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Genetic diversity of *Streptococcus equi* subsp. *zooepidemicus* isolated from horses

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ABSTRACT

Streptococcus equi subsp. *zooepidemicus* (SEZ) is an opportunistic and zoonotic pathogen of horses. In this study, genetic intraspecies variability of SEZ obtained mainly from respiratory and genital samples of horses was investigated by analysis of the 16S–23S rRNA intergenic spacer region (ISR) and of the 16S rRNA gene. 16S–23S ISR rRNA type A1 was predominant, although a high rate of multiple products (30.5%) was obtained. Phylogenetic analysis of the 16S rRNA gene detected three genogroups (I, II and III). 16S rRNA variable regions V1 and V2 are the most important regions for evaluating SEZ intraspecies variability, but at least V1-V5 regions should be considered to avoid mistakes. Analysis of all 16S rRNA sequences available in databases assigned human SEZ to groups I and III but not to group II. These results show a high genetic variability in SEZ collected from different specimens of horses from various regions of Italy.

1. Introduction

Streptococcus equi subsp. *zooepidemicus* (SEZ) is a beta-haemolytic streptococcus belonging to the Lancefield group C. SEZ is considered an opportunistic pathogen of horses and can cause different diseases in humans [1–4]. SEZ can be isolated from horses without clinical signs or with diseases ranging from mild to severe pneumonia, pleuropneumonia, strangles-like diseases or endometritis, but also from dogs, cats, and poultry, and recently it has been detected in ticks [5–9]. Among the other *S. equi* subspecies, *S. equi* subsp. *equi* (SEE) specifically infects equids and is not responsible for zoonoses [10], while *S. equi* subsp. *ruminatorum* (SER) is occasionally reported in humans [11]. Zoonoses are caused also by the other C-streptococcus *S. dysgalactiae*, which includes two subspecies: *dysgalactiae* (SDSD) and *equisimilis* (SDSE). Recent molecular studies based also on 16S rRNA gene sequence analysis suggest that strains isolated from animals should be assigned to SDSD, while strains pathogenic for humans should be assigned to SDSE and can be discriminated by PCR [12–14]. Genetic exchange among zoonotic beta-haemolytic streptococci of animals and of humans may occur and may influence host specificity or virulence [15,16]. Examples of gene loss in the genome of SEZ have been identified due to mutation and deletion, and gene gain has been described through the acquisition of mobile genetic elements that have probably shaped the host tropism and pathogenic specialization of SEE [17].

Different techniques have been used in the last twenty years to investigate SEZ, suggesting that they are a genetically heterogeneous group. Genetic intraspecies variations have been evaluated so far in SEZ collected from horses in some countries but not in Italy [18–22]. The aim of this work was to investigate the sequence variations of 16S–23S rRNA intergenic spacer regions (ISR) gene and of the 16S rRNA gene of SEZ isolated from different clinical specimens of Italian horses, even in comparison with sequences of SEZ from animals and humans available in databases.

2. Materials and methods

2.1. Samples

Archival samples of beta-haemolytic streptococci of group C submitted to the laboratory for PCR-based identification [23] were used in this study. DNA samples of 95 SEZ obtained in 2005–2010 from different clinical specimens of horses from 26 different stables located in five regions of Italy were available (Table S1). A total of 45 (47.4%) samples were from the reproductive tract, 46 (48.4%) from the respiratory tract and 4 (4.2%) from other sources (2 milk, 1 faeces and 1 cutaneous wound) (Table 1 and Table S1).

2.2. 16S–23S rRNA intergenic spacer gene typing

16S–23S rRNA ISR gene was investigated by PCR similarly as

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Table 1
Origin of SEZ samples.

	Sample	Symptoms	No symptoms	Total
Reproductive	uterine swab	31	2	**33**
n = 45	uterine flushing	7		**7**
	vaginal swab	2	2	**4**
	epididymis	1		**1**
Respiratory	tracheal wash	18	2	**20**
n = 46	BAL	8	2	**10**
	nasal swab	12		**12**
	GPL	3		**3**
	lung	1		**1**
Other	cutaneous wound	1		**1**
n = 4	stool	1		**1**
	milk	2		**2**
TOTAL		**87**	**8**	**95**

Legend: BAL = broncho-alveolar lavage; GPL = guttural pouch lavage.

