IDENTIFICATION OF LANCIFIELD SEROGROUP G *STREPTOCOCCUS CANIS* BY PCR-RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS (PCR-RFLP) OF 16S RIBOSOMAL RNA GENE

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ABSTRACT

In this study *S. canis* and 12 various species and serogroups of streptococci including: *S. agalactiae, S. dysgalactiae* subsp. *dysgalactiae* (serogroup C and L), *S. dysgalactiae* subsp. *equisimilis* (serogroup G), *S. uberis, S. parauberis, S. phocae, S. suis, S. equi* subsp. *equi, S. equi* subsp. *zooepidimicus, S. porcinus* and *S. pyogenes* were used and identified reliably by PCR-restriction fragment length polymorphism analysis (PCR-RFLP) of 1.43 kb of 16S ribosomal RNA gene using universal oligonuclotide primers and subsequent digestion with the restriction endonucleases including *Rsa*I, *Msp*I and *Ava*II. The PCR-RFLP results showed that *Rsa*I restriction RFLP pattern of *S. canis* appeared different with all streptococci species baring the *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidimicus*. The *Msp*I restriction RFLP pattern of *S. canis* could be differentiated from *S. agalactiae, S. dysgalactiae* subsp. *dysgalactiae* (serogroups C and L), *S. dysgalactiae* subsp. *equisimilis* (serogroups G), *S. phocae, S. suis, S. porcinus* and *S. pyogenes*. The *Ava*II restriction RFLP pattern of *S. canis* could be distinguished from *S. dygalactiae* subsp. *dysgalactiae* (serogroup C and L), *S. dygalactiae* subsp. *equisimilis* (serogroup G) *S. parauberis, S. phocae* and *S. suis*. In conclusion, PCR-RFLP method using restriction endonucleases *Rsa*I, *Msp*I and *Ava*II could be useful method for identification of *S. canis* from *S. dysgalactiae* subsp. *dysgalactiae* (serogroup C and L), *S. dysgalactiae* subsp. *equisimilis* (serogroup G) and other related streptococci species. It can be concluded that RFLP method might help to determine the prevalence of *S. canis* in animal and human infections .

Keywords: Streptococcus canis, *Streptococcus* species; 16S rRNA gene; PCR-RFLP

INTRODUCTION

The Lancifield serogroup G β-hemolytic streptococci consist of *Streptococcus cani*s and *Streptococcus dysgalactiae* subsp. *equisimilis*. *S. canis* under certain circumstances will cause opportunistic infections mainly in dogs, cats, rarely in cows and other animals like mink, mice, rabbits, foxes and sporadically isolated from different cases of human (1, 2, 3). In dogs, *S. canis* associated with infections of the urogenital and respiratory tracts and otitis externa (4, 5, 6). In cats it is associated with lymphadenitis , arthritis, ulceration to toxic shock-like syndrome, chronic respiratory infection, necrotizing sinusitis and meningitis (7, 8, 9, 10). Also different reports described subclinical mastitis in cow due to isolation of *Streptococcu*s group G or *S. canis* from bovine mammary gland infections (11, 12, 13). *S. canis* has also been recovered from human with wound infection and bacteremia from infections associated with meningitis, peritonitis, neonatal septicemia, adult septicemia, and cellulitis (14, 15, 16, 17). Consequently, *S. canis* could be considered as zoonotic bacteria and cross-infection with bacteria occurred between animals and humans (13; 18, 19). In contrast, pyogenic streptococci of Lancefield groups C (*S. dysgalactiae* subsp. *equisimilis*) and group G were predominantly isolated from human infections (20). According to Vieira *et al* (21) and Vandamme *et al* (22), *S. dysgalactiae* could be classified into four groups related to phenotypic and genotypic characterizations. The *S. dysgalactiae* subsp. *dysgalactiae* alpha-hemolytic Lancifield serogroup C and *S. dysgalactiae* subsp. *equisimilis* beta-hemolytic, serogroup C, G and L. According to Hassan *et al* (23) *S. dysgalactiae* Lancifield serogroup C, L and G can be identified using species oligonucleotide primers with specificity for the 16S-23S rRNA intergenic spacer region.

The Lancifield serogroup G beta-hemolytic streptococci could be biochemically and enzymaticlly differentiated including acid production from trehalose and lactose, esculin hydrolysis, fibrinolysin production, α-galactosidase, β-galactosidase, and β-D-glucuronidase

activities and CAMP-like factor phenomena (2, 3). The CAMP and CAMP like phenomena was a benefit test to differentiation between streptococcal isolates. This procedure is used principally to confirm group B streptococci by production of a

characteristic zone of hemolysis when grown in nearness to *S. aureus*, occasionally these phenomena in *S. canis* not always appeared to be perfectly clear, other than with *S.dysgalactiae* the CAMP phenomena always showed negative reaction.

