



Detection of Multi-Drug Resistant *Enterococcus* Spp. in Farmed Pangasius Ponds Using Poultry Waste as Feed

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Abstract

The alarming rise of multi-drug resistant (MDR) bacteria pathogenic to humans and animals poses a major threat to the public health sector. Enterococci are a complex group of bacteria that causes nosocomial infections in humans. It has been widely acknowledged that the environmental loading of antibiotics from hospitals, livestock farms, aquaculture sector and sewage plants create a hot spot for the emergence of antimicrobial resistance (AMR). The sediment, water and fish samples from Pangasius culture ponds using raw and cooked poultry waste as feed, and normal fish feed were screened for the presence of MDR *Enterococcus faecalis*. AMR was analysed by Kirby-Bauer disc diffusion method. Among the 83 enterococci isolates, 65 % of the isolates were *E. faecalis*, all of which exhibited multi-drug resistant phenotypes. The isolates showed maximum resistance to macrolide, tetracycline and lincosamide (100 %) class of antimicrobials. The present study reflects the impact of erroneous practices followed by certain farmers in spreading AMR in aquaculture and environment and the occurrence of MDR enterococci in aquaculture farms is a matter of serious concern. Awareness programmes among farmers, better regulations, regular surveillance and encouraging adoption of better management practices can go a long way to limit the spread of such MDR superbugs.

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Introduction

AMR is one of the major public health concerns of the modern world. AMR has created great barriers in the treatment and control of highly contagious diseases, in both resource-constrained and resource-rich countries (Carrique-Mas et al., 2015; Mc Nulty et al., 2016). AMR evolves when the disease causing microorganisms (bacteria, viruses, fungi, and parasites) withstand the exposure to a drug or group of drugs and continue to grow and spread. Frequent antimicrobial usage for both human and animal health can lead to the selection and dissemination of AMR microorganisms and resistant determinants in the environment (Cheng et al., 2019). In the past, AMR infections were predominantly associated with hospitals and health care settings, but over the last decade, resistant infections have been commonly seen in the wider community too.

Aquaculture is one of the fastest growing food producing sectors in the world. In India, during the last decade, aquaculture has gradually yet steadily transformed into a profitable business. Currently, the country ranks second in the world in total fish production with an annual production rate of about 9.06 million metric tonnes (FAO, 2018). In aquaculture, antibacterial agents are used for both prophylaxis and therapeutic purpose. When it comes to livestock (poultry, cattle, pig, sheep), antibiotics are not only used when they show certain clinical signs of disease but also in advance, to suppress the chance of developing diseases (metaphylaxis) and

also to promote growth. However, the over-use of antimicrobial agents in livestock and aquaculture has led to the emergence of reservoirs AMR bacteria in animals and the surrounding environment (Harris et al., 2012; Landers et al., 2012).

Enterococci are Gram-positive bacteria, and a major colonizer of human and animal gastrointestinal tracts. It is becoming a global concern in human health as a cause of nosocomial infections. Clinical enterococcal isolates display a wide range of AMR mechanisms including inactivation of curative agents, alteration of drug targets, overexpression of efflux pumps, action of biofilm and more importantly, they can transfer resistant genetic material to a broad range of recipients (Champagne et al., 2011). Majority of the enterococcal infections are complicated by the intrinsic resistance to a number of antibiotics including β -Lactams and low concentrations of lincosamides and aminoglycosides as well as acquired resistance to high concentrations of aminoglycosides, glycopeptides, tetracyclines and macrolides (Kristich et al., 2014). The presence of MDR Enterococci in the faecal material of animals has become a serious issue in human and veterinary medicine. *Enterococcus* spp. of veterinary, human and food origin have also been used as indicator organisms for surveillance of AMR. Generally, 90% of all enterococcal infections were caused by *Enterococcus faecalis* (Torres et al., 2018). However, no information is available on the antibiotic susceptibility profile of *E. faecalis* isolated from aquaculture ponds using poultry waste. Majority of the aquaculture farmers are unaware of the presence of antibiotic growth promoters, problems of drug resistant bacteria in raw poultry waste and the problems related to the AMR. The aim of this study was to analyse the influence of poultry waste used in Kerala as feed in aquaculture farms on the resistance of *E. faecalis* recovered from three different types of aquaculture farms, as well as to determine the AMR profile of *E. faecalis* from these aquaculture farms. The types of aquaculture farms consisted of farms fed with raw poultry waste, cooked poultry waste and normal fish feed.

