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Ribotyping of *Staphylococcus caprae* isolated from goat milk

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Abstract – The usefulness of the API-STAPH system and a method based on ribosomal fingerprinting was evaluated by studying 89 coagulase-negative staphylococci isolated from goat milk. The bacteria were supposed to belong to the species *Staphylococcus caprae*. The results obtained from the API-STAPH system showed variations in their phenotypic features. DNA cleavage with *EcoRI* yielded six ribotypes and three distinct patterns were generated when the DNA of the strains was digested with *HindIII*. Forty-six strains were correctly characterized as *S. caprae* by the two methods. In addition, 37 isolates having atypical biochemical profiles with the API-STAPH system were confirmed as being *S. caprae* by the ribotyping. Three strains remained unclassified by both methods. Ribotypes generated by *HindIII* were found to be the most informative for species determination, whereas the number of bands generated by *EcoRI* indicated the usefulness of this enzyme in *S. caprae* typing. © Inra/Elsevier, Paris

coagulase-negative-staphylococci / goat / API-STAPH system / ribotype / *Staphylococcus caprae*

Résumé – Ribotypage de *Staphylococcus caprae* isolé de lait de chèvre. L'utilisation du système API-STAPH et l'étude du ribotype ont été mis en œuvre pour confirmer l'appartenance à l'espèce *S. caprae* de 89 souches de staphylocoques à coagulase négative isolées de lait de chèvre. Les résultats obtenus lors de l'identification par le système API-STAPH ont montré une hétérogénéité des profils biochimiques. L'étude des ribotypes après digestion des ADN par les endonucléases *EcoRI* et *HindIII* a permis d'obtenir respectivement six et trois ribotypes différents. Quarante-six souches ont été ainsi correctement identifiées *S. caprae* par les deux méthodes. Trente-sept souches sur 40 ayant un profil biochimique atypique, ont pu être identifiées par la méthode du ribotype. Les trois autres souches sont restées non identifiées. Les profils obtenus avec

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HindIII permettent la détermination de l'espèce *S. caprae*, alors que ceux obtenus avec *EcoRI* permettent une classification plus précise des souches au sein de cette espèce. © Inra/Elsevier, Paris

staphylococci à coagulase négative / chèvre / système API-STAPH / ribotype / *Staphylococcus caprae*

1. INTRODUCTION

Coagulase-negative staphylococci (CNS) are often present in goat milk samples collected from apparently normal udder halves and from halves with sub clinical mastitis [22]. *Staphylococci* have been associated with clinical and/or sub clinical mastitis and have been much less widely studied in farm animals [11]. This is particularly true for CNS, which are an important cause of acute and sub acute mastitis in cattle [10], in goats [22, 23] and in ewes [16]. However, the significance of CNS in udder infections in goats and other animals is unclear.

Staphylococcus caprae was first described in 1983 and this species has been isolated from goat's milk [12]. This CNS species is often isolated from goat milk [7, 9, 17, 28] and seems to be associated with this host. Attempts to recover *S. caprae* from other animals (i.e. cows, ewes) have proven to be unsuccessful [4, 26]. However, isolation of this bacterium from humans affected with dermatitis, urinary tract infection [19] and other infections [29] has been reported.

Kloos and Schleifer [20] and Devriese et al. [13] have designed schemes which they proposed for CNS differentiation and identification based on their respective biochemical properties. Although these schemes have been able to appropriately identify most species, their application in routine diagnosis of CNS can be ineffective in some instances, particularly for CNS strains isolated from animals [2].

Molecular methods have been developed and proposed for bacterial typing. One of these, designed as ribosomal RNA

(rRNA) gene restriction pattern analysis (ribotyping) has been extensively applied in a wide range of bacterial species [15], including staphylococcal species typing and identification [3, 7, 14].

We isolated 89 strains of CNS which we thought belonged to the *S. caprae* species because of their biochemical features. They were isolated from goat milk sampled in three different areas of France. The aim of the present study was an extensive analysis of these isolates by ribotyping in order to confirm the previous biochemical findings suggesting that they were strains of *S. caprae* or demonstrating that they were other staphylococci species.

