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Research Article

Antibiotic Susceptibility Pattern of *Enterococcus* spp. Isolated from Poultry Feces

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ABSTRACT

Enterococci, especially *Enterococcus faecalis* and *faecium*, have emerged as an important nosocomial pathogen and represent a serious threat to patients with impaired host defenses. *E. faecalis* and *faecium* are part of the normal intestinal microbial flora of poultry and man under most conditions, they are considered as an opportunistic pathogen. In the current study, an investigation of *Enterococcus* spp. isolated from poultry feces and their antibiotic susceptibility pattern was studied, due to the worldwide attachment with poultry by human being. Samples were collected from different sites of Allahabad, India, 80 samples collected screened for the presence of *E. faecalis* and *E. faecium* and identified based on cultural and biochemical characteristics. Thirty-five isolates were identified as *E. faecalis* (57.37%), while 26 were *E. faecium* (42.62%). The pathogens isolated were tested for their susceptibility toward 10 different commonly prescribed antibiotics. Most of the isolates showed resistance toward antibiotics under study. *E. faecalis* strain suggested a higher percentage of possibility of infection estimated by 15% in comparison with *E. faecium* as it was found to be less in a screening. The high resistance rate also indicates the negative impact of the antibiotic therapy. To evaluate the extent of transmission and impact of such transmission on the effectiveness of the antibacterial use in human medicine, further study is imperative. Periodic monitoring of antibiotic resistance pattern to detect any change in it would be necessary for the effective treatment against these pathogens. *Enterococci* revealed an alarming rate of resistance to the standard antimicrobial agents used for therapy and raised MIC values to vancomycin. The importance and infection control were stressed.

Keywords: *Enterococcus faecalis*, *Enterococcus faecium*, poultry feces, vancomycin resistance

INTRODUCTION

Enterococci are members of the normal intestinal microflora in humans and animals, and they are common in environments affected by animal and human fecal material. These organisms are not considered primary pathogens, but due to their ability to acquire high-level resistance to antimicrobial agents, enterococci have emerged as nosocomial pathogens worldwide.[1] Concern has especially been focused on enterococci that show high-level resistance to the glycopeptide antibiotic vancomycin (vancomycin-resistant enterococci) (VRE), which recently has been the drug of the last resort against multiresistant enterococci and methicillin-resistant *Staphylococcus aureus*.[2]

Genetical similarities between animal and human originated enterococci have been reported and role of natural transmission of enterococci from food animals and contaminated foods to human tract cannot be ruled out.[3] Enterococci cause food intoxication through the production of biogenic amines and worrisome opportunistic infections due to the virulence traits.[4] Some strains are resistant to many antibiotics, but antibiotic resistance alone cannot explain the virulence of enterococci.[3] The differentiation of apparently safe and non-safe enterococci strains is not simple, especially due to effective horizontal gene transfer mechanisms.[5] *Enterococcus faecalis* and *Enterococcus faecium* are the most relevant species of *Enterococcus* genus with regard to clinical aspects.[6]

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Vancomycin as a definition is an antibiotic at the end of the last resort for the treatment of Gram-positive microorganisms such as MRSA and the multidrug-resistant *Staphylococcus epidermidis* [Figure 1].[7-10]

In addition to the emergence of VRE,[11] clinical isolates of MRSA strains with decreased susceptibility to vancomycin[12] (vancomycin intermediate-resistant *S. aureus*) and more recently with high-level vancomycin resistance [Figure 1].[13]

Avoparcin is a glycopeptide antibiotic that has been used as a growth-promoting agent for food animals in many countries, except the United States and Canada. In human medicine, glycopeptide-resistant enterococci (GRE) have become an increasingly serious problem in the treatment of nosocomial infections.[14] In Europe, GRE have been isolated from animals, meat, environment, and healthy humans outside hospitals.[15-17]

The widespread use of glycopeptides in hospitals has led to the emergence of VRE which is a major concern for health-care professionals. VRE is frequently reported from hospitals in the USA and Europe.[19] There is a paucity of information on vancomycin resistance in enterococci from our country.[20] Since chicken meat and products are highly consumed by human beings and influx of virulence genes from enterococci of chicken origin to human intestinal tract is a possible route; therefore, it is desirable to study the antibiotic susceptibility pattern of *Enterococcus* spp. to ensure the safe consumption of chicken and their products.[21] In this study, the incidence of *Enterococcus* spp. has been isolated, identified, and tested for their antibiotic susceptibility pattern.

