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ORIGINAL ARTICLE

Antibiotic resistance and virulence of faecal enterococci isolated from food-producing animals in Tunisia

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Abstract Antimicrobial agents exert a selection pressure not only on pathogenic, but also on commensal bacteria of the intestinal tract of humans and animals. The aim of this work was to determine the occurrence of different enterococcal species and to analyse the prevalence of antimicrobial resistance and the mechanisms implicated, as well as the genetic diversity in enterococci recovered from faecal samples of food-producing animals (poultry, beef and sheep) in Tunisia. Antimicrobial resistance and the mechanisms implicated were studied in 87 enterococci recovered from 96 faecal samples from animals of Tunisian farms. *Enterococcus faecium* was the most prevalent species detected (46 %), followed by *E. hirae* (33.5 %). High percentages of resistance to erythromycin and tetracycline were found among our isolates, and lower percentages to aminoglycosides and ciprofloxacin were identified. Most of the tetracycline-resistant isolates carried the *tet*(M) and/or *tet*(L) genes. The *erm*(B) gene was detected in all erythromycin-resistant isolates. The *ant*(6)-Ia, *aph*(3′)-Ia and *aac*(6′)-*aph*(2″) genes were detected in nine aminoglycoside-resistant isolates. Of our isolates, 11.5 % carried the *gelE* gene and exhibited gelatinase acitivity. The *esp* gene was detected in 10 % of our isolates and the *hyl* gene was not present in any isolate. The predominant species (*E. faecium* and *E. hirae*) showed a high genetic diversity by repetitive extragenic palindromic (REP)-PCR. Food animals might play a role in the spread through the food chain of enterococci with virulence and resistance traits to humans.

Keywords Enterococci · Tunisia · Animals · Antibiotic resistance · Resistance genes · Virulence

Introduction

The selective pressure caused by the intensive use of antimicrobial agents in human and veterinary medicine, livestock, aquaculture, and agriculture food technology, associated with the mechanisms of bacteria for genetic transfer, could have contributed to the emergence and wide spread of resistance mechanisms in bacteria of different ecosystems (Hammerum 2012; Nilsson 2012; Lebreton et al. 2013).

Enterococcus spp. colonize the gastrointestinal tract of humans and many animals, and are also commonly found in soil and sediments, beach sand, plants, food and waters (Muller et al. 2001; Aarestrup et al. 2002; Guardabassi et al. 2004; Fisher and Phillips 2009; Byappanahalli et al. 2012; Klibi et al. 2012; Silva et al. 2012; Klibi et al. 2013). Furthermore, they are also important cause of nosocomial opportunistic infections in humans and have been reported in sporadic infections in animals (Fisher and Phillips 2009). Over the last decades, antibiotic multiresistance has increased in enterococci; this can be explained by the massive use of antibiotics both in the human health care system and in agriculture (Murray 1998; Arias and Murray 2008; Top et al. 2008; Clementi and Aquilanti 2011; Hammerum 2012). In fact, enterococci might act as a reservoir of antimicrobial resistance genes that could be transmitted to other pathogenic

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bacteria through the exchange of plasmids and conjugative transposons, and for this reason might represent a worldwide problem in public health (Arias and Murray 2008; Clementi and Aquilanti 2011; Hammerum 2012).

Enterococci are intrinsically resistant to a number of antimicrobial agents, but they can also acquire resistance to other antimicrobial agents, such as quinolones, macrolides, tetracyclines, streptogramines and glycopeptides (Murray 1990; Werner et al. 2008; Fisher and Phillips 2009). The selection of vancomycin-resistant enterococci was associated with the use of avoparcin, a growth promoter used in animal production in Europe but not in Tunisia, which led to its ban in the European Union in 1997 (Werner et al. 2008; Nilsson 2012). Although food-producing animals are not always a source of enterococci for humans, they may cause transmission of their resistance genes to human bacteria. Therefore, prevalence of resistant enterococci, especially vancomycin-resistant enterococci, in food-producing animals has become a serious problem (Hammerum 2012; Nilsson 2012). Antibiotic resistance has previously been studied in faecal enterococci from food animals, as well as wild and pet animals in different countries (Aarestrup et al. 2000; Butaye et al. 2001; Poeta et al. 2005; Jackson et al. 2010; Kojima et al. 2010; Santos et al. 2013), but this type of study is very scarce in Tunisia. The aim of the present study was to determine the occurrence of different enterococcal species and their genetic diversity, as well as to analyse the prevalence of antimicrobial resistance and virulence genes in enterococci recovered from faecal samples of food-producing animals in Tunisia.

