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Determination of intraspecies variations of the V2 region of the 16S rRNA gene of *Streptococcus equi* subsp. *zooepidemicus* isolated from pigs, monkeys and humans on the island of Bali Indonesia

Siti Isrina Oktavia Salasia

Clinical Pathology Department, Faculty of Veterinary Medicine, Gadjah Mada University, Sekip Unit II, Yogyakarta 55281, Indonesia

Abstract

The 16S rRNA gene of 15 *Streptococcus equi* subsp. *zooepidemicus* isolated from pigs, monkeys, and humans was amplified by polymerase chain reaction and subsequently digested with the restriction enzyme HincII. A restriction profile with two fragments size of 1250 bp and 200 bp could be observed for all *S. equi* subsp. *zooepidemicus* isolated from pigs and monkeys indicating a sequence variable within the V2 region of the 16S rRNA gene of the remaining *S. equi* subsp. *zooepidemicus* human isolates. The sequence of the V2 region of *S. equi* subsp. *zooepidemicus* isolated from pigs and monkeys revealed an identical (100 per cent similarity) sequence with the V2 region of *S. equi subsp. equi* ATCC (reference strains). The sequence of the *S. equi* subsp. *zooepidemicus* isolated from humans appeared to be similar (88.5 per cent similarity) to *the S. equi* subsp. *equi* reference stains. A single amplicon of the spacer region gene encoding the 16S-23S rRNA of 950 bp could be observed for *S. equi* subsp. *zooepidemicus* isolated from pigs and monkeys, and an amplicon of 650 bp for *S. equi* subsp. *zooepidemicus* human isolates.

Keywords: *Streptococcus equi* subsp. *zooepidemicus* – 16S rRNA gene – V2 region – 16-23S rRNA intergenic spacer region

Introduction

In 1994 beta-hemolytic streptococci of serological group C were isolated from infection processes of pigs and monkeys on the island of Bali. The isolates appeared to be associated with cases of meningitis, bronchopneumonia and septicaemia. The occurrence of the bacteria in infections of different hosts raises the question about the route of infection and the possibly existing epidemiological relation in outbreaks with this microorganism. In a previous study the β-hemolytic streptococci were identified as *Streptococcus equi* subsp. *zooepidemicus* (Soedarmanto *et al.*, 1996; Salasia, 1998). More recently, these bacteria were isolated from the tonsils of people working at the monkeys resort, in Bali (Salasia *et al.*, 2002).

The identification of streptococci currently relies on the determination of phenotypic characteristics including serological grouping by use of Lancefield antisera. However, developments in nucleic acid technology such as polymerase chain reaction (PCR) and the analysis of 16S rRNA have resulted in new methods that can be used for identification of bacteria. A sequence comparison of 16S rRNA, a molecule most suited for these purposes, has shown that some seg-

ments of the molecule are highly conserved while other vary. The variability of the rRNA sequences are sufficiently stable to allow investigations of phylogenetic relationships. Bentley and Leigh (1995) summarized the sequence of the highly variable V2 region of reference strains of 31 species of genus *Streptococcus* also including the sequence of a reference strain of *S. equi* subsp. *equi*. According to these authors the highly variable V2 region of the 16S rRNA molecule shows enough stable differences to design species-specific oligonucleotide probes. However, according to Bentley and Leigh (1995) it is not known to what extent intraspecies variations of the V2 region might occur, particularly in heterogenous species. Up to now no sequence variations were found among isolates of the species *S. uberis*, *S. parauberis* and *S. agalactiae* (Bentley and Leigh, 1995; Lämmler *et al.*, 1998a; Lämmler *et al.*, 1998b). However, *S. zooepidemicus*, the causative agent of a variety of infections in animals and humans appeared to be closely related to *S. equi* and according to a proposal of Farrow and Collins (1984), this species was reclassified as *S. equi* subsp. *zooepidemicus*. According to Abdulmawjood and Lämmler (2000) the V2 region of *S. equi* subsp. *zooepidemicus* showed some intraspecies variations. Sequencing the V2 region of *S. equi* subsp. *zooepidemicus* isolated from infections of horses revealed to four variants. Three variants were closely related to each other and to the sequence of *S. equi* subsp. *equi* strains and were designated as 16S rRNA type 1. One variation was not related to the sequence of the *S. equi* subsp. *equi* strains and was designated as 16S rRNA type 2.

