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MULTI-DRUG RESISTANCE AND PUBLIC HEALTH SIGNIFICANCE OF *ENTEROCOCCUS FAECIUM* ISOLATED FROM APPARENTLY HEALTHY AND CLINICALLY DIARRHEIC RUMINANTS IN EGYPT

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ABSTRACT

Enterococci are Gram-positive cocci found in various animate sources, living animals, and humans. The current study was designed to identify *Enterococcus spp.* from farm ruminants and define their antimicrobial resistance and virulence components to assess their potential public health concern. One hundred and sixty-four fecal samples were collected from 28 apparently healthy ruminants, as well as 136 diarrheic ones. The collected samples were laboratory-investigated via Enterococcus species standardized isolation and identification protocol. All presumptively identified isolates were further cultured on Vancomycin-resistant enterococci (VRE) chromogenic agar media, then the obtained isolates were further biochemically tested for virulence assessment. The antimicrobial resistance profile was elucidated by the disc diffusion method. The *Enterococcus spp.* discrimination was achieved via polymerase chain reaction. The results revealed the isolation of VRE from 61 diarrhea samples; (44.85%), without any VRE isolate obtained from the apparently healthy samples. Twenty-five out of the 61 (40.9%) isolates were catalase negative. Only 22 (36.1%) catalase-negative isolates were bile esculin positive. Finally, 18 (29.51%) of the bile esculin-positive were salt tolerant and beta-hemolytic on horse blood agar media. Those 18 isolates were examined for vancomycin resistance, resulting in 7 sensitive, 4 intermediate, and 7 resistant isolates. The seven vancomycin-resistant-confirmed isolates were further identified at the species level as *E. faecium*. It has concluded that the obtained *E. faecium* isolates harbored antibiotic resistance and virulence components, which elucidate their ability to be potential human pathogens and constitute public health concern.

***Keywords*:** VRE, *Enterococci*, MDR, ESKAPE, Public concern, Bovine diarrhea

INTRODUCTION

Enterococci are Gram-positive cocci that can grow and live in a variety of hostile environment. They can be found in soil, food, water, and a wide range of living animals. They comprise a substantial amount of the typical gut flora in the gastrointestinal tracts of humans and other animals (Hammerum, 2012). Van Tyne and Gilmore, (2014) and Fiore *et al.* (2019) stated that enterococci included opportunistic infections that cause severe illnesses in both immune-compromised

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humans and animals, and also implemented in food-borne infection (Vehreschild *et al.,* 2019).

Worldwide, the incidence of antibiotic-resistant bacteria is rising due to the misuse of antibiotics in medicine and as growth promoters in animal husbandry (Franz *et al.,* 1999; Muteeb *et al.,* 2023). Antibiotic-resistant bacteria are also transmitted by random mutations and horizontal gene transfer (HGT), which results in the acquisition of antibiotic-resistance genes (Hasan *et al.,* 2018). Unfortunately, enterococci became rapidly resistant to several classes of clinically relevant antibiotics, hampering effective treatment (Olsen *et al.,* 2012). The risk of passing resistance genes to the human gut microbiota is implied by the frequent isolation of antibiotic-resistant entero-cocci from fermented food products (Freitas *et al.,* 2021). Such transmission might increase antibiotic-resistant genes' prevalence and lateral transfer, thereby impairing human health.

Recently, *E. faecium* has been considered a member of the ESKAPE group; (*Enterobacter spp., Staphylo-coccus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudo-monas aeruginosa,* and *E. faecium*) pathogen. According to Lee *et al.* (2019), ESKAPE is an abbreviation for a class of potentially fatal nosocomial infections that effectively avoid the effects of antimicrobials and serve as a model for pathogenesis, transmission, and resistance. Globally, vancomycin-resistant enterococci (VRE) are the nosocomial pathogens that cause the most infections reported (Mwikuma *et al.,* 2023). Otherwise, they haven't received the same attention as other GIT commensals like *Salmonella, Shigella, Campylobacter, E. coli,* and *Staphylococci*. The threat of morbidity, mortality, and economic consequences of antibiotic resistance to public health prompted several countries to create multi-sectoral national action plans (Bhardwaj *et al.,* 2019).

