High prevalence of vancomycin non-susceptible and multi-drug resistant enterococci in farmed animals and fresh retail meats in Bangladesh

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Abstract
The emergence of antimicrobial resistant Enterococcus spp., a main cause of untreatable nosocomial infection, in food animals and dissemination to humans is a public health risk. The study was performed to determine the prevalence and antimicrobial resistance, and virulence characteristics of Enterococcus faecalis and Enterococcus faecium in food animals and meats in Bangladesh. Enterococcus spp., were confirmed using sodA gene specific PCR, and antimicrobial resistance and virulence properties were characterized by PCR. Enterococcus spp. were recovered from 57% of the collected samples (n = 201/352). Farm samples yielded significantly higher (p ≤ 0.05) prevalence (62%) than that of retail meat samples (41%). Enterococcus spp. were recovered from 57% of the collected samples (n = 201/352). Farm samples yielded significantly higher (p ≤ 0.05) prevalence (62%) than that of retail meat samples (41%). E. faecalis (52%) is most frequently isolated species. Greater proportions of isolates exhibited resistance to tetracycline (74%), erythromycin (65%) and ciprofloxacin (34%). Fifty-one isolates are vancomycin non-susceptible enterococci (VNSE), of which forty-seven are MDR and twenty are linezolid resistant, a last line drug for VNSE. Virulence factors such as gelatinase (gelE), aggregation factor (asa1) and sex pheromone (cpd) are detected along with vancomycin resistance gene (vanA, vanB and vanC2/C3) in VNSE isolates. The high prevalence of MDR enterococci in food animals and retail meats may cause consumers infections with concomitant reduction of available therapeutic options.

Keywords Enterococcus spp. · Food safety · Livestock · Multi-drug resistance · Virulence · Antimicrobial resistance

Introduction
Enterococci are Gram-positive bacteria belonging to the lactic acid bacterial group which consists of over 50 diverse species, and inhabit gastrointestinal tract of humans and a wide variety of warm-blooded animals as well as insects (Santestevan et al. 2015). Farm animals (i.e. chicken) harbouring a range of Enterococcus spp. is itself a target of many diseases; such as, arthritis, spondylitis, femoral head necrosis, osteomyelitis, lameness and paralysis, endocarditis, bacteraemia, encephalomalacia, focal necrosis of the brain, ascites and pulmonary hypertension (Velkers et al. 2011).
The spectrum of enterococci-caused infections in humans includes urinary tract infections, peritonitis, endocarditis, neonatal sepsis or even life-threatening infections such as septicaemia and meningitis, and with about 80–90% of all enterococcal clinical infections being caused by Enterococcus faecalis and Enterococcus faecium (Arias and Murray 2012). Additionally, enterococci are often used as indicators of fecal contamination in food and water, and are better able than coliforms to survive in harsh environments (Lebreton et al. 2014).

Enterococci have low susceptibility to beta-lactams and intrinsic resistance to several antimicrobial classes including sulphonamides, cephalosporins, and low concentrations of aminoglycosides. Indeed, enterococci are able to acquire and exchange genetic elements, including antimicrobial resistance (AMR) genes, and thereby serve as reservoirs of transferable resistance circulating in Gram positive bacteria (Hollenbeek and Rice 2012; Wang et al. 2015). Acquisition of resistance to antimicrobials such as vancomycin and linzolid in enterococci is of considerable clinical concern, and is the development of multi-drug resistance (MDR) which could restrict treatment options. Vancomycin resistance is conferred by van genes such as vanA, vanB, vanC1, vanC2/ C3, vanD, vanL, vanM and vanN (Ahmed and Baptiste 2018). Epidemiological studies have described the prevalence of MDR enterococci in different sources including human, animal, food of animal origin and even in environmental sources such as soil and surface water (Bennani et al. 2012; Cassenego et al. 2011; López-Salas et al. 2013; Torres et al. 2018). The emergence and rapid spread of MDR poses a serious therapeutic challenge for effective antimicrobial therapy in human infections, due to scarcity of newer antimicrobial agents as well as fewer remaining therapeutic options caused by Gram positive organisms (Aslam et al. 2012; Wang et al. 2015). Enterococcal strains having virulence genes colonized in chickens are not only at risk of infection, but are also a potential source of microorganisms spreading to the environment and humans. This is extremely probable due to human involvement in many phases of chicken hatching and farming (e.g., individual immunization, hand separation of chicks, and flock monitoring throughout rearing—contact with bioaerosol) or the risk of transmission via the food chain (Stępień-Pyśniak et al. 2021). An additional concern is the presence of virulence genes and diverse virulence factors have been reported in enterococci that increase their ability to colonize hospitalized patients and contribute to infections in humans (Mannu et al. 2003).