Materials and Methods

For the study, sediment, water and fish samples were collected from 11 different aquaculture farms from three different districts (Palakkad, Alappuzha, and Wayanad) in Kerala, India. Out of 11 aquaculture farms, eight farms used raw poultry waste as

feed (AF1), two farms used cooked poultry waste (AF2), and one farm followed normal recommended fish feed (AF3). Samples from fish farm AF3 were used as control. *Pangasianodon hypophthalmus* was the species cultured in all the selected sampling fish farms. All samples were obtained between September 2019 and February 2020. Fish samples with an average weight of 500 g were collected from sampling site and transported live to the laboratory in polythene bags containing oxygenated water. Sediment and water samples were aseptically collected in sterile bottles from the selected aquaculture farms and transported on ice to the laboratory for further processing.

Under sterile conditions, samples (sediment 1 g, fish tissue 1g, water 1 mL) were serially diluted in 9 mL of 0.85 % NaCl and inoculated onto KF streptococcal agar media (Himedia, Mumbai, India) using spread plate technique (Sanders, 2012). The plates were incubated at 37°C for 24 h. On the basis of morphological features enterococcal colonies were randomly picked from the plate and isolates were purified by sub culturing on nutrient agar plates (Himedia, Mumbai, India). To establish if they belonged to the genus *Enterococcus*, selected colonies were further amplified on Bile Esculin agar (Himedia, Mumbai, India) plates and incubated for 24h at 37°C. The purified bacterial strains were phenotypically identified based on morphological, physiological and biochemical characteristics (Devriese et al., 1995), among these, three isolates were selected for confirmatory identification for *E. faecalis* using 16S rRNA gene sequencing (Ryu et al., 2013). Analysis of the sequenced data was done using Basic Local Alignment Search Tool (BLAST) program of National Center for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov/BLAST>). Among these sequenced data, one nucleic acid sequence was submitted to GenBank.

The antibiotic susceptibility pattern of 54 *E. faecalis* isolates, identified phenotypically, was tested by Kirby-Bauer disc diffusion method (Bauer et al., 1966). Nine antibiotics from nine classes were selected for the test based on their use in poultry and importance to human health. The antibiotic discs used were: ampicillin AMP/10 μ g, gentamicin GEN/10 μ g, clindamycin CD/2 μ g, ciprofloxacin CIP/5 μ g, erythromycin E/15 μ g, tetracycline TE/30 μ g, vancomycin VA/30 μ g, chloramphenicol C/30 μ g and tigecycline TGC/15 μ g (Himedia Mumbai, India). For antimicrobial susceptibility testing,

bacterial cultures grown in trypticase soy broth to an optical density of 0.5 McFarland standard corresponding to 1.5×10^8 cfu/ml were harvested by centrifugation. The cells re-suspended in sterile 0.85% saline, were plated on Mueller Hinton agar. Up to four antibiotic discs were placed on each plate and incubated at 35°C for 24 h. After incubation the organisms were classified as sensitive, intermediate or resistant according to the inhibition zone diameter, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2021). The standards of European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2021) were used where CLSI standards were not available. The Minimum inhibitory concentration (MIC) of all the bacterial isolates were determined by HiComb™ MIC strips (Himedia, Mumbai, India). The epidemiological cut-off (ECOFFs) values of CLSI (2021) were used to categorize MIC data for *E. faecalis* against the following antimicrobials (resistance breakpoints mg/L are in parentheses): ampicillin (≥ 16), gentamicin HLAR (>500), ciprofloxacin (≥ 4), erythromycin (≥ 8), tetracycline (≥ 16), vancomycin (≥ 32), chloramphenicol (≥ 32) clindamycin (≥ 2). *E. faecalis* isolates showing resistance to three or more classes of antibiotics were designated as multi-drug resistant (MDR).

