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Ribotyping and rapid identification of *Staphylococcus xylosus* by 16–23S spacer amplification

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Abstract

Ninety-five strains of *Staphylococcus xylosus* isolated from goat milk, French sausage or mice were analyzed together with 35 *Staphylococcus* type strains by 16–23S spacer amplification and ribotyping. The results obtained by PCR amplification of the 16–23S spacer region permitted the distinction of each type strain and additionally generated a DNA banding pattern characteristic for 93 of the 95 *Staphylococcus xylosus* strains. Ribotyping proved to be an efficient epidemiological tool for *Staphylococcus xylosus* species as it clustered the 95 strains into 23 distinct types.

Keywords: *Staphylococcus xylosus*; Intergenic rDNA spacer; Ribotyping; Identification

1. Introduction

Coagulase negative staphylococci (CNS) have often been isolated from bovine and goat milk [1,2]. They have previously been regarded as minor pathogens [3] but the proportion of mastitis due to CNS has increased markedly during the last decade [4]. Within the CNS, *Staphylococcus hyicus*, *Staphylococcus simulans*, *Staphylococcus epidermidis*, *Staphylococcus chromogenes* and *Staphylococcus xylosus* were most frequently isolated [1,4]. Their identification is usually based upon conventional biochemical methods (i.e. API STAPH and ID 32 systems of bioMérieux, Marcy l'Etoile, France) [2,5] but these tools have proven to be unreliable for the identification of veterinary strains [4]. The development of molecular biological methods has extensively increased microbiologists possibilities in bacterial identification and typing [4]. The purpose of the present study was to evaluate the performance of those based on the ribosomal RNA operon polymorphisms (16–23S rRNA intergenic spacer (Its) and ribotyping) as tools for identification or as epidemiological markers for *S. xylosus*.

2. Materials and methods

2.1. Bacterial strains, growth conditions and identification

The strains investigated in the present study are listed in Table 1. The study included a total of 95 *S. xylosus* isolates recovered from goat milk, French sausage, human skin and mice. Additionally, 35 *Staphylococcus* type strains, comprising *S. xylosus* type strain, were analyzed. Bacteria were grown for 24 h at 37°C on Brain Heart infusion agar plates (Merck, Darmstadt, Germany) and identified by biochemical methods as described previously [8] and by the API-Staph bioMérieux system.

2.2. PCR amplification of the 16–23S intergenic spacer

Bacterial genomic DNA treated with 50 mg of RNase (Merck) per ml was extracted and purified as described previously by Renaud et al. [9]. DNA samples were diluted to a concentration of 12 ng ml^{-1} prior to 16–23S spacer amplification according to the method described by Jensen et al. [7]. A 5-ml aliquot of the reaction mixture was combined with 2 ml of loading buffer (25% bromophenol blue, 25% xylene cyanol and 50% glycerol) and the preparation was electrophoresed on a 1.5% agarose (Metaphor TEBU®, France) gel in 1×TBE buffer (90 mM Tris, 2 mM EDTA, 90 mM boric acid (pH 8)) for 5 h at 3 V cm^{-1}. The gels were stained with ethidium bromide and

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photographed on a UV transilluminator. DNA fragment sizes were computer determined using the Taxotron® (Institut Pasteur, Paris) software, by interpolation from the sizes of Biomarker EXT 50–2000 bp (Eurogentec, Seraing, Belgium), which was used as a molecular mass marker.

2.3. Ribosomal RNA (rRNA) gene restriction patterns (ribotyping)

2.3.1. Enzymatic DNA restriction

Four mg of total DNA extracted as above was cleaved with 10 U of *Hin*dIII at 37°C for 4 h. Separation of DNA restriction fragments was done by electrophoresis in a 0.8% (w/v) agarose gel (Seakem LE, TEBU) in 1×TAE buffer (40 mM Tris, 1 mM EDTA (pH 8)) for 16 h at 40 V.