Methods

Sampling

Faecal samples of 96 farm animals (39 beef, 36 sheep and 21 poultry) were analysed for enterococci recovery (one sample per animal). Samples were collected during September–December 2011 in 21 different farms in the North of Tunisia. For isolation, a suspension of the faecal sample was inoculated on Slanetz-Bartley agar plates and incubated for 48 h at 37 °C. One colony per sample was selected and identified by morphological characteristics, Gram's stain, catalase test, bile-esculin reaction and by hypersalin test. Furthermore, seven enterococcal species were identified by PCR using specific primers for $ddl_{E.\ faecalis}$ (D-Ala-D-Ala ligase), $ddl_{E.\ faecium}$ (D-Ala-D-Ala ligase), *vanC-1* (D-alanyl-D-serine ligase), *vanC-2* (D-alanyl-D-serine ligase), *mur-2eh* (muramidase), *mur-2ed* (muramidase) and *MU* (superoxide dismutase) genes specific for *Enterococcus faecalis*, *E. faecium, E. gallinarum, E. casseliflavus*, *E. hirae*, *E. durans* and *E. mundtii*, respectively, as previously described (Torres et al. 2003; Jackson et al. 2004; Arias et al. 2006). In cases of non-clear results with species-specific PCR, the identification was performed by PCR and sequencing of 16S rDNA gene (Galkiewicz and Kellogg 2008).

Antibiotic resistance

Antibiotic susceptibility testing was performed by the disk diffusion method as previously recommended (CLSI, 2012). The following antibiotics were tested (μg/disk): vancomycin (30), teicoplanin (30), tetracycline (30), chloramphenicol (30), erythromycin (15), trimetoprim-sulfamethoxazole (1.25/23.75), pristinamycin (15), ciprofloxacin (5) and ampicillin (10). Detection of high-level aminoglycoside resistance was performed with high charge disks of gentamicin (500 μg), kanamycin (1,000 μg) and streptomycin (500 μg), according to Antibiogram Committee of the French Society for Microbiology (CA-SFM, 2010). Minimal Inhibitory Concentration (MIC) determination of erythromycin, gentamicin and vancomycin was performed by the agar dilution method. *E. faecalis* strain ATCC 29212 was used as quality control strain.

Enterococcal resistant isolates were tested by PCR for detection of the following resistance genes: *erm*(B), *erm*(A) and *erm*(C) (in erythromycin resistant isolates), *tet*(M) and *tet*(L) (in tetracycline resistant isolates), *aac*(6′)-*aph*(2″) (in gentamicin resistant isolates), *ant*(6)-Ia (in streptomycin resistant isolates), and *aph*(3′)-IIIa (in kanamycin resistant isolates), using previously reported primers and conditions (Table 1). All PCR reactions included positive controls from the strain collection of the University of Tunis (Tunisia).

Virulence genes

The presence of virulence genes in all enterococcal isolates was tested by PCR, using primers and conditions described in Table 1. Genes tested were as follows: *gel*E (gelatinase), *ace* (accessory colonization factor), *hyl* (glycosil-hydrolase) and *esp* (extracellular surface protein). Gelatinase production was detected by inoculating the enterococci on tryptic soy agar plates containing 3 % gelatin (Klibi et al. 2007).

Molecular typing

Isolates were grouped by combined analysis of repetitive extragenic palindromic (REP) typing using primer BOXA1R (5′-CTACGGCAAGGCGACGCTGACG-3′) and $(GTG)_5$ (5′GTGGTGGTGGTGGTG-3′) (PRIMM, Milan, Italy) as reported by Colombo et al. (2009) and Versalovic et al. (1994), respectively. Rep-PCR patterns were analysed by GelCompar II software using the UPGMA algorithm and the Dice similarity coefficients (V.6.5, Applied Maths, Belgium).

