

Zoonotic Importance of Vancomycin-Resistant *Enterococcus faecalis*

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Abstract

A worst-case scenario for nosocomial infections led to the rise of vancomycin-resistant enterococci (VRE). This study assesses the occurrence of *Enterococcus faecalis* in 200 fecal samples and 133 milk samples collected from cattle and buffalo. About 70 human samples were collected as stool samples and hand swabs from animal clinics and farms. Isolation and identification of the bacteria were performed using conventional cultural techniques, and biochemical identification and were confirmed by PCR amplification. Antimicrobial susceptibility against six antimicrobial agents commonly used in humans and animals was tested using the disc diffusion method. The resistance of *E. faecalis* to vancomycin (VAN) was confirmed by PCR targeting the *vanA* and *vanB* genes. The occurrence of *E. faecalis* in cattle and buffaloes was 8.1% and 15.5% respectively. Normal milk samples showed 12.8% of *E. faecalis* while abnormal milk samples were 37.5%. *E. faecalis* infection was recorded at 25.7% in workers' contact. Antimicrobial-resistant to Vancomycin was 11.1%, 14.2%, 10.5%, and 50% in cattle, buffalo, milk, and human respectively. Resistance genes (*van A* and *van B*) were detected in all Vancomycin-resistant strains. This study showed that *E. faecalis* which is vancomycin-resistant can be discovered not only in milk and animal products but also in human contact, which is a matter of public health.

Keywords: *E. faecalis*, vancomycin-resistant, human, milk.

Introduction

The *Enterococcus* genus includes bacteria that are considered commensal, but under the right

environmental circumstances, they can also develop into opportunistic pathogens. In the digestive tracts of humans, animals, birds, insects, and

plants, commensal *Enterococcus* species are typically present. Additional environmental sources include water, soil, and food with animal origins including beef, pork, and chicken. (Domig et al., 2003). At this time, enterococci have emerged as one of the most significant nosocomial pathogens, with a high mortality rate of up to 61% (Lopes et al., 2005). Less frequently, enterococci colonize the genitourinary tract, oral cavity, and skin.

Additionally, these bacteria are well known for effectively recruiting and transferring factors that influence antibiotic resistance (Dubin et al., 2017). Several last-resort medications, including vancomycin, become ineffective against different enterococci strains (Bender et al., 2018).

According to data, (Hammerum et al., 2012), The most clinically significant species is *Enterococcus faecalis* and the strains from animals may pose a risk to humans, for instance, it is believed that some nations' human VRE outbreaks were influenced by the rise of vancomycin-resistant enterococci (VRE) as a result of excessive usage of the vancomycin derivative avoparcin as a growth promoter in farm animals (Nilsson et al., 2012). VRE prevalence in hospitals has been considered to be low, although rising rates (>10%) in stool and clinical samples have lately been recorded (Bouchillon et al., 2004).

Years after the ban, samples of animals used for food production were still yielding vancomycin-resistant enterococci, particularly those bearing the van operon (specifically the *vanA* gene) (Hammerum et al., 2012). Several genes control how resistant *Enterococcus* is to vancomycin (*vanA*, *vanB*, *vanC*, *vanD* and *vanE*). These genes are turned on when vancomycin is present, and this causes the synthesis of cell wall precursors with a low affinity for the drug (Murrery, 1998).

Until it was forbidden in 1997, the widespread use of avoparcin as a growth enhancer in the agricultural sector was linked to the frequent isolation of VRE from farm animals (Bonten et al., 2001).

The potential for enterococci to be transmitted from the infected udder to humans is another cause for concern. There may be a chance for pathogenic and antimicrobial-resistant enterococci to enter the food chain and spread to people due to the rising consumption of raw, unpasteurized milk and products made from this milk (Rocha et al., 2014).

Our study's objective is to identify instances of Vancomycin-resistant *E. faecalis* in animals, milk, and human interaction with these animals that raise concerns for the public's health.

Materials and methods

All procedures were carried out in conformity with the applicable rules

and regulations. The Faculty of Veterinary Medicine's Research Ethics Committee at Cairo University, Egypt, examined and approved the study protocol.

Preparation of the samples

Animal samples: From March to June 2019 about 200 rectal swabs samples were collected from 90 cattle and 110 buffaloes in veterinary hospitals and farms in Giza and El-Menofya, Egypt, whether they had diarrhea or appeared well.