2.3.2. Southern blotting

DNA fragments were transferred to a neutral nylon membrane (MagnaGraph nylon membrane, Micron Separation, Westboro, MA, USA) with the Trans-Vac TE 80 blotter apparatus (Hoefer Scientific Instruments, San Francisco, CA, USA), with 20×SSC (3 M NaCl plus 0.3 M sodium citrate (pH 7.0)) used as the transfer solution. The transferred DNA fragments were immobilized onto the membrane by UV cross-linking at 0.120 J cm^{-2} with the Fluo-Link apparatus (Bioblock, Illkirch, France).

2.3.3. Hybridization conditions

A 16S rDNA probe was generated with the following universal primers: forward primer GAG AGT TTG ATC ATG GCT CAG and reverse primer AAG GAG GTG ATC CAG CCG CA [12]. Twenty-five ng of *S. xylosus* type strain genomic DNA was amplified in 50 ml final volume mix containing 10 mM Tris HCl, 50 mM KCl, 0.75 mM $MgCl_2$, 200 mM of each deoxynucleotide triphosphate, 0.9 mM of each primer and 1 U thermostable DNA polymerase (Goldstar *Taq* DNA polymerase, Eurogentec). The reaction mixture was pre-incubated at 94°C for 5 min, followed by 25 cycles of 94°C for 1 min, 62°C for 1 min and 72°C for 2 min. One mg of purified PCR product was labelled with the DIG-High Prime labelling kit (Boehringer Mannheim, Meylan, France). Prehybridization was carried out in rolling tubes containing 20 ml of hybridization fluid (5×SSC, 1% casein, 0.1% *N*-lauroyl sarcosine, 0.2% SDS) at 68°C for 1 h, followed by 16 h of hybridization at 68°C with 1 mg of heat-denatured 16S-rDNA probe in 10 ml of hybridization fluid. The washing and immunoenzymatic detection of hybrids were performed as recommended by the manufacturer of the DIG detection Kit (Boehringer Mannheim).

2.3.4. DNA fragment size determination

Images of the blots were video captured for computer determination of the fragment sizes with the Taxotron program. The molecular sizes of the different fragments were determined by interpolation from the sizes generated by *Hin*dIII-digested DNA of *Staphylococcus caprae* 5N [10].

2.4. DNA–DNA hybridization

This test was performed to prove the affiliation of some doubtful strains particularly those characterized by Its type 2 and 3 to the *S. xylosus* species. For this analysis *S. xylosus* type strain (CCUG 7324), three *S. xylosus* strains of diverse origins (goat milk, French sausage and mouse), three negative controls: *S. caprae* and *Staphylococcus equorum* type strains and *Escherichia coli* strain were used in comparison to strains belonging to Its type 2 and 3. A colorimetric DNA–DNA hybridization method in microplates with covalently immobilized DNA was performed according to the method described by Rasmussen [11] and Kaznowski [12]. *S. xylosus* type strain DNA was used as a probe and labelled with biotin (Roche Diagnostics, Meylan, France) according to the manufacturer's instructions. For an evaluation of DNA similarities the OD, measured at 450 nm, of the well of reference DNA was calculated as 100%.

3. Results

3.1. Bacterial identification

All the field isolates were identified as *S. xylosus* by

Table 1
Ribotypes and Its types of the *S. xylosus* strains studied

Its type	Ribotype	Number of strains	Origin of strains
1	H1	2	goat milk
	H2	41	goat milk
	H3	10	goat milk
	H4	9	goat milk
	H6	1	goat milk
	H6	3	mice
	H7	3	goat milk
	H8	2	goat milk
	H9	1	goat milk
	H9	1	human skin
	H10	3	goat milk
	H11	2	goat milk
	H12	1	goat milk
	H13	1	goat milk
	H14	2	goat milk
	H15	1	goat milk
	H16	1	goat milk
	H17	1	goat milk
	H18	1	mice
	H19	1	goat milk
	H20	1	goat milk
	H21	2	French sausage
	H22	1	French sausage
	H23	1	French sausage
2	H5	1	goat milk
3	H2	1	goat milk

