Genotypic Characterization of Coagulase-negative Staphylococci Isolated from Sheep Milk in Slovakia

Ivana Pilipčincová[1], Mangesh Bhide[1], Eva Dudriková[2], Milan Trávniček[3]

[1] Department of Microbiology and Immunology, University of Veterinary Medicine, 04181, Kosice, Slovakia
[2] Department of Milk Hygiene and Technology, University of Veterinary Medicine, 04181, Kosice, Slovakia
[3] Department of Epizootology and Preventive Veterinary Medicine, University of Veterinary Medicine, 04181, Kosice, Slovakia

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Abstract

Hitherto very few reports are available presenting identification and molecular characterization of the coagulase negative staphylococci (CNS) from sheep milk in the subclinical stage of mastitis. Furthermore, very scanty data are available on the epidemiological status of CNS in different Slovak provinces. Milk samples from 54 sheep farms located in eastern Slovak region were screened. A total 240 CNS were identified with series of biochemical testes (STAPH-API) and subjected further for genotyping with the help of pulse field gel electrophoresis (PFGE). The most frequently occurring CNS species according the biochemical characterization were: *S. epidermidis* (36.3 %), *S. caprae* (21.3 %), *S. hominis* (6.6 %), *S. chromogenes* (6.3 %), *S. xylosus* (5.8 %), *S. warneri* (5.0 %) and *S. capitis* (4.6 %). Further PFGE-based characterization of these isolates revealed six pulsotypes of the *S. epidermidis,* two of *S. caprae,* three of *S. chromogenes,* nine of *S. hominis,* five of *S. capitis* and seven *of S. xylosus*. These results contribute to knowledge of the epidemiological situation of the CNS from the subclinical form of mastitis in Slovakia.

CNS, PFGE, pulsotype, mastitis, sheep

Clinical and subclinical mastitis presents potential health hazard due to the increase in total bacterial count in milk (Fthenakis and Jones 1990; Gonzalo et al. 2006). In case of subclinical mastitis, coagulase negative staphylococci (CNS) are the most frequently isolated bacteria from milk (Ariznabarreta et al. 2002; Bergonier et al. 2003; Burriel 1997, 1998; Deinhofer and Pernthaner 1993; Gonzalo et al. 2002; Lafi et al. 1998; Pengov 2001; Watkins et al. 1991). Traditionally, CNS have been considered to show low pathogenicity for the mammary gland of domestic ruminants. However, the importance of these bacteria as the main aetiological agent of mastitis in sheep has been documented (Fthenakis and Jones 1990; Poutrel 1984). The primary impact of CNS-mediated subclinical mastitis is an increased number of somatic cells (Gougoulis et al. 2008; Leitner et al. 2000; Santos et al. 2008). Furthermore, CNS may sensitize and increase susceptibility of mammary cells to other infections (Jarp 1991; Oliver and Jayarao 1997; Seegers et al. 2003; Waage et al. 1999).

CNS produce few virulence factors; however, they can cause infections in healthy host tissue. They are opportunists and adhere to metal devices to produce a protective biofilm. Production of biofilm reduces the organism's susceptibility to antimicrobials (John and Harvin 2007; Vuong et al. 2003). Widely used antibiotics including penicillins, particularly semi-synthetic penicillins, cephalosporins, macrolides, aminoglycosides and tetracyclines are ineffective to control CNS (Cerca et al. 2005; de Allori et al. 2006). The ability to resist antimicrobials and produce biofilm enables CNS to persist on metal devices, milking equipments as well as on the milker's hands, which serve a major source of staphylococcal spread.