To determine possible existence of epidemiological relationships of the gene encoding the 16S rRNA of the *S. equi* subsp. *zooepidemicus* isolated from pigs, monkeys and humans on the island of Bali was amplified by PCR and subsequently digested with the restriction enzyme *Hinc*II. In addition a segment of the 16S rRNA gene, including the V2 region, was sequenced and compared with the sequence of *S. equi* subsp. *equi* reference strains, *S. equi* subsp. *zooepidemicus* reference strains and with reference strains of the species *S. agalactiae*. The isolates were additionally investigated by amplifying the 16S-23S rRNA intergenic spacer region.

Materials and Methods

Bacterial isolates

A total number of 22 streptococci were used in this study. The cultures included the *S. equi* subsp. *zooepidemicus* strains W60, 500 and 631, the *S. equi* subsp. *equi* strains 19 and CF32, the group B streptococcal reference strains 24/60 X (ATCC 49449) and 25/60 R (ATCC 49448), kindly provided by Prof. Dr. C. Lämmler, Institut für Tierärztliche Nahrungsmittelkunde, Professur für Milchwissenschaftler, Justus-Liebig-Universität Gießen, Germany. In addition ten *S. equi* subsp. *zooepidemicus* strains isolated from clinically and subclinically diseased pigs (kindly provided by Dr. I W.T. Wibawan, Fakultas Kedokteran Hewan, Institut Pertanian Bogor, Bogor), three *S. equi* subsp. *zooepidemicus* isolated from infected monkeys (Balai Penyidikan Penyakit Hewan, Denpasar, Bali), and two *S. equi* subsp. *zooepidemicus* isolated from tonsils of humans in Bali, Indonesia (Salasia, 2000) were used. The *S. equi* subsp. *zooepidemicus* isolates were identified biochemically according to Farrow and Collins (1984) and serogrouped with a commercial streptococcal grouping system (Latex Reagent, Oxoid, Wesel, Germany).

PCR amplification of the 16S rRNA gene and enzymatic digestion

The analysis of restriction fragment length polymorphisms of the 16S ribosomal RNA gene of the cultures was performed as described previously (Lämmler *et al.*, 1998a; Lämmler *et al.*, 1998b). The oligonucleotide primers, synthesized by MWG-Biotech (Ebersberg, Germany) had the sequence, 5´-GAG AGT TTG ATC CTG GCT CAG CA-3´ for primer 1 and the 5´-CGG GTG TTA CAA ACT CTC GTG GT-3´ for primer 2. The sequence of the primers were designed by Bentley and Leigh (1995).

The reaction mixture (25 µl) contained 1 µl primer 1 (10 pmol l^{-1}), 1 µl primer 2 (10 pmol l^{-1}), 19.5 µl SuperMix (Gibco BRL, Eggenstein, Germany), and 1 µl *Taq* DNA polymerase (5U µl l^{-1}). The DNA (2.5 µl) preparation was subsequently added to each reaction tube. For DNA preparation, colonies of the bacteria were suspended in TE buffer (10 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA, pH 8) containing mutanolysin (50 U, Sigma, Deisenhofen, Germany) for 90 minutes at 37°C and 10 µl proteinase K (14,8 mg ml^{-1}, Sigma) for 120 min at 56°C. After boiling for 10 minutes at 100°C, the suspension was centrifuged (10,000 g, 5 s) and subsequently cooled before use. The tubes were then subjected to 30 cycles on a thermal cycler (COY, TempCycler, USA) with the following programme: 1x3 min precycle at 93°C, 30x90 s at 93°C, 90 s at 53°C and 90 s at 72°C followed by a final extension incubation of 72°C for 5 min. The presence of PCR products was determined by electrophoresis of 10 µl of the reaction product in a 2 per cent agarose gel, with tris acetat-electrophoresis buffer TAE (0,04 mol Tris, 0,001 mol EDTA, pH 7,8) and a 100 bp DNA ladder (Gibco) as molecular marker. The PCR products were further analysed after restriction endonuclease digestion with the restriction enzyme *Hinc*II (Biolabs, Schwalbach, Germany). For this, 15 µl of the PCR product was incubated with 10 U of the enzyme for 2 hours at 37°C (Abdulmawjood and Lämmler, 2000).