Antimicrobial resistant enterococci have been observed in food animals and food of animal origin, such as poultry, duck, swine and cattle (Vignaroli et al. 2011), which is an indication of fecal contamination; meat products are therefore at risk of becoming contaminated during the slaughtering process (Boehm and Sassoubre 2014; Tyson et al. 2018; Wang et al. 2015). The literature suggests food animals as reservoir of antimicrobial resistant enterococci, and contaminated products could enter the food chain (Aslam et al. 2012; Hammerum et al. 2010; Hoelzer et al. 2017; Ogier and Serror 2008) in the absence or lack of strict food safety measures in place. Moreover, enterococci from farm animals and food of animal origin can transfer their resistance genes, located on mobile genetic elements, to human endogenous flora as well as transient bacteria, including pathogens (Vignaroli et al. 2011).

The irrational use of antimicrobials as growth promoters in livestock and poultry has been identified as a risk factor for the emergence of antimicrobial resistant Enterococcus spp. (Hoelzer et al. 2017). The role of livestock in contributing to antimicrobial and multidrug resistance by antibiotic treatment of large numbers of animals raise public health concerns and lead to a particular interest in virulence characteristics of circulating Enterococcus strains. In Bangladesh, there are over 250 antibiotics registered for human and 100 antibiotics registered for veterinary medicine use according to the Directorate General of Drug Administration (DGDA), Bangladesh database (DGDA 2018). However, vast majority of the veterinary medicinal products registered are categorized by the World Health Organization (WHO) as Critically Important Antibiotics (CIAs) in human medicine (WHO 2019). In Bangladesh, the prevalence and diversity of enterococci such as E. faecalis and E. faecium in livestock, poultry and in animal origin food products is poorly defined (Banik et al. 2018). Furthermore, little is known about the antibiotic resistances and virulence genes harboured in these bacteria.

**Objectives**

The aim of this study was to determine the prevalence of enterococci in farm animals and retail meat, as well as the presence of vancomycin resistance genes and virulence factors in VNSE isolates.

**Materials and methods**

**Sample collection and isolation of Enterococcus spp.**

A total of 352 samples were collected from selected farms (layer poultry: 07, broiler breeder: 03, cattle: 04, goat: 03 and, camel: 03) and at retail markets (broiler:14, cattle: 10 and, goat: 08) of Savar, Gazipur, and Dhaka from 2016 to 2017. The sampling and population size frame for the farm component constituted poultry (n = 136; N = ~10,000), cattle (n = 35; N = 451), goat (n = 29; N = 242) and camel (n = 30; N = 120). With the proper ethics committee approval
by Bangladesh Livestock Research Institute (ARAC: 01/10/2016:01), cloacal swabs were collected from poultry using sterile swab, and for cattle, goat, and camel samples approximately 5 g of freshly excreted fecal contents was collected into 25 ml PBS. For meat products, approximately 5 g of chicken meat (n = 60; N = –960), beef (n = 32; N = 300) and mutton (n = 30; N = 300) were collected aseptically into 10 ml PBS, and stored on ice for transport to the laboratory within 3–5 h. All samples were processed using routine bacterial culture for enterococci as per standard protocols (Kuiken et al. 2005) and as outlined below. In brief, samples (cloacal swab or 1 ml of homogenized meat) were cultured in selective pre-enrichment broth (brain heart infusion broth [BHI]; BD Difco™, USA) supplemented with 5% NaCl at 37 °C for 24–48 h. Primary isolation of enterococci was done using kanamycin aesculin azide (KAA) agar (Oxoid, UK) at 37 °C for 18–24 h and further screening was done by transferring 3–5 characteristic brown colonies from KAA agar to 5% sheep blood agar (Oxoid, UK) for the next 24 h at 37 °C, followed by biochemical and temperature tolerance test (i.e. 10 °C then to 40 °C) (Guerrero-Ramos et al. 2016). Final confirmation of species identification was done for one or two selected pure isolates for each primary positive sample, using uniplex polymerase chain reaction (u-PCR I) and multiplex polymerase chain reaction (m-PCR I) based on enterococci specific the superoxide dismutase gene (sodA). Genus specific primer for Enterococcus spp., and species-specific primer for *E. faecalis* and *E. faecium* were used for molecular confirmation of enterococci isolates according to previously published protocol (Jackson et al. 2004; Poyart et al. 2000) with a slight modification. DNA extraction was performed using conventional boiling method (Huq et al. 2012). List of primer and amplified target DNA size used in this study are provided in supplementary material 1.