Unlike coagulase-positive staphylococci such as *S. aureus*, epizootiological studies focused on CNS are sporadic. Among CNS, *S. epidermidis* is the most commonly isolated

Address for correspondence:
Dr. Mangesh Bhide, Ph.D.
Laboratory of Biomedical Microbiology and Immunology
Department of Microbiology and immunology,
University of Veterinary Medicine
Komenského 73, 04181, Košice, Slovakia

Phone: +421 904 461 705
Fax: +421 556 323 173
E-mail: mangeshbhide@hotmail.com
http://www.vfu.cz/acta-vet/actavet.htm

staphylococcal species in the subclinical form of mastitis (Aarestrup et al. 1997; Birgersson et al. 1992). Some authors have reported the prevalence of *S. epidermidis* even higher than 50% of the total CNS isolated from the milk (Ariznabarreta et al. 2002; Moroni et al. 2005a; Moroni et al. 2005b). However, some authors (Chaffer et al. 1999; Taponen et al. 2007) have reported abundance of other species such as *S. simulans*, *S. chromogenes* and *S. haemolyticus*.

The popularity of and consumer preference for sheep milk, cheese and related milk products is increasing continuously. This has brought the need to monitor the subclinical form of mastitis. The study and evaluation of the epidemiological situation of CNS in sheep is thus pivotal to avoid the threat of possible milk-born pathogens as well as to control mastitis.

Among different methods of CNS characterization pulse field gel electrophoresis, PCR based assays and biochemical characterizations are commonly used methods. In particular, PFGE has profound discrimination power with excellent reproducibility. To date no comprehensive study has been done on the isolation and characterization (genotyping and pulso-typing) of coagulase negative staphylococci from sheep in Slovakia. On this background, this study was aimed to determine the prevalence and genetic diversity of CNS in the milk of subclinically affected sheep from eastern regions of Slovakia.

Materials and Methods

Animals and samples

Milk samples (10 animals/farm) were collected from 54 different farms located in 7 regions of eastern Slovakia (Table 1) in the years 2005-2007. The farms were located 15–200 km apart from each other. 59.3% sheep were of Tsigai breed, 37% were Valachian and 3.7% were Tsigai × Merino. Majority (94.5%) of the farms practiced manual milking. Before milk collection, the udder was palpated carefully and milk was tested with NK test to rule out sampling from a sheep with clinical mastitis. During sample collection, teats were cleaned and disinfected, first streams of milk were discarded and 10 ml of milk were collected in sterile tube. Samples were taken from both teats. Milk samples were transported on ice and processed within 24 h.

Ten µl of the sample were inoculated on Columbia blood agar (Oxoid, UK) and incubated at 37 °C for 24 h. Colonies were screened morphologically (colony characteristics and Gram staining) as well as biochemically (catalase and glucose fermentation - Hugh-Leifson test, Oxoid, UK).

Based on Gram staining and biochemical properties, suspected colonies on the blood agar, representing genus *Staphylococcus* were picked and subcultured on Baird-parker agar enriched with egg yolk (Oxoid, UK), a selective medium for staphylococci. Colonies on the Baird parker agar were subcultured and subjected for further biochemical tests.

Biochemical characterization of the CNS

Staphylococcal colonies were further screened for coagulase activity according to the manufacturer's instructions (Oxoid, UK). Coagulase negative colonies were further subjected to biochemical analysis with the help of API-STAPH 32 ID commercial test kits (Bio-Mérieux, France).

Pulsed field gel electrophoresis

PFGE was performed as described previously (Fugett et al. 2006). Shortly, bacterial cells were suspended in TE buffer (Tris HCl 1M, EDTA 0.5M, OD ~ 1.3, all chemicals: Sigma, USA) and cells were lysed with lysis buffer (Tris HCl 1 M, EDTA 0.5 M and N-Lauroyl-Sarcosine 10%) for 10 min at 37 ºC. Agarose blocks containing cell lysate were prepared (1.2% low melting agarose, 10% SDS, 1% proteinase K) as described earlier (Escudero et al. 2000) and blocks were subjected for the enzymatic digestion. Enzyme digestion of DNA was done either by *SmaI* or *BspI* (Fermentas, Amherst, NY) at 37 ºC overnight as per the supplier's instructions. A contour clamped homogenous electric pulse-field apparatus (Bio-Rad, Richmond, USA) was used for DNA fragment separation. Digested DNA was separated by pulse time ramped from 3 to 40 s, for 20 h at 200 volts and 14 ºC. Separated DNA was visualized by ethidium bromide and analyzed by using Bio-Numerics software (USA). Phylogenetic analysis and dendrogram was constructed on the basis of arithmetic average clustering (UPGMA).