Molecular methods such as PCR- restriction fragment length polymorphism analysis (PCR-RFLP) have been used successfully for the differentiation, identification and subtyping of different bacteria at the species level. The PCR-RFLP had been previously used for the identification of the 16S rRNA gene of *S. agalactiae* (24, 25, 26), for differentiation of *S. uberis* and *S. parauberis* (27, 28, 29), for identification of the serologically heterogeneous species *S. porcinus* and *S. phocae* (30, 31), for identification of *S. iniae* (synonym *S. shiloi*), *S. difficile*, *S. porcinus*, *S. uberis* and *S. parauberis* (32), and for demonstration of intraspecies variations of *S. equi* subsp. *zooepidemicus* (24). Hassan *et al* (12) identified *S. canis* strains isolated from bovine mastitis milk by RFLP-PCR method. Barsotti *et al* (33) and McDonald *et al* (34) used PCR-RFLP analysis for 16S-23S rRNA intergenic spacer region for differentiation and identification of *S. mitis* and *Streptococcus* species causing bovine mastitis, respectively. However, the sequence diversity of *groESL* genes among *Streptococcus bovis* group isolates was analysed by using RFLP technique (35). RFLP of ribosomal RNA genes were used for identification of *S. parauberis* (*S. uberis* type II), as well as genotypic identification of viridans streptococci and identification of the virulent and a virulent strains of *S. suis* by ribotyping (36, 37, 38, 39). Scheidegger *et al* (40) used RFLP analysis of a PCR-amplified fragment of the 16S rRNA gene for identification and differentiation of 21 different enterococcal species. Reinoso *et al* (41) identified *S. agalactiae*, *S. dysgalactiae*, *S. uberis*, *S.equinus* and *E. faecalis* on the species level using 16S rRNA RFLP. Jans *et al* (42) developed a specific identification assay for the *Streptococcus bovis/Streptococcus equinus* complex using multiplex PCR of the 16S rRNA gene followed by RFLP method.

this study described a relative and simple PCR-RFLP-based identification method which can be used to discriminate *S. canis* and other species of *Streptococcus*. This approach might help to determine the prevalence of *S. canis* in animal and human infections.

MATERIALS AND METHODS

Computer-simulated RFLP analysis of 16S rRNA genes

To select which endonucleases enzymes are appropriate to determine the characteristic DNA fragment sizes for identification and differentiation of *Streptococcus* species, the computer program Clone Manager 4.0 (Version 4.1, Fa. Scientific Educational Software, USA) was used. This program was kindly obtained from (Professur für Milchwissenschaften, Institut für Tierärztliche Nahrungsmittelkunde, Justus-Liebig Universität Gießen, Gießen, Germany).For this purpose, the 16S rRNA gene sequences (1.43 kb) obtained from NCBI GenBank (the National Center for Biotechnology Information), website (www.ncbi.nlm.nih. gov/genbank) were analyzed using the Clone Manager 4.0. The date of 16S rRNA gene sequences were accomplished and analyzed with the program during 2011-2012 in the Department of Veterinary Public Health, College of Veterinary Medicine, University of Mosul, Mosul, Iraq. The predicated Restriction patterns of 16S rRNA gene sequences of *Streprococcus* species were investigated to determine the characteristic DNA fragment sizes. The 16S rRNA gene sequences which analyzed including, *S. canis* ATCC 43498 (GenBank accession number AB002483), *S. dysgalactiae* ATCC 27957 (AF015928), *S. agalactiae* JCM 5671 (AB023574), *S. uberis* ATCC 27958 (U41048), *S. parauberis* 1348 (X89967), *S. porcinus* (X58315), *S. suis* (AF009475), *S. phocae* (AF235052), *S. equi* (AB002516) with size 1.43 kb. The complete sequence (1.43 kb) of *S. porcinus* and *S. pyogenes* even now are not published in NCBI GenBank.

Streptococcus species strains

The following *streptococcus* species were used in this study. *S. canis* (n = 28), *S. dysgalactiae* subsp. *equisimilis* (serogroup G) (n = 11), *S. dysgalactiae* subsp. *dysgalactiae* (serogroup C) (n = 6), *S. dysgalactiae* subsp. *dysgalactiae* (serogroup L) (n = 6), *S. agalactiae* (n = 36) including reference strains 090 (Ia), H36B (Ib), 18RS 21(II), 6313 (III), 3139 (IV), SS1169 (V), NT6 (VI), 7271 (VII), JM9-130013 (VIII), Compton 24/60 (X), Compton 25/60 (R), BS30 (Rib), 335 (cα), 70339 (cβ) and A909 (Ia/c), *S. equi* subsp. *equi* (n = 3), *S. equi* subsp. *zooepidemicus* (n = 4), *S. uberis* (n = 17) including reference strains NCDO 2022, NCDO 2038 and NCDO 2086, *S. parauberis* (n = 4) including reference strain NCDO 2020, *S. porcinus* (n = 9) serogroup E, P, U and V with reference strains ATCC 3564, ATCC 12390, ATCC

12391 and ATCC 35649, *S. phocae* (n = 8) including reference strains 8399 H1 (NCTC 12719) and 8190 R2, *S. suis* (n = 6) and *S. pyogenes* (n = 4). All isolates of each of the above bacteria were kindly obtained from strains collection of (Institut für Tierärztliche Nahrungsmittelkunde, Professur für Milchwissenschaften), Giessen University, Giessen, Germany.