PCR amplification and sequencing of the V2 region of the 16S rRNA gene

The sequence of the primers used for amplification of the V2 region was designed by Bentley and Leigh (1995). The oligonucleotide primers, synthesized by MWG-Biotech (Ebersberg) had the sequence 5´-GAG AGT TTG ATC CTG GCT CAG CA-3´ for primer 1 and 5´-TTA CCG CGG CTG CTG GCA CGT-3´ for primer 2. The PCR was conducted with the programme mentioned above. The PCR product was purified by Qiagen PCR purification kit (Qiagen GmbH, Hilden, Germany) and sequenced by Eijkman Institute for Moleculare Biology, Jakarta, using ABI 377A DNA Sequencer (ABI PRISM, USA). The sequence data were further studied with manual allignment.

PCR amplification of the 16S-23S rRNA intergenic spacer region

The spacer region was amplified with oligonucleotide primers described by Chanter *et al.* (1997). The oligonucleotide primers, synthesied by MWG-Biotech (Ebesrberg) had the sequence 5´-GGT ACC TTA GAT GTT TCA GTT C-3´ for primer 1, and 5´-TTG TAC ACA CCG CCC GTC A-3´ for primer 2. The PCR was conducted with the programme mentioned above.

Results and Discussion

The size of the amplified 16S RNA gene product of all 22 strains investigated was approximately 1450 bp relative to the DNA size marker. The amplified product was digested with the restriction enzyme *Hinc*II.

After digestion with *Hinc*II two fragments with sizes of approximately 1250 bp and 200 bp could be observed for all 13 *S. equi* subsp. *zooepidemicus* strains isolated from pigs and monkeys, the *S. equi* subsp. *equi* strains 19 and CF32, and the *S. equi* subsp. *zooepidemicus* strains W60, 500 and 631. The amplified product of the *S. equi* subsp. *zooepidemicus* isolated from humans, the *S. equi* subsp. *zooepidemicus* strain 631 and both *S. agalactiae* reference strains were not digested with this restriction enzyme (Fig. 1).

The amplified gene product of a segment of the 16S rRNA gene including the V2 region with size of 560 bp of 13 selected strains were sequenced. The sequence analysis showing 200 bases are summarized in Fig. 2. Comparing the sequence of the 26 bases of the V2 region revealed an identical (100 per cent similarity) of the V2 region of the number *S. equi* subsp. *zooepidemicus* strains isolated from pigs and monkeys with the sequence of *S. equi* subsp. *equi*. The sequence of the two *S. equi* subsp. *zooepidemicus* strains isolated from humans appeared to be similar to the sequence of *S. equi* subsp. *equi* with the exception of three bases (88.5 per cent similarity). The sequence of the *S. equi* subsp. *zooepidemicus* strains 500 and W6 appeared to be similar to the sequence of *S. equi* subsp. *equi* with the exception of one base (96.2 per cent similarity) and two bases (92.3 per cent similarity), respectively. According to Abdulmawjood and Lämmler (2000) the 16S rRNA of the variants with an identical or closely related sequence to the sequence of *S. equi* subsp. *equi* were designated as 16S rRNA type 1. The sequence of the *S. equi* subsp. *zooepidemicus* strain 631 differed in 9 bases compared to the sequence of *S. equi* subsp. *equi* strains (65.4 per cent similarity) indicating the 16S rRNA type 2 (Abdulmawjood and Lämmler, 2000). The sequence of both *S. agalactiae* strains differed in eight bases (69.2 per cent similarity) to the sequence of the *S. equi* subsp. *equi* strains (Fig. 3).

PCR-amplification of the 16S-23S intergenic spacer region produced a single amplicon of approximately 950 bp for both *S. equi* subsp. *equi* strains, *S. equi* subsp. *zooepidemicus* strains isolated from pigs and monkeys, and the *S. equi* subsp. *zooepidemicus* strain 500. A single amplicon with a size of approximately 780 bp could be observed for the *S. equi* subsp. *zooepidemicus* strains W60 and 631, and a single amplicon of approximately 650 bp for the *S. equi* subsp. *zooepidemicus* strains isolated from humans and both *S. agalactiae* strains (Fig. 4). The results from the amplification of the 16S rRNA gene and the subsequent digestion with the restriction enzyme *Hinc*II and the size of the 16S-23S rRNA spacer region are summarized in Table 1.

