

Uncovering linezolid resistance in *Enterococcus* isolates from Tunisian traditional healthy chickens: A One health surveillance study

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Abstract

Traditional poultry farming constitutes an important sector of Tunisian rural economy. The objective of this study was to understand resistance dynamics of these production systems by determining the antimicrobial phenotypes and genotypes as well as virulence factors of *Enterococcus* strains collected from chicken ceca.

One hundred and seven individual traditional chicken ceca were collected between October 2020 and July 2021 from eight different domestic poultry farms in northern Tunisia. Samples were plated on Slanetz–Bartley agar and one *Enterococcus* representative colony was selected by sample and identified by MALDI-TOF mass spectrometry. Antibiotic resistance and virulence genes and multilocus-sequence-typing of selected enterococci was performed by PCR and sequencing.

105 enterococci to six different species were identified. Polymerase Chain Reaction and sequencing were used to determine antibiotic resistance, virulence genes and multilocus-sequence-typing of selected enterococci. Acquired linezolid resistance was detected in *Enterococcus* isolates of seven samples (6.5%), all of them carried the *optrA* gene; these isolates were identified as follows: 3 *E. faecalis* (assigned to ST476, ST40 and ST249), 2 *E. faecium* assigned to new ST2516 and 2 *E. gallinarum*. The virulence genes *agg*, *hyl* and *gelE* were detected with variable distribution among these linezolid resistant isolates. High rates of tetracycline (82.9%) and ciprofloxacin resistance (81%) was observed among enterococci: The following resistance genes were found in resistant isolates: *tet(M)*, *tet(L)*, *ermB*, *catA*, *fexA*, *fexB* and *aac(6')-Ie-aph(2'')-Ia*.

This study reports a valuable insight on the low noise spreading of resistance to linezolid, a last-resort antibiotic, in rural farming systems. It highlights the urgent need to reinforce specific surveillance in such low-monitored environments to limit its public health impact.

Background

Being the most consumed meat in the country, traditional poultry farming constitutes one of the important sectors in Tunisia, whether for personal consumption or for profit [1]. Many consumers, especially in the north of Tunisia, have opted to set up their own poultry farm in a traditional way in order to combat inflation and guarantee a good quality consumer product. In this area, data on the occurrence of antibiotic-resistant enterococci in traditional livestock farming in Tunisia remains very scarce. Without close monitoring, rigorous surveillance and clarified control measures, the occurrence of antibiotic-resistant bacteria in traditional poultry farming could potentially contribute to the low noise spread of humans and animals' infections [2]. Recently, a surveillance study conducted by our research group highlighted that intensive livestock farming serves as a reservoir for vancomycin and linezolid-resistant enterococci [3]. This finding underscores the urgent need for comprehensive monitoring of antibiotic usage policies in both of traditional and intensive poultry farming to mitigate the risk of spreading antibiotic-resistant enterococci.

In poultry farming, enterococci are a component of the normal microbiota of the gastrointestinal tract of animals and humans [4]. Among the bacteria of this genus, the two species *E. faecium* and *E. faecalis* are recognised to be opportunistic pathogens that can change, in some circumstances, from being commensal organisms to seriously infecting both of human and animals [5, 6]. They have been previously linked to various infections, including those of the respiratory and gastrointestinal tracts, endocarditis and septicaemia [7]. In

addition, the rising emergence of antibiotic-resistant *Enterococcus* strains, particularly those resistant to last line antibiotics such as vancomycin, poses a substantial public health challenge in modern medicine since these infections spread quickly and are more difficult to treat [8].

Linezolid is an antibiotic belonging to the oxazolidinone class, mainly used to treat infections caused by multidrug-resistant (MDR) Gram-positive bacteria, notably those associated to *E. faecalis* and *E. faecium* strains [9]. The mechanism of action of this last line antibiotic involves inhibiting bacterial protein synthesis by binding to the 50S ribosomal subunit. It specifically binds to the bacterial ribosome (23S part of the 50S subunit) and prevents the formation of the functional 70S initiation complex which is an essential element of the DNA replication process [10]. This antibiotic, considered a last-line treatment antibiotic, is usually used in the fight against resistant bacterial strains, especially given the remarkable increasing resistance to vancomycin [11]. However, recent studies reported the detection of linezolid-resistant *Enterococcus* strains in intensive poultry farming, in Tunisia and worldwide [3, 12, 13]. These investigations underscore increasing concerns about the efficacy of this last-line treatment antibiotic and highlights the need for stricter control measures in some agricultural practices, especially in traditional livestock breeding in rural area which are often poorly controlled [14].