DNA preparation

All *Streptococcus* strains used in this study were grown 20-22 h in 5% sheep blood agar contained 0.1% ecsulin (Oxoid, Wessel, Germany), five to 10 colonies of the bacteria were suspended in 100 µl TE buffer [10 mmol/l Tris-HCl, 1mmol/l EDTA, (pH 8.0)] for genomic DNA extraction according to a procedure detailed previously (43).

PCR amplification of the 16S rRNA gene

The PCR amplification protocol of 16S rRNA gene of the bacterial cultures was performed as described previously for *S. uberis*, *S. parauberis*, *S. agalactiae* and *S. equi* subsp. *zooepidemicus* (24). Briefly, The oligonucleotide primers AR1 and AMII, synthesized by MWG-Biotech (Ebersberg, Germany). The primer AR1 had the sequence 5'-GAGAGTTTGA TCCTGGCTCAGGA-3' was obtained from Bentley and Leigh (44), the primer AMII had the sequence 5'-CGGGTGTTACAAACTCTCGTGGT-3' was designed as described (24). The PCR reaction mixture (30 µl) contained 1 µl primer AR1 (10 pmol/µl), 1 µl primer AMII (10 pmo/µl), 0.6 µl dNTP (10 mmol, MBI Fermentas, St. Leon-Rot, Germany), 3 µl 10x thermophilic-buffer (Promega, Mannheim, Germany), 1.8 µl $MgCl_2$ (25 mmol) (Promega), 0.2 µl *Taq* DNA polymerase (5U/µl, Promega) and 19.9 µl aqua dest. Finally 2.5 µl DNA preparation was added to each reaction tube. Than PCR reactions were performed using the following program, one denaturation cycle of 95°C, 4 min. and 30 cycle of 95°C, 90sec; 58°C, 90sec; 72°C 90sec; and terminated by one cycle of 72°C, 5 min in thermalcycler Techne-Progene (Thermodux, Wertheim, Germany). The presence of PCR products was determined by electrophoresis of 8 µl of the reaction product in a 2% standard agarose (MBI
Fermentas, St. Leon-Rot, Germany) with Tris acetate-electrophoresis buffer (TAE) [(0.04 mol/l Tris, 0.001 mol/l EDTA, pH 7.8)] and a 100 bp DNA ladder (Gibco BRL, Eggenstein, Germany) as molecular marker followed by staining for 5 min with 5

µl/ml ethidium bromide solution (Sigma, Deisenhofen, Germany). The amplicons were then visualized under a UV trans-illuminator (Biorad, Munich, Germany).

Restriction fragment length polymorphism analysis of 16S r RNA PCR products

The PCR-RFLP analysis of the 16S rRNA gene was performed as described previously (24). The PCR product was further analyzed using restriction endonuclease digestion *Rsa*I, *Msp*I and *Ava*II (BioLabs, Schwalbach/Taunus, Germany). For this purposes 18 µl or 22 µl of the remaining PCR products were incubated with 1µl (10 U/µl) of *Rsa*I or 2 µl (10 U/µl) of *Msp*I or *Ava*II, respectively. The mixture was incubated for 2 h at 37°C in water bath. The DNA fragments were separated by electrophoresis in 2 % high resolution MetaPhor® Agarose (Cambrex Bio Science Rockland, Inc/Biozym, Heaaisch-Oldendoef, Germany) with Tris acetate-electrophoresis buffer (TAE) and followed by staining for 5 min with 5 µl/ml ethidium bromide solution (Sigma). The DNA fragments were then visualized under a UV trans-illuminator (Biorad, Munich, Germany).

RESULTS

Predicated RFLP of 16S rRNA gene sequence using Clone Manager 4.0 program

Table 1 shows the expected restriction fragment of *S. canis* and other streptococci species after selecting the endonucleases enzymes (*Rsa*I, *Msp*I and *Ava*II) according to Clone Manager 4.0 program. The length of the 16S rRNA gene sequence (1.43 kb) relative to the DNA size marker for *S. canis*, *S. dysgalactiae* subsp. *dysgalactiae* (serogroup C), *S. dysgalactiae* subsp. *equisimilis* (serogroup G), *S. dysgalactiae* subsp. *dysgalactiae* (serogroup L), *S. agalactiae*, *S. equi* subsp. *equi*, *S. equi* subsp. *zooepidemicus*, *S. uberis*, *S. parauberis*, *S. porcinus*, *S. phocae*, *S. suis* and *S. pyogenes* investigated. Complete sequence (1.43 kb) of *S. porcinus* and *S. pyogenes* were absent in NCBI GenBank, therefore there is no results of predicated restriction fragment.

Analyzed of 16S rRNA gene sequence using restriction enzymes

The amplified PCR products (1.43 kb) of *streptococcus* species were digested with the restriction enzymes *Rsa*I, *Msp*I and *Ava*II. After digestion with *Rsa*I the 24 strains (85.7%) of *S. canis* 16S rRNA gene appeared in restriction patterns with

fragment sizes of 900, 210 and 145 bp. The remaining four strains (14.3%) of *S. canis* showed fragment sizes of 625, 265, 210 and 145 bp. The first characteristic restriction patterns of *S. canis* differed from those of the species *S. dysgalactiae* subsp. *dysgalactiae* (serogroup C), *S. dysgalactiae* subsp. *equisimilis* (serogroup G), *S. dysgalactiae* subsp. *dysgalactiae* (serogroup L), *S. agalactiae*, *S. uberis* and *S. parauberis*. *S. pocinus*, *S. suis* and *S. phocae* species. Identical patterns where observed for 16S rRNA gene of *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus*, which was similar to the restriction pattern of 16S rRNA gene of *S. canis* (Figure 1 and table 2).