However, there hasn't been much global surveillance or research on multidrug-resistant (MDR) enterococci. Additionally, recommended treatments may be less effective due to the elevated frequencies of β-lactam and glycopeptide resistance in *E. faecium* and aminoglycoside resistance in both *E. faecium and E. fecalis* (Schell *et al.,* 2020). Enterococci may be responsible for the spread of vancomycin resistance to several human and veterinary infections, which led to serious risks of treatment failure. Some of the resistant clinical isolates have come from inanimate ambient elements, human food, and food-producing animals (Madoshi *et al.,* 2018).

Although a lot of data has been gathered regarding the antibiotic resistance of enterococci isolated from clinical sources, the levels of antibiotic resistance of enterococci isolated from animals used to produce human food have not yet been fully recorded (Torres *et al.,* 2018; Torres *et al.,* 2024). It has been determined that it is necessary to evaluate the prevalence of antibiotic-resistant *E. faecium* in animals that are frequently incorporated into human food. Its inherent resistance to antimicrobial agents and its ability to acquire and share new antibiotic resistance traits cause more and more therapeutic problems. For the treatment of infections caused by vancomycin-resistant *E. faecium* (VREF) isolates, a limited arsenal is currently available. However, acquired resistance to these novel drugs; fosfomycin, linezolid, and fluoro-quinolones is possible and has been already detected in vitro and in vivo (Gousia *et al.,* 2015).

The present study has been conducted to assess the prevalence of enterococci, as well as their virulence characteristics and antibiotic susceptibility profile. Additionally, to identify the phenotypic vancomycin-resistance potentials of enterococci from ruminant farm animals,

especially cattle. This could help clarify the present and prospective public health risks and allow for additional investigation of their conjugative transfer capacities within the same and other hosts.

MATERIALS AND METHODS

Sampling site and procedures

The present study was conducted in two main Egyptian governorates, Assiut and Cairo, as they constitute the main geographical districts for dairy cows' farms in Egypt, between January and December 2023. A total number of 164 fecal samples were collected from 28 apparently healthy: (3 calves, 8 dairy cows, 3 buffaloes, 4 sheep, and 10 goats) and 136 diarrheic ones (5 calves and 131 dairy cows).

Skilled veterinarians collected samples from clinically diagnosed diarrheal animals. The veterinarian collected 10 milliliters of composite fecal samples under completely sterile conditions during a single visit to each farm. Samples were then transported to the laboratory in an icebox for microbiological investigation.

Isolation and Identification

The fecal samples were enriched in brain-heart infusion broth (Himedia, India) for 18 hr at 37°C. One hundred microliters of the enriched sample were spread onto bile esculin agar plates and incubated aerobically for 48 hours at 37°C while being monitored daily. Following streaking onto bile esculin plates, at least three colonies exhibiting characteristics of Enterococcus species were purified (Lindell and Quinn, 1975), and then the purified colonies were stored in glycerin at -70°C.

The presumptive black colonies, which have the typical appearance of Enterococcus species, were checked for morphological appearance using Gram-staining.

CHROM agar VRE plates were prepared as per the manufacturer's instructions. Each obtained isolate (the characteristic black colonies) was further streaked on CHROM agar VRE media in triplicates. As described, pink to mauve colonies represent vancomycin-resistant *E. fecalis* or *E. faecium* while blue colonies represent *E. gallinarum* or *E. casseliflavus* (Cuzon *et al.,* 2008).

The purified pinks to mauve colonies were subjected to biochemical reactions including catalase, bile-esculin, salt tolerance, and hemolysis on horse blood agar for identification (Ruoff *et al.,* 1990; Chuard and Reller, 1998; Manero and Blanch, 1999 and Bourne *et al.,* 2001).