Table 2
Sizes of PCR products of 16–23S intergenic spacer amplification obtained for the 35 *Staphylococcus* type strains

Strain reference (CCUG)[a]	*Staphylococcus* species	Sizes of the Its products (bp)
18000	*S. epidermidis*	548, 529, 460, **333**
32416	*S. arlettae*	574, 555, 467, **344**
15606	*Macrococcus caseolyticus* (*S. caseolyticus*)	528, 477, 421, **294**
32418	*S. felis*	676, 568, 511, **467**
25348	*S. lugdunensis*	663, 609, 589, **569**
36975	*S. saprophyticus subsp. bovis*	567, 531, 467, 407, **361**
35071	*S. hominis*	561, 531, **481**, **440**, 374
37246	*S. aureus subsp. anaerobius*	658, 584, 537, 469, **431**
30109	*S. equorum*	644, 572, 509, 492, **375**
34143	*S. cohnii subsp. urealyticus*	642, 560, 507, 482, **369**
30107	*S. delphini*	1028, 933, **731**, **625**, 537
7325	*S. warneri*	709, 629, 598, 506, **480**
15604	*S. caprae*	551, 541, 488, 454, 398, **341**
35142	*S. capitis subsp. urealyticus*	581, 533, 420, 393, 349, **310**
7326	*S. capitis subsp. capitis*	605, 538, 511, 443, 404, **318**
3706	*S. saprophyticus subsp. saprophyticus*	648, 557, 512, 491, **445**, 372
15602	*S. hyicus subsp. hyicus*	749, 685, 608, 562, 494, **454**
7323	*S. haemolyticus*	695, 593, 542, 508, 452, **422**
15605	*S. carnosus*	547, 509, 469, 417, 393, 332
33636	*S. vitulus*	555, 514, 474, 444, 395, **337**
1800	*S. aureus subsp. aureus*	792, 710, 626, 536, 487, **453**
15599	*S. lentus*	578, 533, 484, 462, 425, **364**
7324	*S. xylosus*	553, 511, 472, 446, 422, **370**
24040	*S. saccharolyticus*	704, 613, 593, 493, **451**, 404
35133	*S. piscifermentans*	587, 496, 471, 408, 355, **321**
4319	*S. chromogenes*	611, 543, 494, 442, 383, **352**
25351	*S. schleiferi subsp. schleiferi*	607, 531, 451, 416, 362, **333**
15600	*S. gallinarum*	643, 552, 491, **458**, 398, 365
7327	*S. simulans*	608, 587, 531, 480, 432, **334**
32420	*S. pasteuri*	705, 615, 565, 519, **477**, 446
6520	*S. intermedius*	691, 634, 569, 534, 491, 458, **388**
30110	*S. kloosii*	706, 589, 546, 505, 474, 435, **357**
33938	*S. pulvereri*	552, 509, 487, 464, 435, 408, **337**
6463	*S. cohnii subsp. cohnii*	601, 576, 508, 451, 399, 340, **309**
15601	*S. auricularis*	608, 578, 531, 497, 445, 392, 360, 309

Boldface indicates the main band of each band pattern.
[a]CCUG: Culture Collection of University of Göteborg.

combination of both API bioMérieux system and conventional procedures [8].