Eight strains (88.9%) of *S. porcinus* 16S rRNA gene appeared in restriction patterns with fragment sizes of 900, 210 and 145 bp and one strain (11,1%) with fragment sizes of 625, 265, 210 and 145 bp. The results of *Rsa*I restriction fragments could be divided the 13 *Streptococcus* species into 6 different RFLP patterns (Table 3).

Table 1: Predicated RFLP of 16S rRNA gene sequence using Clone Manager 4.0 program of different *Streptococcus* species. The 16S rRNA gene sequence cleaved with *Rsa*I, *Msp*I and *Ava*II endonucleases enzymes.

Species	code of strain	NCBI accession number	Estimated RFLP pattern (size bp)[1]		
			***Rsa*I**	***Msp*I**	***Ava*II**
S. canis	ATCC 43498	AB002483	891, 212, 146, 143	555, 315, 163, 125, 120, 91	892, 311, 229
S. agalactiae	JCM 5671	AB023574	629, 355, 262, 146	555, 163, 163, 154, 125, 120, 91	892, 311, 229
S. dysgalactiae subsp. *dysgalactiae*	ATCC 43078	AY121359	629, 355, 262, 146	474, 163, 154, 150, 125, 120, 91, 81	892, 311, 182, 70
S. dysgalactiae	ATCC 27957	AF015928	629, 355, 262, 146	474, 163, 154, 150, 125, 120, 91, 81	892, 311, 182, 70
S. dysgalactiae subsp. *equisimilis*	ATCC 35666	AB096755	629, 355, 262, 146	474, 163, 154, 150, 125, 120, 91, 81	892, 311, 182, 70
S. uberis	ATCC 27958	U41048	700, 121, 183, 146, 143	555, 317, 163, 125, 120, 91	892, 311, 229
S. parauberis	1348	X89967	700, 355, 183, 146	555, 317, 163, 125, 120, 91	1203, 229
S. phocae NCTC	12719	AF235052	899, 359, 148	563, 164, 163, 156, 125, 121, 93	1066, 233, 148
S: suis	5428	AF009475	629,355, 262,146	555, 317, 288, 211	597, 311, 295, 299
S. equi subsp. *equi*	ATCC 43079	AB002516	835, 355, 146	499, 304, 211. 164, 125	892, 311, 173
S. equi subsp. *zooepidimicus*	ATCC 43079	AB002516	896, 212, 146, 143, 99	560, 304, 163, 125, 120, 109	951, 311, 234
*S. porcinus***	NCDO 600 (T)	X58315	-	-	-
*S. pyogenes***	ATTC 19615	Y12924	-	-	-

** = partial sequence of *S. porcinus* (NCBI accession number X58315) and *S. pyogenes* (NCBI accession number Y12924) in NCBI GenBank.

[1] = Estimated sizes of fragments of less than 50 base pair (bp) are not provided