Antimicrobial Susceptibility Testing (AST)

The obtained isolates were tested for vancomycin resistance by the disc diffusion method. The confirmed vancomycin-resistant Enterococcus spp. isolates were tested against other antimicrobials widely used to treat human and animal diseases, including diarrhea cases. The Clinical and Laboratory Standards Institute's (CLSI) guidelines were followed in the analysis and interpretation of the results (Khan *et al.,* 2022). A total of thirteen different antibiotics (HIMEDIA), belonging to 10 different antibiotic classes, were employed such as β-lactams; ampicillin 10µg (AMP) and penicillin G 10 units (P), glycopeptides; vancomycin 30µg (VA), macrolides; erythromycin 15µg (E), ansamycins; rifampicin (RIF) 5µg, phenicols; chloramphenicol 30µg (C), and tetracyclines; (tetracycline 30µg (TE) and doxycycline (DO), phosphonic; fosfo-mycin 20µg (FO), oxazolidinones; linezolid 30µg (LZ), nitrofuran; nitro-furantoin 300µg (NIT), fluoroquinolones; norfloxacin 10µg (NX) and ciprofloxacin

5µg (CIP). Each AST was performed two times to confirm and ensure the reproducibility of the results. According to the World Health Organization (WHO), isolates resistant to three or more antibiotic classes were considered multidrug-resistant (MDR) (Magiorakos *et al.*, 2012).

Molecular Identification

Polymerase chain reactions were used to detect Enterococcus species and validate the traditional techniques for isolating and identifying the genus Enterococcus. The QIAamp DNA mini-Kit was used to extract DNA from the isolates, following the manufacturer's instructions. Two microliters of 10x Taq PCR buffer, 1.6µL of 2.5 mM dNTPs mixture, 1µL of 10pmole/µL of each primer (F and R), 1.5µL template (20ng/µL), 0.2µL KOMA-Taq (2.5 U/µL), and distilled water (HPLC grade) were used to complete the amplification conditions in a 20µl reaction volume. The Emerald Amp GT PCR Mastermix (TAKARA) kit was used for the amplification of the genes of 16S rRNA of the genus Enterococcus, as well as 16S rRNA of both *E. faecium* and E. faecalis at 30 cycles (Dutka-Malen *et al.*, 1995 and Cheng *et al.*, 1997). The primer sequences, amplicon sizes, and cycling conditions for PCR are mentioned in Table (1).

Table 1: Target genes, primer sequences, amplicon sizes and cycling conditions for PCR

Specificity	Primers sequences	PCR product size (bp)	primary denaturation	secondary denaturation	Annealing	Extension	final extension
E. faecalis	F:ATCAAGTACAGTTAGTCTTTATTAG R:ACGATTCAAAGCTAACTGAATCAGT	941	94°C for 10 min	94°C for 45 sec	50°C for 45 sec	72°C for 45 sec	72°C for 7 min
E. faecium	F:TTGAGGCAGACCAGATTGACG R:TATGACAGCGACTCCGATTCC	658	94°C for 10 min	94°C for 45 sec	50°C for 45 sec	72°C for 45 sec	72°C for 7 min

Statistical analysis

All data collection, tabulation, analysis, and presentation procedures have been done using the different Microsoft Excel drop-down menu features.

RESULTS

Isolation and identification

The cultured samples onto chromogenic media revealed sixty-one VRE as pink or purple colonies (Figure 1), detected from diarrheic samples (1 calf and 60 dairy cows) without any isolates obtained from the apparently healthy samples.

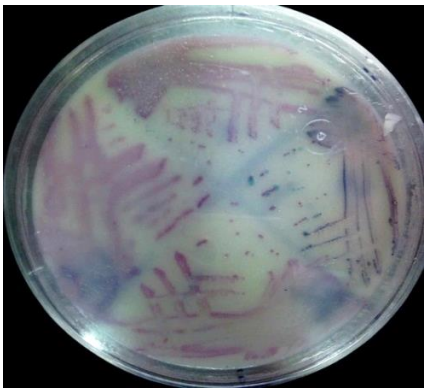

Figure 1: Pink or purple colonies of vancomycin-resistant enterococci growing on CHROM agar VRE.

