

# Identification and biotyping of coagulase positive staphylococci (CPS) in ripened French raw milk cheeses and their *in vitro* ability to produce enterotoxins

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## SUMMARY

The biochemical characteristics of CPS isolates collected from different varieties of French raw milk cheeses were investigated, together with their ability to produce staphylococcal enterotoxins (SEs) using the Transia (total SEs) and the Oxoid (typing of enterotoxins A to D) SE detection kits. Results indicated that isolates suspected to belong to the CPS group, could be divided into 3 groups comprising *Staphylococcus aureus* (82.2 %) ; *S. intermedius*, (13.3 %) and unexpectedly 4.5 % of coagulase negative staphylococci isolates. Within the group of *S. aureus*, isolates of biotype C were significantly more prevalent than other biotypes, in all the cheese varieties studied. Isolates belonging to the human A biotype were found at a very low level in all the cheese varieties. The study of the production *in vitro* of enterotoxins showed that SED was the most frequently produced (35 isolates were producers, all biotypes included). Cumulative identification of the other SEs gave 5 SEA producers (found exclusively in biotype A or of undetermined biotype isolates), 3 SEB producers and 11 SEC producers. Moreover, three isolates of bovine C biotype produced one or more enterotoxins that could be detected with the kit Transia, but are neither enterotoxins A, B, C nor D. In a very few cases, combined SEA + SED, SEB + SED, SEC + SED production were observed

**KEY-WORDS :** Coagulase positive staphylococci - raw milk - French cheese - biotypes - staphylococcal enterotoxins production.

## RÉSUMÉ

**Identification et biotypage de staphylocoques à coagulase positive dans des fromages au lait cru affinés et leur capacité à produire des entérotoxines *in vitro*.** Par H. LAMPRELL, L. VILLARD, J.-F. CHAMBA, E. BEUVIER, E. BORGES, F. MAURIN, G. MAZEROLLES, Y. NOEL et A. KODJO.

Les caractéristiques biochimiques d'isolats de staphylocoques à coagulase positive isolées de fromages au lait cru affinés ont été étudiées de même que leur capacité à produire des entérotoxines staphylococciques (SEs). Si la majorité des isolats appartenait à l'espèce *Staphylococcus aureus* (82,2 %) et parfois à l'espèce *S. intermedius* (13,3 %), 4,5 % des isolats se sont révélés appartenir à des espèces à coagulase négative. L'analyse des isolats de *S. aureus* a montré que les isolats de biotype C étaient majoritairement retrouvés dans les différentes variétés de fromage à l'inverse de ceux qui appartiennent au biotype A humain. L'étude de l'entérotoxigenèse *in vitro* a révélé que SED était l'entérotoxine majoritairement rencontrée (35 isolats producteurs, tous biotypes confondus). L'identification cumulée des autres entérotoxines a montré 5 isolats producteurs de SEA, détectés exclusivement dans les biotypes A ou indéterminés, 3 isolats producteurs de SEB et 11 isolats producteurs de SEC. Par ailleurs 3 isolats producteurs de SE non A, non B, non C, et non D ont été détectés avec le kit Transia. De manière tout à fait exceptionnelle, une production combinée d'entérotoxines SEA + SED, SEB + SED ou SEC + SED a été observée.

**MOTS-CLÉS :** Staphylocoques à coagulase positive - lait cru - fromages français - biotypes - production d'entérotoxines staphylococciques.

## Introduction

Staphylococcal enterotoxins (SEs) are exoproteins which, when produced in food that is then ingested by humans, give rise to symptoms of acute gastroenteritis [2]. Several types of staphylococcal enterotoxins have been identified on a serological basis and are named SE A through U (SEA through SEU) with SEA to SEE being the most frequently encountered due to their detection by usual standard kits [1, 3, 14, 16].