Table 2
Correspondence between PCR product codes and size of the products obtained by different PCR reactions.

Reaction*	Product size (bp)	Product code
A	142	A1
	311	C1
B	261	B1
	424	D1
C	145	A2
	314	C2
D	264	B2
	427	D2

* With primers described by Newton et al. [21].

described previously [21]. In particular, four PCRs (named A, B, C and D) were carried out with sets of primers targeting different regions of the 16S–23S RNA ISR [20]. A code was assigned to each PCR product on the basis of the size of the product obtained in each reaction (Table 2). If more than one PCR product was identified, multiple intergenic spacer categories were assigned, as previously reported [21,24]. PCR reactions were carried out in 50 µl total volume containing 25 µl of TaqPCR mastermix 2X (Qiagen GmbH, Germany), 20 nmol of each primer, 2 µl of the DNA template and water up to 50 µl final volume. The amplification conditions were 95 °C for 5 min, 40 cycles at 95 °C for 1 min, 56 °C for 2 min, 72 °C for 2 min and a final extension step at 72 °C for 7 min. The presence of PCR products was determined by electrophoresis of 10 µl reaction products in 2% agarose gel containing 0.5 µg/mL of ethidium bromide with Tris-borate-EDTA buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA pH 8.3). The images were captured and analysed by Kodak Gel Logic System 100. On the basis of the PCR reactions, the strain was assigned precise types named A1, A2, B1, B2, C1, C2, D1 or D2. In case of unexpected results, PCRs were repeated and unexpected PCR products were sequenced (BMR Genomics, Padua, Italy). A representative selection of the sequences was deposited in GenBank (Accession Numbers MK621015-MK621017). The resulting sequences were investigated by nBLAST and were subsequently aligned and compared with the most homologous sequences and with sequences used as representative examples of the 16S–23S ISR types [20].

2.3. 16S rRNA gene typing

Samples were used for PCR amplification and sequencing of 16S rRNA gene with primers 27-F (AGA GTT TGA TCM TGG CTC AG) and Strep-R (ACC TGT CAC CCG ATG TAC CGA AGT A), obtaining 1112 bp products of the 16S rRNA gene variable regions V1-V6. The PCR mixture consisted of 25 µl of 2X TaqPCR mastermix (Qiagen GmbH, Germany), 20 nmol of each primer, 300 ng of the DNA template and water up to 50 µl final volume. The PCR conditions were 94 °C for 3 min, 35 cycles at 94 °C for 1 min, at 60 °C for 1 min, and at 72 °C for 1 min, with a final extension at 72 °C for 7 min. The PCR products were resolved by electrophoresis of 10 µl reaction products in a 1.5% agarose gel as described above and they were sequenced by an external laboratory (BMR Genomics, Padua, Italy). A representative selection of sequences were deposited in GenBank (Accession Numbers MK614219-MK614257).

Nucleotide sequences were manually checked and edited with the program BioEdit. Considering that SEZ sequences available in databases show some nucleotidic variations only in V1-V5 regions of 16S rRNA gene and that many partial sequences including these regions are present in GenBank, phylogenetic analysis was carried out by using 825 bp sequences covering these regions to include the highest number of sequences (Table S2). The sequences were aligned by MUSCLE [25] and phylogenetic trees were inferred with the program MEGA 7.0.21 [26]. The best-fitting nucleotide substitution models were estimated and the Jukes-Cantor model with a gamma distribution with invariant sites was used with bootstrap values based on 1000 repetitions. Phylogeny was estimated by both the neighbour-joining algorithm and the maximum likelihood method.

On the basis of the preliminary results obtained, further phylogenetic analyses have been carried out by including shorter sequences available in databases and covering only V2 or only V1-V3 regions.

Correlations among groups were evaluated by the Fisher's exact test.

