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Research Article

EMERGING THREAT OF MULTIDRUG RESISTANT *ENTEROCOCCUS* IN THE PORK SUPPLY CHAIN: A STUDY IN ANDHRA PRADESH, INDIA

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ABSTRACT

This study aimed to examine the multidrug resistance patterns and prevalence of *Enterococcus* species in samples obtained from ten pig farms and ten pork retail outlets located in Andhra Pradesh. The resistance of 497 *Enterococcus* isolates to 16 different antibiotics was examined. *Enterococcus faecalis* (n=261) was the most frequently identified species, followed by *E. faecium* (n=139), *E. gallinarum* (n=61), and *E. casseliflavus* (n=36). Numerous antibiotics showed significant rates of resistance; erythromycin, amikacin, and streptomycin had the highest rates, with 80.08%, 79.67%, and 48.49%, respectively. There were also noteworthy resistance rates for ofloxacin (32.99%), moxifloxacin (36.61%) and tetracycline (40.04%). Additionally, resistance rates of 19.51% and 20.12% to penicillin-G and vancomycin, respectively, were noted. These results draw attention to the alarmingly high incidence of multidrugresistant *Enterococcus* species in Andhra Pradesh pig farming and retail pork industries.

Keywords: *Enterococcus*, Farmers, MDR, Pigs, Pork retail outlets

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As a prominent pathogen linked to hazardous hospital acquired infections, enterococci have become increasingly important (Hayes *et al.*, 2003; Hershberger *et al.*, 2005). According to Peters *et al.* (2003), they rank as the third most frequent cause of nosocomial bacteremia and the second most frequent source of nosocomial infections. Animals, food products, and inanimate environments have been suspected of being sources for several resistant clinical isolates, even though the role of non-human sources and reservoirs in the spread of *Enterococcus* strains remains unsettled (Thal *et al.*, 1995; Hershberger *et al.*, 2005). It's been suggested that food animals could act as a reservoir for enterococci and a source of resistance genes that people can contract through the food chain (Hayes *et al.*, 2003; Hershberger *et al.*, 2005). As of right now, we understand that bacteria resistant to antibiotics are chosen from among the intestinal flora of animals, contaminate foods derived from animals, and spread their resistance to other gut bacteria in humans, whether or not those bacteria are harmful (Van den Bogaard *et al.*, 2000). Accordingly, the intestinal flora of healthy animals and people is the most significant reservoir for resistant bacteria and resistance genes (Van den Bogaard *et al.*, 2000). According to Kludtson *et al.* (1992), Van den Bogaard *et al.* (1997) and Van den Bogaard *et al.* (2000), the prevalence of antibiotic resistance detected in indicator bacteria isolated from human and animal faecal samples is regarded to be a good indicator of the selective pressure of antibiotic usage. Furthermore, it has been suggested that one of the top priorities for public health should be to monitor the presence of lowlevel antibiotic resistance in microbes, especially in food animals (Van den Bogaard *et al.*, 1997; Van den Bogaard *et al.*, 2000). This study set out to determine the prevalence of multidrug resistance (MDR) in isolates of enterococci from swine, swine farmers, swine farm environments, and porcine retail outlets in Andhra Pradesh. The isolates included *E. faecalis*, *E. faecium*, *E. gallinarum* and *E. casseliflavus*.

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MATERIALS AND METHODS

In ten chosen pig farms and 10 retail pork stores in Andhra Pradesh, sampling was done between November 2021 and June 2023 (Fig. 1). Five piglets, five weaner pigs, and five adult pigs were randomly chosen from each farm, and 150 pigs from all ten farms were subjected to veterinary observation during the collection of oral, rectal and faecal samples (a total sample size of n=450). In addition, twenty swine farmers who worked on the involved farms provided twenty nose swabs, twenty faecal, twenty urine and twenty hand swab samples. It was suggested to the human subjects to gather midstream capture urine samples. In addition to samples from humans and animals, 140 environmental samples were gathered from different sources. The 80 samples from retail pork outlets, including swabs from cutting boards, knives, and hands in addition to pork samples, 30 samples from feed sources (including kitchen waste, concentrate and mix), and 30 samples from storage tanks, tap water sources, and trough water sources. The attending veterinarian both directly saw each sample during the sampling process and filled out a questionnaire to get background information about each one.