These toxins are usually produced by coagulase positive *Staphylococcus* (CPS) species, mainly *Staphylococcus aureus* strains which are themselves subdivided in several biotypes based upon biochemical characteristics. Staphylococci are able to readily multiply in many foods, however, in France, dairy products are probably the most implicated, with SEA and SED being the SEs most often associated [6, 13-19]. Literature regarding the enterotoxigenicity of *Staphylococcus aureus* strains isolated from cow dairy pro-

ducts indicate a wide range of enterotoxigenic strains depending either on the authors or on the methods used. BRUN and BES [2] have indicated values ranging from 2.6 to 9.4 % against about 16 % for ROSEC *et al* [19]. In addition, studies on enterotoxigenic strains of CPS have indicated that SEA is found mainly in biotype A strains of *S. aureus* of human origin [1, 12, 19]. Thus, contamination of food products may occur during phases of manufacturing and or handling of final products.

In this study, we investigated the biochemical characteristics of CPS strains isolated from different varieties of French raw milk cheeses, together with their ability to produce SEs (SEA through SEE).

## Materials and methods

### BACTERIAL ISOLATES

Isolates supposed to belong to CPS on the basis of their morphological aspects on the surface of Baird Parker agar plus rabbit plasma fibrinogen (RPF) were collected. Petri dishes were received in the laboratory throughout the period 2001 to 2002. These isolates, as listed in table I, were recovered during normalized routine analysis of different varieties of French semi-hard (uncooked pressed) cheeses made from raw cows' milk. A total of 1036 isolates (one or two colonies per plate) were isolated from the following cheese varieties : Saint Nectaire, Reblochon, Tomme de Savoie, Cantal, Salers and Laguiole.

### SPECIES AND BIOTYPE DETERMINATION

All the isolates identified as CPS on rabbit plasma were tested, for species assignment, using the following characteristics: coagulase activity in human plasma, beta hemolysis, pigment production, clumping factor, and finally maltose and d-trehalose acidification. Isolates belonging to *S. aureus* species were then subjected to biotype determination, using the HAJEK and MARSALEK' scheme [11] modified by DEVRIESE [8] and ISIGIDI *et al.* [12]. Namely, isolates were tested for staphylokinase, pigment, beta hemolysis production, coagulase activity on bovine plasma and finally, susceptibility to crystal violet.

### ENTEROTOXIN DETECTION

Selected isolates of CPS were grown on BHI broth (Brain Heart Infusion broth, Biokar Diagnostics, France) for 24 h at

37°C. Following incubation, the cultures were centrifuged at 10000 g for 10 min. at 4°C (Beckman centrifuge). The culture supernatant was recuperated and 10 fold dilutions in PBS Tween were prepared. The culture supernatants and the dilutions were sterilised by filtration (millipore filters, porosity 0.22 µm). Aliquots of each were distributed into 1.5 mL tubes (100 µL per tube for total enterotoxins and 200 µL per tube for individual enterotoxins) and frozen until analysis. For total enterotoxins, the Transia kit (Diffchamb, France) was chosen as it is the official French method for the detection of enterotoxins in food products. The tests were carried out on 100 µL of the diluted supernatants, according to the manufacturers instructions. Individual enterotoxins (A to D only) were identified using the kit Oxoid (Unipath, England). This kit was chosen for typing the enterotoxins, as it is one of 3 official methods recommended by the French Ministry of Agriculture, Food and Fisheries for auto-controls. The non-diluted supernatants (due to a lower detection threshold) were analysed, according to the manufacturers instructions.

## Results

### IDENTIFICATION OF STAPHYLOCOCCUS SPECIES

Isolates suspected to belong to the CPS group, as observed on Baird Parker plus RPF plates, could be divided into 3 groups : the first comprised the species *S. aureus* with a total of 852 isolates (82.2 %), the second was represented by the species *S. intermedius*, with 138 isolates (13.3 %) and the third contained 46 (4.5 %) isolates that were unexpectedly found to be coagulase negative staphylococci (CNS), for which typing was not carried out.