A total number of 25 out of the 61 obtained isolates were catalase-negative (1 calf, 24 dairy cows). Twenty-two (1 calf and 21 dairy cows) of the catalase-negative isolates were bile-esculin positive; with the

formation of black color (Figure 2). The brief isolation and identification of obtained enterococci were mentioned in Table (2).

Antimicrobial susceptibility testing

Those 18 obtained isolates were tested for vancomycin - resistance by disc diffusion method, resulting in 7 sensitive, 4 intermediates, and 7 resistant (7 dairy cows) isolates. The seven confirmed vancomycin-resistant isolates showed different MDR antibiotic patterns, as illustrated in Figures (4 and 5).

Eighteen isolates (1 calf, 17 dairy cows) that showed the bile esculin positive were salt tolerant and beta-hemolytic on horse blood agar media (Figure 3).

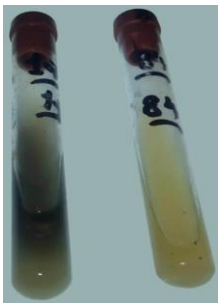

Figure 2: VRE positive colonies showing a black colour on bile-esculin media.

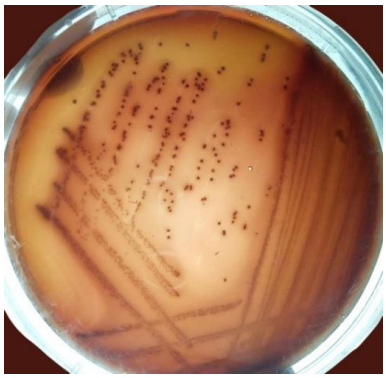

Figure 3: VRE isolates showing β- hemolysis activity on horse blood agar.

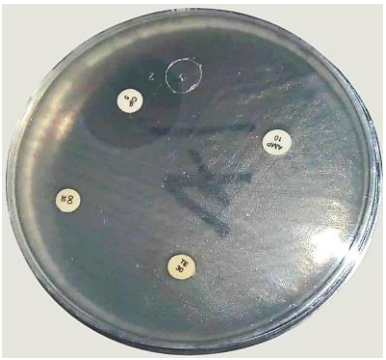

Figure 4: The antibiotic senstivity profile of a VRE isolate showing resistance to ampicillin (AMP), doxycycline (DO), and tetracycline (TE) but susceptible to ciprofloxacin (CIP).

Table 2: A brief description of obtaining enterococci isolates

Samples origin	Number of samples	Number of VRE isolates	Number of catalase-negative isolates	Number of bile-esculin positive isolates	Number of salt-tolerant and β – hemolytic isolates
Diarrheic calves	5	1	1	1	1
Diarrheic dairy cows	131	60	24	21	17
Total	136	61	25	22	18

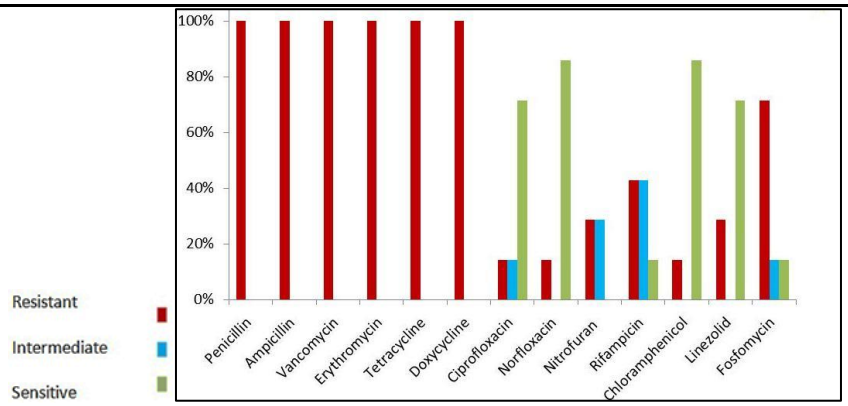

Figure 5: Antibiotics profile of the seven tested *Enterococcus* spp.