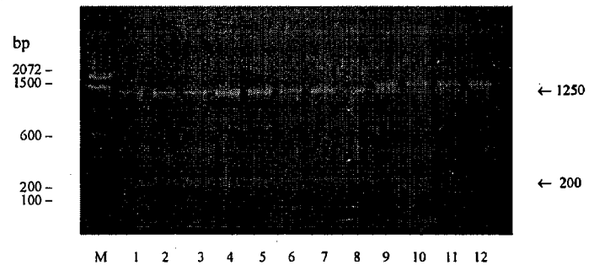

Fig. 1. Typical fragments of the PCR amplified 16S rRNA gene after digestion with the restriction enzyme *Hinc*II of the *S. equi* subsp. *zooepidemicus* strains isolated from pigs (lanes 1-4: 01, 02, 6.4a, and 18L), monkeys (lanes 5-6: 6.70 and 6.72), and humans (lanes 7-8: M6 and M9), the *S. agalactiae* reference strains (lanes 9-10: ATCC 49448 and ATCC 49449), and both *S. equi* subsp. *equi* strains (lanes 11-12: CF 32 and 19). M = a 100 base pair ladder served as size marker.


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1 GCGAACGGGTGAGTAACGCGTAGGTAACCTAGCTT - Se. 19
1 GCGAACGGGTGAGTAACGCGTAGGTAACCTAGCTT - Se. CF32
1 GCGAACGGGTGAGTAACGCGTAGGTAACCTAGCTT - Sz. 02
1 GCGAACGGGTGAGTAACGCGTAGGTAACCTAGCTT - Sz. 18L
1 GCGAACGGGTGAGTAACGCGTAGGTAACCTAGCTT - Sz. 6.72
1 GCGAACGGGTGAGTAACGCGTAGGTAACCTAGCTT - Sz. 6.73
1 GCGAACGGGTGAGTAACGCGTAGGTAACCTAGCTT - Sz. 500
1 GCGAACGGGTGAGTAACGCGTAGGTAACCTACCTT - Sz. W60
1 GCGAACGGGTGAGTAACGCGTAGGTAACCTACCTC - Sz. M6
1 GCGAACGGGTGAGTAACGCGTAGGTAACCTACCTC - Sz. M9
1 GCGAACGGGTGAGTAACGCGTAGGTAACCTACCTT - Sz. 631
1 GCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTC - Sa. (R)
1 GCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTC - Sa. (X)

36 ATAGCGGGGGATAACTATTGGAAACGATAGCTAAT - Se. 19
36 ATAGCGGGGGATAACTATTGGAAACGATAGCTAAT - Se. CF32
36 ATAGCGGGGGATAACTATTGGAAACGATAGCTAAT - Sz. 02
36 ATAGCGGGGGATAACTATTGGAAACGATAGCTAAT - Sz. 18L
36 ATAGCGGGGGATAACTATTGGAAACGATAGCTAAT - Sz. 6.72
36 ATAGCGGGGGATAACTATTGGAAACGATAGCTAAT - Sz. 6.73
36 ATAGCGGGGGATAACTATTGGAAACGATAGCTAAT - Sz. 500
36 ATAGCGGGGGATAACTATTGGAAACGATAGCTAAT - Sz. W60
36 ATAGCGGGGGATAACTATTGGAAACGATAGCTAAT - Sz. M6
36 ATAGCGGGGGATAACTATTGGAAACGATAGCTAAT - Sz. M9
36 ATAGCGGGGGATAACTATTGGAAACGATAGCTAAT - Sz. 631
36 ATAGCGGGGGATAACTATTGGAAACGATAGCTAAT - Sa. (R)
36 ATAGCGGGGGATAACTATTGGAAACGATAGCTAAT - Sa. (X)