To assess whether a correlation exists between biotyping (API-STAPH test) and pulsotyping (PFGE), kappa (K) test was employed by using Win-Episcope software 2.0 (CLIVE, UK).

Results

Based on Gram staining and catalase test, we differentiated staphylococci from micrococci (Gram positive cocci and catalase positive n = 259; gram positive cocci, catalase negative

n = 5). With the help of selective Hugh-Leifson medium staphylococci (n = 244) were further differentiated from micrococci (n = 10) and *Kocuria* spp. (n = 5). With the help of coagulase test and Baird-Parker selective medium 4 *Staphylococcus aureus* colonies and 240 CNS colonies were differentiated.

Further biochemical characterization with the help of API-STAPH 32 ID test differentiated CNS in to *S. epidermidis* (n = 87; 36.3 %), *S. caprae* (n = 51; 21.3 %), *S. hominis* (n = 16; 6.6 %), *S. chromogenes* (n = 15; 6.3 %), *S. xylosus* (n = 14; 5.8 %), *S. warneri* (n = 12; 5.0 %), *S. capitis* (n = 11; 4.6 %), *S. Sciuri* (n = 2; 0.8%), *S. kloosii* (n = 2; 0.8%), *S. cohnii cohnii* (n = 1; 0.4%), *S. auricularis* (n = 1; 0.4%) and *S. lugdunensis* (n = 2; 0.8%). The occurrence of CNS in different Slovak regions is presented in Table 1.

Table 1. Prevalence of major CNS species isolated from sheep milk from various provinces in Slovakia

Province	*S. epidermidis*	*S. caprae*	*S. hominis*	*S. chromogenes*	*S. capitis*	*S. xylosus*	*S. warneri*
Poprad	33%	44%	19%	13%	42%	13%	27%
Prešov	21%	28%	6%	7%	0%	7%	46%
Stará Ľubovňa	14%	2%	19%	0%	29%	0%	18%
Svidník	5%	2%	19%	67%	0%	67%	0%
Stropkov	13%	4%	24%	0%	0%	0%	9%
Vranov	13%	18%	0%	13%	0%	13%	0%
Humenné	1%	2%	13%	0%	29%	0%	0%

API-STAPH based biotyping sub-divided *S. epidermidis* strains into 20 biotypes (1^{epi} to 20^{epi}) whereas, *S. caprae, S. hominis* and *S. xylosus* showed 7 biotypes each. Other CNS species were divided in to various biotypes as follows: *S. warneri* 1^{war} to 10^{war}, *S. chromogene* 1^{chrom} to 8^{chrom}, *S. capitis* 1^{tis} to 11^{tis}, *S. sciuri* 1^{scr}, *S. lentus* 1^{len} to 5^{len}, *S. simulans* 1^{sim} to 2^{sim}, *S. haemolyticus* 1^{hem} to 3^{hem}, *S. saprophyticus* 1^{sap} to 2^{sap}, *S. kloosii* 1^{kl}, *S. cohnii cohnii* 1^{cc}, *S. auricularis* 1^{aur} and *S. lugdunensis* 1^{lug}. All CNS with more than 3% prevalence (n = 206) were characterized further with the help of PFGE.

With the help of PFGE by using *SmaI* enzyme, 80 *S. epidermidis* strains were characterized. Isolates showing REDP *(restriction endonuclease digestion profiles)* pattern with more than 80% homology were clustered into 5 pulsotypes (pulsotype A to E) which further clustered into 39 sub-pulsotypes ($A1^{epi}$, $B1^{epi}$ etc., Plate VIII, Fig. 1). Some REDP patterns were unique (< 80% similarity) from those 39 sub-pulsotypes and thus designated as $X1^{epi}$ to $X10^{epi}$. All REDP patterns of *S. epidermidis* showed 12 to 23 DNA fragments with molecular mass from 14 to 450 kb. 71% of the *S. epidermidis* isolates formed B and D pulsotype. Pulsotype B was the most commonly found (38.5%) with 15 sub-pulsotypes ($B1^{epi}$ - $B15^{epi}$) followed by pulsotype D (7 sub-pulsotypes $D1^{epi}$ - $D7^{epi}$). Occurrence of other pulsotypes namely A, C and E was 7.7%, 5.1% and 7.7%, respectively.