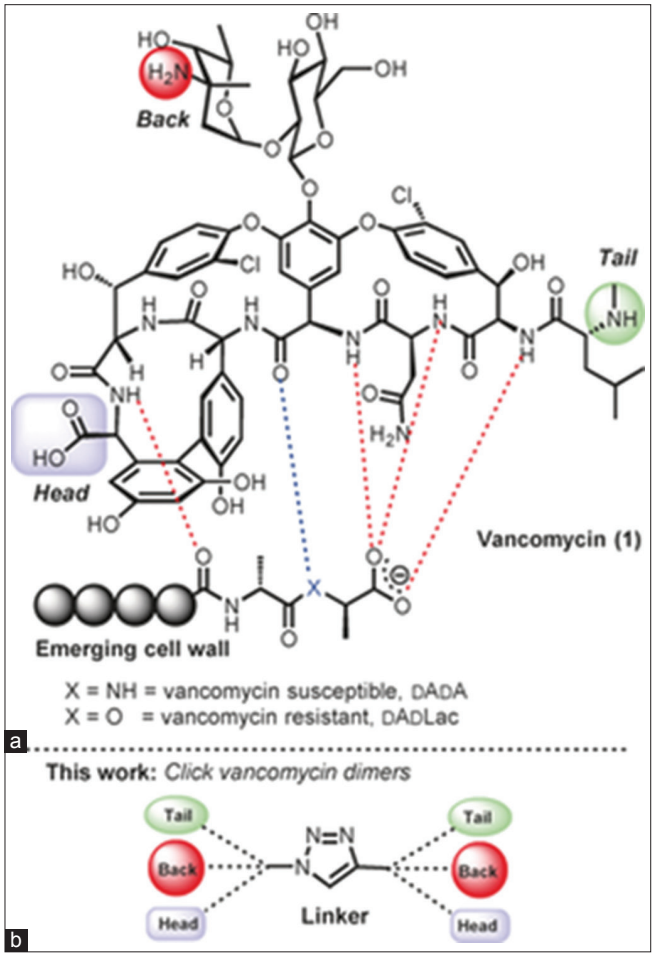

Figure 1: (a) Structure of vancomycin 1 and a binding scheme between vancomycin and a cell wall precursor analog, DADA. (b) A cartoon illustration of the type of Click vancomycin dimers[18]

MATERIALS AND METHODS

Sample Collection

Fresh poultry fecal samples were collected from (Mahewa Purab Patti, Naini, Atala, and Civil Lines) Allahabad, India, and used in the present study. A total of 80 fecal samples (20 from each site) were collected in pre-autoclaved glass sample bottles and immediately transported to the laboratory for further studies.

Isolation of *Enterococcus* spp.

About 0.5 g of each fecal sample was diluted in 4.5 ml of sterile physiological water and roughly homogenized. Serial dilutions ranking from 10^{-3} to 10^{-7} prepared. Afterward, 100 μL of each dilution has been plated in duplicate into a Bile Esculin Azide Agar (BEA) medium and incubated for 24–48 h at 30°C, pH 7.1 ± 0.2. The isolates identified using standard morphological, cultural, and biochemical tests.[22] After incubation time, samples were inspected, and those containing from 50 to 100 colonies were selected for preliminary identification based on morphological and physical characteristics like Gram staining and production of catalase, oxidase, and acid from glucose fermentation.

Morphological Characteristics

Various cultural and morphological characteristics of the isolates have been examined, namely, color, elevation, odor, and surface of the colonies on BEA agar plates.

Biochemical Tests

Slide catalase test

A glass rod has been used to pick up a colony from a culture plate and has been placed in a drop of 3% hydrogen peroxide on a glass slide. A positive catalase reaction would show gas bubbles.

