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Antimicrobial-resistant *Escherichia coli* and *Enterococcus* spp. isolated from Miranda donkey (*Equus asinus*): an old problem from a new source with a different approach

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Abstract

Purpose. The Miranda donkey (*Equus asinus*) is an endangeredasinine from Miranda do Douro region, located in the north east of Portugal. We studied the antimicrobial resistance and virulence genes in *Escherichia coli* and *Enterococcus* spp. isolates from these animals.

Methodology. In March 2014, a total of 66 faecal samples were recovered from independent animals. Antibiotic resistance was determined by the disc diffusion method. Carriage of genes coding for antibiotic-resistant and virulent factors was analysed by PCR.

Results. A total of 66 *E. coli* and 41 enterococcal isolates were detected, with *Enterococcus faecium* (61 %) and *Enterococcus hirae* (24 %) being the most prevalent species. For enterococcal isolates, high percentages of resistance rates to tetracycline (68.3 %), quinupristin/dalfopristin (51.2 %) and ciprofloxacin (48.8 %) were observed. The genes *erm*(A) and/or *erm*(B), *tet*(M) and/or *tet*(L), *vat*(D) and/or *vat*(E) and *aph*(3′)-*IIIa* were also found. The most frequent virulence gene detected was *gel*(E), followed by *ace*, *cpd* and *hyl*. *Escherichia coli* isolates were highly resistant to streptomycin (78 %), whereas 39 % of them exhibited resistance to aminoglycosides and tetracycline. Genes *sul*1 and/or *sul*2 were detected in 66.7 % of trimethoprim/sulfamethoxazole-resistant isolates. The virulence genes detected were *fim*(A) (46 %) and *cnf*1 (27 %).

Conclusion. To the best of our knowledge, this is the first report showing antibiotic resistance among *Escherichia coli* and *Enterococcus* spp. isolates from the Miranda donkey in Portugal, indicating possible antibiotic-resistant bacterial reservoirs. However, the detection of these resistances presents a low risk for other animals and human beings in that rural area.

INTRODUCTION

Antibiotics are widely used not only to treat bacterial infections in humans and animals (during common veterinary practice) but also in animal production. Since the 1990s, antibiotics have been considered one of the most important discoveries of the century [1, 2]. According to Van Boeckel *et al.* [3], the global consumption of antimicrobial drugs will increase by 67 % between 2010 and 2030. In general, we consider that the two main factors for antibiotic resistance are overuse and misuse of antimicrobials [4, 5]. Farming [6], the transport of goods, and human migration [7, 8] all contribute to the dissemination of resistance genes. According to Ventola [9], 80 % of antibiotics sold in the USA are widely used as supplements in livestock to promote healthy growth, prevent infections and to obtain a high-quality product.

The selective pressure created by this excessive use of antibacterial drugs affects the normal microbiota of humans and other animals [10, 11]. Thus, antimicrobial resistance (AMR) observed in animals from different habitats, wild marine and terrestrial animals, has been increasing in recent years and it could be transferred horizontally between bacteria [12, 13]. According to Martínez [14], the high prevalence and diversity of resistance genes are associated with a higher probability of transfer among them. So, resistance genes in an external environment have a central role in the emergence of multi-resistant pathogens [5].

Different animal species could potentially act as reservoirs of antibiotic resistance genes (ARGs) with the capacity of dissemination in the environment, and these can ultimately be transferred to humans [15]. Multidrug-resistant organisms persist and spread worldwide, causing clinical failures in the treatment of infections and public health crise [4, 16]. As AMR is a threat to human and animal health worldwide, it is important to reduce the risks and limit access to antibiotics [17].

In the past, the Miranda donkey (*Equus asinus*) was of enormous importance in agricultural practice and transportation in rural areas of Miranda do Douro (north east of Portugal), but nowadays with agricultural mechanization, the rural exodus and additionally 35–50 % of the females producing infertile foals (hybrid from equine x asinine or asinine x equine), it has become an endangered endemic species [18, 19]. This breed is adapted to the mountainous terrain of rural areas with very low population density and poor soil [20]. The Miranda donkey breed represents a suitable model to better understand resistant microorganism dynamics and the prevalence of resistant genes.

Escherichia coli and *Enterococcus* spp. are commensal inhabitants of the intestinal microbiota in warm-blooded animals. These strains can act as reservoirs of antibacterial-resistant genes that could be transmitted to other pathogenic bacteria. AMR is widespread world wide and it is a key public health concern [16, 17, 21, 22]. In some countries, like Brazil, Russia, India, China and South Africa, among others, the predicted increase in antimicrobial consumption will be 99 %, up to seven times the estimated population growth [3].

The use of common antibacterial drugs renders therapy inefficient against resistant bacteria and, when the immune system is impaired, increased human mortality can be the result [23, 24]. Cantas *et al.* [4] defined some procedures aimed at reducing this problem, including decreasing antibiotic administration, using antibacterial drugs only for bacterial infections and minimizing treatment for problematic reservoirs (such as manure from intensive animal production or hospital effluent) among others. If the community can conserve the efficiency of existing drugs, resistance will be lower among new antimicrobials developed [1]. According to Spellberg [25], 'to prescribe a powerful antibiotic is easy, to protect it is hard'. Hence, it is crucial to study the genetic mechanisms involved in the development and dissemination of AMR [6, 26, 27].

Enterococcus spp. have attracted particular clinical interest in recent years. The majority are not virulent but there are reports of opportunist *Enterococcus* spp. responsible for nosocomial infections [28]. These authors also showed that *Enterococcus faecium* and *Enterococcus faecalis* are responsible for high levels of infection in humans and animals, including bacteraemia, endocarditis and pyelonephritis. Although they are intrinsically resistant to a wide range of antibiotics (β-lactams, aminoglycosides and ampicillin), they may develop other genetic mechanisms of drug resistance. Moreover, the prevalence of antimicrobial-resistant bacteria, particularly vancomycin-resistant *Enterococcus* (VRE), has increased which can compromise clinical treatment [10, 29]. Among the different kinds of antimicrobial agents used clinically, one of the most important are the β-lactams [30, 31]. A review by Al Atya *et al.* [32] analysed five *E. coli* strains producing extended-spectrum β-lactamases isolated from French children with urinary infections.