2. MATERIALS AND METHODS

2.1. Bacterial strains

Eighty-nine staphylococcal isolates were collected following culture of goat milk specimens aseptically sampled in herds from three different areas in France (14 isolates from Poitou-Charentes, 41 isolates from Ardèche and 34 from Rhône-Alpes). Clinical mastitis was not observed throughout the study. Two human isolates of *S. caprae* (kindly provided by Professor J. Etienne, Centre National de Référence des Staphylocoques, Lyon, France) and eight other type species (*S. caprae* ATCC 35538, *S. xylosum* ATCC 35663, *S. simulans* ATCC 27848, *S. chromogenes* ATCC 43764, *S. capitis* ATCC 35661, *S. gallinarum* ATCC 35539, *S. cohnii* ATCC 35662, *S. epidermidis* ATCC 14990) were included in the study for comparison purposes.

2.2. Species identification

The isolates identified as *S. caprae* were characterised as previously described [2] using

biochemical identification on standardised API-STAPH strip systems using 19 substrates (Bio-Mérieux, France).

2.3. DNA preparation

Extraction and purification of the total DNA from the staphylococcal strains were carried out as previously described [24]. DNA samples (8 µg) were cleaved separately with *EcoRI* and *HindIII* endonucleases (Eurogentec, Seraing, Belgium), according to a slightly modified version of the manufacturer's instructions. DNAs were digested for 4 h with 2U (in total) of each endonuclease per 1 µg of DNA. Half of the quantity of enzyme (1U) was used for the first 2 h of incubation at 37 °C, then the remaining half was added for a further 2 h of incubation. This procedure, routinely used in our laboratory, usually ensured complete digestion of most of the DNA. Restriction fragments were separated by electrophoresis in 0.8 % (w/v) agarose gel (Appligène, Illkirch, France) in TAE buffer (0.04 M Tris Acetate 0.001 M EDTA buffer) at 2.5 V/cm. *MluI* digestion of DNA from *Citrobacter diversus* (kindly provided by P.A.D. Grimont, Institut Pasteur, Paris) was used as a molecular size marker.

2.4. Southern blotting and hybridization

DNA fragments were vacuum transferred to neutral nylon membrane, (Amersham, Les Ulis, France), and were UV cross-linked at 0.125 joules/cm². The Southern blots were hybridized at 60 °C with 1 µg/mL of a 16S-23S rRNA probe labelled with acetylaminofluorene (AAF), (Eurogentec) in 10 mL of total hybridization fluid. DNA fragments encoding rRNA genes including those generated by digested *Citrobacter diversus MluI* were visualized by immunoenzymatic detection according to the specifications of the manufacturer. The DNA fragments of digested *Citrobacter diversus MluI* which were used in all blots as size markers included: 16 751, 12 481, 7 330, 6 551, 5 751, 5 097, 4 404, 3 022, 2 777, 1 695, 1 443, 1 170 bp.

2.5. Analysis of ribotyping

The fragment size of the ribotype patterns were calculated from migration distances and analysed using the TAXOTRON software (Institut Pasteur, Paris, France).

3. RESULTS

3.1. Biotyping

Forty-nine of the 89 isolates were identified according to biochemical characterisation, recorded in a five digit profile number, as *S. caprae*. Forty strains were given an atypical profile according to the API-STAPH database, which could lead to false identification (*table 1*). In this last group, six different clusters were found on the basis of their phenotypic properties.

3.2. Ribotyping

3.2.1. *EcoRI* hybridization pattern analysis

Six ribotype patterns designated as ribotype 1 to 6, were found when the DNA from the 89 isolates was cleaved with *EcoRI* endonuclease. The *EcoRI* rDNA banding patterns varied between 10 and 18 bands per lane, with fragment sizes between 0.9–9.3 kb.

Comparison of *S. caprae* (type strain and wild-type isolates) hybridization patterns with other different staphylococci type species analysed through the study demonstrated the presence of species-specific ribotypes (*figure 1*). Ribotype 1 which regrouped 14 strains (8 from Ardèche, 5 from Rhône-Alpes and 1 from Poitou-Charentes), showed 12 bands, similar to the hybridization pattern of the *S. caprae* type strain (0.9; 1.0; 1.2; 1.3; 1.3; 1.7; 2.2; 3.1; 3.3; 3.6; 4.3 and 4.8 kb). The ribotypes 2, 3 and 6 which regrouped

Table I. API-STAPH profile codes of staphylococci isolated from goat milk.