Nitrate Reduction Test

This test has been used to identify whether an organism that was able to reduce nitrate into nitrite and further into ammonia or molecular nitrogen. The isolates have been inoculated into nitrate broth media and incubated at 37 ± 2°C for 24 h. Nitrate reagent (Solution A) sulfanilic acid + acetic acid and Solution B – α-naphthylamine + acetic acid added to observe the color change into red indicate positive result.

Sugar fermentation test

This test has been performed to determine the ability of *Enterococcus* spp. to ferment various carbohydrates under anaerobic condition in fermentation tube. For this purpose, basal media have been prepared and 4.5 ml of this media have been transferred into test tube along with inverted Durham's tubes and autoclaved at 121°C for 15–20 min. Different carbohydrates, namely, glucose, lactose, galactose, mannitol, sorbitol, sucrose, maltose, xylose, and dextrose have been prepared in 1% concentration in distilled water and autoclaved at 10 lbs/inch^2 separately. Then, 0.5 ml aliquot from sterile

stock solution of each sugar added in 4.5 ml of basal medium. One loopful of isolated strains inoculated in each sugar solution and incubated at 37°C for 24–48 h. After incubation, the tubes have been observed for color change and gas production. Uninoculated tube period has been taken as control.

Starch Hydrolysis Test

Starch agar media were prepared and autoclaved at 121°C for 15–20 min. Then, the plates have been streaked with the bacterial isolates and incubated at 37°C for 24 h. After incubation period, the plates flooded with iodine solution (1 M) and observed for clear zone around the colonies. Formation of clear zone around the colonies indicates positive starch hydrolysis.

Motility Test

This test has been done to check the motility of the bacteria. Tube containing motility agar has been stab inoculated and incubated at 37°C for 24 h. Growth around the stab line indicates a positive test. No growth around the stab line indicates a negative test.

Indole Production Test

Test tubes containing peptone broth will be inoculated with test organism and incubated at 37°C + 1°C for 24–48 h. After incubation, the 10 drops of Kovac's reagent will be added to it. A red color ring on the top of the peptone water indicated positive result.

Growth at various temperatures

The isolates have been streaked on the BEA agar plates and incubated at three different incubation temperatures, i.e., 10, 45, and 50°C for 24 h. After incubation period, the plates have been observed for microbial growth.

Study of antibiotic susceptibility pattern of identified bacterial isolates

The antibiotic susceptibility of the isolated and identified *Enterococcus* species has been assessed by the disc diffusion method.[23] For this test, melted and cooled Muller-Hinton agar has been poured in sterilized Petri dishes and swabbed with overnight culture of *Enterococcus* isolates. Under aseptic conditions, antibiotics placed onto the surface of inoculated plates. Following overnight incubation at 37°C, zone of inhibition for each antibiotic measured (in mm).

To determine the susceptibility pattern of isolated *Enterococcus* spp., the following antibiotics were used which are as follows: Chloramphenicol (30 μg), penicillin G (10 μg), erythromycin (15 μg), vancomycin (30 μg), teicoplanin (30 μ), ciprofloxacin (5 μg), gentamycin (10 μg), streptomycin (10 μg), ampicillin (10 ˜g), and tetracycline (30 μg).

RESULTS AND DISCUSSION

Incidence of *Enterococcus* spp. isolated from Different Sites in Allahabad City

In the present study, among 80 samples collected from poultry feces of Allahabad region, 61 bacterial isolates were obtained, of which 35 were identified as *E. faecalis* (57.38%) and 26 were *E. faecium* (42.62%) [Figure 2]. The percentage incidence of *Enterococcus* spp. was, however, found to be observed that there is a significant difference in primary screening of *E. faecalis* strain, suggesting a higher percentage of possibility of infection estimated by 15% in comparison with *E. faecium* as it was found to be less in a screening, isolation, and most importantly the pathogenicity of it at their level of opportunistic pattern in contrast with *E. faecalis*. Among both *E. faecium* and *E. faecalis*, some strains produced β-hemolysin with the incidence of this trait being higher for *E. faecalis* strains (21.3%) than for *E. faecium* strains (8.3%). Hemolysin plays an important role in enterococcal virulence, as it may increase the severity of the infection [Table 1].[24]

The identification of enterococci isolated from the commercial poultry production environment did not reveal any unusual species, although eight isolates require more discriminate analysis before definitive identification. While multiple isolates were occasionally recovered from the same sample, the elimination of isolates with indistinguishable antibiograms from the same farm provided a collection that was conservative in its estimation of diversity but did not substantively affect the relative proportions of species isolated.