The main purposes of this study were to determine the prevalence of antibiotic resistance, to assess the presence of virulence genes and to analyse the population structure of enterococci and *E. coli* isolates from faecal samples from the Miranda donkey in north east Portugal.

METHODS

Animals and sampling

The animals used in this study were 34 jacks and 32 jennies from the population of the Miranda donkey (*Equus asinus*), an endangered Portuguese breed living in the north east of the country. Samples were collected in March 2014 using standardized procedures. One faecal sample per animal was obtained rectally, giving a total of 66 samples which were dispatched immediately to the Microbiology Laboratory of the University of Trás-os-Montes and Alto-Douro (UTAD).

E. coli and *Enterococcus* spp. isolation

At the laboratory the faecal samples were spread onto Levine agar plates, then either supplemented or not supplemented with $2\,\mu g\,ml^{-1}$ cefotaxime, in order to recover susceptible and resistant *E. coli* isolates. They were then incubated for 24 h at 37 °C. One colony per sample with standard *E. coli* morphology was subjected to standard biochemical methods (Gram-staining, catalase, oxidase, indol, methyl-red, Voges–Proskauer, citrate, urease and triple sugar iron). Species identification was confirmed by matrix-assisted laser desorption/ionization time-of-flight [MALDI-TOF (Bruker)].

Faecal samples were also inoculated on Slanetz–Bartley agar plates with or without $4\,\mu g\,ml^{-1}$ vancomycin for enterococcal isolation. They were then incubated for 24 h at 37 °C, and colonies with typical enterococcal morphology were submitted to biochemical testing, such as catalase and

kanamycin–esculin reaction. Finally, enterococcal species were identified by MALDI-TOF.

Antimicrobial analysis

Bacterial isolates were tested for antimicrobial susceptibility as recommended by the Clinical and Laboratory Standards Institute, by the disc diffusion method of Kirby–Bauer according to CLSI [33] using the 16 following antibiotics for *E. coli*: ampicillin (10 µg ml^{-1}), amoxicillin+clavulanic acid (20 +10 µg ml^{-1}), cefoxitin (30 µg ml^{-1}), cefotaxime (30 µg ml^{-1}), ceftazidime (30 µg ml^{-1}), aztreonam (30 µg ml^{-1}), imipenem (10 µg ml^{-1}), gentamicin (10 µg ml^{-1}), amikacin (30 µg ml^{-1}), tobramycin (10 µg ml^{-1}), streptomycin (10 µg ml^{-1}), nalidixic acid (30 µg ml^{-1}), ciprofloxacin (5 µg ml^{-1}), trimethoprim/sulfamethoxazole (1.25+23.75 µg ml^{-1}), tetracycline (30 µg ml^{-1}) and chloramphenicol (30 µg ml^{-1}).

Similarly, the antimicrobial susceptibility of enterococcal isolates was tested for 11 antibiotics, using a protocol based on the work of Billington *et al.* [34]: vancomycin (30 µg ml^{-1}), teicoplanin (30 µg ml^{-1}), ampicillin (10 µg ml^{-1}), streptomycin (300 µg ml^{-1}), gentamicin (120 µg ml^{-1}), kanamycin (120 µg ml^{-1}), chloramphenicol (30 µg ml^{-1}), tetracycline (30 µg ml^{-1}), erythromycin (15 µg ml^{-1}), quinupristin/dalfopristin (15 µg ml^{-1}) and ciprofloxacin (5 µg ml^{-1}).

DNA extraction and quantification

Escherichia coli isolates were submitted to boiling for DNA extraction, and we used the InstaGene Purification Matrix (Bio-Rad) for *Enterococcus* spp. In order to quantify the nucleic acid concentration and level of purity, the absorbance readings were taken at 260 and 280 nm (Spectrophotometer ND-100, Nanodrop).

Antibiotic resistance and virulence genes characterization

The carriage of resistance genes was analysed and identified by PCR, using primers and suitable conditions for each species [10]. The 41 *E. coli* and the 41 *Enterococcus* spp. isolates that were resistant to one or more antibiotics were selected for the next step.

The presence of ARGs was tested in the *E. coli* isolates that exhibited resistance to tetracycline [*tet*(A), *tet*(B) and *tet*(C)], streptomycin [*aad*(A)], trimethoprim/sulfamethoxazole (*sul*1, *sul*2 and *sul*3), ampicillin [*amp*(C)], gentamicin [aac(6′)], amikacin [*aac*(3′)] and tobramycin [ont(4′)] [35]. The ARGs tested in enterococci were *erm*(A), *erm*(B), *erm*(C) and *erm*(T) (in erythromycin-resistant isolates), *tet*(K), *tet*(L), *tet*(M) and *tet*(O) (in tetracycline-resistant isolates), *vat*(D) and *vat*(E) (in quinupristin/dalfopristin-resistant isolates), *aph*(3′)-IIIa (in kanamycin-resistant isolates), *van*(A) and *van*(B) (vancomycin-resistant isolates), *aac*(6′)-*aph* (2″) (in gentamicin-resistant isolates) and *cat*(A) (in chloramphenicol-resistant isolates) using primers and respective reaction conditions [36, 37].

The presence of virulence genes was tested in *E. coli* [*cnf*1, *fim*(A), *aer*, *pap*(C), *stx*, *pap*(G)III] [38, 39] and

Enterococcus spp. [*ace*, *agg*, *cpd*, *gel*(E), *fsr*, *hyl*, *esp*, *cyl*(A), *cyl*(B), *cyl*(L) e *cyl*(M)] [40–42], respectively. Positive and negative controls were used in all PCR assays, from the strain collection of UTAD.

Population structure

The identification of the major phylogenetic group among the isolates was determined by PCR [43].The genetic diversity of all isolates was assessed by PFGE in a CHEF DR II apparatus (Bio-Rad). To separate the digested fragments of enterococci (*Sma*I) and *E. coli* (*Xba*I), different protocols were applied (2 to 28 s for 24 h and 5 to 40 s for 20 h, respectively). Dendrogram analysis of PFGE profiles was performed using the Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) method based on Dice similarity by Phoretix 5.0 software (Nonlinear Dynamics).