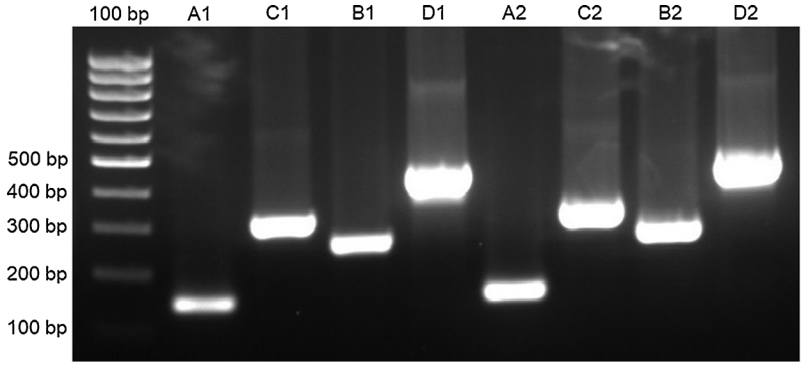

Fig. 1. Electrophoresis of the 16S–23S rRNA gene ISR PCR products and corresponding types. Line 1: 100 bp ladder (MiniSizer 100 bp DNA Ladder, Norgen Biotek, Canada); lines 2-3: products of reaction A; lines 4-5: products of reaction B; lines 6-7: products of reaction C; lines 8-9: products of reaction D.

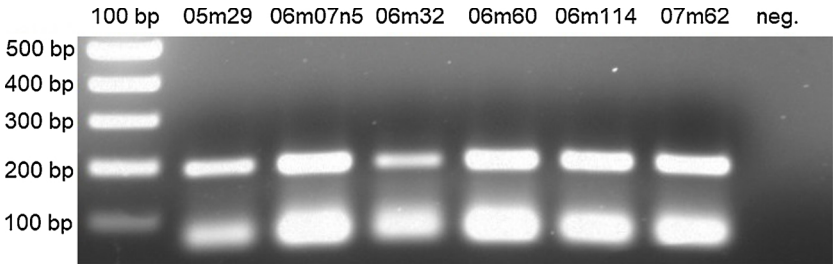

Fig. 2. Electrophoresis of the 16S–23S rRNA gene ISR PCR products of reaction C showing unspecific products of about 200 bp instead of 145 bp (type A2) or 314 bp (type C2). Primer dimers under 100 bp are also evident. Line 1: 100 bp ladder (MiniSizer 100 bp DNA Ladder, Norgen Biotek, Canada); lines 2-7: SEZ samples; line 8: negative control.