i) Isolation and identification of bacteria

After being enriched in brain heart infusion (BHI) broth with 6.5% NaCl, all of the samples that were gathered plated onto kanamycin esculin azide (KAA) agar. Based on their morphological characteristics, including Gram staining (+ve), oxidase test (-ve), hippurate hydrolysis (+ve), catalase (-ve), Voges-Proskauer test (+ve), and esculin hydrolysis (+ve), distinctive transparent colonies surrounded by black haloes on KAA agar were identified as presumptive *Enterococcus* spp., after incubation at 37º C for 24 hours (Forbes *et al.*, 2007). PCR was used to further validate that these isolates belonged to the *Enterococcus* genus (Poyart *et al.*, 2000). Using PCR and previously published techniques, four species *viz*., *E. faecalis*, *E. faecium*, *E. gallinarum* and *E. casseliflavus* were identified (Dutka-Malen *et al.*, 1995; Jackson *et al.*, 2004).

ii) Antibiotic Susceptibility test

Using commercial antimicrobial discs (HiMedia Pvt. Ltd., Mumbai) and the disk diffusion method, the antibiotic susceptibility of *Enterococcus* strains was evaluated in compliance with the Clinical and Laboratory Standards Institute (CLSI, 2008) recommendations. The bacterial strains were evaluated against a panel of antimicrobial drugs that are regularly used in swine farms and are commonly used to treat enterococcal infections. Amikacin (AK, 30 µg), gentamicin (GEN, 10 µg), streptomycin (S, 30 µg), ciprofloxacin (CIP, 5 µg), moxifloxacin (MO, 5 µg), ofloxacin (OF, 5 µg), vancomycin (VA, 30 µg), teicoplanin (TEI, 30 µg), erythromycin (E, 15 µg), linezolid (LZ, 30 µg), furazolidone (FR, 50 µg), penicillin-G (P, 1U), piperacillin (PI, 100 µg), chloramphenicol (C, 30 µg), colistin (CL, 10 µg) and tetracycline (TE, 30 µg). *E. faecalis* ATCC 29212, the reference strain, was utilized as control strain.

RESULTS AND DISCUSSION

A total of 566 Enterococcus strains (392 from pigs, 69 from farmers, 45 from farm environments and 60 from pork retail outlets) were isolated. It was determined that these strains belonged to the following four species: *E. faecalis* (n = 261), *E. faecium* (n = 139), *E. gallinarum* (n = 61) and *E. casseliflavus* (n = 36). PCR was used to confirm the identity of these species, focusing on the sodA genes as reported by Jackson *et al.* (2004) and Dutka-Malen *et al.* (1995) for all strains of *E. gallinarum*, *E. faecalis*, *E. faecium* and *E. casseliflavus*. The overall 84.47% rate of prevalence for *Enterococcus* spp. in the current study was similar to the 94.6% prevalence rate reported by Chandra and Garg (2006). Hayes *et al.* have claimed greater incidence rates of up to 99% (2003). Krocko *et al.* (2011) and Thu *et al.* (2019) reported prevalence rates of 33.33% and 75%, respectively, which disagree with these findings. In comparison to farms evaluated by Krocko *et al.* (2011) and Thu *et al.* (2019), the increased incidence rate identified in the current study may be due to variations in geographic location, climate circumstances, or cleanliness practices on farms.

The current study outcomes for *Enterococcus* spp. prevalence in pork were 70.00% (14/20), which was in close agreement with the 93.28% and 90% prevalence rates reported by Hayes *et al.* (2003) and Quednau *et al.* (1998). In contrast, a lower prevalence rate of 33.33% was observed by Pavia *et al.* (2000) from retail establishments in Catanzaro, Italy. The study also observed 100.00% prevalence of *Enterococcus* spp. in pig faecal samples closely matched Beshiru *et al.* (2017) findings. The study revealed that samples from farms A and B in Benin City had enterococci prevalence rates ranging from 80-99%. Iweriebor *et al.* (2015), on the other hand, discovered a lower prevalence rate of 80.00% in 400 faecal samples taken from two piggery farms in the South African province of the Eastern Cape. Variations in farm management approaches, climatic circumstances, geographic regions and cleanliness practices could be the cause of the disparities in *Enterococcus* prevalence rates in pig farms.