Multilocus sequence typing (MLST) schemes were applied for PFGE-unrelated strains of *Ent. faecium* (www.mlst.net) and *E. coli* (http://mlst.ucc.ie/mlst/dbs/Ecoli). The phylogenetic relationship of the MLST alleles was assessed by analysis of the concatenate sequence of the different alleles by the neighbour-joining and maximum-likelihood methods with 1000 replicates, using MEGA 6 software. The eBURST algorithm was used to generate a cluster diagram using data of the 966 strains from the *Ent. faecium* MLST database and the seven new sequence-typing (STs) described in the present study, as well as 5804 strains from the *E. coli* MLST database and the three new STs described in the present study.

RESULTS

Bacterial isolation and identification

A total of 66 *E. coli* and 41 *Enterococcus* spp. isolates were identified from the 66 collected faecal samples. Among *Enterococcus* spp. isolates, six different species were detected by MALDI-TOF, the most prevalent being *Ent. faecium* (61 %), followed by *Enterococcus hirae* (24 %), *Enterococcus mundtii* (5 %), *Ent. faecalis* (5 %), *Enterococcus gallinarum* (2.5 %), and *Streptococcus equinus* (2.5 %).

E. coli antibiotic resistance

Antibiotic resistance was detected in 41 out of the 66 *E. coli* isolates, and the results are shown in Table 1. The highest rate of resistance was detected for streptomycin, in 78 % of the isolates. Other high resistances rates were detected for aztreonam (31.7 %), penicillin (34.2 %) and tetracycline and aminoglycosides (both with 39 %). Lower rates were observed for cephalosporins and quinolones (2.4 %), and none of the *E. coli* isolates were resistant to chloramphenicol and cefoxitin. Furthermore, 14.6 % of these bacterial isolates were resistant to more than five antimicrobial agents.

The ARGs detected in the 41 resistant isolates are shown in Fig. 1(a). The genes *sul*1 and/or *sul*2 were detected in 66.7 % of trimethoprim/sulfamethoxazole-resistant isolates. The genes *str*(A) or *str*(B) were found in 41.5 % of streptomycin-resistant isolates, while genes *tet*(A) or/and *tet*(B), related to

Table 1. Prevalence of antibiotic resistance detected in *E. coli* isolates from faecal samples from the Miranda donkey

Antibiotic	No. of resistant isolates	Prevalence (%)
AMC	2	4.9
ATM	13	31.7
CTX	1	2.4
CTZ	1	2.4
AMP	14	34.2
IMP	8	19.5
CIP	1	2.4
CN	4	9.8
TET	16	39
AK	16	39
TOB	7	17.1
STR	32	78
NA	1	2.4
SXT	12	29.3
CHL	0	0
FOX	0	0

%: Percentage of antibiotic-resistant isolates tested from a total of 41 samples. AMC, amoxicillin and clavulanic acid; ATM, aztreonam; CTX, cefotaxime; CTZ, ceftazidime; AMP, ampicillin; IMP, imipenem; CIP, ciprofloxacin; CN, gentamicin; TET, tetracycline; AK, amikacin; TOB, tobramycin; STR, streptomycin; NA, nalidixic acid; SXT, sulfamethoxazole/trimethoprim; CHL, chloramphenicol; FOX, cefoxitin.

an active efflux system, were found in 50 % of tetracycline-resistant isolates. The gene *fim*(A), which encodes the type-1 fimbrial subunit, was detected in 19 out of 41 isolates (46 %) and *cnf*1 was detected in 11 isolates (27 %). The coexistence of genes *fim*(A) and *cnf*1 was observed in two isolates (4.8 %).

Enterococcus spp. antibiotic resistance

As shown in Table 2, higher resistance levels were observed for tetracycline (68.3 %), quinupristin/dalfopristin (53.7 %),

ciprofloxacin (48.8 %), teicoplanin (36.6 %) and erythromycin (34.2 %). All *Ent. faecalis* isolates were intrinsically resistant to quinupristin/dalfopristin. However, lower rates of aminoglycosides resistance were detected for gentamicin (2.4 %) and streptomycin (4.9 %). The most prevalent *Enterococcus* species was *Ent. faecium* (61 %) and, as expected, the rates of resistance in *Ent. faecium* and *Ent. hirae* were higher than in other bacterial species. The genes *tet*(M) and/or *tet*(L) were found in 8 out of 18 tetracycline-resistant *Ent. faecium* (Table 3). The genes *tet*(M), *tet*(K) or *tet*(L) were not found in *Ent. mundtii* or *Ent equinus*. The gene *erm*(A) was found in one *Ent. faecium*, and the combination *erm*(A)+*erm*(B) in only one *Ent. hirae* erythromycin-resistant isolate. The gene *tet*(K) was found in four tetracycline-resistant *Ent. hirae* and the combination *tet*(M)+*tet*(L) in one of these. All kanamycin-resistant *Enterococcus* were positive for the gene *aph*(3′)-IIIa, while among 20 quinupristin/dalfopristin-resistant *Enterococcus* spp. only one did not have the genes *vat*(D) or *vat*(E), although all isolates showed some level of resistance (Fig. 1b). The most detected virulence gene was *gel*(E) in five isolates, followed by *ace*, *cpd* and *hyl* (one each in three *Enterococcus* spp. isolates).

E. coli population

Five out of 41 *E. coli* isolates (12 %) fit into the B2 phylogenetic group, and 19 into group D (46.3 %). Interestingly, eight of the *Escherichia coli* isolates classified in phylogenetic group D carry the gene *fim*(A) gene while and six isolates of this group had the gene *cnf*1.

