Short communication: Enterotoxigenic potential of coagulase-negative staphylococci isolated from bovine milk in Poland

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ABSTRACT

Our objective was to assess the enterotoxigenic potential of coagulase-negative staphylococci (CNS) isolated from bovine milk in Poland. We analyzed CNS isolates collected from 133 bovine milk batches from dairy farms in the Western Pomerania district during 2 milking seasons. A total of 163 isolates were screened by multiplex/duplex PCR for the presence of 18 of 25 enterotoxin genes identified so far in *Staphylococcus aureus*. The CNS strains presumed to be potentially enterotoxigenic were identified at the species level based on MALDI-TOF mass spectrometry analysis. Based on the presence of an amplicon matching D, G, or O enterotoxin genes, we initially identified 32 of the 163 CNS isolates tested as potentially enterotoxigenic. However, only 8 of these strains were confirmed as such. All 8 of these CNS strains, identified as *Staphylococcus haemolyticus*, harbored the *seg* genes, a prerequisite for enterotoxin G production, but so far not connected with staphylococcal foodborne poisoning cases. None of the CNS bovine milk isolates tested was a potential producer of classical A to E staphylococcal enterotoxins. Results of our surveys revealed a low prevalence of enterotoxigenic CNS among the milk isolates from dairy farms in the Western Pomerania district, Poland, suggesting that they pose only a mild health risk in milk. In our opinion, confirmed formation of nonspecific amplicons leading to false-positive results excludes multiplex/duplex PCR as the sole method for assessing the enterotoxigenic potential of CNS.

Key words: coagulase-negative staphylococci, enterotoxin, enterotoxigenic potential, bovine milk, multiplex/duplex PCR

Short Communication

Coagulase-negative staphylococci, presently comprising 44 species and 24 subspecies (<http://www.bacterio.net/staphylococcus.html>), constitute the majority of organisms in the *Staphylococcus* genus. Widespread in the environment, with some considered opportunistic pathogens, these bacteria are also frequent contaminants of food, including milk. When in food and under conditions supporting their growth, CNS, similar to coagulase-positive staphylococci, may affect food quality and safety. However, in contrast to *Staphylococcus aureus*, which produces 25 enterotoxins and is the main cause of staphylococcal foodborne poisoning worldwide (Benkerroum, 2018), knowledge on the enterotoxigenic potential of CNS representatives is scant so far (Podkowik et al., 2013). This limited and ambiguous knowledge on the enterotoxigenic potential of CNS and their share in staphylococcal foodborne poisoning statistics results from differences in the scope of research and methods applied, the types and number of toxins considered, the diversity in origin and number of CNS strains tested, and so forth (Rodríguez et al., 1996; Zell et al., 2008; Park et al., 2011; de Freitas Guimarães et al., 2013).

Staphylococcal enterotoxins and staphylococcal enterotoxin-like toxins are superantigens differing in structure, including their amino acid composition (168–257 amino acids), molecular weight (19–28 kDa), coding gene sites, and systems regulating gene expression (Hu and Nakane, 2014). All are soluble in water and physiological saline solutions, resistant to low pH, and resistant to most of the proteolytic enzymes, including trypsin and pepsin (Hu and Nakane, 2014). Thermostability allows enterotoxins to retain their biological activity while in food and when subjected to thermal processing. For example, staphylococcal enterotoxin A stays biologically active when exposed to 121 or 130°C for 28 min or 4 s, respectively (Argudín et al., 2010).

The frequent, concurrent presence of CNS with coagulase-positive staphylococci in food and the prevalence of CNS over coagulase-positive staphylo-

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cocci (Chajęcka-Wierzchowska et al., 2015), with the possible transfer of genes' encoding for enterotoxins, might signify a higher than commonly accepted share of enterotoxigenic CNS in staphylococcal foodborne poisoning statistics.

The aim of our work was to assess the scale and diversity of the enterotoxigenic potential among the CNS isolated from bovine milk in Poland. We analyzed 163 CNS isolated from bovine milk collected from the contract milk suppliers of one dairy plant in the Western Pomerania district, Poland, during 2 milking seasons, in 2011. The DNA of the CNS isolates tested was isolated by the column method using the PCR template preparation Genomic Mini kit (A&A Biotechnology, Gdynia, Poland), following the manufacturer's instructions. To confirm the tested isolates as belonging to the *Staphylococcus* genus, we used the genus-specific *tuf* gene (Martineau et al., 2001), according to the procedure described by Filleron et al. (2014).