Antibiotic Susceptibility Pattern

The antimicrobial susceptibility test was performed by disc diffusion method or Kirby–Bauer technique.[23] *E. faecalis* was tested for their sensitivity against various antibiotics. Most of the antibiotics were found to be sensitive, vancomycin (30 μg) was resistance (10%), and some of them were found to be intermediately resistance against *E. faecalis* [Table 2].

E. faecalis was found to be sensitive to five antibiotics, namely, penicillin G (10 μg), erythromycin (15 μg), ciprofloxacin (5 μg), streptomycin (10 μg), and tetracycline (30 μg), (50%). It was found to be resistant to vancomycin (30 μg) (10%). It was found to be intermediately resistant to gentamycin (10 μg), ampicillin (30 μg), teicoplanin (10 μg), and chloramphenicol (30 μg) (40%) [Figure 3]. Among the antibiotics, vancomycin, an inhibitor of cell wall synthesis is of major concern as it is one of the last antibiotics broadly effective against clinical infections caused by multidrug resistance pathogens.[25]

E. faecium was found to be sensitive to penicillin G (10 μg), ciprofloxacin (5 μg), and tetracycline (30 μg), and it was resistant vancomycin (30 μg) and streptomycin (10 μg). Furthermore, it was found to be intermediately resistant to chloramphenicol

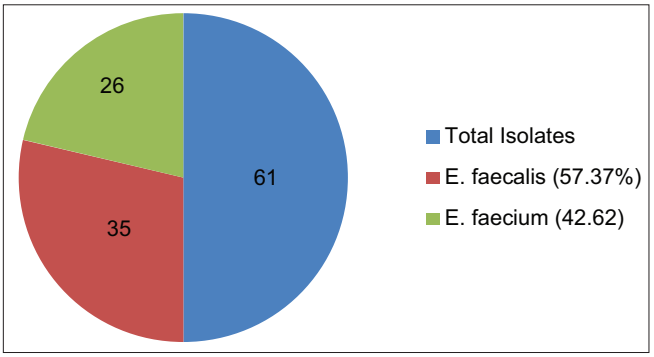

Figure 2: Incidence of *Enterococcus* spp. percentages isolated from different sites of Allahabad city

Table 1: Morphological characteristics and biochemical tests of *Enterococcus spp.*

Parameter	*Enterococcus spp.*	
	E. faecalis	***E. faecium***
Cultural characteristics	Entire, flat, non-mucoid, white	Transparent, elevated, smooth, and entire margin
Gram reaction	+ve	+ve
Shape	Cocci	Cocci
Arrangement	Monococci	Monococci
Growth at	+ve	+ve
10°C		
40°C		
50°C		
Growth at pH 9.6	+ve	+ve
Biochemical tests		
Nitrate residuals	+ve	+ve
Catalase	+ve	+ve
Starch hydrolysis	−ve	−ve
Indole test	−ve	−ve
Motility	−ve	−ve
Esculin hydrolysis	+ve	−ve
Sugar fermentation		
Glucose	A^+G^-	A^-G^-
Lactose	A^-G^-	A^-G^-
Maltose	A^-G^-	A^-G^-
Sucrose	A^-G^-	A^-G^-
Mannitol	A^+G^-	A^-G^-
Galactose	A^-G^-	A^-G^-
Dextrose	A^+G^-	A^+G^-
Sorbitol	A^-G^-	A^-G^-
Xylose	A^+G^-	A^+G^-