SmaI enzyme REDP clustered 50 *S. caprae* isolates into two pulsotypes viz. A^{cap} (18 fragment REDP with molecular masses 14 to 450 kb) and B^{cap} (19 fragment REDP with molecular mass 14 to 450 kb). In case of *S. caprae* the REDP patterns confirmed genetic homogeneity with 98% pattern similarity (Plate IX, Fig. 2).

When *BspI* enzyme was used to restrict *S. chromogenes* genome, 3 pulsotypes (A^{chrom}, B^{chrom}, C^{chrom}) were observed (15 to 17 fragment REDP with molecular masses 14 to 240 kb, Plate IX, Fig. 3). With the help of similar enzyme used to restrict *S. hominis* DNA (n = 16), nine genetically heterogeneous pulsotypes, A^{hom} to I^{hom}, were observed with 40% homology cut-off. The restriction pattern of *S. hominis* consisted of 8 to 16 fragments with molecular mass 14 to 450 kb (Plate X, Fig. 4). *S. capitis* (n = 7) and *S. xylosus* (n = 9) isolates were clustered into the 5 and 7 pulsotypes respectively (Plate X and XI,

Figs 5 and 6). Among *S. warneri* isolates (n = 11) considerable heterogeneity was observed. All *S. warneri* isolates represented separate pulsotype (Plate XII, Fig. 7).

Discussion

The predominance of *S. aureus* among the mastitis causing agents was recorded earlier in cattle and sheep, and has been characterized geno-phenotypically (Annemuller et al. 1999; Buzzola et al. 2001; Dingwell et al. 2006; Jorgensen et al. 2005; Lim et al. 2004; Sommerhauser et al. 2003; Zadoks et al. 2002). However, epidemiological data and characterization of the CNS causing clinical/subclinical mastitis of sheep from Slovakia are scanty. Characterization of the Slovak isolates revealed that the *S. epidermidis* pulsotypes were equally distributed in all the Slovak regions under study. This indicates the ability of this pathogen to disseminate easily in a wide geographic area. Similar conclusion was also found elsewhere (Miragaia et al. 2002; Nunes et al. 2005) wherein the wide range of *S. epidermidis* genotypes were distributed in hospitals of different countries. Furthermore, the presence of identical *S. epidermidis* genotypes, called clones, was found in several hospitals in different countries (Dominguez et al. 1996; Miragaia et al. 2002; Nouwen et al. 1998; Silva et al. 2001; Villari et al. 2000). The presence of genetically homologous strains of *S. haemolyticus* at eight cattle farms located in five different regions, and *S. warneri* at three goat farms was also reported (Bjorland et al. 2005). The 98% genetic similarity among *S. caprae* isolated from seven provinces of eastern Slovakia indicates the distribution of a clonal/homologous strain in this geographical region. Parallel to our results, in a previous study (George and Kloos 1994) only one pulsotype (either A^{cap} or $B^{cap)}$ occurred at different farms. In our study, *S. warneri* strains were also genetically homologous (100% homology), however, *S. xylosus*, *S. capitis*, *S. hominis* and *S. chromogenes* were genetically diverse CNS. The high heterogeneity among *S. xylosus* isolates was also reported earlier (Martin et al. 2006).

The reason behind the wide distribution of the homologous CNS strains versus diversity is explained earlier (Miragaia et al. 2002). The authors put forth a hypothesis of dissemination that correlates wide distribution of the homologous CNS strains with their affinity to skin and mucous membrane. Other researchers (de Allori et al. 2006; Poutrel 1984; Zadoks et al. 2002), have suggested that the same staphylococcal strain can spread successfully in a wide geographic area or among different hosts like cattle, sheep, human etc., if it has the ability to produce adhesives or slime.