**Antimicrobial susceptibility assay**

Kirby-Bauer disk diffusion assay was performed according to Clinical and Laboratory Standards Institute standard methods M02-A12 and M07-A10 (Tuohy et al. 2000). Standard strain *E. faecalis* ATCC 29212 was used for quality control of in-house produced Mueller-Hinton agar plates and susceptibility testing because of its availability and high reproducibility during routine testing. *E. faecalis* and *E. faecium* isolates susceptibility was tested using a panel of eight antimicrobials commonly used in veterinary and human medicine in Bangladesh (DGDB 2018) belong to phenicols (chloramphenicol: 30 μg), quinolones (ciprofloxacin: 5 μg), macrolides (erythromycin: 15 μg), oxazolidinones (linezolid: 30 μg), glycopeptides (vancomycin: 30 μg), penicillins (penicillin G: 10 μg), nitrofurans (nitrofurantoin: 300 μg), and tetracyclines (tetracycline: 30 μg). Among the all isolated enterococci a subset of pure *E. faecalis* and *E. faecium* culture (n = 117) representing individual positive samples were selected for antibiogram study. Isolates non-susceptible to three or more antibiotic classes were defined as MDR. Isolates non-susceptible to 7 or 8 of the antibiotics were termed as possible-Extremely Drug Resistant (XDR) isolates according to published criteria (Magiorakos et al. 2012). Isolates phenotypically non-susceptible to vancomycin were termed as vancomycin nonsusceptible enterococci (VNSE).

**Vancomycin resistance genes and virulence factors identification**

The presence of the vancomycin resistance genes *vanA*, *vanB*, *vanC* and *vanC2/C3* was determined using a multiplex PCR (m-PCR II) according to previously published protocol. Published PCR primers were also used to assess the presence of ten virulence factors: aggregation substance (asA1), gelatinase (*gell*), cytolysin (*cyl*), enterococcal surface protein (*esp*), hyaluronidase (*hyl*), collagen-binding protein (*ace*), transmembrane protein (*fsr*), endocarditis specific antigen (*efa*), aggregation protein (*agg*), and sex pheromones (*cpd*) (Strateva et al. 2016; Vankerckhoven et al. 2004; Zoletti et al. 2011). In case of resistance gene and virulence factors we used our internal positive control isolates based on trial-error and their reproducibility. We used no positive control for *cylA*, *hyl* and, *efa* gene rather depended on established primer and thermal cycle from previous publications. All PCR primers used are given in (Supplementary material 1).

**Statistics and data analysis**

Descriptive statistics and univariate analysis were performed using SPSS Statistics 20 software (IBM corp, USA) and The Survey System 12.0 (Creative Research Systems, USA). Descriptive cross tabulation statistics and frequency statistics were used for prevalence data analysis. 95% confidence interval (95% CI) for individual prevalence ratio with known population size was determined using the standard error (SE), z- score and sample fraction (f) whereas in case of unknown population, 95% CI value was estimated applying bootstrapping method with replacement statistics with 1000 bootstrap samples. Comparison of prevalence between parameters of the study was characterized based on Fisher’s exact test (F test). A p value <0.05 was considered significant. Inhibition zone data in antibiotic susceptibility assay were analyzed using BacLink and WHOMET-2019 software (WHO Collaborating Centre for Surveillance of Antimicrobial Resistance, USA) (Stelling and Kulldorff 2007). For this analysis the cattle, goat, and camel samples were combined to form a group termed ‘Livestock’ as farming system for these species are largely similar in Bangladesh. Moreover, same butcher shop sells all these meat species sharing
the same slaughtering facilities and equipment and similar processing techniques. Comparative analysis of resistance against dual antibiotics was done using data analysis tool Morpheus. A comparison of pair-wise resistance patterns among antibiotics is calculated using a heat map similarity matrix and dendrogram cluster based on the one minus Pearson correlation coefficient and average linkage method (Morpheus 2018).

**Results**

**Prevalence of Enterococcus spp. and distribution**

A total of 211 Enterococcus spp. comprising 115 (105 single and 10 co-isolation) E. faecalis, 36 (26 single and 10 co-isolation) E. faecium and 60 enterococci not assigned to a species by PCR were isolated from 352 collected samples, summarized in Table 1 and detailed in Supplementary material 2. Overall prevalence of Enterococcus spp. was 57% (95% CI [52–62]). In poultry, farm samples yielded significantly higher recovery of enterococci compared to fecal samples (Table 1). In case of livestock a greater proportion of meat samples were positive for Enterococcus compared to fecal samples (Table 1). In farm, a significantly greater proportion of poultry isolates were culture positive for Enterococcus spp. than poultry meat (Table 1).

**Antimicrobial susceptibility**

High prevalence of VNSE (44%; 51/117) was observed in both farm (41%; 35/86) and meat (52%; 16/31) isolates, in addition, resistance level was also noteworthy for tetracycline (74%; 87/117) and erythromycin (65%; 76/117). Moreover, 17% isolate (20/117) was resistant to linezolid which includes 14 (16%) and 6 (19%) isolates from farm and meat respectively. MDR was observed in 80% (93/117) of the isolates with a similar proportion for both E. faecalis (79%; 68/86) and E. faecium (81%; 25/31). One E. faecium isolate and seven E. faecalis isolates were possible-XDR, having resistance to ≥7 antibiotic classes. Almost all the E. faecalis and E. faecium from poultry, including poultry farms (n = 57/60) and poultry meat (n = 9/9) were MDR. The full antibiotic resistance patterns and profiles towards the eight antimicrobials tested is presented in Table 2.