The enterotoxigenic potential of the CNS isolates was assessed based on the presence of genes necessary for the production of any of 18 staphylococcal enterotoxins from among the 25 identified so far. An assessment was conducted using multiplex/duplex PCR analysis (Fijałkowski et al., 2014) with pairs of primers standard for *S. aureus* (Jarraud et al., 2002; Holtfreter et al., 2007). Positive results obtained in this analysis, namely the presence of amplicons in the electrophoregrams that were the same or almost the same size as positive controls, were evaluated by a single PCR.

Lack of the same amplicons in a single PCR called for an analytical explanation of false-positive results. To explain the presence of nonspecific amplicons in multiplex/duplex PCR electrophoregrams, we paired each primer with others in the group of amplicons not confirmed by a single PCR. Furthermore, the PCR amplicons formed were subjected to sequencing (Genomed, Warsaw, Poland) to verify and compare their nucleotide sequences with enterotoxin gene standards. As positive controls, *S. aureus* strains harboring the enterotoxin genes under surveillance were used (Table 1). In addition, CNS strains presumed to be potentially enterotoxigenic were identified at a species level using MALDI-TOF MS (Bruker Daltonik, Bremen, Germany).

Our results from the genomic DNA analysis, conducted by multiplex/duplex PCR and addressing genes encoding 18 enterotoxins, indicated that 32 of the 163 isolates were potentially enterotoxigenic (Table 2). The only amplicons appearing were those exactly or almost matching those for the *sed* ($n = 7$) and *seg* ($n = 8$) genes encoding the D and G enterotoxins, respectively, or the *selo* ($n = 17$) gene encoding the O enterotoxin.

Only 8 of these 32 CNS strains were confirmed as being enterotoxigenic. All were carriers of *seg* genes and were identified as *S. haemolyticus* by the MALDI-TOF MS analysis. The homology of *seg* gene nucleotide sequences between the CNS milk isolates and reference *S. aureus* was 99%.

The presence of nonspecific amplicons leading to false-positive results was noted for 24 of the 32 CNS tested, with 7 of these 24 CNS strains found to be carriers of the *sed* enterotoxin gene (duplex I PCR) and the other 17 identified as potential producers of the O enterotoxin (multiplex II PCR; Table 1).

For CNS strain 502, identified as *S. warneri*, a product of 493 bp in size exactly matched the size of the enterotoxin D gene. This product was created by a linkage between both strands of the sek primer (sek-1 and sek-2) and the strain's DNA (Table 2). For the other 6 CNS strains initially presumed to be potential carriers of the D enterotoxin gene, the nonspecific amplicon was an artifact of the linkage between single strands sek-1 and sed-2 of the sek and sed primers and the strains' DNA. Of these 6 CNS strains, only 3 were identified at the species level: *S. schleiferi*, *S. xylosus*, and *S. chromogenes* (Table 2). Moreover, none of the nucleotide sequences of the DNA of these 7 CNS strains corresponded to the reference sequence for the D enterotoxin.

Seventeen other CNS strains were falsely identified by the multiplex II PCR as carriers of the *selo* gene. Nonspecific amplicons were 189 bp in size, which almost matched the typical size for the enterotoxin O gene (180 bp; Table 1). By pairing each of the 5 pairs of primers with each other, we determined that the false-positive products were due to the linkage of single see-2 and sem-2 strands of the see and sem primers to the DNA of these CNS strains. Moreover, a nucleotide sequence analysis of this amplicon showed no resemblance to sequences of the *selo*, *sell*, *selm*, *seb*, and *see* genes in the 17 CNS strains, all of which were identified as *S. epidermidis*.

Although attention to CNS as a food contaminant has increased in the past few years, current knowledge of their toxigenic properties is still fragmentary and the results are questionable. In our study, only 5% of the 163 CNS bovine milk isolates were enterotoxin gene positive, with all 8 of the positive CNS strains harboring the *seg* gene only. The results of other studies, assaying different numbers and types of enterotoxin genes and applying different analytical methods, indicated that 0 to 80% of CNS strains tested were potentially enterotoxigenic (Nemati et al., 2008; Park et al., 2011; Fijałkowski et al., 2014; Bertelloni et al., 2015; Siqueira et al., 2017; Corbeil et al., 2019).