The key objective of this investigation is to determine both the phenotypic and genotypic profiles of *Enterococcus* isolates collected from caecal samples of healthy chickens within different traditional poultry farming systems in Tunisia. It will also focus on identifying and characterizing *Enterococcus* strains that exhibit resistance to different antibiotic classes, including those used as last-line therapy antibiotics such as linezolid. These data will involve elucidating the underlying mechanisms of antimicrobial resistance and determining the different genetic lineages associated to these resistant isolates.

Material and methods

1. Sample processing and antimicrobial susceptibility testing

Sampling of cecum material was obtained from 107 traditional healthy chickens (one sample per animal) from eight different domestic poultry farms in Northern Tunisia, with the number of sampled chickens ranging from 11 to 16 per farm. All the chickens that were analysed in this study were intended for the personal consumption of the breeders and were occasionally sold to consumers. Sampling was conducted at the time of routine slaughter performed by professional slaughterers as part of standard local practice. No anesthesia or euthanasia procedures were performed, supervised, or overseen by the research team; caecal samples were collected aseptically immediately after slaughter and transported to the laboratory under refrigerated conditions for microbiological analysis. Samples were recovered during the containment period of SARS-CoV-2 crisis, from October 2020 to July 2021. These samples were transported to the laboratory and processed within 24 hours of the collection. The chicken cecal content was obtained in sterile spittoons and then plated on Slanetz and Bartley (SB) medium (BIOKAR, France) non-supplemented with antibiotic. After plates incubation at 37°C for 24–48 hours, one representative of each typical *Enterococcus* colony was selected for further analysis. Identification was carried out via conventional biochemical tests and confirmed by MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) (Bruker Daltonik GmbH, Bremen, Germany).

Antimicrobial susceptibility to 14 antibiotics was determined according to the disk diffusion method, by referring to the 2021 Clinical Laboratory Standards Institute M100 (according to a corresponding year; 31st edition guidelines and breakpoints). CASFM 2021 criteria were used for antimicrobial susceptibility to trimethoprim–sulfamethoxazole. The tested antimicrobial agents, purchased from Bio-Rad, were as follows (µg/disk): linezolid (30), vancomycin (30), teicoplanin (30), kanamycin (1000), gentamicin (120), streptomycin (300), ampicillin (10), trimethoprim–sulfamethoxazole (1.25 + 23.75), chloramphenicol (30), erythromycin (15), tetracycline (30), pristnamycin (15), ciprofloxacin (5), rifampicin (5) and fosfomycin (200). Minimal inhibitory concentrations (MICs) of vancomycin were determined by the use of E-test (Bio-Rad) for the two *E. gallinarum* resistant strains.

2. Detection of *Enterococcus* antibiotic resistance genes

The mechanisms of acquired resistance to linezolid were tested by PCR using the specific primers of the genes *cfr*, *optrA* and *poxTA* [15, 16]. The PCR-positive products were analyzed through sequencing by CarthaGenomics Advanced Technologies (Tunisia).

The detection of additional antimicrobial resistance genes was carried out by PCR, selected based on the bacterial resistance phenotype. The targeted genes included those encoding tetracycline resistance [*tet(M)*, *tet(L)*, *tet(O)*, and *tet(K)*], erythromycin resistance [*erm(A)*, *erm(B)*, *erm(C)*, and *msr(A)*], aminoglycoside resistance [*aac(6)-Ie-aph(2)-Ia*, *aph(3)-IIIa*, and *ant(6)-Ia*], pristnamycin resistance [*vat(D)* and *vat(E)*] and phenicol resistance [*fexA*, *fexB*, *catA*, *catB* and *cfr*], as previously reported [17–19]. The positive controls employed in this study were provided by the OneHealth-UR Research Group of the University of La Rioja and the Bioresources, Environment, and Biotechnology Lab collection from the Institut Supérieur des Sciences Biologiques Appliquées de Tunis, as referenced in previous studies [3, 20].