**Detection of vancomycin resistance genes**

A total of 51 VNSE isolates were further characterized to determine the presence of vancomycin resistance genes (Table 3). Twenty isolates were PCR positive for at least one vancomycin resistance gene, of which nine isolates were phenotypically resistant to vancomycin. Nonetheless, eleven isolates harbored at least one vancomycin resistance gene without being phenotypically resistant. The most commonly detected vancomycin resistance genes were vanA and vanC2/3.

**Detection of virulence genes**

The 51 VNSE isolates were also tested for the presence of ten virulence factors. The gelatinase gene (gelE) was observed in 79% isolates (42), aggregation factor (asa1) in 38% isolates (20), and the sex pheromones (cpd) in 66% isolates (35) (Table 3). Other virulence factors, including cytolsin, surface protein, hyaluronidase, collagen-binding protein, transmembrane protein, endocarditis specific antigen, and aggregation protein were not detected among these isolates.

**Discussion**

To assess the prevalence of AMR enterococci in healthy finisher livestock and poultry, and their products in Bangladesh, we screened 230 samples from livestock and poultry at farms and 122 meat samples at retail markets. Our findings indicate that Enterococcus spp. are relatively common in poultry and livestock animals in Bangladesh, though the overall prevalence of E. faecalis was higher than E. faecium, which proves its greater colonization potential as discussed in other studies (Agudelo and Huycke 2014). Nonetheless, poultry has been demonstrated as the leading source of Enterococcus spp. in several studies conducted elsewhere including Bangladesh (Banik et al. 2018; Ali et al. 2014; Stępień-Pyśniak et al. 2016). The prevalence of E. faecalis was significantly higher in poultry farms than at other livestock farms, but retail livestock meat yielded a higher prevalence of E. faecalis than poultry meat. This is likely to be indicative of post-slaughter contamination as well as the absence of, or lack of enforcement of, food safety regulations during the slaughtering process and meat product handling in Bangladesh.

Poultry and livestock are essential to food security in Bangladesh, the potential dissemination of resistant Enterococcus spp. in the food production continuum and strengthening of food safety regulations needs to be addressed. In the context of One Health and to further inform food safety interventions in Bangladesh, it is also important to identify the major reservoirs and their dissemination downstream of the production continuum, which was the intent of our study. Our data indicates that more than half of the samples were contaminated with Enterococcus spp. and the most predominant species was E. faecalis, the third most commonly...
## Table 1

Prevalence of enterococci in corresponding farm and fresh meat samples

| Isolates                  | Poultry (n=196)       | Livestock (n=156)    | Poultry farm vs Live-stock farm | Poultry meat vs Live-stock meat |
|--------------------------|-----------------------|----------------------|---------------------------------|---------------------------------|
|                          | Farm (n=136)          | Meat (n=60)          | p-value | Risk Estimate | OR | 95% CI | OR | 95% CI | OR | 95% CI | OR | 95% CI |
| Entero-coccus faecalis   | 50 (36.8%) 28.7–44.9% | 12 (20.0%) 10.0–31.6% | .021    | 2.3 1.1–4.8  | 18 (19.1%) 11.7–27.7% | .006    | 0.4 0.2–0.7 | .415 | 1.2 0.8–1.9 | .005 | 2.4 1.3–4.6 | .018 | 0.4 0.2–0.8 |
| Entero-coccus faecium    | 10 (7.4%) 3.7–12.5%   | 6 (10.0%) 3.3–18.3%  | .575    | 0.7 0.2–2.1  | 08 (8.5%) 3.2–14.9% | .317    | 2.7 0.6–13.6 | .682 | 1.3 0.6–2.9 | .805 | 0.9 0.3–2.2 | .160 | 3.3 0.6–17.2 |
| E. faecalis & E. faecium | 5 (3.7%) 0.7–7.4%     | 0 (0.0%) 0–0%       | .326    | – –         | 4 (4.3%) 1.1–8.5% | .649    | 2.7 0.3–24.8 | .755 | 0.8 0.2–2.8 | 1.0 | 0.9 0.2–3.3 | 1.0 | – –         |
| Other Enterococci        | 19 (14.0%) 9.7–19.5%  | 3 (5.0%) 1.1–11.3%  | .055    | 1.9 0.8–3.7  | 28 (29.8%) 23.7–36.6% | .125    | 1.8 0.7–3.9 | .335 | 1.3 0.8–1.8 | .008 | 2.8 1.0–4.7 | .022 | 0.7 0.2–1.4 |
| Overall                  | 84 (61.8%) 53.7–68.5% | 21 (35.0%) 18.9–41% | .028    | 2.1 1.1–4.1  | 58 (61.7%) 52.8–69.3% | .087    | 0.5 0.2–1.1 | .381 | 1.1 0.7–1.8 | .018 | 2.1 1.1–3.4 | .095 | 0.5 0.2–1.1 |