Table 1 Primers used in PCR reactions for the detection of genes implicated in antibiotic resistance and virulence

Primer name	Sequence (5′→3′)	T_m (°C)	Reference
aac(6′)-*aph*(2″)-*Ia*	F- CCAAGAGCAATAAGGGCATA R- CACTATCATACCACTACCG	60	Del Campo et al. 2003
ant(6)-*Ia*	F- ACTGGCTTAATCAATTTGGG R- GCCTTTCCGCCACCTCACCG	58	Del Campo et al. 2003
erm(B)	F- GAAAAGRTACTCAACCAAATA R- AGTAACGGTACTTAAATTGTTTAC	52	Sutcliffe et al. 1996
erm(A)	F- TCTAAAAAGCATGTAAAAGAA R- CTTCGATAGTTTATTAATATTAGT	52	Sutcliffe et al. 1996
erm(C)	F- TCAAAACATAATATAGATAAA R- GCTAATATTGTTTAAATCGTCAAT	52	Sutcliffe et al. 1996
tet(M)	F- GTTAAATAGTGTTCTTGGAG R- CTAAGATATGGCTCTAACAA	55	Aarestrup et al. 2000
tet(L)	F- CATTTGGTCTTATTGGATCG R- CAATATCACCAGAGCAGGCT	50	Aarestrup et al. 2000
aph(3′)-*IIIa*	F- GCCGATGTGGATTGCGAAAA R- GCTTGATCCCCAGTAAGTCA	60	Del Campo et al. 2003
ace	F- GAATTGAGCAAAGTTCAATCG R- GTCTGTCTTTTCACTTGTTTC	56	Klibi et al. 2007
gel(E)	F-AGTTCATGTCTATTTTCT TCAC R-CTTCATTATTTACACGT TTG	56	Klibi et al. 2007
hyl	F-GAGTAGAGGAATATGTTAGC R- AGGCTCCAATTCTGT	56	Klare et al. 2005
esp	F- TTGCTAATGCTAGTCCACGACC R-GCGTCAACACTTGCATTGCCGAA	63	Klibi et al. 2007

Results and discussion

Enterococci were recovered from 87 of the 96 faecal samples of farm animals tested (91 % recovery) and one isolate per sample was recovered (39 from beef, 31 from sheep and 17 from poultry). Species detected were the following ones: *E. faecium* (40 isolates, 46 %), *E. hirae* (29 isolates, 33.5 %), *E. casseliflavus* (six isolates, 7 %), *E. gallinarum* (five isolates, 5.8 %), *E. faecalis* (four isolates, 5 %) and *E. mundtii* (three isolates, 3.5 %). All these isolates were identified by species-specific PCRs as indicated in the 'Methods' section (except seven isolates with no clear results, for which PCR and sequencing of 16S rDNA gene was performed for identification).

The detection of *E. faecium* and *E. hirae* as the predominant enterococcal species in faeces of food-producing animals in this study shows strong similarities with data from pigs and wild boars, previously reported by others (Kuhn et al. 2003; Poeta et al. 2005). *E. faecium* and *E. hirae* have also been referred to as frequent species in the faecal content of other animals (Butaye et al. 2001; Guardabassi et al. 2004; De Leener et al. 2005; Poeta et al. 2005; Silva et al. 2012).

Table 2 shows the percentage of resistance to antibiotics detected among our enterococcal isolates. Moderate resistance rates were detected for erythromycin (MIC>4 μg/ml) and tetracycline in *E. faecium* (30 % and 15 %, respectively) and *E. hirae* isolates (13.8 % in both cases), lower than data reported in faecal enterococci of wild animals in Portugal (Poeta et al. 2005) or even in enterococci from domestic animals (Stovcik et al. 2008). This difference might be

Table 2 Antibiotic resistance among enterococci recovered from food-producing animals in this study

Antibiotic	Number of isolates (%) resistant to antibiotics		
	E. faecium (n=40)	*E. hirae* (n=29)	Other species[b] (n=18)
Tetracycline	6 (15 %)	4 (13.8 %)	3 (17 %)
Erythromycin	12 (30 %)	4 (13.8 %)	3 (16 %)
Pristinamycin	1 (2.5 %)	0	1 (5.5 %)
SXT[a]	40 (100 %)	29 (100 %)	18 (100 %)
Vancomycin	0	0	0
Teicoplanin	0	0	0
Gentamicin	1 (2.5 %)	0	1 (5.5 %)
Streptomycin	3 (7.5 %)	1 (3.5 %)	0
Kanamycin	4 (10 %)	1 (3.5 %)	1 (5.5 %)
Ciprofloxacin	9 (22.5 %)	7 (24 %)	5 (25 %)
Chloramphenicol	0	0	0
Ampicillin	0	0	0

[a] Trimethoprim-sulphametoxazole

[b] Other species: *E. casseliflavus* (n=6), *E. gallinarum* (n=5), *E. faecalis* (n=4) and *E. mundtii* (n=3)

associated with a lower use of these antibiotics in animal husbandry in Tunisia, although no data on this aspect are available. Pristinamycin resistance was detected in one *E. faecium* isolate and ciprofloxacin resistance was detected in different enterococal species (22-25 %).