- = not analyzed

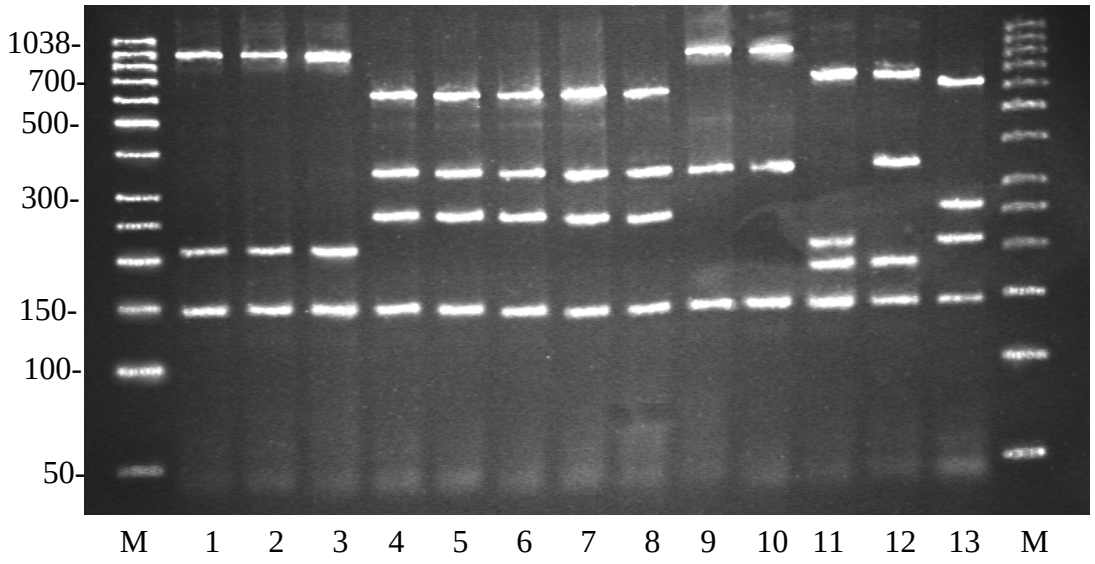

Figure 1. Typical restriction fragments of the PCR amplified 16S rRNA gene after digestion with *Rsa*I enzyme. PCR fragments Lanes (1 through 13), *S. canes*, *S. equip* subsp. *equip*, *S. equip* subsp. *zooepidimicus*, *S. agalactiae*, *S. dysgalactiae* subsp. *dysgalactiae* (group C), *S. dysgalactiae* subsp. *equisimilis* Group G), *S. dysgalactiae* subsp. *dysgalactiae* (group L), *S. suis*, *S. phocae*, *S. porcinus*, *S. uberis*, *S. parauberis* and *S. pyogenes*, respectively M = GeneRular^{TM} 100 bp DNA Ladder (MBI Fermentas).

Digestion of the 16S rRNA gene of *S. canis* with *Msp*I revealed a uniform restriction pattern 570, 310, 160, 140, 90 for all 28 *S. canis.* The same pattern was observed also in *S. uberis*, *S. parauberis*, *equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus*. Identical characteristic restriction patterns 570, 160, 140, 90 were determined for all *S. dysgalactiae* subsp. *dysgalactiae* (serogroup C), *S. dysgalactiae* subsp. *equisimilis* (serogroup G), *S. dysgalactiae* subsp. *dysgalactiae* (serogroup L) strains, which were differed from all other *Streptococcus* species. *S. phocae* and *S. pocinus* strains showed other different species specific restriction patterns 570, 160, 150, 140, 90. *S. agalactiae*, displayed restriction fragments 570, 160, 140, 90 but *S. suis* gave 570, 210, 160, 140, 70 and *S. pyogenes* (Figure 2). The results of *Msp*I restriction fragments divided the 13 *Streptococcus* species in to 6 different RFLP patterns (Table 3).

After digestion of the 16S rRNA gene with *Ava*II, *S. canis, S. agalactiae, S. uberis, S. porcinus, S. equi* subsp. *equi, S. equi* subsp. *zooepidimicus, S. pyogenes* revealed a uniform restriction pattern (900, 310, 240). A characteristic restriction fragments were observed for *S. dysgalactiae* subsp. *equisimilis* (serogroup G) and *S. dysgalactiae* subsp. *dysgalactiae*

(serogroup C and L). Characteristic restriction patterns were distinguished also for *S. parauberis* and *S. phocae* (1210, 230) and 1210, 230, 91, respectively. *S. suis* gave restriction fragments 590, 310, 290 (Figure 3). The fragment sizes and RFLP patterns of all strains are summarized in table 2 and table 3.

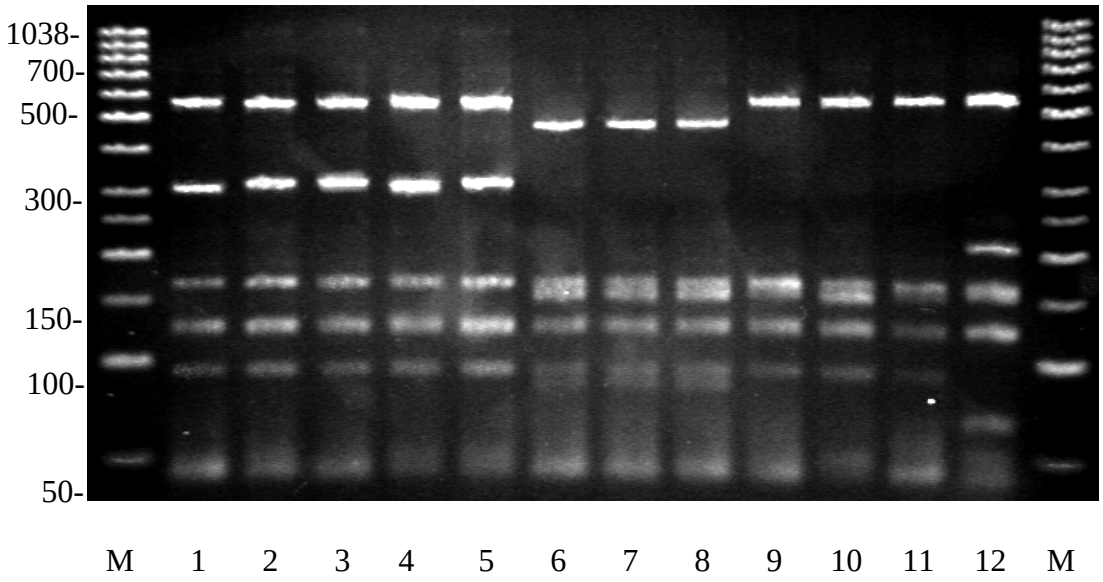

Figure 2. Typical restriction fragments of the PCR amplified 16S rRNA gene after digestion with *Msp*I enzyme. PCR fragments (Lanes (1-12) of *S. canis*, *S. uberis*, *S. parauberis*, *S. equi* subsp. *equi*, *S. equi* subsp. *zooepidimicus*, *S. dysgalactiae* subsp. *dysgalactiae* (group C), *S. dysgalactiae* subsp. *equisimilis* Group G), *S. dysgalactiae* subsp. *dysgalactiae* (group L), *S. phocae*, *S. porcinus*, *S. agalactiae* and *S. suis* respectively. M = Fig.1.