Significant p-value are marked as bold font

**OR** Odd Ratio
Table 2  Antibiotic resistance of enterococci among tested poultry, poultry meat, livestock and livestock meat isolates

| Enterococci Sources | Isolates               | Antimicrobials |
|---------------------|------------------------|----------------|
|                     |                        | CHL | CIP | ERY | NIT | LID | PEN | TET | VAN | MDR |
| Poultry             | *Enterococcus faecalis* (n=46) | 9 (20%) | 1 (17%) | 38 (83%) | 6 (13%) | 8 (17%) | 21 (46%) | 44 (96%) | 9 (20%) | 43 (93%) |
|                     | *Enterococcus faecium* (n=14)  | 1 (7%) | 6 (43%) | 11 (79%) | 2 (14%) | 0 (0%) | 3 (21%) | 13 (93%) | 1 (7%) | 14 (100%) |
| Overall (n=60)      |                        | 10 (17%) | 23 (38%) | 49 (82%) | 8 (13%) | 8 (13%) | 24 (40%) | 57 (95%) | 10 (17%) | 57 (95%) |
| Poultry Meat        | *E. faecalis* (n=5)   | 2 (40%) | 2 (40%) | 5 (100%) | 2 (40%) | 3 (60%) | 2 (40%) | 4 (80%) | 3 (60%) | 5 (100%) |
|                     | *E. faecium* (n=4)    | 1 (25%) | 2 (50%) | 4 (100%) | 1 (25%) | 1 (25%) | 2 (50%) | 4 (100%) | 1 (25%) | 4 (100%) |
| Overall (n=9)       |                        | 3 (33%) | 4 (44%) | 9 (100%) | 3 (33%) | 4 (44%) | 4 (44%) | 8 (89%) | 4 (44%) | 9 (100%) |
| Livestock           | *E. faecalis* (n=15)  | 0 (0%) | 2 (13%) | 7 (47%) | 0 (0%) | 3 (20%) | 0 (0%) | 7 (47%) | 2 (13%) | 9 (60%) |
|                     | *E. faecium* (n=11)   | 0 (0%) | 0 (0%) | 6 (55%) | 0 (0%) | 3 (27%) | 1 (9%) | 5 (45%) | 2 (18%) | 6 (55%) |
| Overall (n=26)      |                        | 0 (0%) | 2 (8%) | 13 (50%) | 0 (0%) | 6 (23%) | 1 (4%) | 12 (46%) | 4 (15%) | 15 (58%) |
| Livestock’s Meat    | *E. faecalis* (n=20)  | 0 (0%) | 7 (35%) | 4 (20%) | 1 (5%) | 2 (10%) | 2 (10%) | 9 (45%) | 0 (0%) | 11 (55%) |
|                     | *E. faecium* (n=2)    | 0 (0%) | 1 (50%) | 1 (50%) | 0 (0%) | 0 (0%) | 1 (50%) | 0 (0%) | 1 (50%) |
| Overall (n=22)      |                        | 0 (0%) | 8 (36%) | 5 (23%) | 1 (5%) | 2 (9%) | 2 (9%) | 10 (46%) | 0 (0%) | 12 (55%) |
| Overall             | *E. faecalis* (n=86)  | 11 (13%) | 28 (33%) | 54 (63%) | 9 (10%) | 16 (19%) | 25 (29%) | 64 (74%) | 14 (16%) | 68 (79%) |
|                     | *E. faecium* (n=31)   | 2 (6%) | 9 (29%) | 22 (71%) | 3 (10%) | 4 (13%) | 6 (19%) | 23 (74%) | 4 (13%) | 25 (81%) |
| Overall Resistance  | (n=117)                | 13 (11%) | 37 (32%) | 76 (65%) | 12 (10%) | 20 (17%) | 31 (27%) | 87 (74%) | 18 (15%) | 93 (80%) |