Results and Discussion

A total of 83 enterococcal isolates were recovered from samples (sediment, water and fish) collected from farm type AF1 (75.9 %), AF2 (19.2 %) and AF3

(4.8 %) (Table 1). Among the 83 enterococcal isolates, 54 nos. (65 %) were identified phenotypically as *E. faecalis*. All the isolates of *E. faecalis* were derived from aquaculture farms fed with raw poultry waste comprising samples from sediment, water and fish. Maximum isolates of *E. faecalis* were derived from the sediment (64.8 %), while 25.9 % of the isolates were recovered from water samples. Least number of isolates were obtained from fish samples (9.2 %). No isolate of *E. faecalis* were obtained from aquaculture pond fed with cooked poultry waste and control farm i.e., aquaculture farm using normal fish feed. Morphological studies revealed that all these isolates formed colonies with red to pink centres on KF Streptococcal agar media and formed creamy transparent colored colonies on nutrient agar. In Bile-Esculin agar plates, they formed black halos around the colonies. All the isolates were found to be Gram positive cocci, non-motile, oxidase, and catalase negative (Table 2). The 16S rRNA gene sequence analysis of three phenotypically identified isolates exhibited 99.95 % sequence homology with *E. faecalis*. One nucleic acid sequence of *E. faecalis* was submitted to GenBank (GenBank accession number: OP435585)

The environmental and water samples usually contain *Enterococcus* spp. due to the distribution of huge amounts of human and animal waste through sewage or non-sewage systems, and Enterococci is also often used as an indicator of faecal contamination of water and food products (Byappanahalli et al., 2012; Rodrigues & Cunha, 2017). Some studies

Table 1. Distribution of *Enterococcus* spp. from different sampling sites of Kerala, India, in 2019-2020

Farm type	No. of farms	Sample type/source	No. of <i>Enterococcus</i> spp. isolates	No. of <i>E. faecalis</i> isolates*
Aquaculture farms fed with raw poultry waste. (AF1)	8	Sediment	38	35
		Water	18	13
		Fish	7	5
Aquaculture farms fed with cooked poultry meat (AF2)	2	Sediment	10	-
		Water	5	-
		Fish	1	-
Aquaculture farm fed with normal fish feed (AF3)	1	Sediment	4	-
		Water	-	-
		Fish	-	-

*Identified phenotypically

Table 2. Biochemical and physiological characteristics of *Enterococcus faecalis* isolated from sediment, water and fish samples (n = 54)

Biochemical test	Characteristics
Gram staining	+ ve
Motility	- ve
Spore forming	- ve
Catalase	- ve
Oxidase	- ve
Voges-Proskauer	+ ve
H ₂ S production	- ve
Metabolism with:	
Adonitol	- ve
Arabinose	- ve
Cellobiose	+ ve
Dulcitol	- ve
Glucose	+ ve
Glycerol	+ ve
Lactose	+ ve
Maltose	+ ve
Mannitol	+ ve
D - Mannose	+ ve
Melibiose	- ve
D - Raffinose	- ve
Salicin	+ ve
Sorbitol	+ ve
Sucrose	+ ve
Trehalose	+ ve
L - Xylose	- ve
Growth at:	
4°C	- ve
10°C	+ ve
45°C	+ ve
50°C	- ve
pH 9.6	+ ve
Growth in:	
6.5% NaCl	+ ve
0.1% Methylene blue milk	+ ve

have found that *E. faecalis* was the most predominant *Enterococcus* species from poultry environment (Aarestrup et al., 2000; Hayes et al., 2004; Diarra et al., 2010; Rehman et al., 2018). Accumulation of poultry waste and faecal matter in the pond bottom