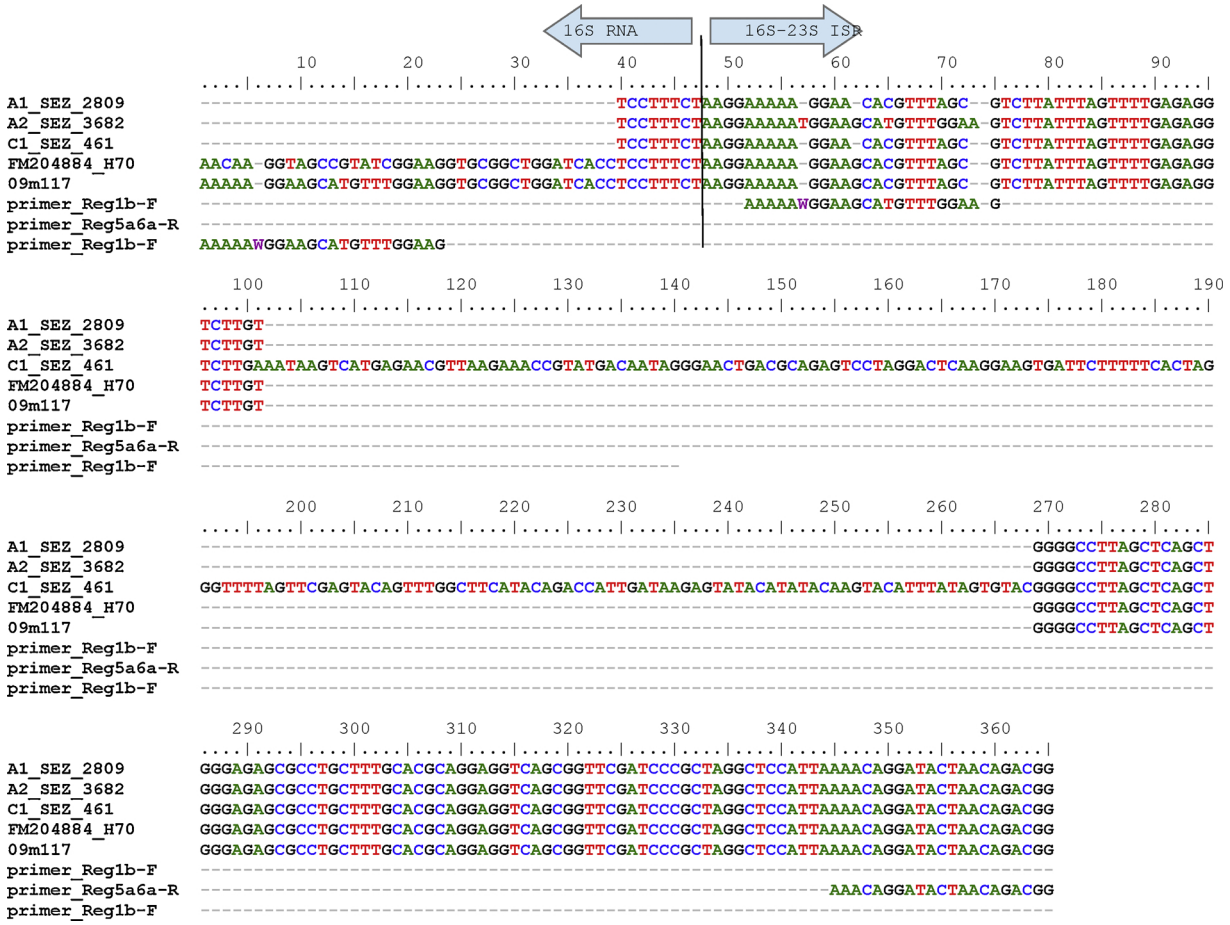

Fig. 3. Alignment of the terminal part of the 16S RNA gene and of the initial part of the 16 S–23 S rRNA intergenic spacer region of some SEZ representative type strains [20], SEZ strain H70, sequence of the 200 bp unspecific product obtained by PCR reaction C and primer forward (Reg1b-F) and reverse (complementary sequence of primer Reg5a6a-R) used in PCR reaction C. The alignment shows that the forward primer designed to amplify the initial tract of the 16S–23S ISR of type A2 strains (positions 52–73 nt) binds to the final part 16S RNA of SEZ H70 (positions 1–23 nt), giving a 195 bp product with the same sequence of A1 type strain SEZ_2809. Sequences of the other unexpected products obtained from the other 13 samples are not reported here because they were identical to the sequence of sample 09m117. SEZ H70 is reported as representative of other strains with identical terminal sequence of the 16S RNA.

3. Results

3.1. 16S–23S rRNA intergenic spacer gene typing

The 16S–23S ISR PCR gave the expected PCR products in 81 samples (Fig. 1) while 14 samples gave an unexpected product in PCR named C. The expected products of this reaction are 145 bp or 314 bp [20,21], while the product obtained from 14 samples was about 200 bp (Fig. 2). In addition, all these samples gave also a product named A1 in reaction A. Sequencing and comparison of the unexpected products showed that the forward primer used in the reaction C (Reg1b) matched to the 16S rRNA gene sequence, 50 bp before the expected site of binding in the 16S–23S ISR (e.g. SEZ strain H70, GenBank accession number FM204884.1, matching from nt 19,563 instead of nt 19613) (Fig. 3). This has occurred because of a close nucleotidic similarity in the two regions of the target DNA. Sequences of the unexpected products

Table 3
Results of 16 S–23 S ISR typing.