Table 1. Enterotoxigenic potential of CNS isolates of the bovine milk origin (when screened by multiplex/duplex PCR¹ and when confirmed)

PCR method	Gene	Primer	Oligonucleotide sequence (5'→3') ²	PCR product (bp)	<i>Staphylococcus aureus</i> reference strain	No. of CNS presumed enterotoxic	
						Initially	When confirmed
Multiplex I	<i>sea</i>	sea-1	gaaaaaagctctgaattgcagggaaca	560	CCM 5756 ³	0	—
		sea-2	caaataaatcgtaattaaccgaaggttc		CCM 5755 ³		
	<i>seh</i>	seh-1	caatcacatcatatgcgaagcag	376	FRI 137 ⁴		
		seh-2	catctaccaaacattagcacc				
	<i>sec</i>	sec-1	cttgatgtatggaggaataacaaacatg	275	CCM 5971 ³		
Multiplex II		sec-2	catatcataccaaaaagtattgccgt		N315 ⁵	17 selo	0
					FRI 137		
	<i>see</i>	see-1	caaagaatgctttaagcaatcttaggc	482	CCM 5972 ³		
		see-2	caccttaccgcaaagctg				
	<i>seb</i>	seb-1	attctattaaggacactaagttaggga	404	CCM 5755		
		seb-2	atccggttcataaaggcgagt				
	<i>selm</i>	sem-1	ctattaatctttgggttaattggagaac	326	CCM 5971		
		sem-2	ttcagtttcgacagttttgtgtcat		N315		
	<i>sell</i>	sel-1	gcgatgtaggtccaggaaac	234	CCM 5971		
		sel-2	catatatgtacgagagttagaaccata		N315		
Multiplex III					FRI 137		
		<i>selo</i>	seo-1	agtttgtgaagaagtcaagtgtaga	180	CCM 5971 N315	
			seo-2	atctttaaattcagcagatattccatctaac			
	<i>seln</i>	sen-1	cgtagcaattagacgagtc	474	CCM 5971 N315	8 seg	8 seg
		sen-2	gattgatytgtatgattatgag				
	<i>seg</i>	seg-1	tctccacctgttgaagg	323	CCM 5971 N315		
		seg-2	aagtgattgtctattgtcg				
	<i>selq</i>	seq-1	acctgaaagcttcaagga	204	CCM 5755		
		seq-2	cgccaacgtaattccac		CCM 5972		
	<i>selj</i>	sej-1	tcagaactgtttccgctag	138	CCM 5756		
		sej-2	gaattttaccaycaaaggtac		CCM 5971		
	<i>sed</i>	sed-1	gaattaagtagtaccgcgctaataatag	493	CCM 5756		
		sed-2	gctgtatttttctccgagagt		CCM 5971		
Duplex I	<i>selk</i>	sek-1	atgccagcgctcaaggc	134	CCM 5755	7 sed	0
		sek-2	agattcatttgaaaattgtagttgattagct				
Duplex II	<i>sei</i>	sei-1	ctygaattttcaacmggtac	461	CCM 5971	0	—
		sei-2	aggcagtcacatctcctg		N315		
	<i>selp</i>	sep-1	gaattgcagggaactgct	182	N315		
Duplex III		sep-2	ggcgggtgtcttttgaac			0	—
	<i>ser</i>	ser-1	agcggtaatagcagaaaatg	363	CCM 5756		
		ser-2	tcttgtagcgttaacgggtttt				
	<i>selu</i>	seu-1	aatggctctaaaattgatgg	215	FRI 137		
		seu-2	atttgatttccatcatgctc				

¹Fijalkowski et al. (2014).²Sequences from Jarraud et al. (2002) and Holtfreter et al. (2007).³Omoe et al. (2005).⁴Wu et al. (2011).⁵Kuroda et al. (2001).

Screening 71 CNS bovine isolates for 12 enterotoxin genes (*sea*, *seb*, *sec*, *sed*, *seg*, *selk*, *sell*, *selm*, *seln*, *selo*, *selp*, *selq*) by single PCR (Corbeil et al., 2019) revealed the presence of 27 amplicons matching those typical for *selo* (9), *sec* (8), *seg* (5), *seb* (4), and *sell* (1) genes. However, when assessed by sequencing, none of these amplicons was confirmed as an enterotoxin gene. In addition, no potentially enterotoxigenic strains were identified from among 102 bovine milk CNS isolates collected from cows with clinical and subclinical mastitis and tested for 17 enterotoxin genes by Nemati et al. (2008). Interestingly, a high percentage of potentially

enterotoxigenic strains among CNS isolates was noted when multiplex PCR was the only method used to confirm it.

According to Bertelloni et al. (2015), among 74 CNS strains isolated from bovine milk of the Tuscan district (Italy) origin and tested for 5 classical enterotoxins (*sea*, *seb*, *sec*, *sed*, *see*), over half (54.1%) harbored 1 or 2 enterotoxin genes, mostly the *sea* gene (41.9%).