*CHL* Chloramphenicol, *CIP* Ciprofloxacin, *ERY* Erythromycin, *NIT* Nitrofurantoin, *LID* Linezolid, *PEN* Penicillin G, *TET* Tetracycline, *VAN* Vancomycin, *MDR* Multi-drug Resistance
| Sample ID | Source           | Isolate         | Resistance Profile | N. Res | VAN | Van resistance gene | Virulence gene |
|-----------|------------------|-----------------|--------------------|--------|-----|---------------------|----------------|
| BCS 016   | Poultry Cloaca   | Enterococcus faecalis | ERY VAN            | 2      | R   | ge/E, cpd           |                |
| LBM 002   | Beef             | E. faecalis     | ERY VAN            | 2      | I   | asa1                |                |
| LBM 011   | Beef             | E. faecalis     | ERY VAN            | 2      | I   | asa1, ge/E, cpd     |                |
| L-En 017  | Camel Fecal      | Enterococcus faecalis | ERY VAN            | 2      | I   | asa1, ge/E, cpd     |                |
| LBM 014   | Beef             | E. faecalis     | CIP ERY VAN        | 3      | I   | asa1, ge/E, cpd     |                |
| LBM 023   | Beef             | E. faecalis     | CIP ERY VAN        | 3      | I   | asa1, ge/E, cpd     |                |
| LBM 028   | Beef             | E. faecalis     | CIP ERY VAN        | 3      | I   | asa1, cpd           |                |
| BBS 009   | Poultry Cloaca   | E. faecalis     | CIP ERY TET VAN    | 4      | I   | ge/E, cpd           |                |
| BBS 012   | Poultry Cloaca   | E. faecalis     | CIP ERY TET VAN    | 4      | I   | cpd                 |                |
| BCS 031   | Poultry Cloaca   | E. faecalis     | CIP ERY TET VAN    | 4      | I   | vanC1, vanC2/3 ge/E, cpd |
| BCS 032   | Poultry Cloaca   | E. faecalis     | CIP ERY TET VAN    | 4      | I   | vanC1, vanC2/3 ge/E, cpd |
| BCS 033   | Poultry Cloaca   | E. faecalis     | CIP ERY TET VAN    | 4      | I   | ge/E                |                |
| LBM 001   | Beef             | E. faecalis     | CIP ERY TET VAN    | 4      | I   | ge/E, cpd           |                |
| LBM 016   | Beef             | E. faecalis     | CIP ERY TET VAN    | 4      | I   | asa1, ge/E, cpd     |                |
| LCM 047   | Chicken Meat     | E. faecalis     | CIP ERY TET VAN    | 4      | I   | ge/E, cpd           |                |
| LCS 006   | Poultry Cloaca   | E. faecalis     | CIP ERY TET VAN    | 4      | I   | ge/E                |                |
| LCS 008   | Poultry Cloaca   | E. faecalis     | CIP ERY TET VAN    | 4      | I   | vanA asa1, ge/E     |                |
| LCS 008   | Poultry Cloaca   | E. faecalis     | CIP ERY TET VAN    | 4      | I   | vanA asa1, ge/E     |                |
| LCS 011   | Poultry Cloaca   | E. faecalis     | CIP ERY TET VAN    | 4      | I   | ge/E, cpd           |                |
| LCS 013   | Poultry Cloaca   | E. faecalis     | CIP ERY TET VAN    | 4      | I   | asa1, ge/E, cpd     |                |
| LCS 032   | Poultry Cloaca   | E. faecalis     | CIP ERY NIT VAN    | 4      | R   | asa1, ge/E, cpd     |                |
| LCS 048   | Poultry Cloaca   | E. faecalis     | CIP ERY TET VAN    | 4      | I   | asa1, ge/E, cpd     |                |
| L-En 009  | Camel Fecal      | E. faecalis     | CIP ERY LNZ VAN    | 4      | I   | vanC2/3 ge/E, cpd   |                |
| L-En 014  | Camel Fecal      | E. faecalis     | ERY TET LNZ VAN    | 4      | R   | vanA asa1, ge/E     |                |
| L-En 015  | Camel Fecal      | E. faecalis     | ERY TET LNZ VAN    | 4      | R   | vanA asa1, ge/E     |                |
| L-En 033  | Goat Fecal       | E. faecalis     | ERY TET LNZ VAN    | 4      | I   | vanC1 asa1, ge/E    |                |
| L-En 033  | Goat Fecal       | E. faecium      | ERY TET LNZ VAN    | 4      | I   | vanC1 asa1, ge/E    |                |
| LMM 015   | Mutton           | E. faecalis     | CIP ERY TET VAN    | 4      | I   | asa1, ge/E, cpd     |                |
| BBS 007   | Poultry Cloaca   | E. faecalis     | CHL CIP ERY TET VAN| 5      | I   | ge/E, cpd           |                |
| BCS 013   | Poultry Cloaca   | E. faecalis     | CHL CIP ERY TET VAN| 5      | I   | ge/E, cpd           |                |
| LCM 037   | Chicken Meat     | E. faecalis     | CHL CIP ERY TET VAN| 5      | I   | ge/E, cpd           |                |
| L-En 027  | Camel Fecal      | E. faecium      | ERY PEN TET LNZ VAN| 5      | R   | vanB asa1, ge/E, cpd|                |
| BCS 029   | Poultry Cloaca   | E. faecalis     | CHL ERY PEN TET LNZ VAN| 6      | R   | vanB asa1, ge/E, cpd|                |
| BCS 035   | Poultry Cloaca   | E. faecalis     | CHL CIP ERY NIT TET VAN| 6      | I   | vanC1, asa1, ge/E   |                |
| BCS 035   | Poultry Cloaca   | E. faecalis     | CHL CIP ERY NIT TET VAN| 6      | I   | vanC1, asa1, ge/E   |                |
| LBM 022   | Beef             | E. faecalis     | CHL CIP ERY PEN TET VAN| 6      | I   | asa1, ge/E, cpd     |                |
| LCM 054   | Chicken Meat     | E. faecalis     | CHL CIP ERY PEN TET LNZ VAN| 6      | R   | ge/E, cpd           |                |
| LCM 057   | Chicken Meat     | E. faecalis     | CHL CIP ERY NIT PEN TET LNZ VAN| 6      | R   | ge/E, cpd           |                |
| LCS 003   | Poultry Cloaca   | E. faecalis     | CHL CIP ERY PEN TET LNZ VAN| 6      | R   | ge/E, cpd           |                |
| LCS 037   | Poultry Cloaca   | E. faecalis     | CHL CIP ERY NIT PEN TET LNZ VAN| 6      | R   | ge/E, cpd           |                |
| LCS 039   | Poultry Cloaca   | E. faecalis     | ERY NIT PEN TET LNZ VAN| 6      | R   | asa1, ge/E, cpd     |                |
| LCS 040   | Poultry Cloaca   | E. faecalis     | CHL CIP ERY NIT PEN TET LNZ VAN| 6      | R   | vanC2/3 ge/E, cpd   |                |
| BCS 030   | Poultry Cloaca   | E. faecalis     | CHL CIP ERY PEN TET LNZ VAN| 7      | R   | asa1, ge/E, cpd     |                |
| LCM 040   | Chicken Meat     | E. faecium      | CHL CIP ERY PEN TET LNZ VAN| 7      | R   | asa1, ge/E, cpd     |                |
| L-En 003  | Camel Fecal      | E. faecalis     | CHL CIP ERY NIT PEN TET LNZ VAN| 7      | R   | vanA, vanC2/3 ge/E, cpd |
| LBM 025   | Beef             | E. faecalis     | CHL CIP ERY PEN TET LNZ VAN| 8      | R   | vanA asa1, ge/E     |                |
| LCM 036   | Chicken Meat     | E. faecalis     | CHL CIP ERY PEN TET LNZ VAN| 8      | R   | asa1, ge/E, cpd     |                |
identified pathogen in hospitals associated with increased mortality (Coque 2008). These findings are consistent with several preceding studies (Ngbede et al. 2017; Poeta et al. 2006; Yoshimura et al. 2000). There is no extensive data available on AMR amongst enterococci in livestock and poultry in Bangladesh because a national AMR surveillance program has not yet been established. However, one study showed high prevalence of enterococci among chickens (Banik et al. 2018) consistent with our study. Two relevant studies in involving human cases have implicated enterococci (Akram Hossain 2016; Suchi et al. 2018).