The present study findings regarding the prevalence of *Enterococcus* spp. in water samples were in agreement with Asha Peter (2013) findings, which revealed a 100% overall prevalence in a sample frame consisting of 170 water samples from Kerala. On the other hand, a lesser incidence of 71% was noted by Montiel *et al.* (2013) in Venezuelan lake water samples. In addition, Tan *et al.* (2018) found that drinking water samples taken from swine farms in Peninsular Malaysia had a lower prevalence of only 7%. The location and timing of the samples may have an impact on the variations in *Enterococcus* prevalence found in water samples from swine farms. The study pig farms increased prevalence of *Enterococcus* in their water samples could be attributed to their unsanitary circumstances. The results of Asha Peter (2013), who reported a prevalence of 100% in a sample frame of 200 human faeces samples from Kerala, was closely followed by the 100% overall prevalence of *Enterococcus* spp. in stool samples from farm workers. In comparison to these findings, investigations utilizing comparable sampling frames of human diarrheal samples revealed lower prevalence rates of enterococci, with rates of 51.55% and 83.33%, respectively, reported by Kudaier (2007) and Biswas (2015). Furthermore, Thu *et al.* (2019) found that hospitalized patients in Thailand and Lao PDR, as well as slaughterhouse workers and butchers in retail markets, had prevalence rates of 25.3% and 94.7%, respectively. Geographical disparities, sample

Fig. 1. The map of Andhra Pradesh state showing the locations of pig farms

PF1 LFC, NTR, CVSc, Gannavaram
PF2 Piggery farm Nuziveed
PF3 Piggery farm Veeravalli
PF4 Piggery farm Buddhavaram
PF5 Piggery farm LPT, NTR, CVSc, Gannavaram
PF6 Pig breeding station, Muktyala
PF7 AICRP on pigs, Tirupati
PF8 Pig breeding station, Vizianagaram
PF9 Piggery farm Buddhavaram
PF10 Piggery farm Chilakaluripet

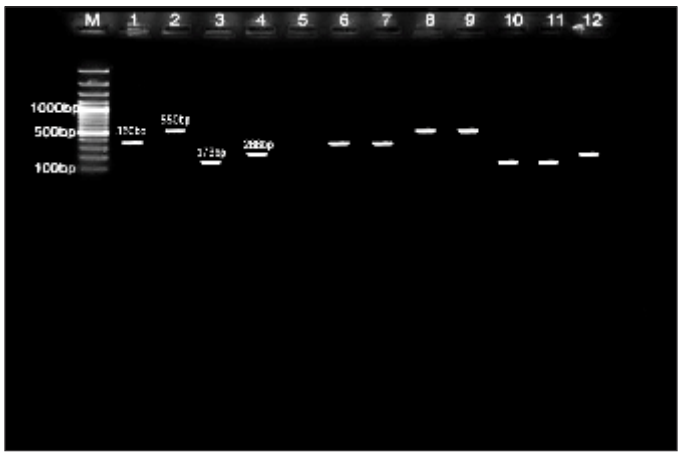

Fig. 3. Gel photograph of PCR showing species specific bands of different *Enterococcus* spp. (*E. faecalis*, *E. faecium*, *E. gallinarum* and *E. casseliflavus*)

Lane M- Molecular weight marker (100-3000bp)
Lane 1 positive control of *E. faecalis* ATCC29212 (360 bp)
Lane 2 positive standard of *E. feacium* ATCC6059 (550bp)
Lane 3 positive control of *E. gallinarum* (173bp)
Lane 4 positive control of *E. casseliflavus* (288bp)
Lane 5 negative control (distilled water)
Lane 6 *E. faecalis* positive isolate from farm worker faecal sample (360bp)
Lane 7 *E. faecalis* positive isolate from piglet sample (360bp)
Lane 8 *E. faecium* positive isolate from rectal swab sample of adult pig (550bp)
Lane 9 *E. faecium* positive isolate from rectal swab sample of adult pig (550bp)
Lane 10 *E. gallinarum* positive isolate from weaner pig faecal sample (173bp)
Lane 11 *E. gallinarum* positive isolate from pork sample (173bp)
Lane 12 *E. casseliflavus* positive isolate from adult pig faecal sample (288bp)