### BIOTYPING OF *S. AUREUS* ISOLATES

Data, as indicated in table I, showed that bovine biotype C was significantly more prevalent than other biotypes. Distribution of these biotypes was 529 (62.1 %), 227 (26.6 %), 35 (4.1 %), 25 (2.9 %), 25 (2.9 %), and 11 (1.3 %) for biotypes C bovine, undetermined, C ovine, A, D, and B respectively for *S. aureus* isolates. Those isolates that could not be determined as being biotypes A, B, C bovine, C ovine or D, according to HAJEK and MARSALEK' scheme [11] modified by DEVRIESE [8] and ISIGIDI *et al.* [12], were classed as having an undetermined biotype. It can be noted that this biotype was often very similar to the C bovine biotype.

No of strains investigated	<i>S. aureus</i> biotypes						Other	
	A	B	C* (bov)	C* (ov)	D	NT**	<i>S. intermedius</i>	Others (CNS)
All cheese varieties	25	11	529	35	25	227	138	46

\*bovine (bov) or ovine (ov) origin ; \*\*NT, non typable strains.

TABLE I. — CPS identification and their respective biotype.

## IDENTIFICATION AND TYPING OF SEs FOR *S. AUREUS* ISOLATES

Data, as shown in table II, indicated that SED was produced at a detectable level more frequently than the other SEs, that is to say 35 occurrences in contrast to 23 for all the other SEs. In addition, this SED production was seen in almost all biotypes, except biotype B isolates, mainly in biotype A (12 occurrences) and biotype D isolates (10 occurrences) of *S. aureus* (see table II). A majority of isolates of undetermined biotype also produced SED (8 isolates). Cumulative identification of the other SEs gives 5 SEA producers (1 biotype A isolate and 4 of undetermined biotype), 3 SEB producers (one of each of the following biotypes : A, D and undetermined), 11 SEC producers and 3 isolates of bovine C biotype that produced one or more enterotoxins that could be detected with the Transia kit but are neither enterotoxins A, B, C nor D. Additionally, combined production of SEs was observed : SEA + SED in 1 biotype A isolate, SEB + SED in 2 isolates of undetermined biotype, and finally, SEC + SED in 1 biotype B isolate and in 2 isolates of *S. aureus* of undetermined biotype. Overall, a greater percentage of biotype A and D isolates were capable of producing enterotoxins *in vitro* (60 et 56 % respectively) in comparison with the other biotypes examined. Finally, we have observed that in one specific cheese variety, only one isolate produced a SE. This SE belonged to the group C of the SEs and harboured the ovine C biotype.

## IDENTIFICATION AND TYPING OF SEs FOR *S. INTERMEDIUS* ISOLATES

6 of the *S. intermedius* isolates analysed for their production of SEs *in vitro* were found to produce SEC, in concordance with the literature [4]. However, 2 isolates were found to produce SED and SEA + SED respectively (see table III).

Type of SE	No. Strains
SEA	0
SEB	0
SEC (C1, C2, C3)	6
SED	1
Others	0
Combined SE's :	
SEA+SED	1

TABLE III. — SE production by the *S. intermedius* strains.

## Discussion

### IDENTIFICATION

In France, standards for dairy product controls involve counting of CPS on specific media such as Baird Parker agar plates. To facilitate the rapid identification of CPS, manufacturers have developed a specific medium containing rabbit plasma fibrinogen (RPF) which makes the localization of CPS on the surface of the agar plates much easier. Thus, identification includes two steps, the first step being the identification of black colonies and the second step being the identification of those black colonies surrounded by a dense halo, significant of coagulase activity. Violation of this «two steps» rule in the observation of SCP strains can lead to mistakes as some coagulase negative Staphylococci (CNS) may also appear as black colonies on BP agar. In our study, this was the case for about 5 % of the colonies isolated. In addition, one should keep in mind that all CPS counted on the Baird Agar

Type of SE	No of strains according to biotype					
	A	B	C(bov)*	C(ov)*	D	Und**
SEA	1					4
SEB	1				1	1
SEC (C1, C2, C3)		1	1	6	3	
SED	12		3	2	10	8
Others			3			
Combined SE's :						
SEA+SED	1					
SEB+SED						2
SEC+SED		1				2

\*bovine (bov) or ovine (ov) origin; \*\* undetermined SEs

TABLE II. — SE production by the *S. aureus* strains investigated with respect to their biotype and source of isolation.