In our work we assessed whether a correlation exists between biochemical characterization (API-STAPH based biotyping) and the macro-restriction pattern based genotyping (PFGE based pulsotyping). We found no evident correlation between biotypes and pulsotypes (Kappa coefficient - K~ 0.6); not all strains showing the same biotype had the same pulsotype and vice versa. In contrast to our observation, a strong correlation between biotyping and pulsotyping was observed in a recent study (Jousson et al. 2007), where the correlation K~ 0.6-0.8 was found for *S. hominis*, *S. warneri*, *S. cohnii* subsp. *urealyticus* and *S. simulans*, and K~ 0.8-1 for *S. epidermidis*, *S. equorum*, *S. haemolyticus*, *S. sciuri* and *S. kloosi.* It is necessary note that the PFGE method is based on macro-restrictions of the whole genome, and the limitation of its discrimination ability may be noticed when polymorphisms present outside of the restriction sites. On the other hand, polymorphism/ mutation within the restriction sites shows genotypic divergence, but not necessarily cause a change in the phenotype. This can be the cause of lesser correlation between phenotyping and genotyping in some CNS.

The work presents prevalence, distribution and characterization (biochemical and molecular) of the CNS isolated from sheep milk from eastern Slovakia. To our knowledge, epizootiological studies in Slovakia related to CNS from sheep milk are very scanty. This

study shows the presence of a wide range of biotypes and pulsotypes present in ovine milk in subclinical mastitis. Moreover, we report the distribution of genetically homologous and heterologous CNS within the geographic area under study.

Genotypová charakterizácia koaguláza negatívnych stafylokokov izolovaných z ovčieho mlieka na Slovensku

V súčasnosti je dostupných veľa literárnych prameňov, v ktorých sú identifikované a charakterizované na molekulárnej úrovni, koaguláza negatívne stafylokoky (KNS) v ovčom mlieku u oviec v subklinickom štádiu mastitídy. Napriek tomu informácie o epidemiologickej situácii v rozličných regiónoch Slovenska sú nedostačujúce. Vyšetrených bolo 54 vzoriek od oviec z farmových chovov na východnom Slovensku. Celkovo u 240 vzoriek boli biochemickými testami (STAPH-API) identifikované KNS a tieto vzorky boli ďalej podrobené genotypizácii pomocou pulse field gélovej elektroforézy. Najčastejšie sa vyskytujúcim KNS druhmi boli podľa biochemickej charakterizácie: *S. epidermidis* (36.3 %), *S. caprae* (21.3 %), *S. hominis* (6.6 %), *S. chromogenes* (6.3 %), *S. xylosus* (5.8 %), *S. warneri* (5.0 %) and *S. capitis* (4.6 %). Napriek predchádzajúcej PFGE charakterizácii týchto izolátov, bolo odhalených šesť pulzotypov u *S. epidermidis,* dva u *S. caprae*, tri u *S. chromogenes,* deväť u *S. hominis, päť u S. capitis* a sedem u *S. xylosus.* Táto práca rozširuje poznatky ohľadne epidemiologickej situácie u KNS počas subklinickej formy mastitídy na Slovensku.