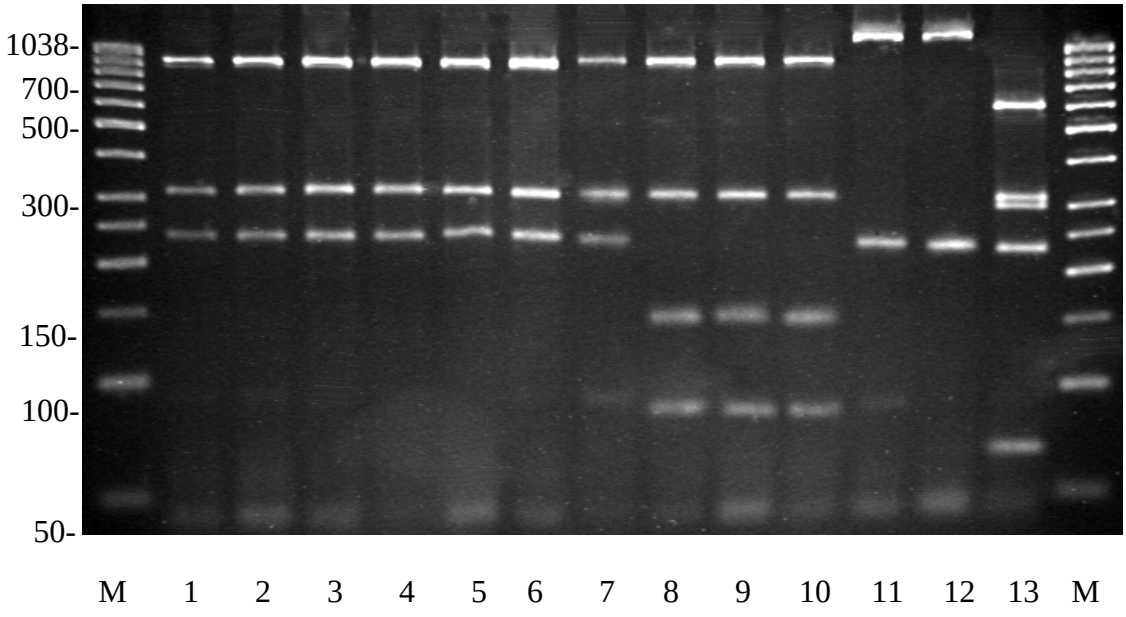

Figure 3. Typical restriction fragments of the PCR amplified 16S rRNA gene after digestion with *Ava*II enzyme. PCR fragments (Lanes (1-13) of *S. canis*, *S. agalactiae*, *S. uberis*, *S. porcinus*, *S. equi* subsp. *equi*, *S. equi* subsp. *zooepidimicus*, *S. pyogenes*, *S. dysgalactiae* subsp. *dysgalactiae* (group C), *S. dysgalactiae* subsp. *equisimilis* Group G), *S. dysgalactiae* subsp. *dysgalactiae* (group L), *S. phocae*, *S. parauberis* and *S. suis*, respectively. M = Fig.1.

Table 2: Recognized DNA fragment size (bp) of the 16S rRNA gene PCR products cleaved with *Rsa*I, *Msp*I and *Ava*II enzymes in different *Streptococcus* species.

	Species	n	Deduced size (bp)[1]		
			***Rsa*I**	***Msp*I**	***Ava*II**
1.	*S. canis*	24 4	900, 210, 145 625, 265, 210, 145	570, 310, 160, 140, 90	900, 310, 230
2.	*S. agalactiae*	36	625, 350, 265, 145	570, 160, 140, 90	900, 310, 230
3.	*S. dysgalactiae* subsp. *dysgalactiae* (serogroup C)	6	625, 350, 265, 145	475, 160, 150, 140, 90	900, 310, 180,70
4.	*S. dysgalactiae* subsp. *equisimilis* (serogroup G)	11			
5.	*S. dysgalactiae* subsp. *dysgalactiae* (group L)	6			
6.	*S. uberis*	17	700, 220, 190, 145	570, 310, 160, 140, 90	900, 310, 230
7.	*S. parauberis*	4	700, 375, 190, 145	570, 310, 160, 140, 90	1210, 230
8.	*S. phocae*	8	890, 350, 150	570, 160, 150, 140, 90	1207, 230, 90
9.	*S: suis*	6	625, 350, 265, 145	570, 210, 160, 140, 70	590, 310, 290
10.	*S. equi* subsp. *equi*	3	900, 200, 150	570, 310, 160, 140, 90	900, 310, 230
11.	*S. equi* subsp. *zooepidimicus*	4			
12.	*S. porcinuss*	8 1	890, 350, 150 690, 350, 200, 150	570, 160, 150, 140, 90	900, 310, 230
13.	*S. pyogenes*	4	629, 262, 222, 144	485, 310, 160, 125	900, 310, 230

n = number of strains

[1] = Deduced sizes of fragments of less than 100 bp are not provided

Table 3. RFLP patterns of 16S rRNA gene of different *Streptococcus* species after digestion with *Rsa*I; *Msp*I and *Ava*II enzymes.