3. Molecular typing of linezolid resistant isolates

The linezolid-resistant *E. faecium* and *E. faecalis* isolates were subjected to multilocus sequence typing (MLST) for detailed genetic analysis, allowing the assessment of the clonal relationships and the epidemiological tracking of these resistant isolates, facilitating a better understanding of their transmission and evolutionary patterns. This involved sequencing internal fragments of seven housekeeping genes specific to each species. For the three *E. faecalis* isolates, the analysed genes were *aroE*, *gki*, *gdh*, *ylqL*, *gyd*, *pstS*, and *xpt*. For the two *E. faecium* isolates, the genes *adk*, *atpA*, *gdh*, *purK*, *gyd*, *pstS*, and *ddl* were sequenced. The sequence types (STs) obtained were compared to the entries in the MLST databases for *E. faecalis* (<https://pubmlst.org/organisms/enterococcus-faecalis/>) and *E. faecium* (<https://pubmlst.org/organisms/enterococcus-faecium/>).

4. Virulence genes detection of linezolid resistant *Enterococcus*

Extracted DNA served as a template for the amplification of virulence genes. The presence of genes encoding the following virulence factors were analysed in the seven *optrA*-positive *Enterococcus* strains through PCR: aggregation substance (*agg*), gelatinase (*gelE*), collagen adhesin (*ace*), hyaluronidase (*hyl*), sex pheromones (*cpd*) and biofilm production (*esp*) as previously described by [21].

Results

Cecum samples (n = 107) of traditional chickens were plated on SB medium non-supplemented with antibiotics and then 105 isolates with typical characteristics of *Enterococcus* genus were recovered. One representative colony per positive sample was analysed. After identification tests our findings showed that *E. faecium* (n = 75) was the most prevalent species in our collection, followed by *E. gallinarum* (n = 14), *E. faecalis* (n = 13), *E. durans* (n = 2) and *E. casseliflavus* (n = 1). All the *Enterococcus* selected isolates were resistant to at least one antibiotic (Table 1). Antimicrobial susceptibility testing showed that 83.8% of the enterococci (88/105) were qualified as multidrug resistance isolates, being resistant to three or more classes of antibiotics.

Table 1

Number (%) of antibiotic-resistant *Enterococcus* isolates collected from healthy traditional chicken cecum

Antibiotic	<i>E. faecium</i> n = 75	<i>E. gallinarum</i> n = 14	<i>E. faecalis</i> n = 13	<i>E. durans</i> n = 2	<i>E. casseliflavus</i> n = 1	Total enterococci ^b n = 105
Linezolid	2	2	3	-	-	7 (6.7%)
Vancomycin	-	14	-	-	1	15 (14.3%)
Teicoplanin	-	-	-	-	-	0 (0%)
Ampicillin	3	2	-	-	-	5 (4.8%)
Streptomycin ^a	12	1	1	-	-	14 (13.3%)
Gentamicin ^a	15	3	5	-	-	23 (21.9%)
Kanamycin ^a	35	4	4	1	1	45 (42.9%)
Chloramphenicol	9	3	3	1	-	16 (15.2%)
Tetracycline	64	13	8	1	1	87 (82.9%)
Erythromycin	48	14	10	1	1	74 (70.8%)
Ciprofloxacin	67	11	7	-	-	85 (81%)
Trimethoprim–sulfamethoxazole	33	8	7	-	-	48 (45.7%)
Pristinamycin	6	4	8	-	-	18 (17.1%)
Fosfomycin	18	1	-	-	-	19 (18.1%)
Rifampicin	11	7	7	-	-	25 (23.8%)
^a High level resistance						
^b Number of isolates (%)						

Seven isolates showed linezolid resistance, representing 6.5% of the total analysed samples (7/107). These isolates were identified as *E. faecalis* (3 isolates), *E. faecium* (2 isolates) and *E. gallinarum* (2 isolates); they

were found in three different farms (Table 2 and Table 3). All these linezolid-resistant isolates harboured the acquired *optrA* gene. The genes *cfr* and *poxA* were not detected in this study.