16S/23S ISR type	Tot	Respiratory	Reproductive	Other
A1	41	22	19	0
A2	5	4	1	0
B1	2	1	1	0
B2	1	0	0	1
C1	7	4	3	0
C2	2	1	1	0
D1	6	1	5	0
D2	2	0	2	0
A1A2	9	5	3	1
A1B2	1	0	0	1
A1C1	1	1	0	0
A1C2	2	1	0	1
A1D2	1	0	1	0
A2D2	1	0	1	0
B1B2	1	0	1	0
B1D2	1	1	0	0
B2C2	1	0	1	0
B2D2	1	0	1	0
C1C2	3	0	3	0
C1D2	1	1	0	0
D1D2	2	0	2	0
A1A2D2	1	1	0	0
A1C1B1	1	1	0	0
A1D1D2	1	1	0	0
A1C1B1A2B2	1	1	0	0
TOT.	**95**	**46**	**45**	**4**

(excluding the first 50 bp) were identical to the 16S–23S ISR sequence of the representative example strain of type A1 [20]. On the basis of these considerations, the unexpected 200 bp products obtained by reaction C were considered as type A1 products. Once this point was resolved, the total number of samples producing a single PCR product was 66 (69.5%), while 29 (30.5%) samples gave two or more products (Table 3).

3.2. 16S rRNA gene typing

The analysis of the sequences of the 16S rRNA gene showed high heterogeneity among different SEZ strains and phylogenetic analysis showed that samples can be divided into three subgroups. The previous classification into group I and group II has been maintained [22]. Samples included in the genogroup I showed sequences almost identical to *S. equi* subsp. *equi* (SEE), while samples included in the genogroup II showed sequences significantly different than SEE but closer to *S. equi* subsp. *ruminatorum* (SER) (Fig. 4). Interestingly, two samples of group I (09m253 and 10m96) showed a V2 region sequence closer to the sequences of group II members than to those of group I members. Nucleotide variations were observed at specific positions and in different combinations (Fig. 5). Considering the sequence of SEZ ATCC 43079 (GenBank EF406034.1) as reference, all group II samples showed variations in regions V1 and V2. Some strains showed the same variations of group II members in V1 region but not in V2 region. On the basis of the phylogenetic results these strains, together with strains previously defined as “intermediate” [22], have been included in a different genogroup named III. Phylogenetic analysis of shorter sequences covering only V2 region (Fig. S3) or V1, V2 and part of V3 regions (Fig. S4) showed that members of group III are not recognised or are only partially recognised depending on the length of the sequences analysed. In addition, the two samples of group I with an unusual V2 sequence (09m253 and 10m96) would be erroneously assigned to group II if only V2 region is examined.