	API-STAPH profile (No. of isolates)										
	A (11)	B (13)	C (18)	D (3)	E (4)	F ^a (24)	G ^a (3)	H ^a (10)	I ^b (1)	J ^b (1)	K ^b (1)
Substrates											
Glucose	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	-	+	+
Maltose	+	+	+	-	-	+	-	+	-	-	-
Lactose	+	+	+	+	+	+	+	+	+	+	+
Threhalose	+	+	+	-	+	+	+	+	+	+	+
Mannitol	-	-	+	-	-	+	+	-	-	+	-
Xylitol	-	-	-	-	-	-	+	-	-	-	-
Melibiose	-	-	-	-	-	-	+	-	-	+	-
Nitrate reductase	+	+	+	+	-	+	-	-	-	-	+
Alcaline phosphatase	+	+	+	+	+	+	+	+	+	+	-
Acetoin production	+	+	+	+	+	+	+	+	+	+	+
Raffinose	-	-	-	-	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-	-	-	-	-
Saccharose	-	-	-	-	-	-	-	-	+	+	-
MDG	-	-	-	-	-	-	-	-	-	-	-
NAG	-	-	-	-	-	-	-	+	+	+	-
ADH	+	+	+	+	+	+	+	+	+	+	+
Urease	-	+	-	+	+	+	+	+	+	+	+

A: 6716101 (*S. caprae* type strain ATCC 35538 API-STAPH profile code); B: 6716103; C: 6516101; D: 6506103; E: 6514103; F: 6736103; G: 6534103; H: 6734143; I: 6416153; J: 6534153; K: 6512103. ^aAtypical API-STAPH profile code; ^bnon-identified API-STAPH profile code (three isolates).

respectively 40 isolates (19 from Ardèche, 18 from Rhône-Alpes and 3 from Poitou-Charentes), 20 strains (14 from Ardèche and 6 from Rhône-Alpes) and three isolates (2 from Poitou-Charentes and 1 from Rhône-Alpes), showed the same or almost the same pattern as ribotype 1 but lacked the 1.73 kb band, or the 1.73 and 2.22 kb bands for ribotype 2 and ribotype 3, respectively. An additional band of 1.49 kb was also found within ribotype 3. Ribotype 4, which regrouped six strains (five from Poitou-Charentes and one from Rhône-Alpes), showed the same 12 bands as ribotype 1 plus a further six bands of size 1.4; 2.4; 2.5; 5.8; 7.5 and 9.3 kb. Ribo-

type 5 which included three strains from Poitou-Charentes, displayed all the bands of the ribotype 1 with three additional bands (2.4; 2.8; and 6.0 kb) (*figure 1*).

3.2.2. *Hind*III hybridization pattern analysis

Three ribotypes, designated 1 to 3, were found after digestion with *Hind*III. DNAs from the samples cleaved with *Hind*III gave an average of ten bands per hybridization pattern with sizes ranging between 0.7 and 15.5 kb. These ribotypes differed one from another by the number and size of the rDNA restriction frag-

