Prevalence and Growth Dynamics of Enterotoxinogenic *Staphylococcus aureus* Isolates in Slovakian Dairy Products

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Abstract

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We investigated the prevalence of enterotoxigenic *S. aureus* in the Slovakian dairy products and compared the reliability of different routine methods of identification. Out of 64 isolates, 44% were confirmed as *S. aureus*. There was only a little correlation in the confirmation by API Staph, VIT-Staphylococcus, and PCR detection. The PCR results revealed that 32% of *S. aureus* isolates possessed the selected gene for SEs enterotoxins (SEA-SEE), with *sea* as the most predominant gene. Neither *seb* nor *sed* genes were found throughout the collection. Additionally, the growth analysis of the isolates was performed in milk at 15°C. The growth parameters were very close to one another and were also compared with the data described in the global databases. These results provided evidence that the growth rate and lag phase duration can be determined with a high degree of reproducibility without regardless of the strain origin.

Keywords: coagulaso-positive staphylococci; staphylococcal enterotoxins; PCR; growth dynamic

In the recent years, an increasing interest in the consumption of traditional dairy foods has been noticed. This is due to the demands of consumers for varieties of cheeses with special and characteristic flavours. In Slovakia, the manufacture of Bryndza cheese made from ewes' lump cheese from raw milk is of great importance to preserve the national gastronomical heritage. However, the milk origin, production environment and conditions of ewes' lump cheese manufacture may act as a source of pathogenic microorganisms, including *Staphylococcus aureus*. Due to its good growth in milk with respect to the conditions of intrinsic and extrinsic factors, *S. aureus* presents a potential or even actual threat to human health. Not only the production of the surface factors, degradative enzymes and toxins, but also the production of heat-stable enterotoxins (SEA-SEV) makes it such a dangerous microorganism (Ote *et al*. 2011). The staphylococcal food-poisoning (SFP) is considered worldwide as the third most important cause of food-poisoning outbreaks (Kérouanton *et al.* 2007; Normanno *et al.* 2007).

The skin, mucosa membranes, teats and udders of milking animals are its important reservoirs, especially if mastitis occurs in the herd. In the case of an infected udder, *S. aureus* can contaminate milk during milking in a density ranging from 1–8 log CFU/ml, mostly about 4 log CFU/ml (Boynukara *et al.* 2008).

Moreover, one third of people are considered as asymptomatic carriers. The organism finds its way into food through hands (infected wounds, skin lesions) or by coughing and sneezing (Asperger & Zangerl 2003). The lack of proper hygienic measures during food processing would also increase the counts of *S. aureus*, especially in manually prepared foods. Therefore, *S. aureus* can also contaminate heat-treated milk and can be subsequently present in cheeses prepared from both raw and pasteurised milk.

In this connection, the aim of this work was to analyse the prevalence of *Staphylococcus aureus* in Slovakian dairy products. Further, enterotoxigenic properties of the isolates with respect to the presence of *sea-see* genes were investigated throughout the

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isolates. Moreover, the statistical evaluation of the growth parameters of *S. aureus* isolates was observed in order to assess the precision of the growth parameters determination. This information could provide the precision and reliability of the subsequent growth predictions related to the behaviour of *S. aureus* not only in the dairy products but also in other foods.

MATERIAL AND METHODS

Food samples. A total of 64 dairy samples were collected from different markets and farmers in Slovakia from September 2009 to December 2010. The set of samples consisted of raw ewes' milk (n = 15), ewes' lump cheeses (n = 30), whey (n = 6), and Slovakian traditional cheeses (n = 13; e.g. bryndza, oštiepok). The samples were transported within 1 h to the laboratory in a cooled box and analysed for the presence of *S. aureus*. The counts of coagulase-positive staphylococci (CPS) were detected by surface spreading of 20 µl of 1 : 10 dilution according to the EN ISO 6888-1:1999.