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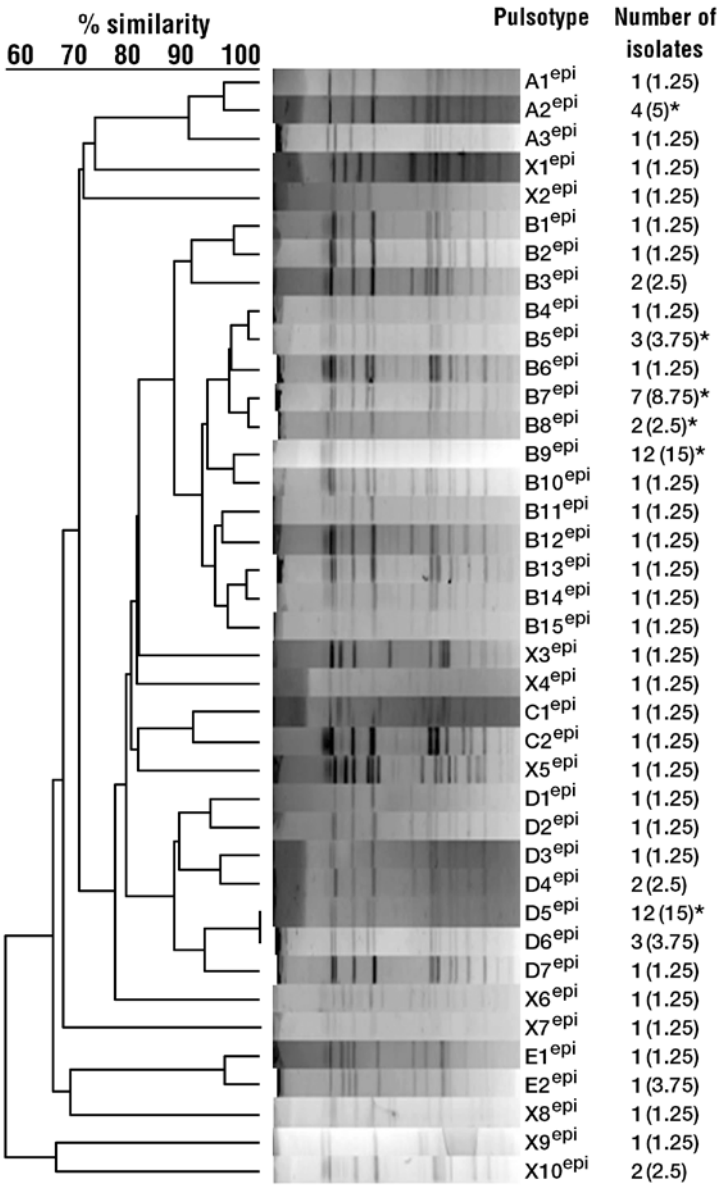

Fig. 1. Pulse field gel electrophoresis analysis of *S. epidermidis*
$A1^{epi}$, $A2^{epi}$ etc. indicate – A1 sub-pulsotype of *S. epidemidis;* * occurrence of sub-pulsotype in two or more provinces; figures in the parenthesis indicate occurrence of the given sub-pulsotype in per cent.

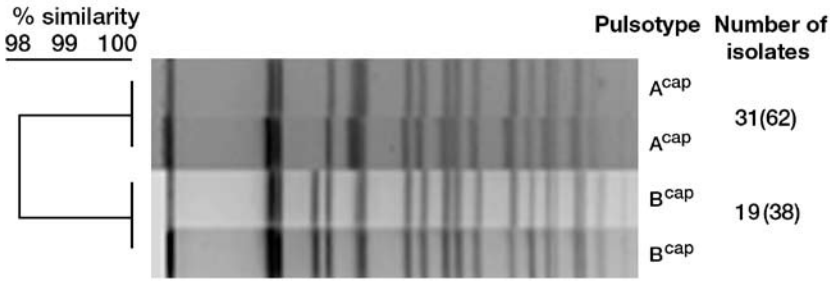

Fig. 2. Pulse field gel electrophoresis analysis of *S. caprae*
A^{cap}, B^{cap} indicate – A and B pulsotypes of *S. caprae;* figures in the parenthesis indicate occurrence of the given sub-pulsotype in per cent.

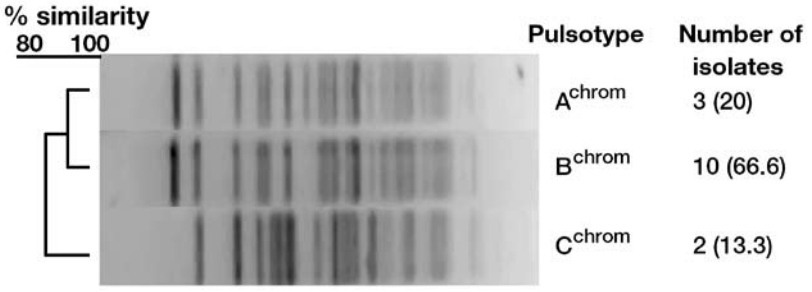

Fig. 3. Pulse field gel electrophoresis analysis of *S. chromogenes*
A^{chrom}, B^{chrom} and C^{chrom} indicate – A, B and C pulsotypes of *S. chromogenes;* figures in the parenthesis indicate occurrence of the given sub-pulsotype in per cent.