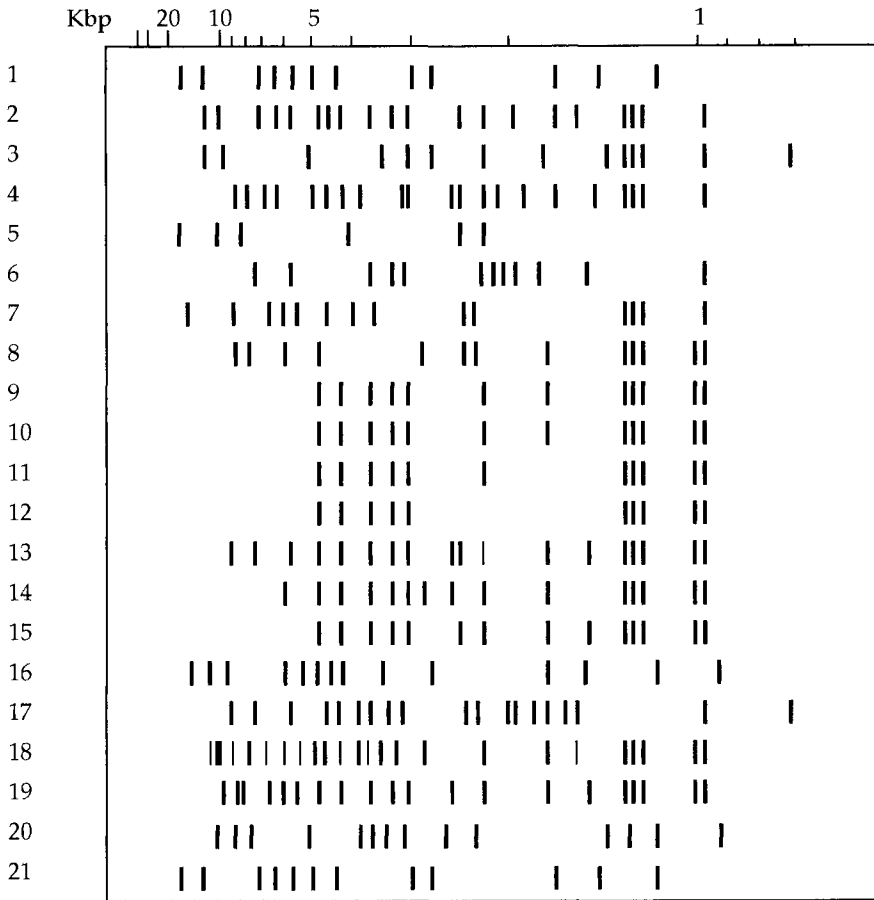


Figure 1. *Eco* RI Size of rRNA gene restriction fragments. Lane 1 and 21, *Citrobacter diversus* used as size markers. Lane 2, *S. xylosum* ATCC 35663. Lane 3, *S. cohnii* ATCC 35662. Lane 4, *S. epidermidis* ATCC 14990. Lane 5, *S. chromogenes* ATCC 43764. Lane 6, *S. simulans* ATCC 27848. Lane 7, *S. gallinarum* ATCC 35539. Lane 8, *S. capitis* ATCC 35661. Lane 9, *S. caprae* ATCC 35538T (Strain type). Lane 10, Ribotype 1 (14). Lane 11, Ribotype 2 (40). Lane 12, Ribotype 3 (20). Lane 13, Ribotype 4 (6). Lane 14, Ribotype 5 (3). Lane 15, Ribotype 6 (3). Lane 16, Atypical 1 (1). Lane 17, Atypical 2 (2). Lane 18, N880526 (human isolate). Lane 19, N920271 (human isolate). Lane 20, *S. aureus* 801-2. Parentheses indicate number of isolates per ribotype.

ments, and this observation made it possible to identify 86 of the isolates as *S. caprae*.

Cleavage with *Hind*III generated nine common bands representing the ribotype

of the *S. caprae* type strain (0.7; 0.8; 1.7; 2.4; 4.1; 4.4; 4.9; 7.9 and 8.8 kb). The comparison between the ribotype of the *S. caprae* type strain and the ribotypes of *S. caprae* isolates analysed in this work showed similarity with ribotype 1 which

regrouped 72 of the field strain *S. caprae* (41 strains from Ardèche, 28 from Rhône-Alpes and three from Poitou-Charentes). When compared to ribotype 1, ribotype 2, including 8 *S. caprae* strains (two strains from Rhône-Alpes and six from Poitou-Charentes), lacked the 3.0 kb band. It also had an additional band of 15.5 kb (figure 2). Ribotype 3, which regrouped six *S. caprae* strains (one strain from Rhône-Alpes and five from Poitou-Charentes), was very similar to ribotype 2 except that an additional band of 0.8 kb was observed. Three strains from the Rhône-Alpes produced three distinct hybridization patterns when their DNA was cleaved with *HindIII* and two distinct hybridization patterns, two of these three strains yielded identical hybridization patterns. The hybridization patterns of these three strains were very different from that of *S. caprae* ATCC type strain (figures 1 and 2). Human strains also gave different patterns with the two restriction enzymes (figures 1 and 2).

3.2.3. Comparison of biotypes, ribotypes and origin of isolates

Data regarding biotypes and ribotypes versus the origin of the isolates are recorded in table II. Analysis of these data indicated that there was no evidence of a relationship between one biotype or ribotype with a given origin. In other words, according to the methods used, the same strains were found in the three herds investigated, whatever their localization. Nevertheless, biotypes D and E (API-Staph scoring 6506103 and 6736103) appeared to be the main biotypes of *S. caprae* isolated from goats, both representing 48 % of total isolates.

4. DISCUSSION

Ribotyping has been proposed as a method for identifying staphylococcal

species and subspecies [6–8, 14]. Other identification methods have been applied, including plasmid profile analysis [1] and antibiotic susceptibility [25]. However, these methods are not always able to discriminate between species. Analysis of the complex patterns obtained after digestion with only total DNA endonucleases is too difficult to interpret and makes this method insufficient for discriminating isolates [21].