Table 2
Genetic characterization of *Enterococcus* isolates recovered from traditional chicken caecum harboring acquired antimicrobial resistance genes

Species	Antibiotic Resistance (number of isolates)	Antibiotic resistance genes detected (number of isolates)
<i>E. faecium</i> (n = 75)	Linezolid (2) Tetracycline (64) Erythromycin (48) Chloramphenicol (9) Gentamicin (15)	<i>optrA</i> (2) <i>tet</i> (M) (41); <i>tet</i> (L) (13); <i>tet</i> (M) + <i>tet</i> (L) (10) <i>erm</i> (B) (44) <i>fex</i> (B) (6) ; <i>catA</i> (1); <i>fex</i> (A) (1); <i>catA</i> + <i>fexA</i> (1) <i>aac</i> (6')-le- <i>aph</i> (2'')-la (12)
<i>E. faecalis</i> (n = 13)	Linezolid (3) Tetracycline (8) Erythromycin (10) Chloramphenicol (3) Gentamicin (5)	<i>optrA</i> (3) <i>tet</i> (M) (3); <i>tet</i> (L) (2); <i>tet</i> (M) + <i>tet</i> (L) (3) <i>erm</i> (B) (10) <i>fex</i> (A) (3) <i>aac</i> (6')-le- <i>aph</i> (2'')-la (5)
<i>E. gallinarum</i> (n = 14)	Linezolid (2) Vancomycin (14) Tetracycline (13) Erythromycin (14) Chloramphenicol (3) Gentamicin (3)	<i>optrA</i> (2) <i>vanC1</i> (14) ^a <i>tet</i> (M) (10); <i>tet</i> (L) (2); <i>tet</i> (M) + <i>tet</i> (L) (1) <i>erm</i> (B) (14) <i>catA</i> (3) <i>aac</i> (6')-le- <i>aph</i> (2'')-la (3)
<i>E. durans</i> (n = 2)	Tetracycline (1) Erythromycin (1) Chloramphenicol (1)	<i>tet</i> (M) (1) <i>erm</i> (B) (1) -
<i>E. casseliflavus</i> (n = 1)	Vancomycin (1) Tetracycline (1) Erythromycin (1) Gentamicin (1)	<i>vanC2/3</i> (1) ^a <i>tet</i> (L) (1) <i>erm</i> (B) (1) <i>aac</i> (6')-le- <i>aph</i> (2'')-la (1)

^aIntrinsic resistance

Table 3
Characteristics of the enterococci with acquired linezolid resistance collected from chicken cecum of traditional farms

Strain	Species	Farm	Origin	Resistance phenotype	Linezolid resistance genes	Other acquired resistance genes	Virulence genes	Sequence type
ET208	<i>E. faecium</i>	Farm 2	E6	LZD GEN KAN CIP TET CHL ERY RIF SXT	<i>optrA</i>	<i>tet(M)</i> <i>tet(L)</i> <i>fex(B)</i> <i>aac(6')-le-aph(2'')-la</i>	<i>hyl gelE</i>	ST2516
ET210	<i>E. faecium</i>	Farm 3	E22	LZD GEN KAN CIP TET CHL ERY SXT	<i>optrA</i>	<i>tet(L)</i> <i>fex(B)</i> <i>aac(6')-le-aph(2'')-la</i>	<i>hyl</i>	ST2516
ET190	<i>E. faecalis</i>	Farm 3	E19	LZD GEN KAN CIP TET CHL ERY SXT	<i>optrA</i>	<i>tet(M)</i> <i>tet(L)</i> <i>erm(B)</i> <i>fex(A)</i> <i>aac(6')-le-aph(2'')-la</i>	<i>hyl</i>	ST476
ET209	<i>E. faecalis</i>	Farm 3	E20	LZD CIP TET ERY RIF SXT	<i>optrA</i>	<i>tet(M)</i> <i>tet(L)</i> <i>erm(B)</i>	<i>hyl</i>	ST40
ET278	<i>E. faecalis</i>	Farm 3	E23	LZD GEN RIF ERY CHL SXT	<i>optrA</i>	<i>erm(B)</i> <i>aac(6')-le-aph(2'')-la</i>	-	ST249
ET206	<i>E. gallinarum</i>	Farm 3	E21	LZD VAN* GEN KAN STR CIP TET CHL PRST ERY SXT	<i>optrA</i>	<i>tet(M)</i> <i>tet(L)</i> <i>erm(B)</i> <i>aac(6')-le-aph(2'')-la</i>	<i>hyl agg</i>	-
ET281	<i>E. gallinarum</i>	Farm 4	E27	LZD VAN* GEN ERY CHL PRST FOS RIF SXT	<i>optrA</i>	<i>tet(M)</i> <i>erm(B)</i> <i>aac(6')-le-aph(2'')-la</i>	<i>agg</i>	-
VAN: vancomycin, LZD: linezolid; GEN: gentamicin, CHL: chloramphenicol, PRST: pristinamycin, FOS: fosfomicin, ERY: erythromycin, RIF: rifampicin, SXT: trimethoprim– sulfamethoxazole, CIP: ciprofloxacin, KAN: kanamycin, TET: tetracycline.								
*VAN resistance is intrinsic in <i>E. gallinarum</i> species due to the presence of the <i>vanC1</i> gene.								