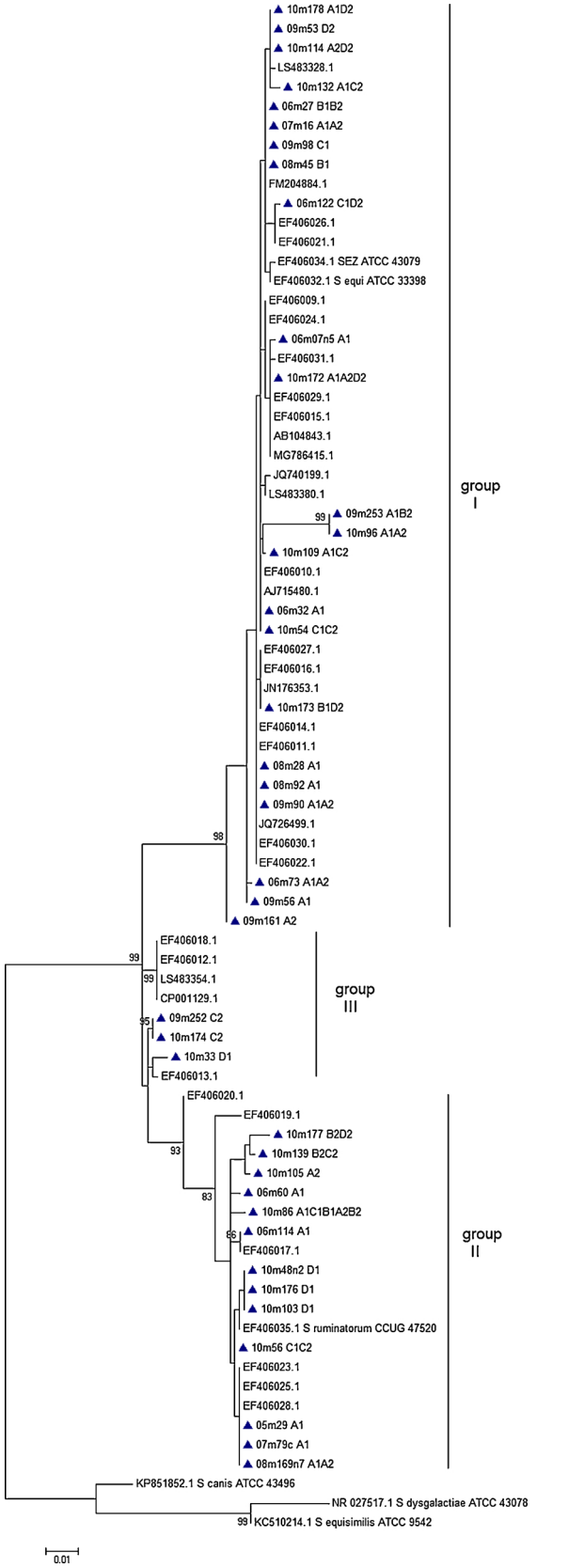

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Fig. 4. Phylogenetic analysis of 16S rRNA gene sequences of SEZ strains obtained in this study (▲) and strains available in GenBank and other databases. Some strains have been selected as representative of the others. Each sample of this study is named with its laboratory code and with its type of 16S–23S rRNA gene ISR obtained. Strains from databases are reported with their GenBank code. *Streptococcus canis* (ATCC 43496) has been used as outgroup. The phylogenetic analysis was performed with maximum likelihood method using Jukes Cantor model with a gamma distribution and with bootstrap values based on 1000 repetitions. Sequences of samples are deposited in GenBank.

4. Discussion

Preliminary studies based on PCR amplification and enzymatic digestion and/or sequencing detected genetic diversity in SEZ, and consequently different techniques have been developed for their subtyping [18–20,27].