A^+: Acid positive, A^-: Acid negative, G^+: Gas positive, G^-: Gas negative, *E. faecalis: Enterococcus faecalis, E. faecium: Enterococcus faecium*

Table 2: Antibiotic susceptibility pattern

Antibiotics	*Enterococcus* spp. clear zone (mm)			
	E. faecalis		***E. faecium***	
Chloramphenicol (30 µg)	15	++	12	++
Penicillin G (10 µg)	26	+++	19	+++
Erythromycin (15 µg)	24	+++	17	++
Vancomycin (30 µg)	10	+	10	+
Teicoplanin (30 µg)	12	++	17	++
Ciprofloxacin (5 µg)	21	+++	21	+++
Gentamycin (10 µg)	12	++	12	++
Streptomycin (10 µg)	18	+++	10	+
Ampicillin (10 µg)	15	++	15	++
Tetracycline (30 µg)	23	+++	24	+++

+++: Sensitive, ++: Intermediate, +: Resistant. Degree of inhibition = +: Moderate inhibition zone (6–9 mm), ++: Strong inhibition zone (10–14 mm) +++: Very strong inhibition zone (15–18 mm or above), –: No inhibition zone, *E. faecalis: Enterococcus faecalis, E. faecium: Enterococcus faecium*

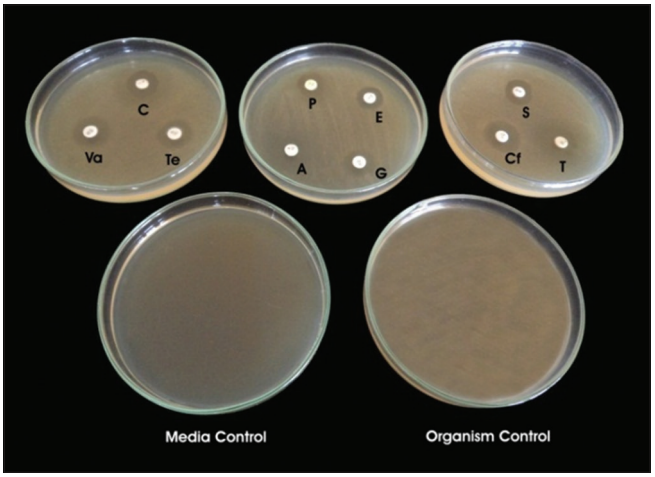

Figure 3: Antibiotic susceptibility pattern of *Enterococcus faecalis* isolates

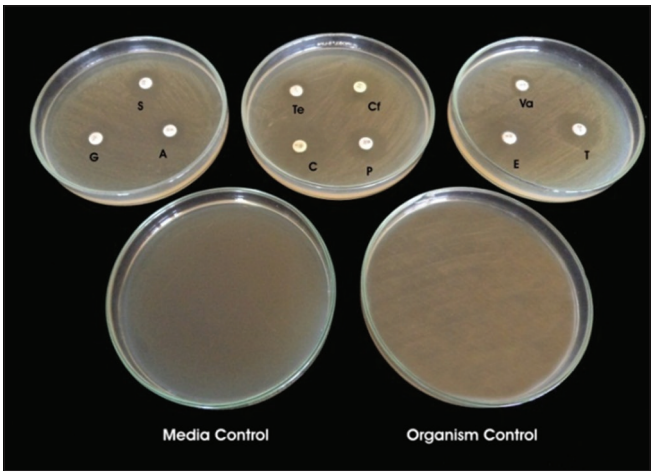

Figure 4: Antibiotic susceptibility pattern of *Enterococcus faecium* isolates

(30 µg), ampicillin (30 µg), teicoplanin (10 µg), gentamycin (10 µg), and erythromycin (15 µg) [Table 2]. Conjugal transfer of vancomycin resistance genes from enterococci to other Gram-positive bacteria has been accomplished *in vitro* [Figure 4]. The Gram-positive organisms include Group A and viridans group streptococci, *Listeria monocytogenes,* and *Staphylococcus aureus.*[26] This gives rise to concern that such transfer in humans under natural conditions might be feasible.