The three linezolid-resistant *E. faecalis* isolates were ascribed by MLST to the lineages ST40, ST249 and ST476. The two *optrA*-positive *E. faecium* isolates showed the same sequence type and were assigned to the new ST2516 (https://pubmlst.org/bigsdbs?page=info&db=pubmlst_efaecium_isolates&id=8202). These two

isolates were collected from the cecum samples of two chickens obtained in two different farms separated by 57 kilometres.

In this study we did not detect enterococci with acquired resistance to vancomycin. Nevertheless, was detected 15 isolates with intrinsic mechanisms of vancomycin resistance: 14 *E. gallinarum* harbouring the *vanC1* gene and 1 *E. casseliflavus* harbouring the *vanC2* gene. The MIC determined by E-test showed that all these strains presented intrinsic low-level resistance to this antibiotic which does not exceed 6µg/ml. All the tested isolates were susceptible to teicoplanin.

In addition to the linezolid and vancomycin resistance, the obtained *Enterococcus* isolates showed high percentage of resistance to tetracycline and ciprofloxacin with 82.9% (87/105) and 81% of isolates (85/105), respectively.

Moderate resistance levels were observed for trimethoprim-sulfamethoxazole (45.7%), kanamycin (42.8%), and rifampicin (23.8%). The resistance rate for gentamicin was of 21.9%, followed by fosfomicin (18.1%). Moreover, lower resistance percentages were found for chloramphenicol (15.2%), streptomycin (13.3%) and ampicillin (4.8%).

The molecular characterisation of *Enterococcus* resistant isolates showed that tetracycline resistance was related to the presence of genes *tet(M)* and/or *tet(L)* (Table 2). Erythromycin resistance was associated to the acquisition of the *ermB* gene and chloramphenicol resistance to the genes *fexA*, *fexB* or *catA* genes encoding to efflux pumps and chloramphenicol acetyltransferase, leading to the inactivation of the antibiotic. Remarkably, one chloramphenicol resistant *E. faecium* isolate harboured two of these genes (*catA* and *fexA*). High-level gentamicin resistance in *E. faecium*, *E. faecalis*, *E. gallinarum*, and *E. casseliflavus* isolates was linked to the expression of the *aac(6')-Ie-aph(2'')-Ia* gene; this gene encodes a bifunctional aminoglycoside-modifying enzyme responsible for the acetylation and phosphorylation of aminoglycosides, which causes the inhibition of the antibiotic antibacterial activity.

Molecular analysis of the virulence factors of the seven linezolid-resistant enterococci isolates revealed that most of them (5/7) harboured the *hyl* gene, encoding for the hyaluronidase production. The gene *gelE* was detected only in one *E. faecium* isolate of the two assigned to the lineage ST2516. However, the genes *ace*, *cpd* and *esp*, which encodes for collagen adhesin, sex pheromones and biofilm production, respectively, were not identified in the tested isolates.

Discussion

Resistance to linezolid was detected in enterococci collected from seven cecum samples of traditional chickens, representing 6.5% of the total analysed samples. These isolates were collected from three of the eight tested traditional farms. To the best of our knowledge, this study reports the first detection of the acquired linezolid resistance gene *optrA* in *E. gallinarum* in African country in traditional chickens. Furthermore, *E. gallinarum* is intrinsically resistant to vancomycin and harbours the *vanC1* gene. Moreover, all *optrA*-positive enterococci were characterized by a multidrug resistance profile and were resistant to aminoglycosides, macrolides, tetracycline and/or chloramphenicol.

This study clearly highlights the presence of numerous circulating strains that have acquired resistance to relevant therapeutic options, as linezolid, considered antibiotics of last resort in cases of enterococcal infection. This prevalence of linezolid resistant isolates in cecum of the traditional poultry (6.5%) was higher than the one recently found in intensive poultry system in a previous study (3.6%) [3]. In both studies the selection of isolates was performed in SB plates non supplemented with antibiotics.