Phenotypical characterisation. 3–5 colonies with typical morphological appearance were selected from each sample. For purification, all colonies were grown aerobically in BHI broth (Sigma Aldrich, Steinheim, Germany) at 37°C for 24 h and repeatedly spread on Baird-Parker agar (Merck, Darmstadt, Germany). From each sample one colony was randomly chosen and identified on the basis of the Gram-staining, microscopic morphology, catalase reaction, ability to coagulate human plasma (tube coagulase test), APIStaph (Biomeriuéx, Marcy l'Etoile, France), VIT-Staphylococcus (Vermicon, Munich, Germany). The VIT-Staphylococcus method is fast method for the detection of *Staphylococcus* species including *S. aureus* without previous growth of culture on the selective media. This is based on the penetration of a specific gene probe into the bacteria cell, marking special signature of the gene sequence with the dye and illuminating them. Subsequently, the samples are examined under fluorescence microscope. Bacteria belonging to the genus *Staphylococcus* light up in green, bacteria belonging to the species *S. aureus* additionally light up in red (Vermicon 2004).

Bacterial strains. The reference strain of *S. aureus* CCM 3953 was used as a positive control and that of *S. epidermis* CCM 4418 as a negative control, both originating from the Czech Collection of Microorganisms.

PCR amplification. The oligonucleotides used in this work, their sequences, annealing temperature, number of cycles needed and size of fragments are summarised in Table 1. PCR amplification was conducted in a solution containing 5 µl HotMaster *Taq* buffer, 1.5 µl dNTP (all deoxyribonucleotides in equimolar concentrations), 0.5 µl of HotMaster *Taq* polymerase (5prime, Hamburg, Germany), 1 µl of each primer (Ecoli, Bratislava, Slovakia), and the DNA template – a 24 h old colony, in a final volume of 50 µl according to Pinto *et al.* (2005), Kérouanton *et al.* (2007), and Rall *et al.* (2008). The negative control of amplification was performed with 5 µl of water instead of DNA template. The reactions were carried out in a MasterCycler Gradient thermal cycler (Eppendorf, Hamburg, Germany). PCR products were electrophoresed through 3% agarose gel (Sigma-Aldrich, Steinheim, Germany) in TAE buffer (40mM Tris-acetate, pH 8.0, 0.5mM Na_2EDTA) stained with 8 µl of GelRed (C-consulting, Bratislava, Slovakia), at 300 mA, 109 V, 500 W for 1 hour. The amplified DNA fragments were visualised by UV transillumination T 2201 (Sigma, St. Louis, USA).

Growth dynamics of isolates. The growth curves of all isolates in milk at 15°C were obtained as previ-

Table 1. Conditions of PCR amplification for *S. aureus* nuclease and enterotoxines

Primer	Sequence	Base pair (bp)	Annealing temperature (°C)	Number of cycles	Reference
nuc-1	GCGATTGATGGTGATACGGTT	270	55	35	Pinto *et al.* (2005)
nuc-2	AGCCAAGCCTTGACGAACTAAAGC				
sea-1	CCTTTGGAAACGGTTAAAACG	127	55	35	Pinto *et al.* (2005)
sea-2	TCTGAACCTTCCCATCAAAAAC				
seb-1	TCGCATCAAACTGACAAACG	478	50	30	Rall *et al.* (2008)
seb-2	GCAGGTACTCTATAAGTGCC				
sec-1	GACATAAAAGCTAGGAATTT	257	55	35	Kérouanton *et al.* (2007)
sec-2	AAATCGGATTAACATTATCC				
sed-1	CTAGTTTGGTAATATCTCCT	317	50	30	Rall *et al.* (2008)
sed-2	TAATGCTATATCTTATAGGG				
see-1	TAGATAAAGTTAAAAAACAAGC	170	55	35	Pinto *et al.* (2005)
see-2	TAACTTACCGTGGACCCTTC				

ously described in our work (Medveďová *et al.* 2009). The growth parameters of the isolates under study were fitted and calculated, respectively, by using the mechanistic modelling technique of Baraniy *et al.* (1993) which is incorporated in the DMFit tools and analysed by statistic tools of the Microsoft Office 2007. The growth parameters were compared with the data in the databases Combase Predictor (IFR, Norwich, UK), and Pathogen Modelling Programme Ver. 7 (Wyndmoor, Pennsylvania, USA).