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High prevalence of VNSE (44%; n = 51) has been observed in this study which is higher than some other studies (Cosentino et al. 2010; Maasjost et al. 2015; Stepien-Pyśniak et al. 2016). Nonetheless, most alarming finding was the co-occurrence of linezolid resistance and VNSE, where most linezolid (90%) resistant isolates were VNSE, which could drive a therapeutic crisis for treating infection caused by VNSE (Bialvaei et al. 2017). In addition, this study detected low susceptibility of isolates to antimicrobials that are concurrently being used for both to treat human infection and for animal production in Bangladesh. In our study, the frequency of resistance to tetracyclines and macrolides were relatively high whereas comparatively low resistance were observed to DNA synthesis inhibitors including nitrofurantoin (10%) and ciprofloxacin (31%), which are consistent with similar studies conducted elsewhere (Cosentino et al. 2010; Maasjost et al. 2015; Stepien-Pyśniak et al. 2016).

MDR prevalence was high in farm and in fresh meat sold at retail markets (i.e. products originating from the same geographical locations where they were raised) and most of the VNSE isolates were also MDR (92%, 47/51). Remarkably, isolates that were possible-XDR were mostly recovered from poultry farms, this also raises the potential public health concern of exposure to and consumption of products from this species. These findings may be reflective of the indiscriminate use of antimicrobials in poultry in Bangladesh, (Ahmed et al. 2019) notably, the high use of CIAs (Imam et al. 2020) and thus support the need for surveillance of AMR and monitoring of AMU to inform changes in usage policy and to better understand AMR and AMU relationships.

The comparative pairwise antibiotic resistance matrix reveals that the combination between chloramphenicol or ciprofloxacin with nitrofuratoin, linezolid or vancomycin may produce better efficiency against multidrug resistant isolates as combined resistance level to those antibiotics is very low compared to the other antibiotics (Figure 1). These findings warrant further research.