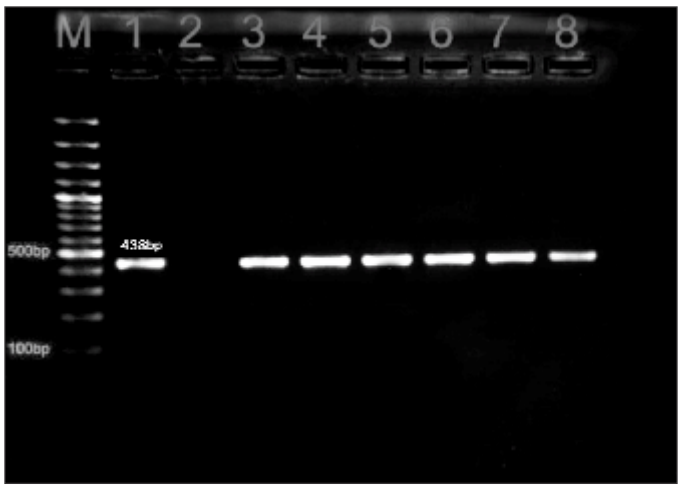

Fig. 2. Gel photograph of PCR showing genus specific bands for *Enterococcus*

Lane M- molecular weight marker (100-3000bp)
Lane 1 positive control of *E. faecalis* ATCC 29212 showing genus specific gene (partial sequence of sodA) (438 bp)
Lane 2 negative control (distilled water)
Lane 3 Genus *Enterococcus* positive isolate from piglets (438bp)
Lane 4 Genus *Enterococcus* positive isolate from weaners (438bp)
Lane 5 Genus *Enterococcus* positive isolate from adult pigs (438bp)
Lane 6 Genus *Enterococcus* positive isolate from pig farm worker (438bp)
Lane 7 Genus *Enterococcus* positive isolate from meat sample (438bp)
Lane 8 Genus *Enterococcus* positive isolate from cutting board swab sample (438bp)

sizes, seasonal fluctuations, and isolation techniques could all be contributing factors to these discrepancies in prevalence rates.

While research on human urine samples from India and Spain revealed lower prevalence rates of 8.92% and 7.38%, respectively, Desai *et al.* (2001) and Miskeen and Deodhar (2001) found that all urine samples from farm workers tested positive for *Enterococcus* spp. These variations in prevalence rates could be caused by a number of things, including variations in geography and the sample size and isolation techniques employed in each study.The results of this investigation are consistent with those of Chingwaru *et al.* (2003), who found that animalorigin foods had high prevalence rates of *E. faecalis* (46.1%), *E. faecium* (29.0%) and *E. casseliflavus* (7.6%). In 37 meat samples of different species, Chandra and Garg (2006) likewise showed significant prevalence rates, with *E. faecalis* being the most prevalent species (73%) followed by *E. gallinarum* (45.9%) and *E. raffinosus* (37.8%). Additionally, they reported reduced prevalence rates for *E. faecium*, *E. durans*, *E. hirae*, *E. mundtii*, *E. solitarius*, *E. pseudoavium*, *E. dispar*, *E. cecoruum* and *E. avium*, among other species. These results demonstrate the prevalence of specific *Enterococcus* species in animalderived foods and recommend the adoption of appropriate hygiene measures.

Of the 14 *Enterococcus* isolates obtained from pork in the current study, 42.85% of the species belong to *E.*

faecalis. With a prevalence rate of 28.57%, *E. faecium* recorded second and *E. gallinarum* and *E. casseliflavus* each accounted for 7.14% of the isolates. These results are in consistent with research conducted on pork samples at various stages of the slaughter process as well as pork products by Klein *et al.* (1998) and Knudtson and Hartman (1993). In addition, both investigations revealed that *E. faecalis* had higher prevalence rates than other *Enterococcus* species. These results imply that *E. faecalis* is frequently present in pig products because of its capacity to endure the stages of processing, storage, and transportation, which increases the risk of foodborne diseases. In the current investigation, *Enterococcus* species were found in all 30 water samples that were analyzed, with 36.66% of the isolates found in the water samples, *E. faecalis* was the most common species found. These findings align with those of Kimiran-Erdem *et al.* (2007) and Alipour *et al.* (2014), who similarly observed high prevalence rates of *E. faecalis* in isolates from surface water (68.6% in Babolsar and 96% in Istanbul, respectively). However, *E. faecium* (37.46%) was found to be the most common species in water samples followed by *E. gallinarum* (34.92%), *E. hirae* (11.74%), *E. casseliflavus* (10.15%) and *E. mundetii* (5.71%) by Enayati *et al.* (2015). Tan *et al.* (2018), in contrast, discovered that water samples from Malaysian pig farms had no *E. faecium* and a lower prevalence rate of *E. faecalis* (7%). The differing prevalence rates of *Enterococcus* species in water samples highlight possible bacterial contamination in water sources and to take the necessary precautions to protect the public health.