RESULTS AND DISCUSSION

All of the 64 isolates were Gram-positive, catalase-positive cocci being organised in grape-like clusters. All showed typical characteristics on Baird-Parker agar, i.e. black, convex, lustrous colonies with the diameter of 1–1.5 mm and a narrow clear zone after 24 hours. Total counts of coagulase-positive staphylococci in each dairy sample and the distribution of the positive *S. aureus* isolates among the analysed samples are presented in Table 2. It is assumed that in properly drawn milk, the counts of *S. aureus* are around 2 log CFU/ml. During the cheese manufacture, *S. aureus* is concentrated in the curd, so the counts in whey are lower than those in milk. An increase about 1.5–3 log during the first 24 h of the cheese-making process may be expected under normal conditions (Asperger & Zangerl 2004).

Identification of isolates. According to the APIStaph profile, 52 out of 64 isolates (81.3%) were tentatively determined as *S. aureus* with the identity to *S. aureus* with 44% (6 isolates), 67% (6 isolates), 89% (13 isolates) or 98% reliability (26 isolates). Among the remaining isolates occurred *S. caprae* (4), *S. lentus* (2), *S. epidermis* (2), *S. hominis* (2), and *S. xylosus* (2) with the identity of more than 90%. Those isolates were excluded from further experiments, since they were also coagulase-negative. These findings are in agreement with Pinto *et al.* (2005) who also confirmed 71% of isolates as *S. aureus* by using the APIStaph system.

According to VIT-Staphylococcus examination, only three isolates out of 52 did not light up red, thus they were not detected as *S. aureus*. These three isolates were also negative for *nuc* gene. However, the rest of isolates that lighted up in red were not all members of the species *S. aureus*, as they were either coagulase-negative or *nuc* negative. Thus, the VIT-Staphylococcus method is really very fast and simple method, but for definitive identification of *S. aureus*, other identification tests are necessary.

S. aureus produces a well described extracellular thermostable nuclease *nuc*, which was previously cloned and sequenced (Palomares *et al.* 2003). This ubiquitous and conserved gene within *S. aureus* species has been used for its differentiation from other closely related coagulase-negative staphylococci (Pinto *et al.* 2005).

In this study, the specific 270 bp amplicon corresponding to this gene was obtained in 28 isolates (44%, Table 2). These results are consistent with the findings of Pelisser *et al.* (2008) who identified 45.5% of *S. aureus* in 200 dairy and meat samples. Rall *et al.* (2008) and Rosengren *et al.* (2010) identified about 70% of strains as *S. aureus* in milk and fresh cheeses samples. On the other hand, in some works (Normanno *et al.* 2007; Ertas *et al.* 2010; Trnčíková *et al.* 2010; Zigo *et al.* 2011), the contamination of mostly raw milk, dairy products, but also meat and meat products by *S. aureus* was bellow 20%.

All the isolates which were *nuc* positive were also coagulase-positive. Besides them, only three other isolates were coagulase-positive but not *nuc* positive, so they could belong to the species *S. intermedius*,

Table 2. The distribution of *S. aureus* among dairy samples

Sample	No. of samples	No. of *S. aureus* positive samples		No. of SE positive isolates	log counts of CPS
		prior to API and coagulase test	based on *nuc* gene detection		
Raw milk	15	9	3	1	2.2 ± 0.3 CFU/ml
Whey	6	5	5	1	1.6 ± 0.2 CFU/ml
Ewes' lump cheese	30	25	14	6	4.5 ± 0.6 CFU/g
Slovak cheeses	13	10	4	0	0.5 ± 0.1 CFU/g
Other	3	3	2	1	na
Total	64	52 (81%)	28 (44%)	9 (32%)	

na – not analysed; CPS – coagulase-positive staphylococci

Table 3. Growth parameters of *S. aureus* at 15°C

	Gr (log CFU/ml·h)	λ (h)	y_0	y_{max}	t_d (h)
			(log CFU/ml)		
Mean	0.071	13.8	2.93	8.17	4.4
s_d	0.011	3.2	0.59	0.29	0.6
VC%	15.5	23.0	20.0	3.6	14.7
Minimum	0.045	4.4	0.78	7.19	2.2
Maximum	0.138	21.4	3.72	9.02	6.6
Medium	0.069	14.3	3.13	8.19	4.3

Gr – growth rate; lag – lag phase; y_0 – initial counts of CPS; y_{max} – counts of CPS in stationary phase; t_d – time to double

S. hyicus or others. There was no correlation between API profile and the presence of *nuc* gene. Even if the identity of isolates according to the API tests was higher than 98%, there were four *nuc* negative isolates and, moreover, three of them were also coagulase-negative. Contrary to that, there also some occurred isolates with API profile of 44% match, but they were coagulase-positive and also *nuc* positive.