contribute to the high prevalence of resistant bacteria in the aquaculture farm. In strongly selective environments, the genetic determinants, which specify resistance to different antibiotic drugs spread at a random rate (Skippington & Ragan 2011). The results of antimicrobial susceptibility testing of all the *E. faecalis* isolates are shown in Figure 1. Maximum resistance was observed against erythromycin, tetracycline and clindamycin (100%). The percentage of resistance against antibiotic ciprofloxacin was also high (88.8%), whereas none of the isolates showed resistance to the glycopeptide vancomycin, β -lactam ampicillin and glycylycline tigecycline. 63% of the *E. faecalis* isolates were resistant to tetracycline at $\geq 64 \mu\text{g/ml}$ and 37% were resistant to tetracycline at $\geq 32 \mu\text{g/ml}$. The MIC values of selected antimicrobials for *E. faecalis* is shown in Table 3. The sediment samples from aquaculture ponds fed with raw poultry waste showed highest proportion of isolates (20.4%) with very high degree of resistance against most of the antibiotics tested. The drug resistance pattern of multi drug resistant *E. faecalis* isolates is represented in Figure 2. All the isolates showed multi-drug-resistance against majority of the critically and highly important antimicrobial drugs. A total of four multiple antibiotic resistance profiles were recorded. The profiles included TE-E-CD-CIP, TE-E-CD, TE-E-CD-C-CIP, and TE-E-CD-C-CIP-GEN which occurred in 72.2%, 11.1%, 11.1% and 5.5% of bacterial isolates respectively (n=54) (Table 4). Isolates derived from sediment samples possessed high multi-drug-resistance pattern (combination of six antibiotics).

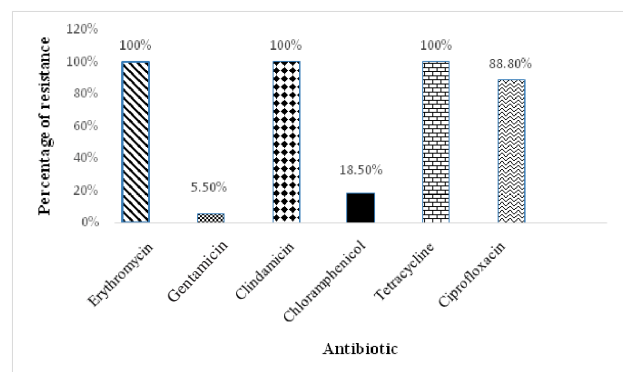


Fig. 1. AMR of *Enterococcus faecalis* isolates from sediment, water and fish samples against different antibiotic classes. E - Erythromycin; GEN - Gentamicin; CD- Clindamycin; C - Chloramphenicol; TE - Tetracycline; CIP - Ciprofloxacin. *E. faecalis* (n = 54).

Table 3. MIC of selected antimicrobials to *E. faecalis* isolates from aquaculture farm fed with raw poultry waste. AMP, ampicillin; E, erythromycin; VA, vancomycin; TE, tetracyclin; CIP, ciprofloxacin; C, chloramphenicol; CD, clindamycin. Resistance breakpoints for *Enterococcus* spp. (in µg/ml) were ampicillin (≥16), erythromycin (≥8), vancomycin (≥32), tetracycline (≥16), ciprofloxacin (≥4), chloramphenicol (≥32), clindamycin (≥2)

Antibiotic	Antibiotic class	Number of isolates for which MIC (µg/ml) was:										Percentage of resistance
		≤0.25	≥0.5	≥2	≥4	≥8	≥16	≥32	≥64	≥120	≥240	
AMP	Penicillin			9	45							0
E	Macrolide							52	2			100
VA	Glycopeptide				44	10						0
TE	Tetracycline							18	36			100
CIP	Fluoroquinolone		2	4	48							88
C	Amphenicol					35	9	10				20.4
CD	Lincosamide				46	8						100

As per the previous reports from Vietnam (Nguyen et al., 2016), polypeptides, penicillin, third-generation cephalosporins, quinolones, aminoglycosides, polymyxins, and many of the aforementioned antibiotics are largely employed in the poultry farms. A similar study in Punjab, India showed the wide use of tetracyclines, fluoroquinolones and cephalosporins in the poultry farms (Brower et al., 2017). Both tetracycline and erythromycin are often used for enteric infections, both in veterinary and human medicine (Aarestrup et al., 2000). High prevalence of resistance to erythromycin, tetracycline and clindamycin resulting from poultry, as observed in the present study, were also reported

from the United States and Canada (Rehman et al., 2018), and high prevalence of resistance to tetracyclines and erythromycin were reported from Nile tilapia farms in Brazil (Guidi et al., 2018). Igbinosa et al. (2016) from Nigeria reported high level resistance to erythromycin, oxytetracycline, chloramphenicol and ciprofloxacin in *Enterococcus* isolates from tilapia fish ponds. They reported that these antimicrobials are habitually used in the fish ponds for prophylaxis, therapeutic purpose, and growth promotion. However, these discharge waters can contaminate another environment directly or indirectly by resistant determinants or antimicrobials (Taneja & Sharma, 2019). Resistance to clindamycin has been reported to be an intrinsic property of *Enterococci*, and is said to be mediated by the product of the *lsa* gene (Dina et al., 2003). Resistance to macrolide class of antibiotics is also a frequent observation among enterococci from poultry envi-