71 ACCGCATAAAAGTGGTTGACCCATGT TAACCA TTT - Se. 19
71 ACCGCATAAAAGTGGTTGACCCATGT TAACCA TTT - Se. CF32
71 ACCGCATAAAAGTGGTTGACCCATGT TAACCA TTT - Sz. 02
71 ACCGCATAAAAGTGGTTGACCCATGT TAACCA TTT - Sz. 18L
71 ACCGCATAAAAGTGGTTGACCCATGT TAACCA TTT - Sz. 6.72
71 ACCGCATAAAAGTGGTTGACCCATGT TAACCA TTT - Sz. 6.73
71 ACCGCATAAAAGTGGTTGACCCATGT TAACTA TTT - Sz. 500
71 ACCGCATAAAAGTGGTTGACCCATGT TAACTA TTT - Sz. W60
71 ACCGCATAAAAGTGTTTAACCCATGT TAAACA TTT - Sz. M6
71 ACCGCATAAAAGTGTTTAACCCATGT TAAACA TTT - Sz. M9
71 ACCGCATAAAAGCCAATGACCCATGT CATTGG CTT - Sz. 631
71 ACCGCATAAGAGTAATTAACACATGT TAGTTA TTT - Sa. (R)
71 ACCGCATAAGAGTAATTAACACATGT TAGTTA TTT - Sa. (X)

                V2 region

106 AAAAGGAGCAACAGCTCCACTATGAGATGGACCTG - Se. 19
106 AAAAGGAGCAACAGCTCCACTATGAGATGGACCTG - Se. CF32
106 AAAAGGAGCAACAGCTCCACTATGAGATGGACCTG - Sz. 02
106 AAAAGGAGCAACAGCTCCACTATGAGATGGACCTG - Sz. 18L
106 AAAAGGAGCAACAGCTCCACTATGAGATGGACCTG - Sz. 6.72
106 AAAAGGAGCAACAGCTCCACTATGAGATGGACCTG - Sz. 6.73
106 AAAAGGAGCAACAGCTCCACTATGAGATGGACCTG - Sz. 500
106 AAAAGGAGCAACAGCTCCACTATGAGATGGACCTG - Sz. W60
106 AAAAGGT GCAATTGCATCACTATGAGATGGACCTG - Sz. M6
106 AAAAGGT GCAATTGCATCACTATGAGATGGACCTG - Sz. M9
106 GAAAGAAGCAAACGCTCCACTATGAGATGGACCTG - Sz. 631
106 AAAAGGAGCAATTGC TTCACTGTGAGATGGACCTG - Sa. (R)
106 AAAAGGAGCAATTGC TTCACTGTGAGATGGACCTG - Sa. (X)

141 CGTTGTATTAGCTAGTTGGTAGGGTAAAGGCCTAC - Se. 19
141 CGTTGTATTAGCTAGTTGGTAGGGTAAAGGCCTAC - Se. CF32
141 CGTTGTATTAGCTAGTTGGTAGGGTAAAGGCCTAC - Sz. 02
141 CGTTGTATTAGCTAGTTGGTAGGGTAAAGGCCTAC - Sz. 18L
141 CGTTGTATTAGCTAGTTGGTAGGGTAAAGGCCTAC - Sz. 6.72
141 CGTTGTATTAGCTAGTTGGTAGGGTAAAGGCCTAC - Sz. 6.73
141 CGTTGTATTAGCTAGTTGGTAGGGTAAAGGCCTAC - Sz. 500
141 CGTTGTATTAGCTAGTTGGTAGGGTAAAGGCCTAC - Sz. W60
141 CGTTGTATTAGCTAGTTGGTGAGGTAACGGCTCAC - Sz. M6
141 CGTTGTATTAGCTAGTTGGTGAGGCAACGGCTCAC - Sz. M9
141 CGTTGTATTAGCTAGTTGGTAGGGTAAAGGCCTAC - Sz. 631
141 CGTTGTATTAGCTAGTTGGTGAGGTAAAGGCTCAC - Sa. (R)
141 CGTTGTATTAGCTAGTTGGTGAGGTAAAGGCTCAC - Sa. (X)