The PFGE results are shown in Fig. 2, representing 56 out of the 66 *Escherichia coli* isolates; the other 10 isolates could not be PFGE typed by DNA degradation. The dendrogram analysis showed a highly unrelated population with at least 20 independent clusters of band patterns. At least one isolate per PFGE cluster was subsequently typed by MLST, corroborating the high genetic diversity of our population, and 10 out the 20 isolates typed by MLST corresponded to previously described STs.
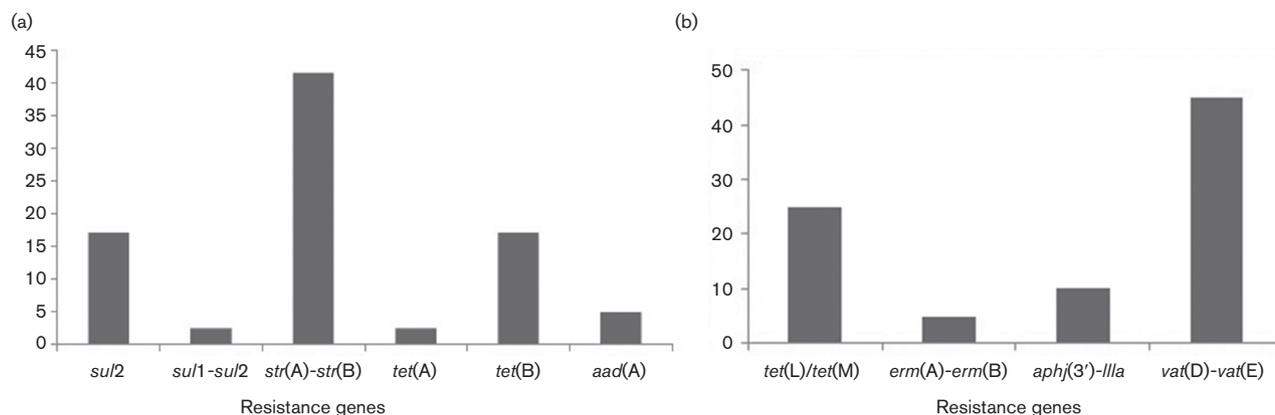

Fig. 1. (a) Distribution of *Escherichia coli* resistance genes isolated from faecal samples from the Miranda donkey. (b) Prevalence of *Enterococcus* spp. resistant genes isolated in the same study.

Table 2. Distribution of *Enterococcus* spp. isolates and prevalence of antibiotic resistance

Antibiotics	*Enterococcus faecium* (*n*=25)	*Enterococcus hirae* (*n*=10)	*Enterococcus faecalis* (*n*=2)	*Enterococcus mundtii* (*n*=2)	*Enterococcus gallinarum* (*n*=1)	*Streptococcus equinus* (*n*=1)	Prevalence (*n*=41)
AMP	8 (32 %)	0	0	0	0	1	9 (22 %)
CN	0	0	0	0	0	1	1 (2.4 %)
STR	0	0	0	0	1	1	2 (4.9 %)
CIP	15 (60 %)	3 (30 %)	0	1	1	0	20 (48.8 %)
TET	17 (68 %)	7 (70 %)	1 (50 %)	2 (100 %)	1	0	28 (68.3 %)
CHL	2 (8 %)	0	0	0	0	0	2 (4.9 %)
VAN	4 (16 %)	1 (10 %)	1	1	1	0	8 (19.5 %)
TEC	11 (44 %)	1	1	1	1	0	15 (36.6 %)
KAH	2 (8 %)	0	0	0	1	0	3 (7.3 %)
ERY	11 (44 %)	1	0	0	1	1	14 (34.2 %)
Q/D	14 (56 %)	4 (40 %)	2 (100 %)	1	1	0	22 (53.7 %)

%, Percentage of antibiotic-resistant isolates tested from a total of 41 samples. AMP, ampicillin; CN, gentamicin; STR, streptomycin; CIP, ciprofloxacin; TET, tetracycline; CHL, chloramphenicol; VAN, vancomycin; TEC, teicoplanin; KAH, kanamycin; ERY, erythromycin; Q/D, quinupristin/dalfopristin.

Furthermore, of the 20 isolates, 14 different STs were identified and 10 of these grouped a single isolate. Isolate 11 corresponded to ST10, which is one of the key hospital-high-risk clones associated with human infections (Fig. 3). The phylogenetic analysis of the concatenated nucleotide sequences grouped the 20 isolates into six major lineages as shown in Fig. 4.

Table 3. ARGs in enterococcal isolates obtained from faecal samples from the Miranda donkey

Enterococcus spp.	Resistance phenotype	No. of isolates	Resistance genes	No. of isolates
Enterococcus faecium	TET	18	*tet*(L)	1
			tet(M)	3
			tet(L)+*tet*(M)	4
	ERY	11	*erm*(B)	1
	KAH	2	*aph*(3′)-IIIa	2
	Q/D	14	*vat*(D)	12
			vat(E)	1
Enterococcus hirae	TET	7	*tet*(K)	4
			tet(L)+*tet*(M)	1
	ERY	1	*erm*(A)+*erm*(B)	1
	Q/D	4	*vat*(D)	1
			vat(E)	3
Enterococcus faecalis	TET	1	*tet*(L)	1
Enterococcus gallinarum	TET	1	*tet*(M)	1
	KAH	1	*aph*(3′)-IIIa	1
	Q/D	1	*vat*(D)	1
Enterococcus mundtii	Q/D	1	*vat*(D)	1
Strep. equinus	KAH	1	*aph*(3′)-IIIa	1

TET, tetracycline; CHL, chloramphenicol; ERY, erythromycin; KAH, kanamycin; Q/D, quinupristin/dalfopristin.

Ent. faecium structure population

The population structure of the *Ent. faecium* isolates was considerably less diverse than that observed for *E. coli*. A total of 10 unrelated PFGE band patterns were discriminated among the 25 *Ent. faecium* isolates (Fig. 5), corresponding only to previously described STs. MLST was applied to 29 isolates, 16 of these corresponding to ST32, and 7 previously undescribed alleles and/or combinations. The newly described ST1164 grouped five isolates, whereas the remaining STs matched only with a single isolate. The phylogenetic analysis demonstrated the existence of six lineages, none of which are related to hospital-associated high-risk clones (Figs 6, 7).

DISCUSSION

The antibiotic resistance crisis is related directly to the misuse and overuse of antibiotics, frequent not only in veterinary medicine [17, 21] and in medical practice [4] but also in animal production [3, 17], as well as the lack of new drugs. A recent study by Cuthbertson *et al.* [44] demonstrated that shortly after the introduction of antimicrobials in human medicine, resistance in pathogenic bacteria (or microbiota) was observed. In that context, the main concern is the possibility of disseminating resistance genes from environmental bacteria to pathogenic ones, which have the potential to infect patients and result in treatment failure. Consequently, due to the increase in drug resistance, these superbugs can multiply explosively and antimicrobials become less effective, especially in individuals with a weakened immune system [5, 45].