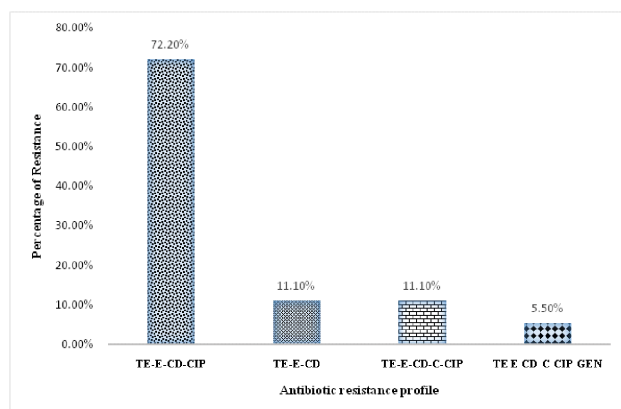


Fig. 2. MDR pattern of *E. faecalis* against different antibiotic classes. GEN, gentamycin; CIP, ciprofloxacin; C, chloramphenicol; CD, clindamycin; E, erythromycin; TE, tetracycline. *E. faecalis* (n = 54).

Table 4. The MDR pattern of multi-drug-resistant *E. faecalis* isolates. TE, tetracyclin; E, erythromycin; CD, clindamycin; C, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin

Antibiotic Resistance profile	Percentage of <i>E. faecalis</i> isolates (n=54) showed MDR
TE-E-CD-CIP	72.2%
TE-E-CD	11.1%
TE-E-CD-C-CIP	11.1%
TE-E-CD-C-CIP-GEN	5.5%

ronment. Some early studies found that use of medicated feed ingredients like virginiamycin and tylosin promote the development of macrolide resistance. Most often, these are used as a feed additive formulation for growth promotion in broiler chicken, turkey and swine (Yoshimura et al., 2000). *E. faecalis* isolates in the present study were not resistant to vancomycin, tigecycline and chloramphenicol. In contrast, resistance to vancomycin has been observed from livestock and related food products in Europe, as a consequence of the use or abuse of the glycopeptide antimicrobial avopracin in food-producing animals (Aarestrup et al., 2000). This is in agreement with the results of Rehman et al. (2018) in a study carried out in Canada showed that tigecycline has good *in vitro* activity against enterococcal isolates, although their use may be limited in certain clinical scenarios due to the emergence of resistance (Arias et al., 2010). Detection of tigecycline resistance in Enterococci is very rare; majority of the reports are from health care settings (Bender et al., 2020).

The results of the present study distinctly showed the presence of *E. faecalis* Enterococci with high AMR from an aquaculture environment and highlights the potential risk of spread of AMR from aquaculture farms using raw poultry waste from industrial farms where antibiotics are used as a cheap production tool. The findings suggests that the illegal practice of dumping raw poultry waste in aquaculture farms solely for profit is a definite contributor to the spread of MDR isolates in the environment. Hence, farmers have to be dissuaded from this practice through awareness programmes and regulatory mechanisms.

A positive outcome of this study was an official ban by the Department of Fisheries, Government of Kerala on the use of poultry waste in aquaculture (ReAct, 2018). The state was one of the first in India to implement the 'One Health Approach' to prevent the misuse and indiscriminate use of antimicrobials in the different sectors through its AMR action plan Kerala Antimicrobial Resistance Strategic Action Plan (KARSAP).

The findings of the study points to the fact that new superbugs can emergence from the aquaculture environment due to improper farming practices with poor concern for biosecurity, and this is a potential hazard eventually for the community at large.

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