Molecular identification

The species-level discrimination of the obtained (7) vancomycin-resistant isolates to species level was confirmed as *E. faecium* by PCR amplification of 16s rRNA at 658 bp (Figure 6).

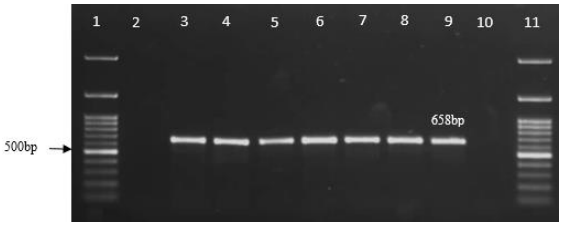

Figure 6: Agarose gel electrophoresis profile of PCR products showing the positive bands of vancomycin-resistant *E. faecium* at 658bp, lanes 1, 11 represent DNA molecular size marker, 100bp ladder then lanes 2, 10 are negative control and lanes 3-9 represent the 7 tested isolates.

DISCUSSION

In recent decades, one of the major issues for worldwide public health has been antibiotic resistance. Therefore, the transmission of antibiotic-resistant bacteria to humans is thought to be the careless or illogical use of medically significant antimicrobials in animal production (Salam *et al.,* 2023; Brüssow, 2024). Diarrhea is one of the most common causes of animal production threats, as it is difficult to treat, and dairy farms are the major users of therapeutically significant antimicrobials (Lee *et al.*, 2024).

Enterococci have become one of the most important organisms that can cause infections over time, particularly in humans, due to their acquisition of virulence factors and resistance to anti-biotics. The most prevalent enterococci species in clinical infections is *E. faecalis*; however, research on the virulence factors and antibiotic resistance of *E. faecium*, as well as its involvement in clinical bovine diarrhea, is still lacking (Aarestrup and McNicholas, 2002).

The current study was conducted to determine the incidence and antibiotic resistance profile of *E. faecium* from dairy cattle.

Our data revealed the occurrence rate of vancomycin-resistant enterococci (VRE) isolates in fecal samples that obtained from dairy cattle suffering from diarrhea was 44.85%, which is greater than previously reported rates of 22% in Turkey, 11.25% in Bangladesh, 10%, in China, 0.2% in Germany, and the United States (Ok *et al.,* 2009; Petersson-Wolfe *et al.,* 2008; Xu *et al.,* 2012; Bag *et al.,* 2022). Nonetheless, reports of higher Enterococci prevalence in clinical mastitis have come from South Korea (86.5%), Egypt (24%), and Belgium (20%) (Devriese *et al.,* 1999; Hammad *et al.,* 2022; Kim *et al.,* 2022).

The observed discrepancies in incidence may be due to geographic location and sample size, as well as sanitary conditions. However, the presence of Enterococci in milk samples is dangerous to human health, since the bacterium can be transmitted to humans by consuming contaminated meat, milk, or their products (Pesavento *et al.,* 2014).

Eighteen isolates (one calf and 17 dairy cows) that tested positive for bile esculin were salt-tolerant and beta-hemolytic on horse blood agar media.

One of the most often used glycopeptides against enterococci is vancomycin. However, with the advent of VRE, its monotherapy is no longer effective in these situations. In cases of low-level resistance, gentamicin or other aminoglycosides are advised in combination therapy with cell wall inhibitors such as penicillin and glycopeptides against enterococci (Kang *et al.,* 2021).

A previous Egyptian study mentioned the isolation rate of VRE in retail raw milk as *Enterococcus faecium* (29.1%) and *Enterococcus faecalis* (12.5%), (Hammad *et al.*, 2022). A low isolation rate of LA has been reported by Pesavento *et al.* (2014) as 3.53% from milk and meat products in Italy. Our study showed a failure of VRE isolation from apparently healthy animals. This contradicts the findings of Madoshi *et al.* (2018), who recovered fifty-eight isolates as *Enterococcus faecalis and Enterococcus faecium* at 43.5% and 38.4%, respectively in Tanzania.