Restriction patterns (RP) after digestion with *Rsa*I; *Msp*I and *Ava*II enzymes							
***Rsa*I**			***Msp*I**			***Ava*II**	
RFLP patterns	Strains and recognized fragment size (bp)		RFLP patterns	Strains and recognized fragment size (bp)		RFLP patterns	Strains and recognized fragment size (bp)
1	*S. canis*[1] *S. equi subsp. equi* *S. equi* subsp. *zooepidimicus* (900, 210, 145)		I	*S. canis* *S. uberis* *S. parauberis* *S. equi subsp. equi* *S. equi* subsp.*zooepidimicus* (570, 310, 160, 140, 90)		A	*S. canis* *S. agalactiae* *S. uberis* *S. porcinus* *S. equi* subsp. *equi* *S. equi* subsp. *Zooepidimicus* *S. pyogenes* (900, 310, 240)
2	*S. agalactiae* *S. dysgalactiae* subsp. *dysgalactiae* (group C and L) *S. dysgalactiae* subsp. *equisimilis* (group G) *S. suis* (625, 350, 265, 145)		II	*S. dysgalactiae* subsp. *dysgalactiae* (group C and L) *S. dysgalactiae* subsp. *equisimilis* (group G) (475, 160, 150, 140, 90)		B	*S. dysgalactiae* subsp. *dysgalactiae* (group C and L) *S. dysgalactiae* subsp. *equisimilis* (group G) (900, 310, 180,70)
3	*S. phocae* *S. porcinus*[2] (890, 350, 150)		III	*S. phocae* *S. porcinus* (570, 160, 150, 140, 90)		C	*S. parauberis* (1210, 230)
4	*S. uberis* (700, 220, 190, 145)		IV	*S. agalactiae* (570, 160, 140, 90)		D	*S. phocae* (1210, 230, 90)
5	*S. parauberis* 700, 375, 190, 145		V	*S. suis* (570,210, 160, 140, 70)		E	*S suis* (590, 310, 290)
6	*S. pyogenes* (629, 262, 222, 144)		VI	*S. pyogenes* (485, 310, 160, 125)			

[1] = the 24 strains of *S. canis* showed this fragments and four strains showed fragments 625, 265, 210, 145 which is not presented in this table

[2] = 8 strains of *S. porcinus* showed this fragments and one strain showed fragments 690, 350, 200, 150,40 which is not presented in this table

DISCUSSION

The PCR-RFLP assay has been described previously in different *Streptococcus* species and subspeices by (24, 27, 33, 35, 41, 42, 45, 46). In all these publication, the authors were used housekeeping gene such as ribosomal RNA and *groESL*, whose nucleotide sequence are more conserved as DNA targets which becoming more practicable for PCR amplifications. According to Jayarao et al. (27, 46) the amplification of the 16S rRNA gene from genomic DNA and the subsequent digestion with endonucleases proved to be a simple and reliable technique for differentiation of various bacterial species and identify microbial pathogens, which is applicable in microbiological diagnostics. Depending to the restriction site predicted by computer program Clone Manager 4.0, the sequences of *S. canis, S. agalactiae, S. dysgalactiae, S. uberis S. parauberis, S. porcinus, S. sius, S. phocae* and *S. equi* subsp. *equi*, no significant difference was obtained between the Clone Manager 4.0 predicted fragments and PCR-restriction endonucleases fragments.

In the present study, digestion of the 16S rRNA gene of *S. canis* with *Rsa*I yielded two different restriction patterns (900, 210, 145 bp) and (625, 265, 210, 145 bp) which were different to those of *S. agalactiae, S. dysgalactiae* subsp. *dysgalactiae* (serogroup C and L), *S. dysgalactiae* subsp. *equisimilis* (serogroup G), *S. uberis, S. parauberis, S. phocae, S. suis* and *S. porcinus*. Comparable to Hassan et al. (12) and Jayarao et al. (27) the restriction pattern (900, 210, 145 bp) appeared to be uniform and allowed a correct identification of these species by RFLP.

Additionally, *Msp*I restriction enzyme showed a characteristic restriction patterns of all *S. dysgalactiae* subsp. *dysgalactiae* (serogroup C and L), *S. dysgalactiae* subsp. *equisimilis* (serogroup G), with four restriction fragments which can successfully used to differentiated with *S. canis*. Moreover, *Msp*I restriction enzyme showed a restriction pattern of *S. canis* differed from *S. agalactiae, S. phocae, S. porcinus, S. suis* and *S. pyogenes*