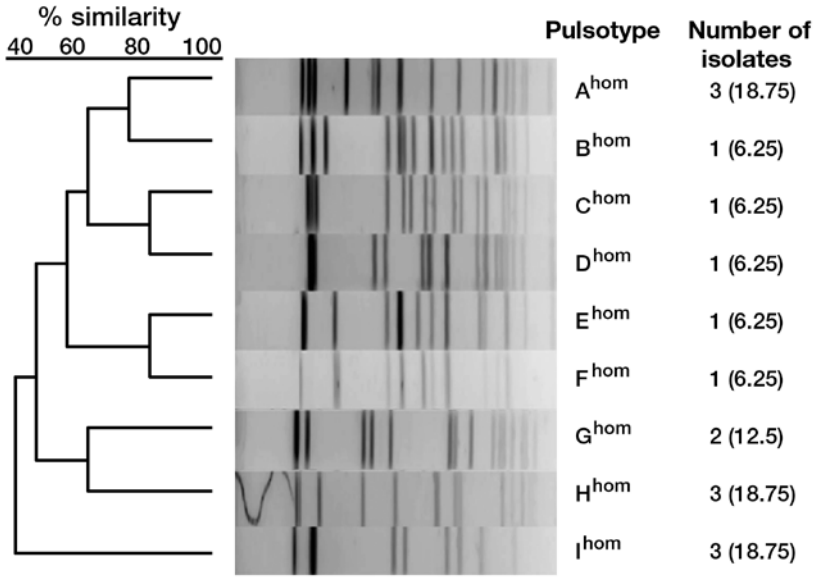

Fig. 4. Pulse field gel electrophoresis analysis of *S. hominis*
A^{hom} to I^{hom} indicate – A to I pulsotypes of *S. hominis;* figures in the parenthesis indicate occurrence of the given sub-pulsotype in per cent.

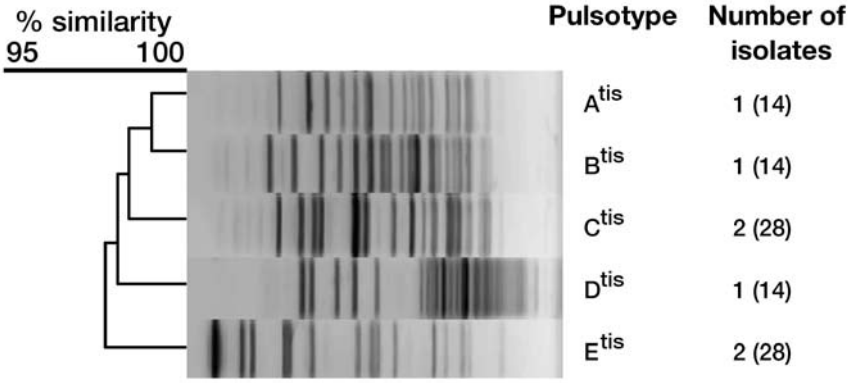

Fig. 5. Pulse field gel electrophoresis analysis of *S. capitis*

A^{tis} to E^{tis} indicate – A to E pulsotypes of *S. capitis*; figures in the parenthesis indicate occurrence of the given sub-pulsotype in per cent.