Moreover, the dissemination of *optrA* gene is no longer exclusive to Gram-positive bacteria. It was also recently reported in Gram negative bacteria, including *Campylobacter coli* isolates collected from Tunisian avian farms [22]. The rapid spreading of this gene between different bacterial species underscores the potential for swift dissemination of linezolid resistance. These findings call for urgent measures to monitor gene transfer dynamics and implement stringent controls to curb the high spread of resistance in both traditional and intensive poultry farming systems.

In the last decade, the worldwide and frequent detection of the positive *optrA* *E. faecalis* ST476 is being alarming. This strain was detected in the Asian, American and European continent from different human and animal origins [23–25]. This study confirms also the presence of *E. faecalis* ST476 strain in the Tunisian traditional poultry breeding for human consumption. These data raise an important public health and worldwide food safety concerns. High surveillance and rigorous monitoring must be given to this clone because the worldwide and low noise spreading of these antibiotic-resistant bacteria through the food chain can pose a direct risk for human health [26].

It is important to mention also that one of our linezolid resistant *E. faecalis* isolates was assigned to the ST249. This sequence type associated to the clonal complex (CC)246, has been previously reported in several countries in poultry and ducks' slaughterhouses, occasionally associated to some diseases like the vertebral osteomyelitis [27]. In Tunisia, the ST249, resistant to vancomycin, was detected in 2021 from routine rectal swab from an 8-year-old child with bone marrow aplasia and residing in a rural area [28]. We report in our study, the detection of the same ST, this time resistant to linezolid and susceptible to vancomycin, during the same period. Special attention should be accorded to ST249 which is detected in diverse sources including humans and traditional poultry farms, with resistance to the last-line antibiotics used to treat enterococci infections.

By analysing the food system of the different animals involved in this study, especially in the farms 2, 3 and 4 where linezolid resistant isolates were detected, the breeders have confirmed that these chickens feed mainly on human food waste and that they were in contact with the water sources surrounding the chicken coop. The breeders have also confirmed that they never used antibiotics during the life cycle and that the duration of domestic breeding of these chickens varied between four to six months before being consumed. For these three farms, the number of chickens exceeds 50 units and breeders sell these chickens when the opportunities arise.

It is important to mention that, during our visit, we did not notice phenotypically any disease in the animals carrying linezolid resistant enterococci. Moreover, the detection of resistance to this last resort treatment during the containment period of SARS-CoV-2 crisis, in a period of partial confinement, suggests that the transmission of these resistant bacteria could be due to the environment surrounding the farms. Potential sources of contamination include nearby water sources, food based on human waste, or even direct contact with the breeders.

The detection of two linezolid resistant *E. faecium* isolates with identical sequence type ST2516 in cecum samples of two chickens of farms located at a distance of 57 kilometres suggests a potential dissemination of this specific strain within the local poultry farming environment. Additionally, both of these two isolates were multidrug resistant and harboured common virulence genes encoding for factors implicated in tissues colonisation and invasion [29]. These results underscore the importance of understanding the implicated mechanisms by which antimicrobial-resistant bacteria can disseminate within and between animal populations, being a potential risk for both animal and human health. The emergence of linezolid-resistant enterococci in poultry farming environments raises also the possibility of transmission to humans through various pathways, including direct contact with contaminated poultry products or environmental exposure.

Table 3 shows the presence of the virulence gene *hyl* in five *optrA*-positive *Enterococcus* isolates. This gene, which encodes a hyaluronidase, degrades a key component of the extracellular matrix in host tissues. Previous studies have clearly confirmed that this hyaluronidase is linked to an increase in the virulence and infectivity of these bacteria [30]. Moreover, the two *optrA*-positive *E. gallinarum* isolates harboured the *agg* gene; this gene encodes an aggregation protein which is involved in adherence to eukaryotic cells, cell aggregation and conjugation [31].

For several years now, the rate of very high resistance to tetracycline, ciprofloxacin and erythromycin in animal breeding farms has become very frequent. Recent studies conducted by our research group on enterococci isolates recovered from the cecum of industrial and intensively farmed poultry showed high percentages of resistance to these antibiotics varying between 65.9 and 74.9% [3]. The present study reports also high resistance levels (70.8% and 82.9%), even though they were recovered in the cecum of poultry raised in traditional chicken farms. Notably, acquired resistance to tetracycline and erythromycin is encoded by the *tet(M)*, *tet(L)*, and *erm(B)* genes. This study confirms the widespread prevalence of these three genes in both of clinical and the veterinary environments in Tunisia [32, 33].