The significant differences in the restriction patterns could be observed, using the enzymes *Ava*II, between *S. canis* and *S. dysgalactiae* subsp. *dysgalactiae* (serogroup C and L), *S. dysgalactiae* subsp. *equisimilis* (serogroup G). Moreover, *Ava*II restriction enzyme showed a restriction pattern of *S. canis* differed from *S. parauberis, S. phocae, S. suis, S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus*. These results are in agreement with these reported by (12, 24, 27). The restriction

patterns of the *S. agalactiae* with latter three enzymes appeared to be uniform and no variations in restriction pattern were observed for the 16S rRNA gene of *S. agalactiae* as well as *S. dysgalactiae* isolated from human and different animal species. In the present study, there were no different restriction patterns between the serogroups of *S. dygalactiae* C, G, and L were observed with *Rsa*I and two other enzymes, corresponding to the reports described by (27). Using *Rsa*I restriction enzyme *S. canis* and *S. porcinus* strains showed two different polymorphisms patterns. This results indicate a sequence variation and RFLP could be possibly used for genotyping of these bacteria.. According to the Khan et al. (29) and Williams and Collins, (36) *S. uberis* and *S. parauberis* differed biochemically from each other only with regard to the test for β-D-glucuronidase enzyme activity. In the present study the restriction patterns of *S. uberis* as well as *S. parauberis* with the *Rsa*I and *Ava*II enzymes showed specific fragment sizes and this indicated that RFLP could be used to differentiate *S. uberis* and *S. parauberis* corresponding to (27, 28, 29, 46). Comparable to Vossen *et al* (31) observed uniform restriction patterns of different serological groups of *S. phocae*, using *Ear*I and *Hinc*II enzymes, in the present study, *S. phocae* could be performed by RFLP analysis using restriction enzyme *Msp*I and *Ava*II. Abdulmawjood *et al* (30) found no interspecies variation for a RFLP analysis of the 16S rRNA gene of serologically heterogeneous *S. porcinus* using the restriction enzyme *Bpi*I, comparable to the present study the differences observed in the *Rsa*I restriction patterns of *S. porcinus* indicate some sequence variations and could possibly be used for genotyping bacteria of this species. According to Abdulmawjood and Lämmler (47) interspecies variation in the sequence of 16S rRNA gene was observed for *S. equi subsp. zooepidimicus*, depending to present result *S. equi subsp. zooepidimicus* could be identified by using *Ava*II enzymes.

CONCLUSIONS

The entirety results of present study indicated that PCR-RFLP method using restriction endonucleases *Rsa*I, *Msp*I and *Ava*II could be useful method for identification of *S. canis* from *S. dysgalactiae* (serogroup C, L and G) and other related *Streptococcus* species. This might help to determine the prevalence of *S. canis* in animal and human infections

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تحديد المكورات السبحية *Streptococcus canis* الزمرة المصلية لانسفيلد (Lancifield serogroup G) بواسطة سلسلة تفاعل البلمره PCR و الـ (RFLP) لجين 16S ribosomal RNA

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الخلاصة

في هذه الدراسة تم استخدام المكورات السبحية *S. canis* و 12 نوع و تحت النوع و زمرة مصليه مختلفه من المكورات ال سبحية وكانت تضم الـ *S. agalactiae* والـ *S. dysgalactiae* subsp. *dysgalactiae* (serogroups C and L) ، والـ *S. dysgalactiae* subsp. *equisimilis* (serogroup G) ، والـ *S. uberis* ، والـ *S. parauberis* ، والـ *S. phocae* ، والـ *S. suis* ، والـ *S. equi* subsp. *equi* ، والـ *S. equi* subsp. *zooepidimicus* ، والـ *S. pyogenes* . استخدمت تقنية الـ PCR-RFLP بوثوق في تحديد أنواع المكورات السبحية المذكوره اعلاه من جين 16S RNA الريباسي وبطول 1.43 كيلوبايت وباستخدام البادئ العام universal oligonuclotide primers ومن ثم لاحقا هضمه بأنزيم الـ *Rsa*I، و أنزيم *Msp*I، وأنزيم *Ava*II. وأظهرت نتائج PCR-RFLP أن أنزيم *Rsa*I أعطى نمطاً مختلفاً للمكورات السبحية *S. canis* عن جميع أنواع المكورات السبحية باستثناء المكورات السبحية *S. equi* subsp. *equi* ، والـ *S. equi* subsp. *zooepidimicus* . وكانت نتيجة نمط أنزيم *Msp*I للمكورات السبحية *S. canis* مختلف عن كل من الـ *S. agalactiae* ، والـ *S. dysgalactiae* subsp. *dysgalactiae* (serogroups C and L)، والـ *S. dysgalactiae* subsp. *equisimilis* (serogroup G) ، والـ *S. parauberis* ، والـ *S. phocae* ، والـ *S. suis*. وأظهرت نتائج PCR-RFLP لأنزيم *Ava*II انه بالإمكان تمييز المكورات السبحية *S. canis* عن باقي الانواع من المكورات السبحية والـ *S. dygalactiae* subsp. *dysgalactiae* (serogroup C and L) ، والـ *S. dysgalactiae* subsp. *equisimilis* (serogroup

(G . وفي الختام يمكن الإستنتاج بأن طريقة الـ PCR-RFLP وباستخدام أنزيم *Rsa*I و *Msp*I و *Ava*II يمكن أن تكون طريقة مفيدة لتحديد المكورات السبحية *S. canis* عن غيرها من الأنواع ذات الصلة. وهذا قد يساعد على تحديد مدى انتشار المكورات السبحية *S. canis* في عدوى الحيوان والإنسان.

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