3.2. Amplification of the 16–23S intergenic spacer

Each of the species of the genus *Staphylococcus* was characterized by the production of four (*Staphylococcus lugdunensis*, *Staphylococcus felis*, *Staphylococcus caseolyticus* (newly named *Macrococcus caseolyticus* [13]), *Staphylococcus arlettae* and *S. epidermidis*) to eight (*Staphylococcus auricularis*) PCR fragments with the majority showing five or six bands. The molecular mass of the PCR products ranged in sizes from 294 to 709 bp except for *Staphylococcus delphini* which generated two higher bands of 933 and 1028 bp (Table 2). Moreover, most of the species showed one main fragment which was the same for two species (*S. epidermidis* and *S. schleiferi* ssp. *schleiferi*). Therefore, the total pattern should be taken into account for the differentiation between species. In this scheme, the *S. xylosus* type strain was characterized by six PCR products of 370, 422, 446, 472, 511 and 553 bp of sizes with the 370-bp fragment as the most intense.

Within field isolates of *S. xylosus*, three Its types were recorded and referred to as Its 1, Its 2 and Its 3, with Its 1 being the main one: 93 out of the 95 strains showed this pattern, the other two patterns comprising only one strain (Fig. 1). This major pattern yielded six DNA fragments of

Table 3
DNA similarity values (%) between each strain and *S. xylosus* type strain (CCUG 7324)

Strains (origin)	Test 1 (%)	Test 2 (%)	Mean (%)
S. xylosus type strain	100	100	100
S. xylosus (goat milk)	85	82	84
S. xylosus (French sausage)	90	92	91
S. xylosus (mouse)	83	74	79
S. caprae	32	25	29
S. equorum	49	56	53
E. coli	10	6	8
Its type 3	81	78	80
Its type 2	86	91	89

approximately 370, 422, 446, 472, 511 and 553 bp with the 370-bp fragment being the most intense. This fragment was also found in the two other Its types.

3.3. DNA–DNA hybridization

DNA similarity values obtained for each strain are listed in Table 3. Assuming that bacteria within the same genomic species have DNA similarity values of at least 70% [14], the strains characterized by Its types 2 and 3 which presented a mean of 89 and 80% DNA homology, respectively, with labelled *S. xylosus* type strain, were affiliated to the *S. xylosus* species.

3.4. Ribotyping

The *Hin*dIII rRNA gene restriction fragments revealed a high degree of heterogeneity as the strains studied were clustered within 23 different unique patterns designated as ribotype H1 to H23 with ribotype H2, H3 and H4 being encountered the most often, clustering 42 strains (44% of the total strains), 10 strains (11%) and 9 (10%) strains, respectively (Table 1). Other ribotypes grouped less than four strains. Ribotypes were considered as unique when they differed by at least one fragment. A computerized normalized schematic representation of these profiles is given in Fig. 2. In some instance, the same ribotype could be observed for strains from different origins. For example, the type strain which was recovered from human skin shared ribotype H9 with a strain isolated from goat milk. Another example was represented by mice strains and a goat milk strain which were grouped in ribotype H6.

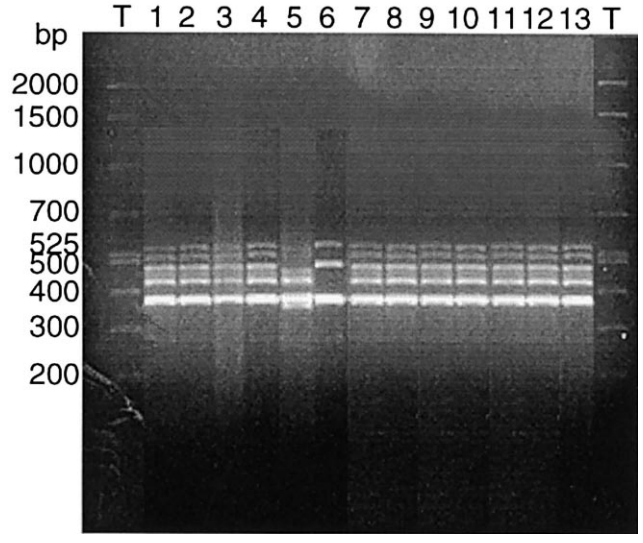

Fig. 1. Amplification of the 16–23S intergenic spacer of representative *S. xylosus* strains. Lane 1 to 6, *S. xylosus* strains from goat milk; lane 7, *S. xylosus* type strain from human skin; lane 8 to 10, *S. xylosus* strains from mice and lane 11 to 13, *S. xylosus* strains from French sausage. Lanes 1 to 4 and 7 to 13 were recorded as Its type 1. Its types 2 and 3 were in lanes 5 and 6, respectively. Biomarker ext. 50–2000 was used as a molecular size marker (T).