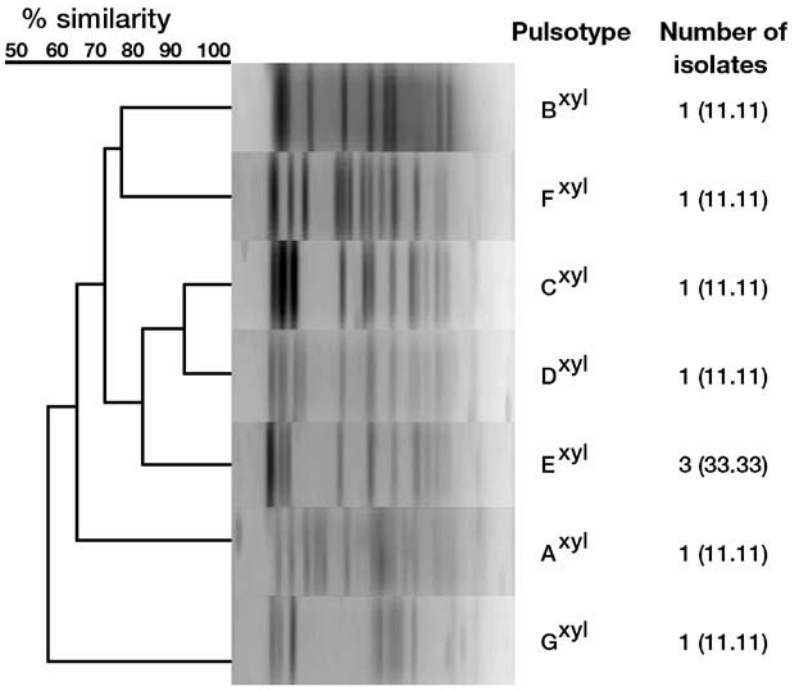

Fig. 6. Pulse field gel electrophoresis analysis of *S. xylosus*
A^{xyl} to G^{xyl} indicate – A to G pulsotypes of *S. xylosus*; figures in the parenthesis indicate occurrence of the given sub-pulsotype in per cent.

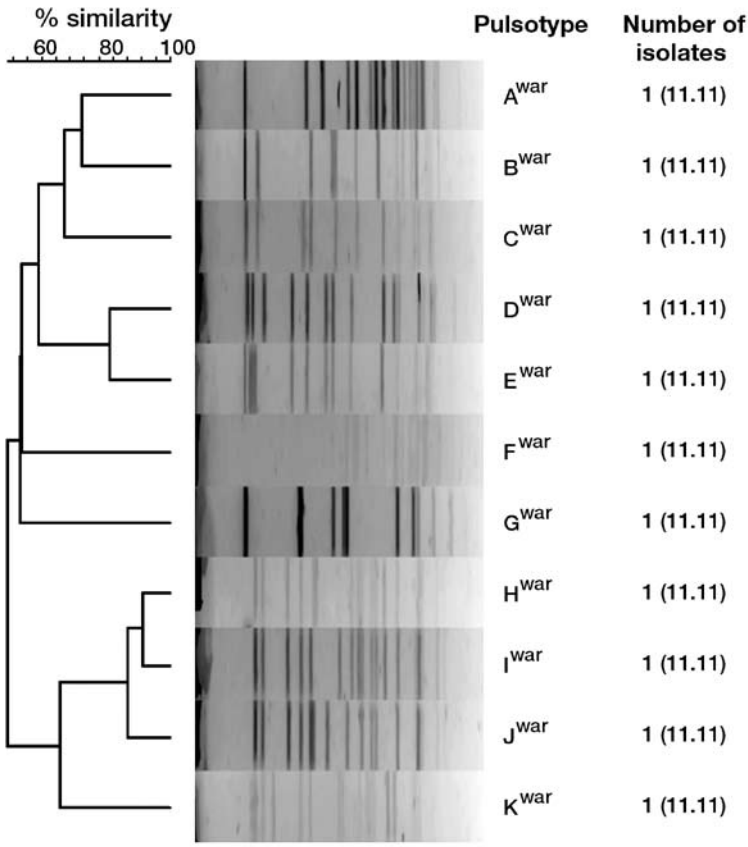

Fig. 7. Pulse field gel electrophoresis analysis of *S. warneri*
A^{war} to K^{war} indicate – A to K pulsotypes of *S. warneri*; figures in the parenthesis indicate occurrence of the given sub-pulsotype in per cent.