176 CAAGGCGACGATACATAGCCGACCT - Se. 19
176 CAAGGCGACGATACATAGCCGACCT - Se. CF32
176 CAAGGCGACGATACATAGCCGACCT - Sz. 02
176 CAAGGCGACGATACATAGCCGACCT - Sz. 18L
176 CAAGGCGACGATACATAGCCGACCT - Sz. 6.72
176 CAAGGCGACGATACATAGCCGACCT - Sz .6.73
176 CAAGGCGACGATACATAGCCGACCT - Sz. 500
176 CAAGGCGACGATACATAGCCGACCT - Sz. W60
176 CAAGGCGACGATACATAGCCGACCT - Sz. M6
176 CAAGGCGACGATACATAGCCGGACC - Sz. M9
176 CAAGGCGACGATACATAGCCGACCT - Sz. 631
176 CAAGGCGACGATACATAGCCGACCT - Sa. (R)
176 CAAGGCGACGATACATAGCCGACCT - Sa. (X)
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Fig. 2. Sequence of a segment (200 bases) of the 16S rRNA gene including the V2 region of the two *S. equi* subsp. *equi* 19 and CF32 strains, 6 *S. equi* subsp. *zooepidemicus* strains isolated from pigs (Sz.02, Sz.18L), monkeys (Sz.6.72, Sz.6.73), and humans (M6, M9), the three *S. equi* subsp. *zooepidemicus* strains 500, W60, 631, and the two *S. agalactiae* reference strains 25/60 R (ATCC 49448) and 24/60 X (ATCC 49449). The differences of the gene sequences compared to the gene sequence of *S. equi* subsp. *equi* were marked separately.

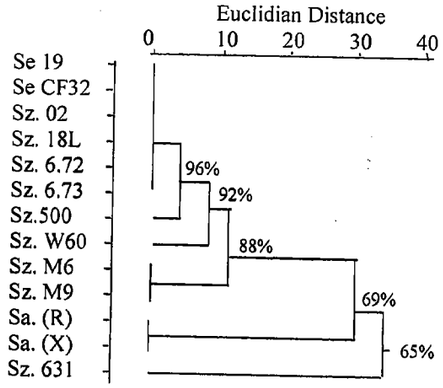

Fig. 3. Dendrogram of similarity of 26 bases of the sequence of the V2 region (position number 77 to 102) of the 16S rRNA genes presented in Fig. 2.

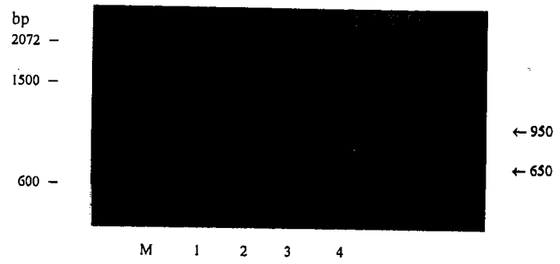

Fig. 4. Typical amplicon of the 16S rRNA-23S rRNA intergenic spacer region of *S. equi* subsp. *zooepidemicus* isolated from pig (18.L, lane 1), monkey (6.72, lane 2), *S. equi* subsp. *equi* strains CF32 (lane 3), and *S. equi* subsp. *Zooepidemicus* isolated from human (M6, lane 4). M = a 100 base pair ladder served as size marker.

Table 1. Size of the amplicons of the 16S rRNA gene, digestion of the amplicon with *Hinc*II, sequence of the gene encoding the V2 region expressed in per cent similarity to the sequence of *S. equi* subsp. *equi* and size of the amplicons of the intergenic spacer region of two *S. equi* subsp. *equi* strains, six *S. equi* subsp. *zooepidemicus* strains isolated from pigs, monkeys, and humans, three *S. equi* subsp. *zooepidemicus* strains and two *S. agalactiae* strains

Strains Code	16S rRNA gene (1450 bp)	Digestion with *Hinc*II	Sequence similarity of the V2 region						Size of the 16S-23S rRNA intergenic spacer region amplicon		
			100%	96%	92%	88%	69%	65%	950 bp	780 bp	650 bp
Se 19	+	+	+						+		
Se CF32	+	+	+						+		
Sz. 02	+	+	+						+		
Sz. 18L	+	+	+						+		
Sz. 6.72	+	+	+						+		
Sz. 6.73	+	+	+						+		
Sz.500	+	+		+					+		
Sz. W60	+	+			+					+	
Sz. M6	+	-				+					+
Sz. M9	+	-				+					+
Sz. 631	+	-					+			+	
Sa. (R)	+	-						+			+
Sa. (X)	+	-					+				+

Se = *S. equi* subsp. *equi*
Sz = *S. equi* subsp. *zooepidemicus*
Sa = S. agalactiae