E. coli and *Enterococcus* spp. have high resistance to adverse environmental conditions and they can transfer extra-chromosomal elements encoding ARG or other virulence factors. For this reason, both are considered a good model for
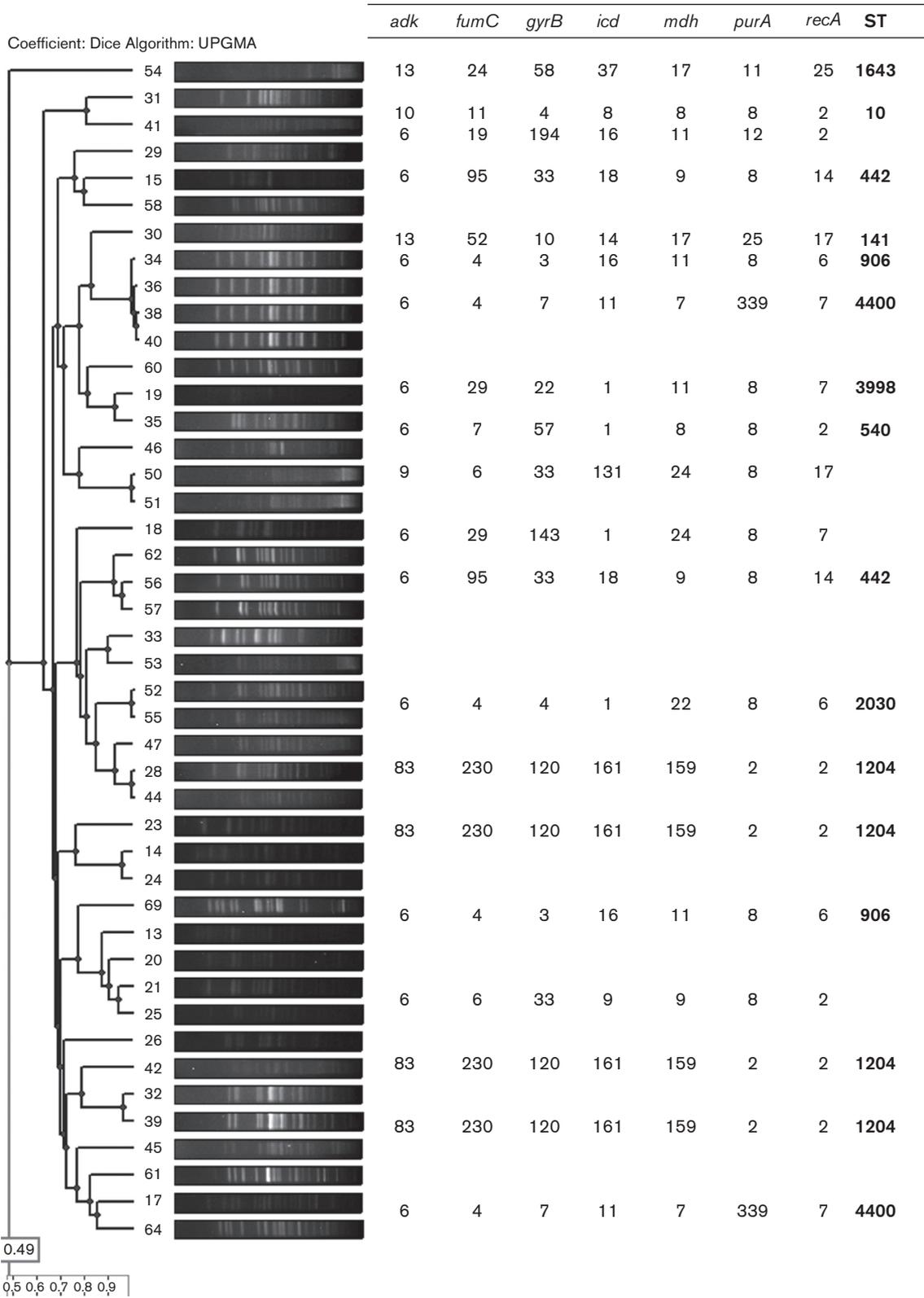
Coefficient: Dice Algorithm: UPGMA

Isolate	adk	fumC	gyrB	icd	mdh	purA	recA	ST
54	13	24	58	37	17	11	25	**1643**
31	10	11	4	8	8	8	2	**10**
41	6	19	194	16	11	12	2	
29								
15	6	95	33	18	9	8	14	**442**
58								
30	13	52	10	14	17	25	17	**141**
34	6	4	3	16	11	8	6	**906**
36	6	4	7	11	7	339	7	**4400**
38								
40								
60								
19	6	29	22	1	11	8	7	**3998**
35	6	7	57	1	8	8	2	**540**
46								
50	9	6	33	131	24	8	17	
51								
18	6	29	143	1	24	8	7	
62								
56	6	95	33	18	9	8	14	**442**
57								
33								
53								
52								
55	6	4	4	1	22	8	6	**2030**
47								
28	83	230	120	161	159	2	2	**1204**
44								
23	83	230	120	161	159	2	2	**1204**
14								
24								
69	6	4	3	16	11	8	6	**906**
13								
20								
21								
25	6	6	33	9	9	8	2	
26								
42	83	230	120	161	159	2	2	**1204**
32								
39	83	230	120	161	159	2	2	**1204**
45								
61								
17	6	4	7	11	7	339	7	**4400**
64								

0.49

0,5 0,6 0,7 0,8 0,9

Fig. 2. Dendrogram representing the diversity of the PFGE patterns of 56 *Escherichia coli* isolates complemented by the isolates typed by MLST and their data.
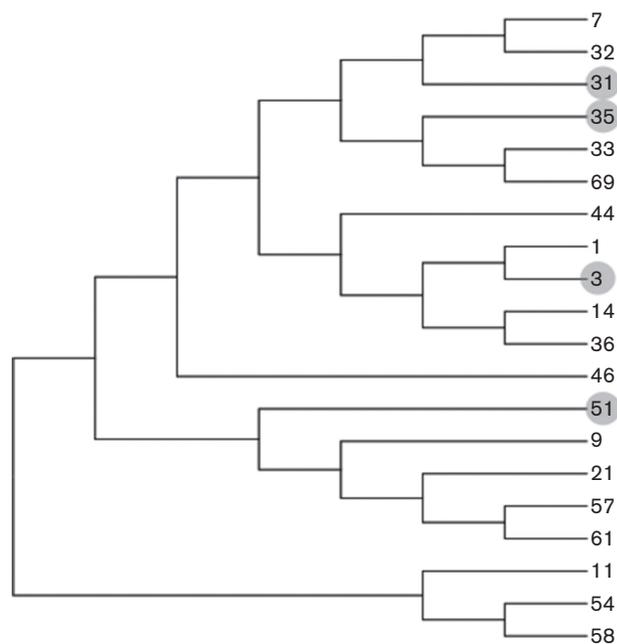
Fig. 3. Phylogenetic relationships of the *Escherichia coli* isolates typed by MLST. Isolates in grey circles represent STs previously not described.

the spread of environmental resistance and they were analysed in this study [5, 14, 46].

