2.5 Detection of vancomycin resistance genes in the staphylococci

2.5.1 Extraction and quantification of bacterial DNA

The template DNA from colony was prepared according to Smyth *et al.* (2001) with minor modifications. The bacterial DNA extracted was quantified by NanoDrop spectrophotometer (NanoDrop technologies, Inc. USA) at 260 nanometer wavelengths using the conversion that one absorbance unit at 260 nm wavelength equals 50 µg DNA per ml.

2.5.2 Detection of vancomycin resistance genes (*vanA* & *vanB*)

The detection of plasmid mediated resistance genes *vanA* & *vanB* was carried out by standard uniplex PCR as described in table 1 and 2. To confirm the targeted PCR amplification, 5 µl of the PCR products from each tube were electrophoresed along with 100 bp DNA molecular weight marker (TAKARA) on 2.0% agarose (low EEO, SeaKem, USA) gels containing 0.5 µg/ml ethidium bromide (Sigma-Aldrich, USA) at 80 V in 0.5X TBE buffer. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system.

Table 1: Details of vancomycin resistance gene (*vanA* & *vanB*) primers.

Sr. no.	Primer details	Product size (bp)	Reference
1.	*VanA* F 5'- GGCAAGTCAGGTGAAGATG- 3′ R 5' ATCAAGCGGTCAATCAGTTC- 3'	713	Azimian *et al.*, 2012 [1]
2.	*VanB* F 5' GTG ACA AAC CGG AGG CGA GGA 3′ R 5′ CCG CCA TCC TCC TGC AAA AAA-3'	430	Saadat *et al.*, 2014 [16]

Table 2: Details of Van-PCR thermal cycling conditions.

Primers	Cycling conditions				
	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension
vanA (F)	94 °C	94 °C	55 °C	72 °C	72 °C
vanA (R)	5 min	1 min	1 min	2 min	5 min
		Repeated for 40 cycles			
vanB (F)	95 °C	95 °C	64 °C	72 °C	72 °C
vanB (R)	10 min	30 s	45 s	30 s	10 min
		Repeated for 35 cycles			

3. Results and Discussion

Result of vancomycin ABST (vancomycin disc, 30 µg concentration) shows that out of total 83 isolates 29 staphylococci were found to be resistant to vancomycin and 54 isolates were found sensitive to vancomycin (fig. 1). As disc diffusion is not currently recommended for vancomycin susceptibility in *S. aureus* due to misleading zone parameters for susceptible and indeterminate strains (CDC, 2015) further isolates were subjected to Epsilometer test (E-test) for the MIC determination of each isolate.

MIC determination was carried out by epsilometer test (E-test) for as per CLSI guidelines in which none of samples were found positive for vancomycin resistance (fig.2). As per international standards only MIC can be gold standard so the here this study agrees for the discrepancy created by disc diffusion method (Dunne *et al.*, 2001; Hubert *et al.*, 1999) [7, 10]. As reviewed in the current study increased prevalence was described gradually by year by year by some authors (Zhang *et al*., 2015; Shariati *et al*., 2020; Wu *et al.*, 2021) [20, 17, 19] which is not in agreement with current results of the study shows that low usage may be responsible for the low prevalence of vancomycin resistance in animal husbandry

sector.

Further for the detection of resistance genes for the vancomycin resistance were carried out by PCR with two different genes (*vanA* and *vanB*) in which out of 83 staphylococcal isolates two were found positive for the presence of *vanB* gene (fig. 3). Comparative result of disc diffusion, MIC and PCR has been shown in table no. 3. Some review study in recent year suggests that the prevalence of VRSA has been increasing threefold (2006-2014) and 1.2-fold (2015–2020) (Wu *et al*., 2021) [19]. The most frequent use of vancomycin for the treatment of MRSA infections, improved diagnostics, insufficient monitoring of definite antibiotic policy, inadequate surveillance for vancomycin-resistance, and a shift in vancomycin-resistance breakpoints since 2006, are some recent potential causes for the emergence or detection of more VRSA strains (Diaz *et al*., 2018) [6].

Table 3: Results comparison of disc diffusion, E-test and PCR.

Isolates	disc diffusion	E-test	PCR
No of resistant isolates	29	0	2
No. of sensitive isolates	54	83	81

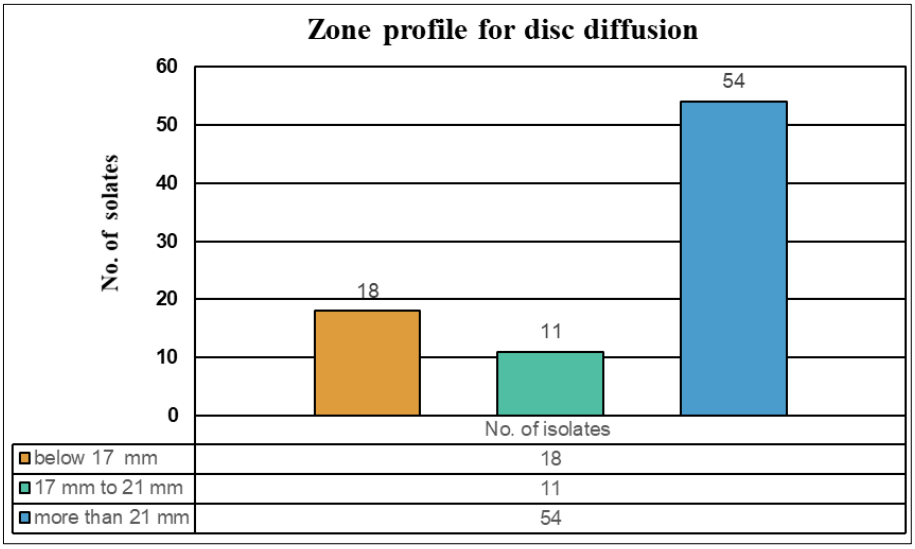

Fig 1: Zone profile for vancomycin disc diffusion method

Fig 2: Epsilometer test for the vancomycin MIC Determination.

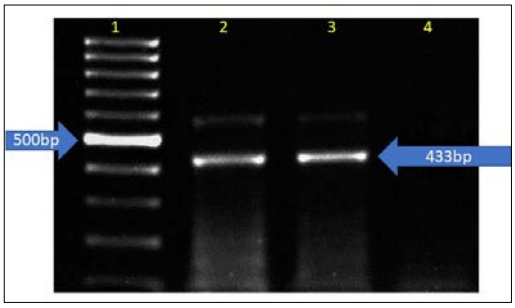

Fig 3: Amplified products for vancomycin resistance (vanB) gene with uniplex PCR. Lane 1: DNA marker 100bp, lane 2: Sample no. 32, lane 3-Sample no. 4, lane 4: Negative control

4. Conclusions

1. Discrepancy produced in the disc diffusion and MIC test suggest that MIC could be the final choice for the susceptibility testing of staphylococci against vancomycin.
2. Molecular detection was carried out in which two isolate was found positive for the presence of *vanB* gene. This isolate was found susceptible on disc diffusion as well as E-test suggest that this gene might not be expressed for the antagonistic effects against vancomycin.

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6. Conflict of Interest statement

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

7. Funding

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8. Ethical Statement

Ethical review and approval were not required for this animal

study because only blood samples collected from organised farm of cattle and buffalos.

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