Milk samples: seventy-five cows and 58 buffalo were used to produce either normal or abnormal milk samples. After the teat orifices were swabbed with 70% ethyl alcohol and the udder was carefully cleaned with clean, sterile water. About 15 ml of milk was aseptically taken from each quarter into a sterile screw-capped bottle after the first two streams were rejected.

Human samples: one hundred and eight stool and hand swab specimens from people in contact with animals in veterinary clinics and farms.

The purpose of the experiment was explained to every employee and before sampling, employees were instructed not to wash their hands.

All samples were transported in ice boxes to ensure a quick transfer to the laboratory at Cairo University's Faculty of Veterinary Medicine for bacteriological analysis.

Isolation and identification of the isolates: Except for milk samples, which were directly incubated for 24 hours at 37°C, all samples (rectal

swabs, stool specimens, and hand swabs) were inoculated in the brain heart infusion broth for pre-enrichment and cultured onto the surface of K.F. streptococcus agar. Before examining any distinctive colonies, the plates were incubated for 46–48 hours at 37° C. Due to the conversion of TTC to formazan, an insoluble red pigment with a range in diameter from 0.3 to 2 mm and yellow haloes, *E. faecalis* shows as red centered colonies. (*Kenner et al., 1961*).

Gram stain was used to stain the films from the pure suspicious colonies, which were then examined under a microscope. Grass-positive cocci include enterococcus. (*Cruickshank et al., 1975*).

According to (*Murray et al., 2003*), biochemical assays such as catalase, oxidase, citrate test, Growth at 45°C, 6.5% NaCl Tolerance Test, Bile Aesculin Test, Tolerance of potassium tellurite, and sugar fermentation were carried out. Finally, the polymerase chain reaction (PCR) was used to identify *E. faecalis* (*Klibi et al., 2015*).

Antimicrobial susceptibility test:

The profile of the isolates' antibiotic resistance was assessed using the Kirby-Bauer disc diffusion method. Following overnight incubation at 37 °C on Mueller-Hinton agar (Oxoid Ltd., Hampshire, UK), the inhibitory zones were quantified, and the results were interpreted in accordance with CLSI recommendations (*CLSI, 2016*). *Enterococcus faecalis* isolates were

tested against six different antibiotics at the equivalent concentrations of clindamycin (CLD), azithromycin (AZ), ciprofloxacin (CF), gentamicin (CN), amoxicillin (AML), and vancomycin (VAN). We bought the CDs from Oxoid Ltd. (Hampshire, UK).

DNA extraction: All *Enterococcus faecalis* isolates were cultivated overnight at 37 °C on brain heart infusion agar. Each plate had a single bacterial colony that was taken out and placed in 200µl of deionized distilled water, genomic DNA was extracted using the boiling process (Wang et al., 1996).

In phenotypically VAN-resistant isolates, PCR amplification of the *vanA* and *vanB* genes encoding

VAN resistance was carried out (14 isolates).

Thermo Scientific, Waltham, USA, provided the 2X DreamTaq DNA PCR Master Mix, 3 l of the extracted bacterial DNA, 25 µl of this mixture, 50 µl of nuclease-free water, and 0.5 µl of each primer at a concentration of 20 pmol for the PCR amplification. Table (1) provides a summary of the primer pairs and cycling settings used in the PCRs. A 1.5 % agarose gel (Sigma, Darmstadt, Germany) stained with 1 g/ml ethidium bromide (Sigma, Darmstadt, Germany) in 1x TAE buffer for 30 min was used to identify 15 microliters of the amplification products, which were then observed under UV light and captured on camera.

Table (1) List of primer pairs and cycling conditions for the *vanA* and *vanB* genes used in this study.