In this study 40 of the 89 CNS isolates were not correctly identified by the phenotypic methods. Ribotyping was used as an additional tool for accurate identification and 37 of these atypical isolates were finally correctly identified using this method. Three of these 40 isolates remained unidentified whatever the method used. Because they did not fit the phenotypic features of *S. caprae*, these isolates should be included in another species. Similar results were also reported previously when a radio labelled probe was used [5].

Digestion with *HindIII* produced fewer DNA fragments than the *EcoRI* cleavage. These results confirmed those of Thompson and Carter et al. [27]. Six ribotypes were found with *EcoRI* and three ribotypes were found with *HindIII*, the same results were recorded by Izart et al. [18]. The biochemical profiles did not make it possible to distinguish between the different taxa. However, we found that isolates included in the same group through biochemical characterization were differentiated into six different clusters with ribotyping when the DNA was cleaved with *EcoRI* and in three different clusters when the DNA was cleaved with *HindIII*. De Buyser et al. [8] used the same endonucleases and concluded that *HindIII* yielded a better discrimination of most of the staphylococcal taxa; however results from these workers indicated that the two following subspecies of *S. intermedius*, *S. intermedius carnivora* and *S. intermedius*

Table II. Ribotypes and biotypes of the 89 *S. caprae* isolates analysed.

API-STAPH profile	<i>Eco</i> RI Ribotypes	No. isolates (%)	Origin of the isolates ^a			<i>Hind</i> III Ribotypes	No. isolates (%)	Origin of the isolates ^a		
			A	R	P			A	R	P
6716 101 (A)	1	2 (2.2)	2	0	0	1	6 (6.7)	4	1	1
	2	1 (1.1)	0	0	1	2	3 (3.3)	0	1	2
	3	3 (3.3)	2	1	0	3	2 (2.2)	0	0	2
	4	2 (2.2)	0	0	2					
	5	2 (2.2)	0	0	2					
	6	1 (1.1)	0	1	0					
6716 103 (B)	1	2 (2.2)	2	0	0	1	10 (11)	9	0	1
	2	3 (3.3)	2	0	1	2	1 (1.1)	0	0	1
	3	5 (5.6)	5	0	0	3	2 (2.2)	0	1	1
	4	2 (2.2)	0	1	1					
	5	0								
	6	1 (1.1)	0	0	1					
6516 101 (C)	1	0				1	17 (19)	16	0	1
	2	17 (19)	16	0	1	2	0	0	0	0
	3	0				3	1 (1.1)	0	0	1
	4	1 (1.1)	0	0	1					
	5	0								
	6	0								
6506 103 (D)	1	0				1	1 (1.1)	1	0	0
	2	0				2	1 (1.1)	0	0	1
	3	1 (1.1)	1	0	0	3	1 (1.1)	0	0	1
	4	1 (1.1)	0	0	1					
	5	1 (1.1)	0	0	1					
6736 103 (E)	1	8 (8.9)	4	3	1	1	22 (24.7)	10	12	0
	2	6 (6.7)	1	5	0	2	2 (2.2)	0	1	1
	3	10 (11.2)	5	5	0	3	0	0	0	0
6534 103 (F)	1	0				1	2 (2.2)	1	1	0
	2	1 (1.1)	0	1	0	2	1 (1.1)	0	0	1
	3	1 (1.1)	1	0	0	3	0	0	0	0
	4	0	0	0	0					
	5	0	0	0	0					
	6	1 (1.1)	0	0	1					
6734 143 (G)	1	2 (2.2)	0	2	0	1	10 (11.2)	0	10	0
	2	8 (8.9)	0	8	0		0	0	0	0
							0	0	0	0
6514 103 (H)	2	4 (4.4)	0	4	0	1	4 (4.4)	0	4	0
							0	0	0	0
							0	0	0	0
Atypical	Atypical	3 (3.3)	0	3	0	Atypical	3 (3.3)	0	3	0
Total		89					89			

^a A: Ardèche, R: Rhône-Alpes, P: Poitou-Charentes.