As SEs can be also produced by non-*S. aureus* species (Trnčíková *et al.* 2010; Zigo *et al.* 2011), all 52 isolates were investigated for SEs genes presence. The SEs encoding genes were obtained only in the confirmed *S. aureus* strains. In the present study, 9 isolates were enterotoxigenic and they might represent a potential danger for consumers if they were present in food and had a chance to multiply up to 10^6 CFU/ml or CFU/g. Five of those isolates possessed the gene for only one SE and the other 4 possessed the genes for two SEs. In the majority of the isolates, the gene for SEA was detected (4), in 3 isolates the combination of *sea* and *sec* genes was found and *see* gene or *sea*/*see* genes combination occurred in one of the isolates. Neither *seb* nor *sed* genes were found throughout the isolates collection isolates.

It was found, that *sea* gene is the most frequent gene among isolates studied by Rall *et al.* (2008) and Ertas *et al.* (2010). However, Normanno *et al.* (2007) and Ote *et al.* (2011) noticed the majority of strains with *sed* gene. It is assumed, that SEA together with SED are the most frequent agents in SFP outbreaks (Normanno *et al.* 2007; Rosengren *et al.* 2010). Furthermore, SEA is predominantly produced by the human strains, so the connection with food contamination during the manufacture is possible (Akineden *et al.* 2008).

On the other hand, the *sec* was the most predominant gene (Akineden *et al.* 2010; Rosengren *et al.* 2010; Trnčíková *et al.* 2010; Zigo *et al.* 2011) as the most important cause of SFP associated with the consumption of dairy products (Normanno *et al.* 2007). This disunity in SEs genes presence among different isolates of *S. aureus* may result from the different ecological niches and geographical origins of strains, different cultivation and detection conditions and kinds of samples investigated.

Growth dynamics of isolates. The growth of all 64 isolates in milk was analysed, with the aim to generalise the growth predictions of *S. aureus*. The temperature of 15°C was chosen due to its connection to artisanal ewes' lump cheese production conditions.

As seen in Table 3, the growth dynamics of *S. aureus* isolates demonstrated very uniform growth character with low statistic variation in each parameter. Despite the fact, that in the cultivation experiments 12–37% of the bound of reliability is tolerable (EN ISO 6888-1: 1999), our results confirmed a very high repeatability of the growth parameters determination.

Values of the growth parameters initiated in the Combase Predictor (Gr = 0.074 log CFU/ml·h, λ = 14.3 h) or in the PMP (Gr = 0.077 log CFU/ml·h, λ = 8.9 h) are very close to those which were determined for the isolates in our study. It should be mentioned that the values of *S. aureus* growth parameters presented in the world databases are based on experiments carried out in non-milk media. So, it can be concluded that the growth dynamics of *S. aureus* strains is not affected by their origin, even not by the culture media, and consequently the growth dynamics can be estimated with 5–30% error in the prediction. In this context, the growth of *S. aureus* under different conditions as it is described by Medveďová *et al.* (2009) can be reliably predicted by means of the predictive models.

CONCLUSION

The obtained results revealed that the dairy products may be still a potential source of enterotoxigenic strains of *S. aureus*. The presented results of the

growth dynamics analysis emphasised the importance of using all tools which may prevent *S. aureus* from reaching the counts above 10^6 CFU/ml and the potential production of enterotoxin. These findings highlighted the need for strict hygienic and preventive measurements not only in the dairy products manufacture, distribution and consumption to avoid human health threat. In this connection, predictions of *S. aureus* growth in relation to the extrinsic and intrinsic factors may serve as an effective tool.

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