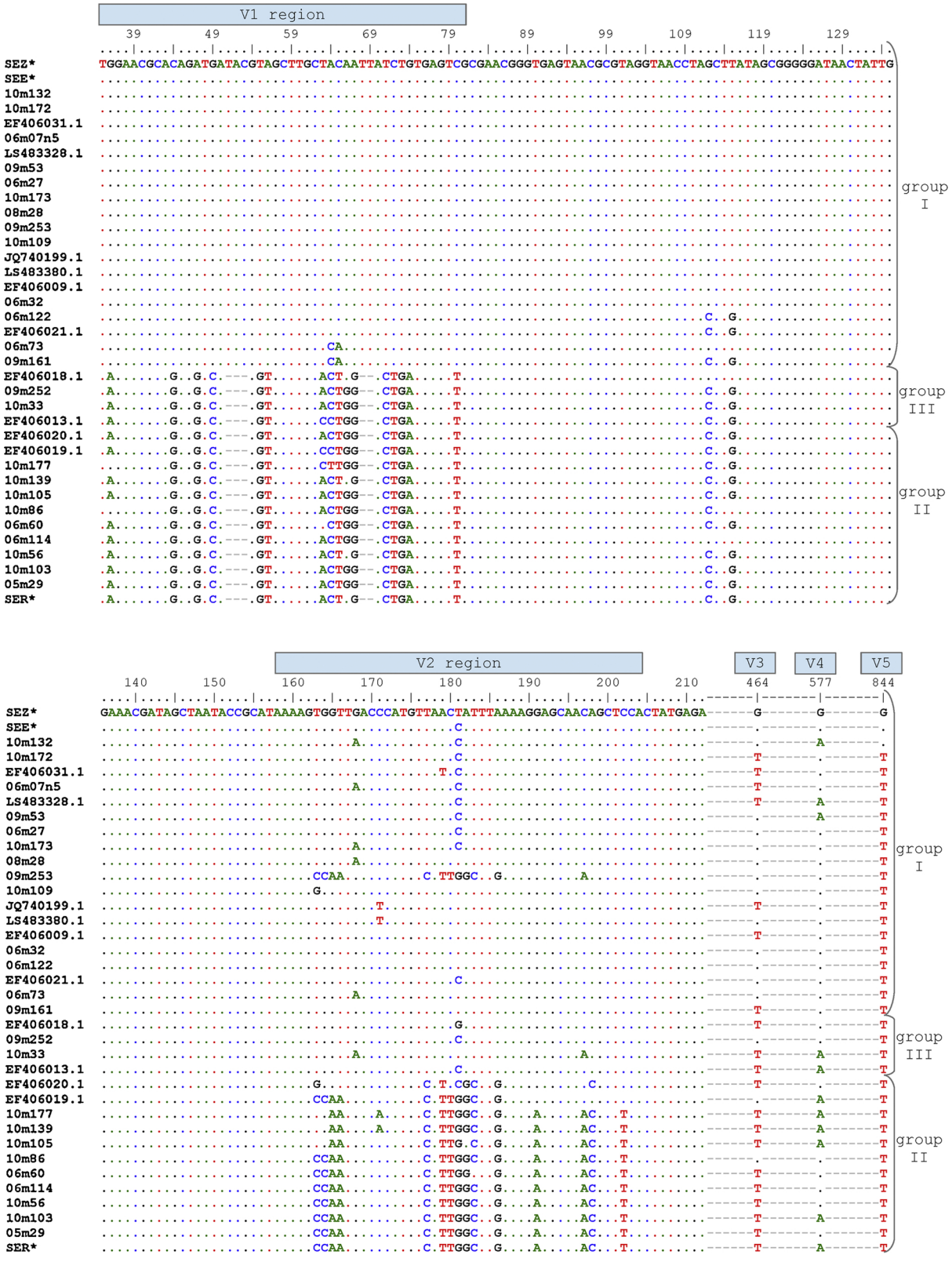
Studies on the 16S–23S rRNA ISR gene of different streptococcal species and subspecies demonstrated that sequence variations of that region allow a molecular typing of SEZ isolates [20,28]. Subsequently, an easy PCR based scheme was used to investigate the variability of the 16S–23S ISR gene of SEZ isolated from ponies or horses [18,24]. In the present work this method was used for screening SEZ strains isolated from different specimens of horses reared in 26 different stables in Italy. PCRs detected at least one strain of each type of 16S–23S ISR rRNA (A1, A2, B1, B2, C1, C2, D1 or D2). In addition, some samples gave unexpected products of about 200 bp in PCR C; analysis of their sequences showed an unexpected binding to the terminal part of the 16S gene and assigned them to the A1 type. Single 16S–23S ISR products were obtained from 69.5% samples and type A1 was the most frequent (43.2%), as previously reported in UK both in ponies and in racehorses [21,24]. Multiple intergenic spacer products (e.g. A1/A2, B1D2, etc.) were obtained from 30.5% samples, most of them were type A1A2, and A1 product was ever present when more than two products were obtained. The proportion of multiple intergenic spacer products obtained (30.5%) and the number of possible combinations observed (n = 17) were much higher than those reported by previous studies [21,24]. These differences could be due to the different origin of SEZ strains examined, because in previous studies SEZ were collected from the respiratory tract of only 29 pony foals or of 198 racehorses from only three training yards at different time points [21,24]. Instead, in the present study SEZ strains were obtained from different anatomical sites of 95 different horses from 26 different horse stables located in five Italian regions. The origin of the samples was very various and this could explain the genetic variability observed.

16S rRNA gene is often used in reconstructing phylogenies due to its slow rates of evolution. Intraspecies sequence variations within the V2 region of the 16S rRNA gene of SEZ had been detected by PCR amplification and enzymatic digestion or by amplification and sequencing of the gene [18,22]. In our study, genetic variability was observed in the 16S rRNA gene sequences at specific positions located mainly in V1-V2 regions, but also in V3-V5 regions. Phylogenetic analysis showed that most sequences obtained in this study were similar but not identical to those available in GenBank, confirming in SEZ the genetic variability of a gene that usually evolves slowly. On the basis of the variability observed mainly in regions V1-V2, samples were assigned to three different groups (I, II and III), similarly as observed before about Swedish SEZ strains [22]. Although different variations were observed, two strains of group I (09m253 and 10m96) showed higher genetic distance from the other members of group I because