The identification of *Enterococcus* species in each of the 30 water samples examined in this investigation is indicative of faecal contamination in environmental water sources. This implies that faeces from either pigs or people may have polluted the tap water, trough water, and storage water. Because *Enterococcus* species are known opportunistic pathogens that can infect immuno-compromised people and can also act as an indicator for the presence of other pathogens, including viruses and bacteria, in the water, faecal contamination of water sources can be a serious public health risk (Bohem *et al.*, 2014). Thus, it is crucial to have the right policies in place to stop faeces from contaminating water sources and to reduce any health hazards that may arise from *Enterococcus* species in aquatic environments. According to studies by Molechan *et al.* (2019) and Iweriebor *et al.* (2015), there has been an increase in the prevalence of highly resistant enterococci in recent years. According to Tan *et al.* (2018), one element that may contribute to the spread of these resistant bacteria in intensive pig farming operations is the closeness of farmers, animals and the farm environment. The 497 *Enterococcus* isolates that were looked at in this study showed a notable degree of antibiotic resistance. The antibiotic resistance of 80.08% was the highest rate of resistance for erythromycin, followed by amikacin (79.67%), streptomycin (48.49%), tetracycline (40.04%), moxifloxacin (36.61%), ofloxacin (32.99%), ciprofloxacin (29.97%), furazolidone (26.35%), gentamicin (23.54%), vancomycin (20.12%), penicillin-G (19.51%), colistin (16.90%), chloramphenicol (16.29%), piperacillin (10.66%), linezolid (10.26%) and teicoplanin (10.26%). Given the serious consequences for both human health and animal welfare, these findings highlight the urgent need for surveillance and control strategies to address the formation and spread of antibiotic resistant enterococci in agricultural settings especially in swine farms.

The unusual high rates of streptomycin (48.49%) and gentamicin (23.54%) resistance shown in this study are especially concerning as they drastically lower the number of enterococcal infection treatments that are accessible. For the treatment of severe enterococcal infections, including endocarditis, ampicillin or penicillin combined with an aminoglycoside is often advised. Gentamicin resistance as described in this study is similar to that of Krocko *et al.* (2011), who likewise found a 25% resistance rate. Nonetheless, Kimiran -Erdem *et al.* (2007) and Messi *et al.* (2006) have reported, respectively, higher and lower rates. A resistance rate of 92.8% was found by Messi *et al.* (2006) to aminoglycosides, however a lower rate of only 2% was recorded by Kimiran-Erdem *et al.* (2007). On the other hand, Kimiran-Erdem *et al.* (2007) and Citak *et al.* (2005) have reported resistance rates of 97% and 88%, respectively, indicating higher levels of streptomycin resistance. These results underline how critical it is to maintain surveillance and create fresh approaches to therapy in order to counteract the rising incidence of antibioticresistant enterococci.

In the current investigation, 80.08% of *Enterococcus* isolates showed alarming erythromycin resistance. The widespread use of macrolide antibiotics, especially tylosin, in animal husbandry practice for the purpose of promoting animal growth and treating illnesses is probably the cause of the high level of erythromycin resistance observed in these bacteria. This result is in line with earlier studies that showed different degrees of erythromycin resistance. For example, resistance rates were reported by Kimiran-Erdem *et al.* (2007) to be 7% and by Cariolato *et al.* (2008) to be 40%. Additionally, erythromycin resistance rate of 66.67% was observed by Zou *et al.* (2011). This study reports the resistance rates were higher than those reported by Valenzuela *et al.* (2008) and Soares *et al.* (2015), recorded resistance rates of 26.66% and 38%, respectively. The current study findings highlight the critical need to use

antibiotics sparingly in animal husbandry procedures in order to prevent the formation and spread of bacteria resistant to these antibiotics.

CONCLUSION

The present study in Andhra Pradesh found a disturbing incidence of multidrug resistant (MDR) *Enterococcus* species in both retail pork shops and swine farms. The public health is seriously at danger due to the prevalence of MDR *Enterococcus* strains carrying different antibiotic resistance genes in the livestock industry. Although the bulk of the strains were hostspecific and regional, this study found ubiquitous strains that were shared by other hosts, underscoring the significance of regional surveillance initiatives. A stringent regulation is required to guarantee the appropriate use of antibiotics in swine husbandry practices in order to minimize the emergence of MDR strains, given their widespread occurrence.

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