+ RPF are not necessarily *S. aureus* species as some colonies may belong to the *S. intermedius* species. Certain publications have described their presence in bovine milk in a range of 0.2 to 2 % in addition to some isolates belonging to the *S. hyicus* species [5]. Again, BP-RPF should be considered as an initial orientation test and specific additional tests are needed for complete identification. Although manufactures indicate that BP + RPF is intended for identification of CPS, instructions should clearly indicate that all CPS do not necessarily belong to the *S. aureus* species.

## BIOTYPING

It has been stated that biotyping of *S. aureus* strains may give an indication of the origin of contamination in food products, as the biotype correlates well with the animal host [12, 17, 18]. In this study, as expected, the majority of the *S. aureus* isolates were found to belong to the C bovine biotype since 62 % of the *S. aureus* isolates belong to this biotype. The undetermined biotype was the second most prevalent biotype as it represents more than 26 % of the isolates investigated. In general, this group of isolates was much closer to the C bovine biotype than to the other biotypes, and lack of beta hemolysis was very often their distinctive characteristic. These results are in total agreement with previous data regarding the origin of isolates in dairy products [10, 12, 17-19]. Data regarding the other biotypes clearly indicate that they were in a significantly lower percentage. Their presence in these cheeses is probably due to the fact that many other animals (i.e. hen, sheep) are also kept on these farms and cross transfer may occur between different animal hosts. This finding is not specific to this study since it has already been reported by previous publications [1, 7]. In addition, the low rate of biotype A isolates is a demonstration of the good sanitary practices of farmers during manufacture and handling of these cheeses.

## SE PRODUCTION

The overall number of *S. aureus* isolates that produced one or more of the SEs was 63 (7.4 %) with SED being the most frequently produced (35 isolates), followed by SEC (11 isolates), which is in accordance with BRUN and BES [2]. Although the rate of SE producing *S. aureus* isolates in dairy products agrees with data usually found in the literature, there is some opposition regarding the frequency of SEC versus SED production since we found SED more frequently than SEC, contrary to that described in previous papers [19]. Biotype A isolates were found to be the most enterotoxigenic, in accordance with previous results [12, 19]. Concerning enterotoxin production by biotype D isolates, very little information is available in the literature [21]. Amongst our isolates, a large majority of these biotype D isolates were capable of producing enterotoxins *in vitro*.

Despite the presence of CPS in the different varieties of semi-hard cheese studied, only a small percentage are potentially enterotoxigenic, which partly explains why SEs are rarely detected, even when CPS populations exceed  $10^5$  cfu/g cheese [16, 20]. Besides the specificity of the *S. aureus* isolates in themselves, cheese technological parameters, such as

acidification kinetics, the starter culture and salting and probably many others still to be explored, may intervene in the growth of *S. aureus* and the production of enterotoxins. In addition, our data indicate that there may be a CPS strain selection mechanism, as most of the isolates (92.6 %) were inapt to produce SEs *in vitro*, in particular for the cheese variety in which only one enterotoxigenic isolate was found.

Concerning *S. intermedius*, in a recent study on veterinary strains, BECKER *et al.* [4] clearly demonstrated, by PCR and immunoassays, that SEC production is a common feature of such strains [4]. Although our data are in agreement with these findings, they also harbor the potential for production of other SEs, as we found some isolates that produced SED or SED + SEA. *S. intermedius* may also therefore be involved in human staphylococcal food poisoning.

This study has shown that only a small percentage of *S. aureus* strains isolated from different varieties of French raw milk cheeses and the milk for fabrication, are able to produce SEs at a detectable level *in vitro*. Therefore, the risk of SEs being found in the final product due to cheese-making conditions, is low. Notably, the biotype C bovine was the most frequently isolated but only 7 of these (1.3 %) were able to produce enterotoxins at a detectable level *in vitro*.

However, it must not be dismissed that many strains of different origin may harbor genes [16, 18] and therefore have the capacity to produce other enterotoxins that are not yet detectable by standard kits.

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