This investigation revealed concerning rates of resistance to aminoglycosides, ranging from 13.3% for streptomycin to 21.9% for gentamicin, and reaching 42.8% for kanamycin. The results of molecular analysis identified the presence of the *aac(6')-Ie-aph(2'')-Ia* gene in high level gentamicin resistant isolates. This gene, which encodes the AAC(6')-APH(2'') bifunctional enzyme, was detected in 12 *E. faecium*, 5 *E. faecalis*, 3 *E. gallinarum* and 1 *E. casseliflavus*. The occurrence of this gene across four of the five identified species of *Enterococcus* in this study could be related to its location on a mobile genetic element (plasmids, transposons and insertion elements) which facilitates horizontal gene transfer not only among isolates of the same species but also in other Gram-positive bacteria including *Pediococcus* and *Staphylococcus* as previously described [34].

Florfenicol is an antibiotic commonly used in veterinary medicine and has recently faced growing resistance, particularly linked to the raising dissemination of the *fexA* and *fexB* genes. Additionally, these genes are often reported in isolates carrying *optrA* or *poxTA*, as they are commonly located on the same mobile genetic elements [35, 36]. These two chloramphenicol-resistant genes, detected in traditional farming in this study, were present in linezolid-resistant *E. faecium* strains. The global spread of *fexB*, not only in *Enterococcus* strains but also in *Staphylococcus* and *Campylobacter* species from farming animals and seafood, raises concerns about future therapeutic limitations [22, 37, 38]. Additionally, previous studies associated the

presence of resistance elements *fexB-poxA* to the transposon *Tn6246*, underscoring the growing difficulty of managing infections across human and animal populations [15].

Conclusion

This study illustrates the key factor driving the spread of *Enterococcus* strains resistant to last-resort antibiotics within the traditional poultry farming sector, where health monitoring is limited. Our findings clearly highlight the important role of the links between animals, environment and eventually human compartments in promoting the circulation of resistance genes and their possible persistence within specific area. In order to manage this situation, we suggest the immediately implementation of rigorous and smart surveillance based not only on a regular regional monitoring throughout time but also a deployment of rapidly and adaptable detection tools. Such an integrated approach, in accordance with the One Health perspective, would allow for a rapid identification of the different factors leading to the low-noise spread of antibiotic-resistant strains, thereby improving our understanding of global resistance dynamics. This strategy will have a direct impact on strengthening prevention and specific management strategies for antimicrobial risk within the poultry sector.

Abbreviations

VAN

Vancomycin, LZD:Linezolid

GEN

Gentamicin, CHL:Chloramphenicol, PRST:Pristinamycin, FOS:Fosfomycin, ERY:Erythromycin, RIF:Rifampicin, SXT:Trimethoprim– sulfamethoxazole, CIP:ciprofloxacin, KAN:Kanamycin, TET:Tetracycline

MDR

Multidrug-resistant

MLST

Multilocus sequence typing

ST

Sequence Typing

MIC

Minimal inhibitory concentrations

Declarations

Ethical Approvals and consent to participate

This research was conducted in accordance with ethical approval granted by the Institut Supérieur des Sciences Biologiques Appliquées de Tunis (Authorization EE-CFS01, 2019). All sampling procedures involving animals were performed in accordance with the relevant guidelines and regulations. All methods were carried out in accordance with relevant guidelines and regulations. Throughout this study, the welfare and rights of animals were rigorously respected in all cases.

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Credit authorship contribution statement

Conceptualization: Ben Slama K, Torres C and Ben Yahia H. Data Curation: Ben Yahia H, Boukhili S. Methodology: Ben Yahia H, Issaoui R and Trabelsi I. Formal analysis: all authors. Writing, review and editing: All authors. Project administration Ben Slama K. Supervision: Ben Slama K and Torres C. All authors have read and approved the final version of the manuscript.

Consent publication

Not applicable

Competing interests

All authors declare that no competing interests

Data availability

All the data related to this current study are available from the corresponding author upon reasonable request. The DNA sequences generated and analysed during the current study are publicly available in the NCBI GenBank database under accession numbers PZ904236 -PZ904269 (*Enterococcus faecalis* isolates ET190, ET209, ET278: PZ904236–PZ904255; *Enterococcus faecium* isolates ET208, ET210: PZ904256–PZ904269).

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