Recent studies in Italy [47] and Greece [48] show that the population of *Equus asinus* in Europe has decreased markedly over the last few decades, but it is also characterized by a high diversity of phenotypes and genotypes. To our knowledge, this is the first report to examine the rates of AMR and the population structure of enterococci and *E. coli* isolates from faecal samples in the Miranda donkey.

In our *E. coli* isolates, high levels of resistance to aminoglycosides, particularly streptomycin (78 %), followed by tetracycline and amikacin (39 %), were detected. These values are higher than those obtained in a study in horses (*Equus ferus caballus*) [49]. On the contrary, similar results were described from two livery stables in north west England [50].

The tetracycline resistance rates of *E. coli* in the present study (using faecal samples) were higher than those in the same strain isolated from raw milk (58.82 %), obtained from a donkey in Iran [51]. According to that study, the genes *tet*(A) or *tet*(B) showed the highest prevalence (64.70 %) while *aac*(3)-IV was the lowest (27.45 %). Among our 16 isolates with tetracycline resistance, the *tet*(B) gene was amplified in seven isolates and one of these was positive for the *tet*(A) gene, according to results observed in Lusitano horses by Moura *et al.* [52]. There are a high diversity of *tet* genes responsible to tetracycline resistance [53]. However, In some of our resistant isolates the *tet* genes were not detected.

According to Costa *et al.* [54], 20 % of *E. coli* isolates from Portuguese pet animals were resistant to tetracycline, and 15 % of these were susceptible to streptomycin. Surprisingly, these levels are lower than the prevalence of antibiotic resistance in our Miranda donkey isolates, probably due to the transmission of resistance through the generations. The genes *erm*(A) and/or *erm*(B), *tet*(M) and/or *tet*(L), *vat*(D) and/or *vat*(E) and *aph*(3′)-*IIIa* were also found to exceed the percentages of resistance to vancomycin obtained from wild animals [55]. These results are in line with what was expected, because wild animals are less close to humans and are less exposed to antibiotics.

Different combinations of genes *sul*1, *sul*2 and *sul*3 were observed from trimethoprim/sulfamethoxazole-resistant isolates obtained from turkey flocks slaughtered in Canada [21]. In the same study, the authors verified that antibiotic resistance was more frequent when turkeys were placed in the same facilities previously used by chickens.

β-Lactamase genes were not detected in this study, although this had already been reported by Gonçalves *et al.* [29] in Iberian lynx (*Lynx pardinus*) in Portugal and in some European countries, as in the case of horses in Germany [56].

The *fim*(A) virulence gene was amplified in 46 % of isolates and *cnf*1 was observed in 22 % of the antibiotic-resistant *E. coli* isolates, findings similar to those from a study in horses by Moura *et al.* [57]. The explanation for these differences may be evolution of the phylogenetic relationship between species and geographical location with extensive livestock production systems. Ruiz *et al.* [39] described other virulence factors in clinical isolates from humans, such as the genes *pap*(C) (55 %) and *pap*(G)III (15 %).

The predominance of phylogenetic groups A and B1 among the *E. coli* isolates from the gut microbiota of animal origin is reported, whereas B2 isolates were the most common in healthy human samples [58]. Interestingly, our *E. coli* isolates belonged to phylogenetic groups B2 (12 %) and D (46.3 %) and, in contrast, Gonçalves *et al.* [59] detected four phylogenetic groups in isolates from Iberian lynx in Portugal.

The high genetic diversity observed in the selected isolates confirms an adequate sampling process. That the absence of ST131 in our collection, and the unique representation of a single isolate belonging to ST10, are remarkable because the prevalence of this high-risk clone has been described in similar animal studies with [60].

de Vaux *et al.* [61] identified *Ent. avium*, *Ent. faecium* and *Ent. pseudoavium* with a sequence similarity of 97.4 % in faecal isolates from French donkeys (*Equus asinus*). In the current study, the predominance of *Ent. faecium* and *Ent. hirae* among the *Enterococcus* spp. isolates is consistent with the study on Iberian lynx [59].