Strangely enough, the highest percentage of potentially enterotoxigenic CNS was noted for strains isolated from bovine milk collected from 2 organic milk farms in Brazil (Siqueira et al., 2017). With only 10

CNS strains subjected to analysis, 8 of them, identified as *S. xylosus* (7) and *S. epidermidis* (1), were presumed to be carriers of *sea* (3), *sec* (4), and *sea* and *sec* (1) genes, respectively.

Among 263 bovine milk CNS isolates from mastitic cows that were screened for genes encoding 18 enterotoxins by multiplex PCR, 82 (31%) were presumed carriers of from 1 to 7 enterotoxin genes (Park et al., 2011). Of these 82 strains, 2 harbored the *seg* gene in their genome (*S. xylosus* and *S. epidermidis*). Similar surveys conducted with 30 *S. xylosus* strains collected from milk from cows with mastitis revealed that 16 of the strains harbored from 1 to 9 enterotoxin genes, mostly the “new” ones, *ser*, *selu*, and *selo*, in particular (Fijałkowski et al., 2014).

According to proximate data, new enterotoxins, including enterotoxin G, are responsible for 5% of staphylococcal foodborne poisoning outbreaks annually (Tang et al., 2011). As yet, an emetic activity of G, I, M, N, and O enterotoxins in humans is still questionable; however, the administration of G enterotoxin caused diarrhea in all rhesus monkeys tested, accompanied by vomiting in 66% of them (Munson et al., 1998).

As we showed through our work, the presence of amplicons on electrophoregrams for CNS strains exactly matching the size typical for the G enterotoxin gene in reference *S. aureus* does not necessarily mean that the tested strain carries enterotoxin genes with exactly the same nucleotide sequence as in the reference strain. As indicated elsewhere (Soares Casaes Nunes et al., 2015), the homology between the *seg* genes of *S. epidermidis* from salami and reference *S. aureus* strain AY158703.1 was 60%. Moreover, 100% nucleotide sequence similarity does not necessarily confirm the case for reference *S. aureus*. The *seg* gene nucleotide sequences of *S. aureus* (SAP097A_021) and *S. aureus* (SAP047A_027), for example, differ by 3 nucleotides in positions 15, 93, and 678 (https://www.ncbi.nlm.nih.gov/nuccore/NC_019010.1?report=fasta&from=15535&to=16314, https://www.ncbi.nlm.nih.gov/nuccore/NC_013331.1?report=fasta&from=20366&to=21145).

To the best of our knowledge, the current study is the first to provide an explanation for the formation of false-positive results in electrophoregrams and to include detailed analytical documentation confirming the appearance of nonspecific amplicons.

Table 2. The experimental explanatory notes for false-positive results (FPR) when CNS enterotoxin genes were detected by multiplex/duplex PCR¹

PCR	Primers used	Primers coupled	Amplicon's emergence	Primer strands responsible for FPR	Amplicon presence	Identification results	
						CNS strains (no.)	<i>Staphylococcus</i> sp.
Multiplex I	see	seo/sel	—	x	x		
	seb	seo/sem	—	x	x		
	sem	seo/seb	—	x	x		
	sel	seo/see	—	x	x		
	seo	sel/sem	—	x	x		
		sel/seb	—	x	x		
		sel/see	—	x	x		
		sem/seb	—	x	x		
		seb/see	—	x	x		
		sem/see	+	sem-1/see-1	—		
			(17)	sem-1/see-2	—		
				sem-2/see-1	—		
				sem-2/see-2	+	197, 199, 236, 244, 245, 249, 250, 251, 304, 317, 352, 357, 399, 402, 406, 447, 564	<i>S. epidermidis</i>
Duplex I		sed/sek	+	sek-1/sed-1	—		
			(7)	sek-2/sed-1	—		
				sek-2/sed-2	—		
				sed-1/sed-2	—		
				sek-1/sek-2	+	502	<i>S. warneri</i>
				sek-1/sed-2	+	217	<i>S. schleiferi</i>
						288	<i>S. xylosus</i>
						625	<i>S. chromogenes</i>
						211, 441, 443	Species not identified

¹— = no amplicon present; + = amplicon present; x = no amplicons (no further steps undertaken). Values in parentheses indicate the number of strains involved.

In our opinion, the formation of nonspecific amplicons leading to false-positive results excludes the multiplex/duplex PCR method from being the sole method for assessing the enterotoxigenic potential of the CNS. Based on our report, unverified results from multiplex PCR, either by single PCR, sequencing, or both, might lead to an overestimation of the enterotoxigenic CNS spread scale in food items.

Our surveys revealed that the prevalence of enterotoxigenic CNS among bovine milk isolates from dairy farms in the Western Pomerania district, Poland, is low, which suggests that they likely pose only a mild health risk to the public.

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