Target gene	<i>vanA</i>	<i>vanB</i>
Primer pairs	5'-GGCAAGTCAGGTGAAGATG3' 5'ATCAAGCGGTCAATCAGTTC3'	5' GTG ACA AAC CGG AGG CGA GGA3' 5' CCG CCA TCC TCC TGC AAA AAA-3'
PCR product (bp)	713	430
Cycling conditions	• Initial denaturation at 94 °C for 5 min. (40 cycles): • Denaturation at 94 °C for 1 min. • Annealing at 55 °C for 1 min. • Polymerization at 72 °C for 2 min. • Final extension step at 72 °C and 5 min. (Azimian et al., 2012).	• Initial denaturation at 94 °C for 10 min. (30 cycles): • Denaturation step at 94 °C and 30 s. • Annealing step at 50 °C and a 45 s. • Polymerization at 72 °C for 30 s. • Final extension step at 72 °C and 10 min. (Saadat et al., 2014).

Result

Table (2) the occurrence of *E. faecalis* among the samples from cattle, buffaloes, and their milk.

Animal species	No. of examined samples	No. of +ve <i>E.s faecalis</i>	Non-diarrheic		Diarrheic		Normal milk samples		Abnormal milk samples	
			No.	+ve	No.	+ve	No.	+ve	No.	+ve
Cattle	110	9(8.1%)	80	6(7.5%)	30	3(10%)	70	7(10%)	5	1(20%)
Buffaloes	90	14(15.5%)	75	10(13.3%)	15	4(26.6%)	55	9(16.3%)	3	2(66.6%)
Total	200	23(11.5%)	15(5%)	16(10.3%)	45	7(15.5%)	125	16(12.8%)	8	3(37.5%)

Table (3) The occurrence of *E. Faecalis* among the human stool specimens and hand swabs.

The examined samples number	Hand swabs		Stool samples				Total
			Non-Diarrheic samples		Diarrheic samples		
	No.	+ve	No.	+ve	No.	+ve	
70	32	8(25%)	20	3(15%)	18	7(38.8%)	18(25.7%)

Table (4) Antibiotic resistance among *E. faecalis* recovered from animals, milk, and human in this study with detection of *vanA* and *vanB* resistant genes.

<i>E. faecalis</i> isolates (no.)	Amoxycillin	Azithromycin	Ciprofloxacin	Clindamycin	Gentamicin	Vancomycin	<i>vanA</i> and <i>vanB</i> genes
Cattle (9)	3(33.3%)	2(22.2%)	2(22.2%)	4(44.4%)	6(66.6%)	1(11.1%)	Detected
Buffalo (14)	7(50%)	3(21.4%)	2(14.2%)	5(35.7%)	10(71.4%)	2(14.2%)	Detected
Milk (19)	10(52.6%)	4(21.1%)	5(26.3%)	2 (10.5%)	8(42.1%)	2(10.5%)	Detected
Human (18)	7 (38.8%)	6(33.3%)	11(61.1%)	8(44.4%)	12(66.6%)	9(50%)	Detected
Total (60)	27(45%)	15(25%)	20(33.3%)	19(31.6%)	36(60%)	14(23.3%)	

Discussion

In the environment, where they can persist and spread, enterococci are routinely isolated from sources like soil, surface waters, and uncooked plant and animal items (*Johnston and Jaykus, 2004*) and in recent years, enterococci have become significant nosocomial pathogens. Approximately 80 to 90 % of all enterococcal infections are attributed to *E. faecalis*, while *E.*

faecium is responsible for about 5% to 10 % of these infections. Their involvement in such infections has increased as a result of their capacity to develop resistance to various antimicrobial agents, which makes them difficult to treat (*Iversen et al., 2002, Kolar et al., 2008 and Dupre et al., 2003*).

According to the findings in the table (2), *E. faecalis* was found in 8.1 % of

the cow samples and 15.5 % of the buffalo samples that were tested.

These findings presupposed the presence of *E. faecalis* in animals, which may be because *enterococcus* species are commonly found outside on vegetables and in water, perhaps because of contamination by animal waste or untreated sewage (**Food and Drug Administration, 2000**).

The frequency of *E. faecalis* in the tested non-diarrheic and diarrheal animal species was (7.5 %, 10 %) in cattle, and (13.3%, 26.6 %) in buffaloes, respectively as shown in Table (2). These results show that *E. faecalis* was more prevalent in both ill and seemingly healthy animals, with a high frequency in ill animals than in supposedly healthy ones, and this is discussed as *enterococcus* species can exist in the body without causing infection where the infection is absent in healthy people (**Bonten et al., 2001**).