Ten (20.8%) *E. faecium* strains and 6 (12.8%) *E. faecalis* strains were susceptible to all antibiotics tested. All *E. faecalis* strains and all but one *E. faecium* strain were susceptible to vancomycin. *E. faecium* strains were mostly resistant to vancomycin (30 µg) (2.1%) and streptomycin (4.2%). While ciprofloxacin (56.3%), followed by penicillin (45.8%), erythromycin (27.1%), chloramphenicol (10.4%), tetracycline (6.3%), and gentamicin (2.1%) showed both intermediate and sensitive activity and none were resistant against ampicillin [Table 2]. In contrast, *E. faecalis* strains were mostly resistant to

vancomycin (30 μg) (2.1%) while chloramphenicol (63.8%), followed by streptomycin (46.8%), tetracycline (44.7%), erythromycin (31.9%), ciprofloxacin (27.7%), gentamicin (25.5%), penicillin (12.8%), and ampicillin (2.1%) were found to be intermediate and sensitive.

The present results of thesis study showed that a larger number of *E. faecium* strains than *E. faecalis* strains were resistant to vancomycin, which may be explained by *E. faecium* being generally more resistant to vancomycin than *E. faecalis*. The incidence of streptomycin and vancomycin resistance for both *E. faecium* and *E. faecalis* was low, indicating that most of the strains tested did not acquire resistance determinants for these antibiotics. In a study of European cheeses, Teuber (1999)[27] also reported a low (4%) incidence of VRE. While the incidence of aminoglycoside-resistant enterococci was low among *E. faecalis* isolates, it was considerably higher for *E. faecium*. These results indicate that especially *E. faecium* strains with acquired, high-level aminoglycoside resistance can occur in traditional cheeses.[28] Frank (1996)[29] reported that isolates were found in 36 poultry flocks and 10 pig herds and among two of the human isolates, whereas no vancomycin-resistant *E. faecium* isolates were found among the isolates from calves.

The pathogens isolated were tested for their susceptibility toward commonly prescribed antibiotics, namely, chloramphenicol, penicillin G, erythromycin, vancomycin, teicoplanin, ciprofloxacin, gentamycin, streptomycin, ampicillin, and tetracycline. From the present study, the following observations were made in incidence of *E. faecalis* in poultry feces samples which were maximum (57.37%) and minimum in *E. faecium* (42.62%). Most of the isolates showed resistance toward a few of antibiotics, suggesting that vancomycin (30 μg) (2.1%) and streptomycin (4.2%) cannot be used against *E. faecium* and *E. faecalis*. The high resistance rate also indicates the negative impact of the antibiotic therapy. To evaluate the extent of transmission and impact of such transmission on the effectiveness of the antibacterial use in human medicine, further study is required. Periodic monitoring of antibiotic resistance pattern to detect any change in it would be necessary for the effective treatment against these pathogens. The presence of high-level resistance to vancomycin eliminates a valuable therapeutic option in the management of serious enterococcal infections. Resistance to glycopeptides in these organisms is caused by synthesis of modified bacterial cell wall precursors that demonstrate decreased affinity for vancomycin and teicoplanin.[30] Enterococci revealed an alarming rate of resistance to the standard antimicrobial agents used for therapy and raised MIC values to vancomycin. The importance and infection control are stressed.

CONCLUSION

The total isolates of *E. faecium* and *E. faecalis* were found to be resistant to vancomycin *E. faecium*. Vancomycin-resistant *E. faecalis* isolates were detected only among isolates from four poultry flocks. The results of this study illustrate that *Enterococcus* spp. from poultry production and processing operations are frequently resistant to multiple antimicrobials and that some of these patterns may very well reflect the use of approved antimicrobials in poultry. This work also establishes a baseline of resistance among *Enterococcus* spp. that will be useful in monitoring the dynamics of resistance longitudinally. Considering some of the current estimates of the extent of antimicrobial use in the poultry production industry for growth enhancement, the increasing potential of such an intensive agricultural operation to affect antimicrobial resistance must be weighed against the reasonable risk that the treatment of human bacterial infections may be compromised.

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