No vancomycin resistance was demonstrated among our isolates, with the exception of six *E. casseliflavus* and five *E. gallinarum* isolates, which showed a vancomycin MIC between 2 and 16 μg/ml. These enterococcal species show intrinsic low-level vancomycin resistance that cannot be transferred horizontally to other microorganisms, and for this reason, the epidemiological interest of this type of resistance is lower. Three isolates (two *E. gallinarum* and one *E. casseliflavus*) showed tetracycline and ciprofloxacin resistance, and one *E. casseliflavus* isolate erythromycin resistance.

Few of our enterococci (four *E. faecium*, one *E. faecalis*, and one *E. hirae*) exhibited high level resistance to gentamicin (MIC≥500 μg/ml), kanamycin or streptomycin (2.3 %, 6.9 %, and 4.6 % of enterococci, respectively). All our enterococci showed susceptibility for ampicillin and chloramphenicol and resistance for trimethoprim-sulfamethoxazole. *E. faecium* was the enterococcal species that showed higher percentages of antimicrobial resistance for erythromycin, kanamycin and streptomycin.

A total of 16 enterococci exhibited resistance to three or more families of antibiotics (18.5 % of isolates), and five isolates (four *E. faecium* and one *E. faecalis*), if we exclude SXT, an antibiotic with very low intrinsic activity on enterococci. The multiresistant phenotype was observed in nine *E. faecium* strains, three *E. hirae*, two *E. gallinarum*, one *E. faecalis* and one *E. casseliflavus* strain from beef (n=9), poultry (n=5), and sheep origin (n=2). No correlation was detected between animal origin and the different phenotypes obtained.

Table 3 shows the antibiotic resistance genes detected in our series of enterococci in relation with their specific antibiotic resistance phenotype. The *erm*(B) gene was found in all the erythromycin-resistant isolates (n=19), whereas *erm*(A) and *erm*(C) genes were not detected. The *erm*(B) gene has also frequently been found by other authors in erythromycin-resistant enterococcal isolates of different species and origins (Aarestrup et al. 2000; Schmitz et al. 2000; Del Campo et al. 2003). Thus, its acquisition could have a predominant role in the development of erythromycin resistance in *Enterococcus*.

The presence of the *tet*(M) gene, associated or not with *tet*(L), was confirmed in all our tetracycline-resistant isolates. Both *tet*(M) and *tet*(L) genes were found in six isolates. *tet*(M) was also the most frequent genetic determinant found in tetracycline-resistant enterococci by other authors (Aarestrup et al. 2000; Del campo et al. 2003), and the association of *tet*(M) and *tet*(L) genes was also reported by Aarestrup et al. (2000) in isolates of human and animal origin.

All six kanamycin-resistant isolates (four *E. faecium*, one *E. hirae* and one *E. faecalis*) harboured the *aph*(3′)-IIIa gene. The *ant*(6)-Ia gene was detected only in one isolate resistant to streptomycin (25 %). The two enterococcal isolates (one *E. faecalis* and *E. faecium*) resistant to gentamicin contained the *aac*(6′)-*aph*(2″) gene. These genes were also found in a previous report, among enterococci isolated from sheep, beef and poultry (Poeta et al. 2006).