Due to the presence of foodborne pathogens and spoilage bacteria in the raw milk samples, consuming raw milk could pose a risk to the general public's health. In our investigation, the overall rate of *E. faecalis* isolation from abnormal milk was 37.5%, higher than the rate from normal milk (12.8%), and more isolates were found in buffalo (16.3%) than cattle (10%). These findings are remarkably similar to those of **Mahami et al. (2011)**, who looked at samples of cow milk and discovered the presence of *Enterococcus faecalis* in 10% of samples. *E. faecalis* was the major

Enterococcus in dairy products, according to investigations by **Andrighetto et al. (2001)**, **Citaki et al. (2005)**, and **Klein et al. (1998)**. These findings show that enterococci may enter milk directly from human or animal excrement or indirectly through contaminated water sources, the exterior of the animal, the milking apparatus, and the bulk storage tank (**Gelsomino et al., 2001**).

Regardless of whether the udder is infected, the presence of *Enterococcus faecalis* in milk is a sign of faecal contamination from milked animals and can happen through the hands of milkers or other human sources (**Catry et al., 2003**). These findings show that enterococci are opportunistic bacteria that can cause bovine mastitis while also being a typical component of dairy animals' gut flora (**Lasa, 2006**).

Enterococcus Pathogenicity genes are more frequently found in *E. faecalis* isolates recovered from inpatients' stool than in isolates from healthy people, suggesting that they are linked to invasiveness and virulence in humans. Numerous epidemiological studies have also frequently linked enterococcal infections with prior colonization of a patient's gastrointestinal tract (**Weinstein et al., 1996**).

E. faecalis was discovered in 25% of human hand swabs, which is consistent with **Wells et al. (1994)** who found that enterococci are frequently found asymptotically

carried on the hands of healthcare workers caring for patients and may act as a reservoir for the spread of these organisms. Additionally, **Duckro et al. (2005)** discovered that 10.6% of chances existed for the transfer of enterococci from a contaminated body or environmental site to another site via worker's hands.

According to these findings, veterinary staff members' hands are the primary method of transmission in veterinary hospitals (**Weese et al., 2004**). If the medical staff does not practice good hand hygiene, enterococci may spread to additional patients or environmental surfaces, continuing the chain of transmission (**Bhalla et al., 2004**).

The prevalence of *Enterococcus faecalis* in the human stool samples from normal and diarrheal stools is shown in Table (3). The outcomes were (15%) & (38.8%), respectively. These findings are consistent with those made by **Aarestrup et al. (2000)**, who looked at stool samples from diarrheal people and discovered that *E. faecalis* was isolated from 38.6% of samples, although **Goossens et al. (2003)** only detected *E. faecalis* in 0.3 percent of stool samples from diarrheic people. As a result, enterococci now commonly coexist with people in their vagina, mouth cavity, and gastrointestinal system. They can infect the urinary tract, circulation, endocardium, abdomen, and biliary tract, resulting in a wide range of illnesses in man (**Jett et al., 1994**).

In fact, enterococci may act as a source of genes encoding antibiotic resistance that can spread to other pathogenic bacteria via the exchange of plasmids and conjugative transposons, and as a result, may pose a global public health issue (**Arias and Murray, 2008; Hammerum, 2012**). Antibiotic resistance testing revealed high levels of vancomycin resistance in human isolates (50%). Cows (11.1%), buffalo (14.2%), and the milk samples have low rates of vancomycin resistance (10.5 %).

According to a study by **Radwan et al. (2018)**, isolates of *E. faecalis* of human origin were 70% more sensitive to the antibacterial drug vancomycin than isolates with animal origin.

The geographic location, local and national antibiotic usage policies, and usage frequency all affect enterococcus' antimicrobial resistance. Uncontrolled use of antibiotics is known to be the primary factor promoting the emergence and spread of resistant bacteria (**Burch, 2005**).

Vancomycin-resistant enterococci (VRE) colonization or infection has been linked to a number of variables, such as length of hospital stay, underlying disease (especially renal failure and neutropenia), liver transplantation, sickness severity, and the presence of feeding tubes (**Boyce, 1994**). After consuming items of animal origin, *E. faecalis* isolates from animals may serve as donors of antibiotic resistance genes

to bacteria that have evolved to live in humans (*Hammerum et al, 2012*). All isolates that were resistant to vancomycin were found to have *van A* and *van B* in our study (table 4). Animal-derived *E. faecium* isolates may not pose a direct threat to humans, but they may provide other pathogenic enterococci with genes for antibiotic resistance. In enterococci of both human and animal origin, (*Ahmed and Baptiste, 2018*) definite the identical mutations of the *vanA* gene encoding vancomycin resistance. This might represent horizontal transmission between enterococci with distinct ancestries.