Streptococcus equi subsp. *zooepidemicus* is well known as an important pathogen of the horse being associated with respiratory tract infections of foals and with uterine infections in mares. In addition this bacteria can be isolated from infections of a wide variety of animals, including pigs, sheep, cows, goats, foxes, birds, rabbits, guinea pigs and monkeys (Stableforth, 1959; Soedarmanto *et al.*, 1996). All of these animals might be potential reservoirs for infections of humans. Cases of human infections with *S. equi* subsp. *zooepidemicus* have been reported and such infections are frequently associated with the consumption of homemade cheese or unpasteurized milk (Colman and Efstratiou, 1985; Edwards *et al.*, 1988; Francis *et al.*, 1993). In addition, the isolation of *S. equi* subsp. *zooepidemicus* from humans has been described from cases of endocarditis (Martinez-Luengas *et al.*, 1982), pneumonia (Rose *et al.*, 1980), meningitis (Ghoneim and Cooke, 1980; Low *et al.*, 1980), septic artritis (Barnham, *et al.*, 1987; Gorman *et al.*, 1987) and cervical lymphadenitis (Köhler and Cedeberg, 1979). The occurrence of the bacteria in infection of these different hosts raises the question about the route of infection and the possibly existing epidemiological relation in outbreaks with this microorganism. Reports on the epidemiology of infections with *S. equi* subsp. *zooepidemicus* are based on serotyping T or M-like proteins (Colman and Efstratiou, 1985; Timoney and Mukhtar, 1992; Walker and Timoney, 1994) and also by genomic typing methods. The latter included DNA fingerprinting techniques (Skjold *et al.*, 1987; Soedarmanto *et al.*, 1996; Bert *et al.*, 1997) and more recently also the PCR amplification of the gene encoding the 16S-23S rRNA intergenic spacer region (Chanter *et al.*, 1997).

In the present study the PCR amplified 16S rRNA gene of *S. equi* subsp. *zooepidemicus* strains isolated from pigs, monkeys, and humans appeared to be heterogenous and useful as an epidemiological marker for this species. Differences in the sequence of the V2 region of the 16S rRNA gene of the isolates could be observed using the restriction enzyme *Hinc*II. The restriction enzyme was selected using the sequence of the V2 region of a *S. equi* subsp. *equi* reference given by Bentley and Leigh (1995). With this restriction enzyme the amplicon of all *S. equi* subsp. *zooepidemicus* strains isolated from pigs and monkeys could be digested. However the human isolates could not be digested with this enzyme indicating sequence variations. This corresponded to the sequence variations of nine of the 39 *S. equi* subsp. *zooepidemicus* strains isolated from horses which were investigated by Abdulmawjood and Lämmler (2000). These findings are in contrast to the V2 region of the species *S. uberis*, *S. parauberis* and *S. agalactiae*. For these species no sequence variations could be observed (Hassan *et al.*, 2000).

According to Abdulmawjood and Lämmler (2000), *S. equi* subsp. *zooepidemicus* could be differentiated into two 16S rRNA types. The 16S rRNA type 1 was closely related to the sequence of *S. equi* subsp. *equi*. In contrast the 16S rRNA type 2 showed no close relation to the sequence of *S. equi* subsp. *equi*. The sequence of the highly variable V2 region of the *S. equi* subsp. *zooepidemicus* isolated from pigs, monkeys and humans of the present investigation revealed that this species belonged to the 16S rRNA type 1. The sequencing results of the V2 region of *S. equi* subsp. *equi*, *S. equi* subsp. *zooepidemicus* and *S. agalactiae* of the present study corresponded to the sequences given by Bentley and Leigh (1995). The sequence of the 16S rRNA type 1 *S. equi* subsp. *zooepidemicus* strain of the present investigation corresponded to the sequence of the *S. equi* subsp. *zooepidemicus* strains given by Abdulmawjood and Lämmler (2000) and different

to the sequence of the 31 streptococal species summarized by Bentley and Leigh (1995). However, according to the present results these 13 type 1 *S. equi* subsp. *zooepidemicus* isolated from pigs and monkeys could be differentiated from both human isolates by restriction fragment polymorphism analysis using the restriction enzyme *Hinc*II. Further differences of the *S. equi* subsp. *zooepidemicus* strains of the isolates from pigs and monkeys and the isolates from humans could be seen in the size of the 16S-23S rRNA intergenic spacer region. The use of this restriction enzyme and the determination of the size of the intergenic spacer region might be helpful in future studies investigating the importance of typing system for *S. equi* subsp. *zooepidemicus*. However, the *S. equi* subsp. *zooepidemicus* isolates obtained from pigs and monkeys seemed to be not identical to these obtained from humans.

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