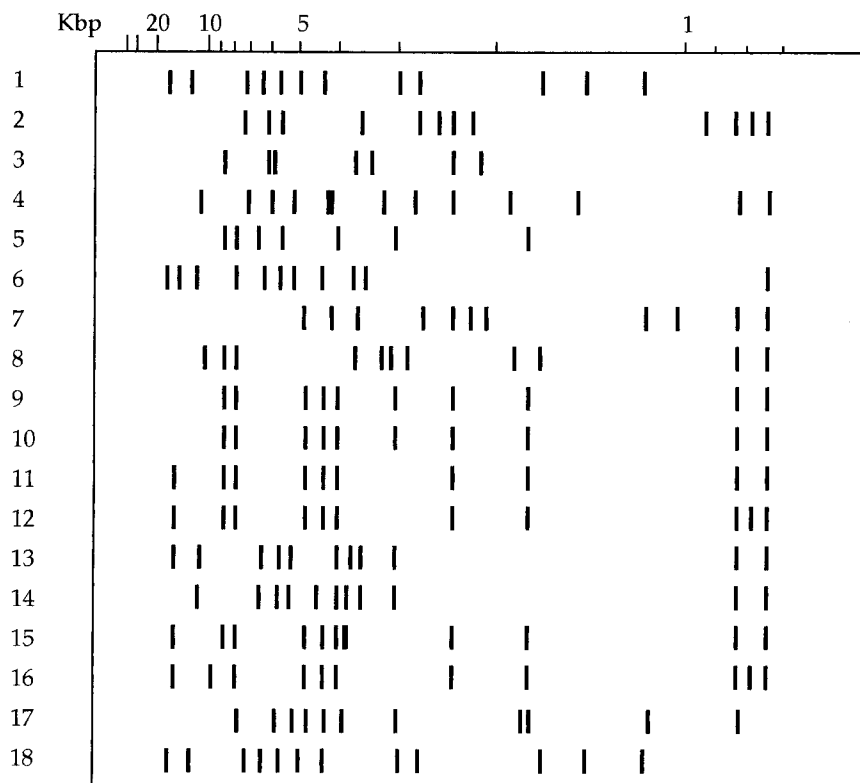


Figure 2. *Hind*III size of rRNA gene restriction fragments. Lane 1 and 18, *Citrobacter diversus* used as size markers. Lane 2, *S. xylosus* ATCC 35663. Lane 3, *S. cohnii* ATCC 35662. Lane 4, *S. epidermidis* ATCC 14990. Lane 5, *S. chromogenes* ATCC 43764. Lane 6, *S. simulans* ATCC 27848. Lane 7, *S. gallinarum* ATCC 35539. Lane 8, *S. capitis* ATCC 35661. Lane 9, *S. caprae* ATCC 35538T (Strain type). Lane 10, Ribotype 1 (72). Lane 11, Ribotype 2 (8). Lane 12, Ribotype 3 (6). Lane 13, Atypical 1 (1). Lane 14, Atypical 2 (2). Lane 15, N880526 (human isolate). Lane 16, N920271 (human isolate). Lane 17, *S. aureus* 801-2. Parentheses indicate number of isolates per ribotype.

intermedius carnivora and *S. intermedius* pigeon were only separated by *Eco*RI.

Two human isolates included in this study were previously characterized by ribotyping by Vandenesch et al. [29]. Our results, obtained when performing ribotyping, showed additional bands, particularly within the *Eco*RI digested fragments, when compared to those previously found. This discrepancy could result from a partial digestion of DNA with this

enzyme due to a high amount of DNA to be digested (8 μ g) or a high background from our non-radio labelled probe. Initial settings of reaction parameters with regards to amount of DNA to be used were satisfactory when using 8 μ g for digestion and vacuum transfer prior to hybridization with our non-radio labelled probe. Less DNA (i.e. 4 to 5 μ g) could be used in a classical Southern capillary transfer, but this would require a considerable increase

in time and quantity of transfer buffer. These remarks point out the need for standardizing between ribotyping methods when the resulting databases are to be compared between laboratories. Be that as it may, the human strains still appeared significantly different from the caprine strains as had been found previously.

The ribotyping method may be of value as an additional tool for the identification of staphylococci when phenotypical characterization is not efficient. Therefore this method can be used before progressing to a DNA/DNA hybridization study or other more specialized investigations. In contrast, its value as epidemiological marker for differentiation of strains of *S. caprae* should be re-evaluated with respect to other molecular tools (i.e. pulse-field gel electrophoresis, randomly amplified polymorphic DNA) since it failed to provide evidence on the relationships between strains and geographic origin in this study.

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