Regarding the presence of virulence factors, PCR amplification revealed that 10.5 % of enterococci (9/87) contained the

Table 3 Antibiotic resistance genes detected by PCR among the enterococcal isolates according to their phenotype of resistance

Antibiotic	Species	Number of isolates	Genes detected (number isolates)	
Erythromycin			*erm*(B)	
	E. faecium	12	12	
	E. hirae	4	4	
	Other species	3	3	
			tet(M)	*tet*(M)+ *tet*(L)
Tetracycline	*E. faecium*	6	4	2
	E. hirae	4	1	3
	Other species	3	2	1
			aac(6′)-*aph*(2″)	
Gentamicin	*E. faecium*	1	1	
	E. faecalis	1	1	
			aph(3′)-IIIa	
Kanamycin	*E. faecium*	4	4	
	E. hirae	1	0	
	E. faecalis	1	1	
			ant(6)-Ia	
Streptomycin	*E. faecium*	3	1	
	E. hirae	1	0	

gene encoding the enterococcal surface protein (Esp), which was previously identified at high frequency among human isolates (35 %), and at lower frequency among meat enterococci (2.5 %) (Klibi et al. 2007; Klibi et al. 2013). The presence of the *esp* gene also suggests a role in colonization and formation of biofilm, and the moderate presence of the *esp* gene among animal faecal isolates could indicate a lower pathogenicity compared with the clinical isolates. The *ace* gene encoding the collagen-binding protein was detected in four isolates (three *E. faecalis* and one *E. faecium*), and the *hyl* gene was absent in all the enterococcal isolates. Fourteen isolates exhibited gelatinase activity (16 %) and the *gel* gene was detected in ten of these isolates; this prevalence is lower than data reported for isolates of poultry (90.9 %) (Hwang et al. 2011) and for isolates from meat origin (40.5 %) (Klibi et al. 2013). This gene was also detected in 26 gelatinase-negative isolates; this might be explained by the presence of a deletion in the *gel* operon (Nakayama et al. 2002; Klibi et al. 2007). The lack of apparent pathogenic potential of these isolates in healthy animals might be due to virulence being multifactorial and associated with different genes (Olsen et al. 2012). Further studies are needed to investigate the pathogenic role of virulence factors in animals. These data could help to determine the prevalence of virulence factors in enterococcal isolates from livestock animals in Tunisia, and indicate that enterococci isolates from food products of animal sources should be considered as a possible reservoir of virulence determinants important for the pathogenic potential of human ones. In fact, Olsen et al. (2012) have reported that poultry isolates of *E. faecalis* increase the virulence gene content of the genes available to human isolates through horizontal gene transfer after food consumption, supporting the zoonotic risk associated with this organism.

Further characterization of wild isolates at strain level was obtained through a PCR approach using oligonucleotides designed to match consensus sequences of repetitive extragenic palindromic (REP) sequences (De Urraza et al. 2000). This technique has been successfully applied to strain typing of both Gram-positive and Gram-negative bacteria, and it seems more specific and reproducible than RAPD-PCR