their variations in the V2 region were closer to those of members of group II or III than of group I. Phylogenetic analysis suggests that SEZ of group I and group II have a distinct evolution and might have speciated from a common ancestral organism with divergent copies of 16S rRNA genes. The finding of some intermediates strains (here named as group III) seems to support this hypothesis and suggests that genetic recombination events might have occurred, although further studies are needed. It is not known if specific genetic subgroups of SEZ are more prone to causing zoonosis than others. SEZ strains of group I show 16S rRNA gene sequences closer to SEE, which does not cause zoonoses, while group II SEZ strains have 16S rRNA gene sequences closer to SER, which occasionally causes zoonoses [11]. Phylogenetic analysis of 16S rRNA sequences available in databases and covering at least the V1-V5 regions showed that group II included only SEZ strains from horses, group III included strains from horses and humans, and group I included strains from humans, horses, dogs, cattle and chickens; furthermore, analysis of the available sequences of SEZ from cats covering only the V1-V3 regions assigned them to groups I or II. It is not clear if belonging to a particular 16S rRNA group may influence the ability of SEZ to infect different animal hosts or humans because most sequences covering V1-V5 regions and deposited in databases (Tab. S2) are mainly from horses (n = 27), only a few are from other animal species (n = 3) or from humans (n = 5), and statistical results are not significant. Further sequencing of the 16S rRNA gene of SEZ from humans and from non-equine animals may contribute to solve this question. Our data show the importance of sequencing at least the V1-V5 regions of the 16S rRNA gene of SEZ strains for correctly defining their genogroup. Exclusive sequencing of the V2 regions [18] (GenBank AF073803-AF073814) not only does not allow distinction among members of group II and III, but may also lead to an erroneous classification; indeed some samples of group I (09m253 and 10m96) showing V2 region sequences typical of group II or group III members would be erroneously assigned to group II instead of group I (Fig. S3). SEZ from cats have been described as sub-grouped on the basis of the sequences of the V2 regions [6]; however the pair of primers used in that study allow amplification and sequencing of V1, V2 and of part of the V3 including the main mutation points, thus sub-grouping is possible for that sequences. However this method seems not able to clearly distinguish members of group III (Fig. S4), thus sequencing of at least V1-V5 regions of the 16S rRNA gene of SEZ is suggested for subtyping.

Sometimes, SEZ collected at the same time from animals living in the same training yard had different 16S rRNA gene sequences and/or different 16S–23S rRNA gene ISR types (e.g. 08m28 and 08m45 or 10m177 and 10m178), confirming that different strains can co-circulate in the same environment. If co-circulation of different strains can increase the rate of genetic mutation in SEZ should be investigated. Significant correlations among variations in the 16S rRNA gene sequences and types of 16S–23S ISR rRNA or source of samples were usually not found ($p > 0.05$), although some exceptions were detected. For example, 16S–23S ISR type D1 SEZ were found only in group II or III but not in group I ($p < 0.05$) and type C2 SEZ were found only in group III ($p < 0.01$); however, further investigations are required for confirming these data.

In conclusion, these results show a high heterogeneity among strains of SEZ collected mainly from respiratory and genital tracts and from other specimens of horses raised in Italy and serve as a basis for further genetic typing investigations with more expensive methods (e.g. Multilocus sequence typing - MLST). For example, a MLST scheme for SEZ subtyping is usually based on 7 houskeeping genes [19], thus the expense for MLST is about 7-fold that for 16S–23S ISR or 16S rRNA typing, but much more variation and many more alleles per locus can be detected by typing multiple loci instead of a single locus.



* SEZ is *S. zooepidemicus* ATCC_43079 (EF406034.1) and has been used as reference strain for evaluating single nucleotides polymorphisms of other strains; SEE is *S. equi* ATCC 33398 (EF406032.1); SRum is *S. ruminatorum* CCUG 47520 (EF406035.1).

Fig. 5. Alignment of the 16S rRNA gene sequences showing all kind of variations observed in V1-V5 regions of samples and of all SEZ available GenBank and other databases. One sample for each type of variation has been considered. Sequences have been aligned and compared to the reference SEZ strain ATCC 43079. Groups have been reported on the basis of the phylogenetic results (Fig. 4).

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cimid.2019.03.012.

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