Since the initial report of VRE in France, the resistance to vancomycin, which has been demonstrated to be plasmid-mediated and transferable, is the most concerning resistance feature to develop in enterococci (Devriese *et al.*, 1995; Gelsomino *et al.*, 2002; Pillay *et al.*, 2018; García-Solache *et al.*, 2019; Hashem *et al.*, 2021)

Hermanovská *et al.* (2016) stated that VRE becomes potential reservoirs and transmitters of the van genes cluster, which encode resistance to vancomycin, are identical in humans and animals. It means that animals, particularly cattle, could be a substantial reservoir of VRE for humans.

The tested 7 VRE isolates in our study showed resistance not only to vancomycin but also to other five distinct antibiotics: ampicillin, penicillin, erythromycin, tetracycline, and doxycycline.

One of the most widely used antibiotics for disease prevention and growth enhancement in animal production is tetracycline. Tetracycline-resistant bacteria emerged as a result of this antimicrobial's misuse (Grossman, 2016). Furthermore, erythromycin and tetracycline are two of the most given antibiotics for human illnesses in many regions of the world (Akhtar *et al.*, 2021). Tetracycline and erythromycin resistance in *E. faecium* can lead to treatment failure and potentially deadly infections in humans.

Rodrigues *et al.* (2022) detected a high resistance rate to tetracycline and erythromycin, (73%). While Makarov *et al.* (2022) reported intermediate resistance of enterococci isolated from cattle against erythromycin (49.3%), and tetracycline (23.4%).

Furthermore, five isolates of *E. faecium* exhibited resistance to fosfomycin (71.43%) which was harmonized with another study reported by Hathcock *et al.* (2023) which mentioned a near resistance rate of enterococci isolated from raw meat (67.86%). Contrarily, low resistance (9%) was observed in *Enterococcus faecalis* strains obtained from bovine subclinical mastitis samples in Brazil (Rodrigues *et al*., 2022).

Currently, most of the previous studies have focused on the mechanism of fosfomycin resistance in Gram-negative bacteria, but limited information regarding the mechanism of resistance in Gram-positive bacteria, especially enterococci (Xin *et al.*, 2022). Many factors, such as bacterial metabolism and the presence of corresponding genes, contribute to antibiotic resistance (Bennett, 2008; Corona and Martinez, 2013).

On the molecular base, all tested seven resistant isolates were confirmed as *Enterococcus faecium* as amplified at 658 bp. Our result was supported by findings of other studies that mentioned that *E. faecium* was the more abundant bacteria in cattle (Kühn *et al.*, 2003; Iweriebor *et al.*, 2016).

The obtained isolate of *E. faecium* represents a risk of spreading these resistance characteristics to other enterobacteria that are clinically significant in infections in humans and animals. Our results demonstrate that *E. faecium* isolates possess the traits of a possible pathogen. Furthermore, the persistence of *E. faecium*

in the lower GIT environment may be associated with these virulence characteristics. Further research into the phenotypic expression of virulence features is advised to determine these possibilities.

CONCLUSION

Enterococci are difficult to be eliminated because they can live and survive in severe circumstances. Good hygiene and thorough cleaning of animal production facilities are also necessary to reduce the number of enterococci and other zoonotic germs that contaminate meat and milk. Similarly, to prevent nosocomial infections with enterococci in the clinical context, appropriate cleaning and hand hygiene are essential. Based on the current findings, combined therapy may be recommended to control *E. faecium*-caused diarrhea due to the organism's broad panel of resistance. Additionally, due to co-resistance, additional antimicrobial drugs utilized in animal production for therapy may favor enterococci that are resistant to vancomycin. To reduce the chance of zoonotic potential resistant enterococci, antimicrobial drugs must be used carefully in animal production. As a result, antibiotic resistance is a global issue. Therefore, to prevent the emergence of resistance, a global strategy on the sensible use of antimicrobial drugs for both human and animal illnesses is needed, and all nations should cease using antimicrobial medicines for growth promotion.