According to (*Ahmed and Baptiste, 2018*) there are multi-resistant *E. faecalis* strains that include vancomycin-resistant strains, which will cause issues because of the absence of any effective therapeutic alternatives.

These results indicate that enterococci constitute a substantial portion of the prevalent bacteria in the mammalian gastrointestinal tract. Once released into the environment by human or animal faeces, they have the extraordinary capacity to tolerate or thrive in unfavorable extra enteric environments, allowing them to colonize a range of habitats. Enterococci are thus present in surface waters, soil, plants, and vegetables in addition to warm-blooded animals. They can colonise raw foods (such as milk and meat) by intestinal or environmental

contamination and multiply in these materials during fermentation due to their resistance to extreme environmental conditions such as high pH, heat, and salinity. This suggests that these bacteria may withstand common food-related conditions.

Conclusion:

The present study reported the presence of *E. faecalis* in cattle, buffalo, and their milk. Human workers contact with these animals also had *E. faecalis* in their stool specimen and hands. Antimicrobial resistance detection to isolated strains revealed vancomycin resistance. VRE in cattle, buffalo, milk, and human in contact with these animals was found to have *van A* and *van B* genes in their isolates, this might represent horizontal transmission between enterococci with distinct origins. further wide-ranging molecular epidemiological investigations are needed to ensure potential zoonotic transmission of VRE in livestock animals. Crucial intrusions to control the transmission of these antibiotic-resistant organisms are needed.

Authors' Contributions:

ZSA: Designed the study and performed the methodology and investigation. MK and FK: Drafted and revised the manuscript. ZSA, FK and MK: Data analysis. All authors have read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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الأهمية الحيوانية المنشأ الأهمية الحيوانية المنشأ للمكورات المعوية البرازية المقاومة للفانكوميسين

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أدى السيناريو الأسوأ لعدوى المستشفيات إلى ظهور المكورات المعوية المقاومة للفانكوميسين
تقيم هذه الدراسة حدوث المكورات المعوية المقاومة للفانكوميسين في 200 عينة برازية و 133 عينة
لين تم جمعها من الأبقار والجاموس. تم جمع حوالي 70 عينة بشرية كعينات براز ومسحات يد من
عيادات ومزارع الحيوانات. تم إجراء عزل البكتيريا والتعرف عليها باستخدام الطرق التقليدية للعزل
و الاختبارات الكيميائية. وتم تأكيدها عن طريق اختبار البلمرة المتسلسل. و بعد ذلك اجري اختبار
حساسية المضادات الحيوية للمعزولات لسنة مضادات للميكروبات شائعة الاستخدام في البشر
والحيوانات باستخدام طريقة نشر القرص. تم تأكيد مقاومة بكتيريا للفانكوميسين بواسطة تفاعل
vanA و vanB. البلمرة المتسلسل الذي يستهدف جينات
نسبة حدوث المكورات المعوية في الأبقار والجاموس 8.1% و 15.5% على التوالي. أظهرت عينات
اللبن الطبيعي 12.8% من بكتيريا المكورات المعوية بينما كانت عينات اللبن غير الطبيعية 37.5%. تم
تسجيل الإصابة ببكتيريا المكورات المعوية بنسبة 25.7% في العينات البشرية للعمال كانت مقاومة
مضادات الميكروبات لفانكوميسين 11.1% و 14.2% و 10.5% و 50% في الأبقار والجاموس
والحليب والإنسان على التوالي
في جميع سلالات الفانكوميسين المقاومة تم اكتشاف جينات المقاومة (van A و van B)
أظهرت هذه الدراسة أن بكتيريا المقاومة للفانكوميسين يمكن اكتشافها ليس فقط في الحليب .
والمنتجات الحيوانية ولكن أيضاً في الاتصال البشري ، وهي مسألة تتعلق بالصحة العامة