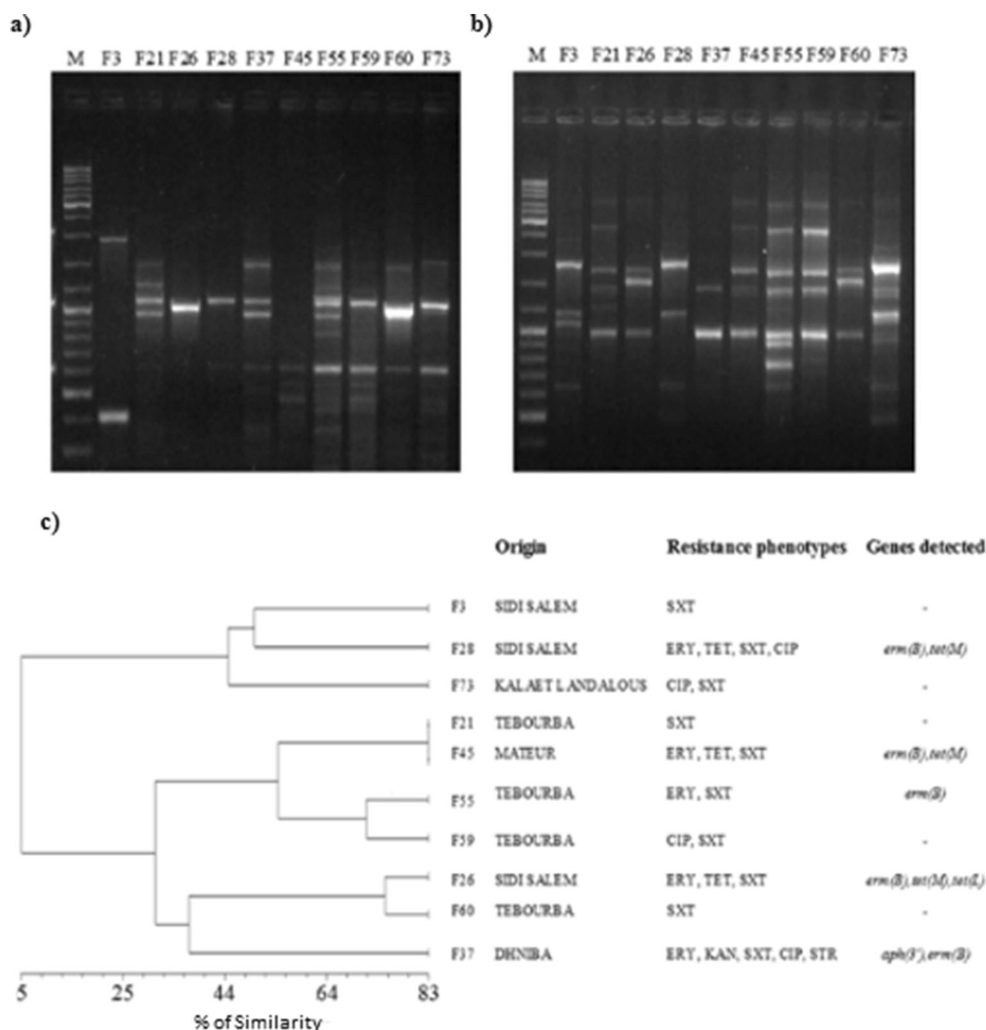

Fig. 1 REP fingerprints of *E. faecium* isolates (n=10). REP-PCR primer: BOXA1R (**a**) and $(GTG)_5$ (**b**); M: DNA ladder mix. Dendrogram obtained for the *E. faecium* isolates from analysis of $(GTG)_5$ - REP-PCR patterns by GelCompar (**c**)

analysis. These methods are frequently used in bacterial taxonomy and they have been successfully applied for reliable and fast differentiation at species level of different bacterial groups including enterococci (Svec et al. 2005; Borgo et al. 2013). Two different primer sets (BOXA1R and $(GTG)_5$) were used for the evaluation of *E. faecium* (n=10) and *E. hirae* (n=12*)* genetic fingerprinting. Isolates tested were selected for their different antibiotic resistance phenotypes. The electrophoretic profiles obtained from Rep-PCR analyses demonstrated that these primers have different discriminatory power (Figs. 1, 2).

For the species *E. faecium*, more heterogeneous electrophoretic profiles were obtained with the primers $(GTG)_5$ (Fig. 1b). This primer clustered nine *E. faecium* groups (and/or strains), while with BOXA1R a low electrophoretic band distribution did not permit a good discrimination at strain-level (Fig. 1a). UPGMA dendrogram obtained with $(GTG)_5$ for *E. faecium* strains showed a presence of two main cluster with 5 % of similarity correlation (Fig. 1c). In the first group, the strains F3, F28 and F73 were separated from the other isolates at a similarity level of 40 % (Fig. 1c). The second cluster was split into different sub-clusters correlated with similarity coefficients ranging from 30 % to 83 %. Also, a better discrimination at strain level was found for *E. hirae* using (GTG)5 REP-PCR (Fig 2b), while BOXA1R electrophoretic profiles did not permit a good separation (Fig. 2a). The UPGMA dendrogram obtained with (GTG)5 profiles (Fig. 2c) indicated a high level of biodiversity, represented by two different strain clusters linked at 8 % similarity level. The main cluster was split off into different sub-clusters correlated with similarity coefficients ranging from 10 % to 80 %. No correlation between ecological niche and isolates was observed. The second cluster bears two isolates, F23 and F67, with 40 % similarity.