Ent. faecium was the most frequently identified enterococcal species in the faecal samples of animals, poultry (71.6 %) and swine (37.7 %) [6], although it was not recovered from bovine samples, *Bos taurus* [62]. However, the rates for *Ent. faecium*
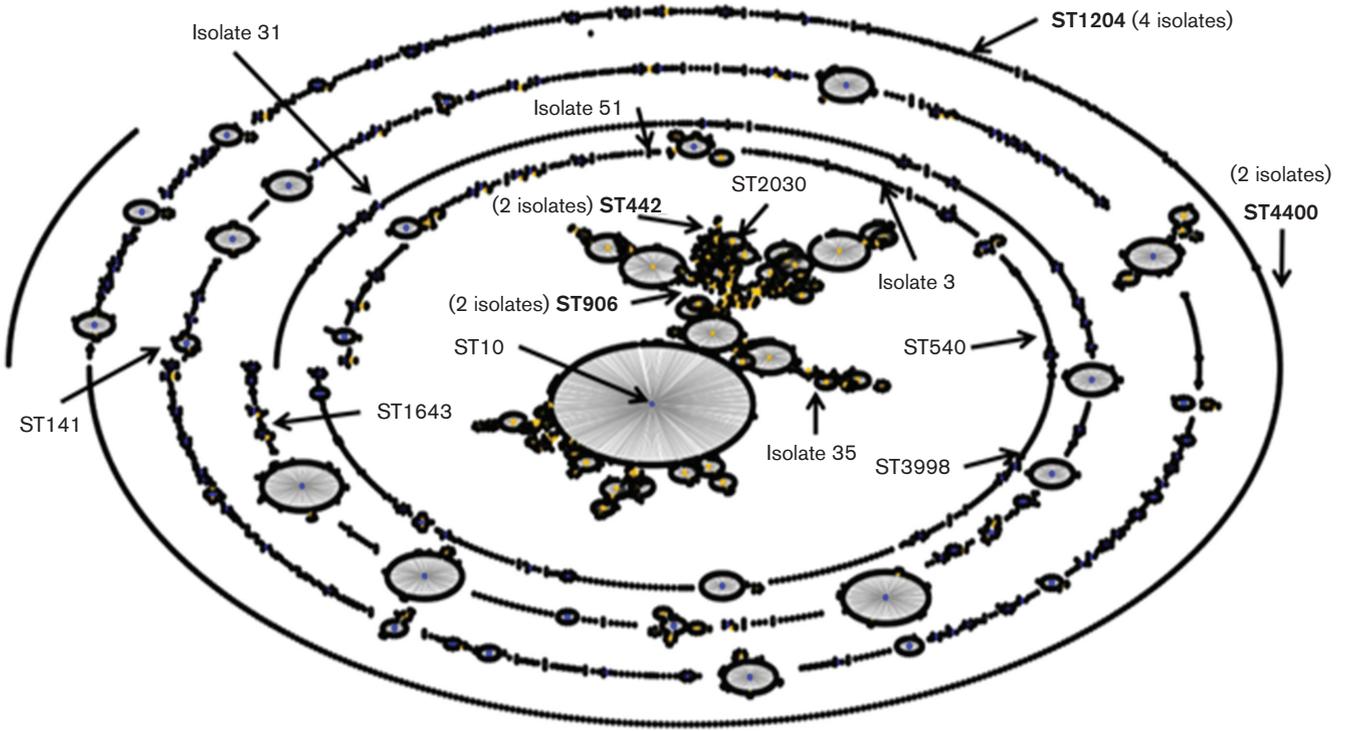
Fig. 4. eBURST of the complete *Escherichia coli* MLST database including 5805 isolates and the three previously unreported STs detected in the present study.

(45.7 %) were higher than for *Ent. hirae* isolates in *Enterococcus* spp. from Portuguese Bísaro pigs [63]. The reason for this diversity can be attributed to differences in geographical location, animal species and their environment, an intensive livestock production system [64].

Enterococcal isolates from the Miranda donkey showed high resistance rates to tetracycline (68.3 %) quinupristin/dalfopristin (51.2 %) and ciprofloxacin (48.8 %), according to a recent study conducted on wild game meat in Spain [55].

In the Miranda donkey, we highlight the finding of 7 out of 41 (approximately 20 %) VRE spp. isolates. Furthermore, similar resistance profiles were reported in other studies based on wild animals in the Azores [65] and the Iberian Peninsula [26]. Interestingly, more than 80 % strains of enterococci of equine origin, in Northern India were VRE [66], and the *van*(A) gene was detected among horses from Costa Rica [52, 57]. Katakweba *et al.* [67] analysed faecal samples from buffalo, wildebeest and zebra in Tanzania,
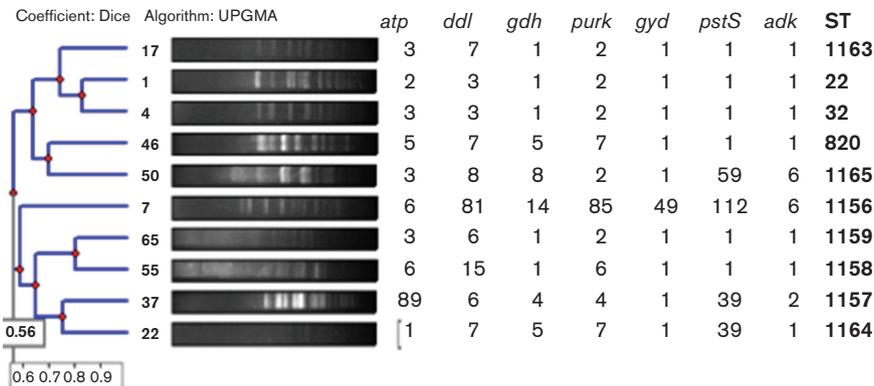

Fig. 5. Dendrogram representing the diversity of the PFGE patterns of 10 *Enterococcus faecium* isolates representative of each band pattern and MLST type.

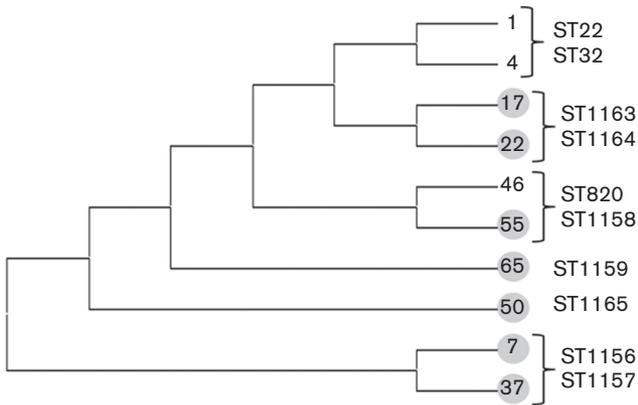**Fig. 6.** Phylogenetic relation of the *Enterococcus faecium* isolates typed by MLST. Isolates in grey circles represent previously undescribed STs.

and they also detected the presence of VRE. In contrast, Poeta *et al.* [68] did not detect VRE from wild animals (birds, mammals and others) in Portugal. Vancomycin resistance can be induced per se, by other glycopeptides or by third-generation cephalosporins [69].

Among the four kanamycin-resistant isolates, we verified that all had the *aph*(3′)-*IIIa* gene because this is an intrinsic resistance. In this study, genes *str*(A) or *str*(B) were found in 41.5 % of streptomycin-resistant isolates, and genes *tet* (A) and/or *tet*(B) in 50 % of tetracycline-resistant isolates. Compared with enterococci isolated from wild animals [55], genes *van*(B), *erm*(B) and/or *erm*(C), *tet*(L) and/or *tet*(M) and *aph*(3′)-*IIIa* were positive by PCR in both studies.