The presence of vanA gene in our study, primarily responsible for vancomycin resistance (Torres et al. 2018) further highlights the widespread dissemination of these resistant strains in animal populations. However, there was a discrepancy in detection of vancomycin resistance gene in our study; it was detected in both resistance and intermediate phenotypes, which may show resistance is not dependent on gene presence alone but it also depends on the gene expression level. We also found the presence of vanC1 and vanC2/3 gene among E. faecalis which was previously thought to be species specific to Enterococcus gallinarum and Enterococcus casseliflavus, respectively (Clark et al. 1998). The vanC gene cluster can be located on plasmids (as well as the chromosome), and can be transferred to other enterococci such as E. faecium and E. faecalis, (Moura et al. 2013; Sun et al. 2014). The location of the vanC gene in the isolates from this study was not determined and may warrant further study in the future. We observed that most genotypic vancomycin non-susceptible enterococci (VNSE) isolates were phenotypically non-resistant to vancomycin indicating vancomycin-variable enterococci (VVE), as described previously (Downing et al. 2015; Thaker et al. 2015). This could be a result of the mutation in van gene cassette including vanSR or vanHAXY gene cluster or any other novel mutations (Hong et al. 2008). This particular finding has impact in clinical settings, where the misidentification in enterococcal infections may result to challenges in the development of an efficacious treatment regimen. The preceding studies found the relevance of VVE with only vanA gene, but in this study, we also found the association of vanB, vanC1 and vanC2/3 with VVE which require further investigation. We also found some vancomycin resistant isolates

### Table 3 (continued)

| Sample ID | Source | Isolate | Resistance Profile | N. Res. | VAN | Van resistance gene | Virulence gene |
|-----------|--------|---------|--------------------|---------|-----|---------------------|----------------|
| LCS 016   | Poultry | Cloaca | E. faecalis        | CHL     | CIP | ERY     | NIT | PEN | TET | LNZ | vanC2/3 | gelE, cpd |
| LCS 018   | Poultry | Cloaca | E. faecalis        | CHL     | CIP | ERY     | NIT | PEN | TET | LNZ | vanC2/3 | asa1, gelE, cpd |
| LCS 029   | Poultry | Cloaca | E. faecalis        | CHL     | CIP | ERY     | NIT | PEN | TET | LNZ | vanA, vanC2/3 | cpd |

*CHL Chloramphenicol, CIP Ciprofloxacin, ERY Erythromycin, NIT Nitrofurantoin, PEN Penicillin G, TET Tetracycline, LID Linezolid, VAN Vancomycin, R: Resistant (Non-susceptible), I Intermediate*
without having any major vancomycin resistance gene which may be due to the other type of resistance mechanism and beyond the scope of our current study. However, this discrepancy in vancomycin resistance pattern may evolve as a new therapeutic challenge for clinical setting. An immediate improvisation in diagnostic technique for VVE and continuous surveillance for VVE is utmost necessary as some other studies suggested the same (Downing et al. 2015; Szakacs et al. 2014).

Moreover, the gene associated with vancomycin resistance and can easily be transferred to another susceptible isolates since there is a probability to be clustered in a mobile genetic element like plasmid or transposons (Torres et al. 2018). This transfer could occur within livestock farms and their environment or throughout the food production chain from farm to fork as evidenced by the detection of MDR E. faecalis and E. faecium from diverse food animal species such as poultry, cattle, and goat and meat products/meat-derived products (Hammerum et al. 2010; Hoelzer et al. 2017).

The high prevalence of virulence indicators like gelatinase, sex pheromones, and aggregation factors were found among the MDR isolates. The presence of these factors in MDR enterococci can contribute to the colonization or formation of bacterial biofilm-like vegetations among immuno-compromised patients through urinary tract or blood and subsequently turned into untreatable urinary tract infection (UTI) or endocarditis respectively (Sharifi et al. 2013).

**Conclusion**

Our study indicated that the livestock and poultry fresh meat are frequently contaminated with *Enterococcus* spp., possibly due to insufficient food safety practices in the slaughtering and meat processing systems in Bangladesh. Mostly poultry meat samples were contaminated with *E. faecalis* indicative that poultry is potentially an important source of resistant enterococci that can infect people via the food chain. The presence of enterococci resistant to WHO’s CIA in food animals can pose an unprecedented threat to public health. The most alarming finding was the co-occurrence of vancomycin and linezolid resistance in enterococci in livestock and poultry. Moreover, the prevalence of gelatinase, pheromones and aggregation factor in MDR enterococci, in the face of poorly enforced (or lack of) food safety regulations in livestock and poultry in Bangladesh could plausibly lead to their widespread dissemination and persistence in nature. This study calls for an urgent need to reduce the use
of WHO’s CIA in livestock and poultry and enhancement of food safety practices at the farms and slaughter plants in Bangladesh to reduce the public health implications of *Enterococcus* spp., particularly VNSE.

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**Code or data availability** On Request.

**Declarations**

**Ethical approval** This study was conducted upon approval by the ethical regulation constructed by Bangladesh Livestock Research Institute (ARAC: 01/10/2016:01).

**Consent to participate** Verbal consent from the authority of each farm was taken before collecting the samples from the farm.

**Consent for publication** We give our consent for the publication of the submitted manuscript.

**Competing interests** None.

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