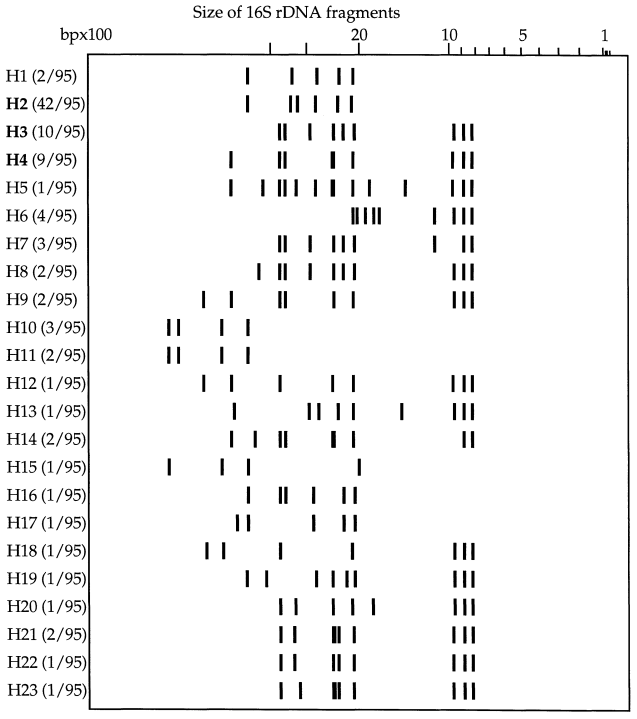

Fig. 2. Schematic representation of the 23 ribotypes obtained for the 95 *Staphylococcus xylosus* strains. Boldface indicates the most frequently occurring ribotypes (number of strains).

4. Discussion

Although CNS are frequently isolated from bovine milk, goat milk and dairy products, their identification at the species level still appears to be difficult in spite of their increased prevalence in mastitis [1,2,4]. The aim of this study was an extensive study of *S. xylosus* by means of ribotyping and Its analysis as tools for its identification and/or typing. In the present study, all the species of the genus *Staphylococcus* produced a characteristic pattern with the amplification of the 16–23S spacer region with a level of diversity which made the differentiation between each species possible. These results are in agreement with previous findings [4,7,15]. Moreover, all the strains of *S. xylosus* included in the study presented a major typical banding pattern, rendering Its useful and practical as an additional or alternative mean for its distinction within CNS. Since Its banding pattern was easy to analyze, it allows rapid screening of unknown isolates.

Assuming that the sensitivity of the ribotyping was highly dependent on the restriction enzymes used to digest chromosomal DNA [16], *Hin*dIII was used in the present study on the basis of previous data which had demonstrated that this enzyme yielded a better discrimination of most of the staphylococcal taxa including *S. xylosus* species [6,17]. The comparative analysis of the *Hin*dIII-hybridization patterns of cellular DNA from 95 *S. xylosus* isolates, using a 1600-bp DNA fragment encoding for 16S rDNA from *S. xylosus* as a probe led to the existence of

23 typical unique patterns. This result indicates a high degree of heterogeneity within this species and confirmed the usefulness of ribotyping as an accurate epidemiological tool [6,16,17].

In conclusion, Its may be considered as an interesting taxonomic marker for this species. It is fast, accurate and therefore useful for a rapid identification of the species *S. xylosus*. In contrast, ribotyping has proved useful in distinguishing the strains within *S. xylosus*.

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