These virulence results are in agreement with the study of Poeta *et al.* [70] in wild boar (*Sus scrofa*), where the *gel*(E) gene was detected in four *Ent. faecium* and two *Ent. faecalis* isolates. Semedo *et al.* [42] studied human and animal clinical isolates and observed that there was a 99 % incidence of the *gel*(E) gene. In another study, the genes *gel*(E) and *cyl*(B) were detected in 24 *Ent. faecium* isolates from artisanal Tunisian fermented meat [71]. That work also showed that the antibiotic resistance had originated in wild meat, representing a further pathway for the resistance of enterococci in other animals as well as in humans, through the food chain.

Among the *Ent. faecium* isolates, ST32 and ST1164 were the most prevalent genetic lineages whereas the other STs only grouped one isolate. The high-risk clone CC17 linked to
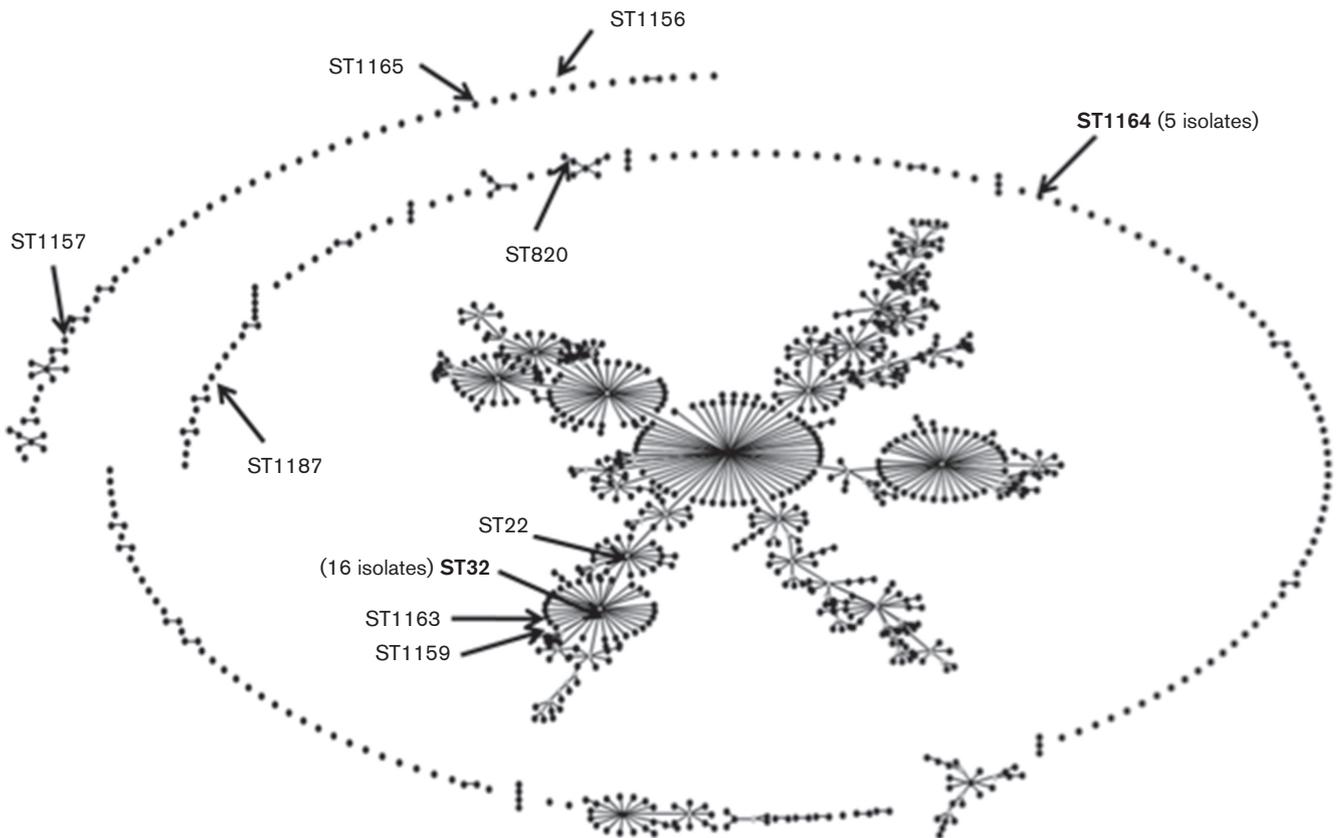

Fig. 7. eBURST of the 966 *Enterococcus faecium* isolates deposited in the MLST website, and the seven new STs described in our study.

human infection was not detected among our collection, although isolates including ST22, ST32, ST1159 and ST1163 may have some genetic relation to CC17.

Although the European Union abolished the use of antibiotics for animal growth promotion in 2006, antibiotic-resistant *Enterococcus* spp. strains are still present and, in general, their numbers continue to increase [72]. According to Alekshun and Levy [16], resistance is very common in farm animals because of the widespread use of antimicrobials. In agreement with studies on other species, this work demonstrates that the Miranda donkey is a potential reservoir of resistance genes [54, 73, 74]. The reduction in numbers of fertile jacks and loss of commercial interest in this breed are among the reasons for the current risk of its extinction [47, 48]. To our knowledge, this is the first report on AMR in the Miranda donkey inhabiting a rural area. This study demonstrated that *E. coli* and enterococcal isolates from faecal samples of the Miranda donkey have significant AMR rates encoded by acquired resistance genes.

Despite living in areas characterized by family farming, this endangered donkey breed presents higher values of resistance than domestic and wild animals in Portugal and also in Spain, respectively. As evidenced by our study, this breed could be a reservoir of AMR but the risk is low as it inhabits rural areas with a low density of population. The development of new antimicrobials is essential, but correct prevention and disinfection measures are also important and should be taken into account. Nevertheless, a key aspect for the future is the control of the misuse and overuse of current and future antimicrobial drugs, in order to avoid fresh antibiotic crises and serious public health concerns [16, 25].

Further studies must be carried out to better understand the antibicrobial status of the Miranda donkey, and the implications for AMR in such rural regions. However, the prevention of infections and the correct use of antimicrobial agents in humans and animals are required in order to avoid current and future resistance to new drugs.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
This study was approved by the AEPGA ethical committee.

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