Ethics approval

The animal-related procedures followed the Ethics Committee of the Faculty of Veterinary Medicine, Cairo University guidelines. Also, there were no invasive techniques, or any level of pain applied to the animals involved in the current work.

Competing interests

All authors declare that there are no competing interests.

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المقاومة المتعددة للأدوية وأهميتها للصحة العامة لبكتريا المكورة المعوية الغائطية *Enterococcus faecium* المعزولة من المجترات السليمة ظاهريًا والمصابة بالإسهال في مصر

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المكورات المعوية هي بكتيريا إيجابية الجرام توجد في مصادر مختلفة مثل التربة والغذاء والماء، وكذلك في مختلف الحيوانات الحية والبشر. صُممت الدراسة الحالية لتحديد أنواع المكورات المعوية من المجترات التي تبدو سليمة ظاهريا أو التي تعاني من الإسهال وتحديد مقاومتها للمضادات الحيوية ومحددات ضراوتها لتقييم أهميتها المحتملة على الصحة العامة. تم جمع مائة وأربعة وستين عينة براز من ٢٨ مجترة سليمة ظاهريا (٣ عجول، ٨ أبقار حلاب، ٣ جاموس، ٤ أغنام، ١٠ ماعز) بالإضافة إلى ١٣٦ حيوان مصاب بالإسهال (٥ عجول، ١٣١ بقرة حلاب). تم فحص العينات التي تم جمعها في المختبر من خلال بروتوكول العزل والتعريف الموحد لأنواع المكورات المعوية. تم تحديد جميع المعزولات المفترضة على أنها انتيروكوكس. تمت زراعتها بشكل أكبر على وسط أجار كروموجيني مخصص لنمو العترات المقاومة للفانكومايسين (VRE)، ثم اختبرت المعزولات الناتجة عن طريق انتاج انزيم الكاتالاز وزراعتها على وسط أجار الدم وإسكولين الصفراء لتقييم الضراوة. تم استبيان وضع مقاومة مضادات الميكروبات للمعزولات الناتجة وفقًا لإرشادات CLSI. تم تحقيق التمييز بين أنواع المكورات المعوية من خلال تفاعل البوليميراز المتسلسل. كشفت النتائج عن عزل المعزولات المقاومة للفانكومايسين VRE من ٦١ عينة إسهال؛ (عجل واحد وستين بقرة حلاب ٤٤.٨٥٪)، دون أي عزل من العينات السليمة ظاهريا. كانت خمسة وعشرون من أصل واحد وستين عزلة تم الحصول عليها سلبية للكاتالاز (عجل واحد، ٢٤ بقرة حلاب)، ومنهم فقط ٢٢ عزلة سلبية الكاتالاز كانت إيجابية لإسكولين الصفراء (عجل واحد و٢١ بقرة حلاب) بينما ١٨ (عجل واحد و١٧ بقرة حلاب) من المعزولات الإيجابية لإسكولين الصفراء كانت إيجابية لمرق الصويا التربسي ٦.٥٪ (TSB) وموجبة للانحلال الدموي بيتا على وسط أجار دم الخيل. تم اختبار هذه المعزولات الـ ١٨ لمقاومة الفانكومايسين بطريقة انتشار قرص المضاد الحيوي مما أدى إلى ٧ معزولات حساسة و٤ متوسطة و٧ مقاومة (كلها من أبقار حلاب). تم التعرف على المعزولات المؤكدة المقاومة للفانكومايسين على مستوى الأنواع على أنها مكورات معوية غائطية *E. faecium* عن طريق تفاعل البوليميراز المتسلسل. وخلصت الدراسة إلى أن معزولات ال *E. faecium* التي تم الحصول عليها تحمل مقاومة للمضادات الحيوية ومحددات الضراوة مما يفسر قدرتها في أن تكون مسببات أمراض بشرية محتملة وتشكل مصدر تهديد للصحة العامة.