The use of REP-PCR allowed good discrimination at the strain level, indicating a high level of biodiversity. It was not possible to relate the biodiversity found in *E. faecium* and *E. hirae* to their origin and their resistance phenotypes. In

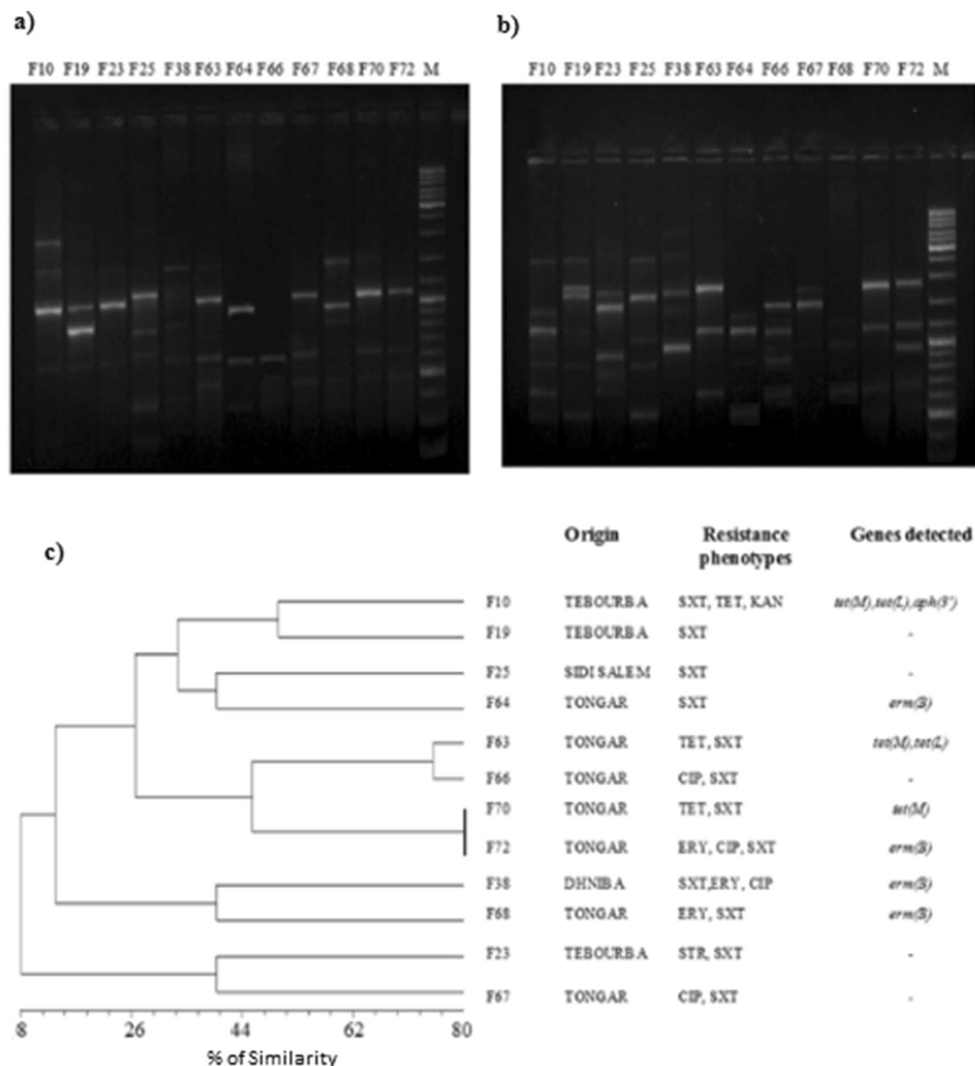

Fig. 2 REP fingerprints of *E. hirae* isolates (n=12). REP-PCR primer: BOXA1R (**a**) and $(GTG)_5$ (**b**); M: DNA ladder mix. Dendrogram obtained for the *E. hirae* isolates from analysis of $(GTG)_5$ - REP-PCR patterns by GelCompar (**c**)

accordance with other studies (Borgo et al. 2013), our results show that $(GTG)_5$-PCR is a good tool for differentiating enterococcal strains isolated from animals.

In conclusion, relatively low levels of antibiotic resistance were detected in enterococcal isolates recovered from farm animals in Tunisia, and the mechanisms of resistance and virulence implicated in resistant isolates are similar to those detected in enterococcal isolates of human origin or in meats (Klibi et al. 2007; Klibi et al. 2013). It is also important to underline the dissemination of genes encoding some virulence factors in tested isolates. The food-producing animals can constitute a reservoir of commensal bacteria with resistance and virulence genes that can be transferred to pathogen bacteria and to humans by contact or through the food chain. Overall, a greater understanding of virulence traits, and especially of increasing antibiotic resistance, is needed in order to fully appreciate the complexity of *Enterococcus* species in causing diseases in animals and humans.

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