DETERMINATION OF STAPHYLOCOCCAL ENTEROTOXINS IN CHEESE BY IMMUNOENZYME ASSAYS

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Abstract - Staphylococcal food poisoning is one of the most common foodborne diseases resulting from the ingestion of staphylococcal enterotoxins (SEs) preformed in foods by enterotoxigenic strains of coagulase-positive staphylococci (CPS), mainly *Staphylococcus aureus*. The presence of enterotoxigenic strains of coagulase-positive staphylococci in raw milk during the production process leads to the contamination of products and outbreaks of alimentary intoxication. The problem of *Staphylococcus aureus* in cheese remains significant on a global level. Domestic cheese contaminated with enterotoxigenic staphylococci can result in the formation of enterotoxin, which can produce foodborne illness when the product is ingested. Due to microbiological contamination, microbiological criteria are tools that can be used in assessing the safety and quality of foods. In order to avoid foodborne illness, the Serbian Regulation on General and Special Conditions for Food Hygiene (Official Gazette of RS, No. 72/10) provides microbiological criteria for staphylococcal enterotoxins in dairy products.

Key words: Domestic cheese, staphylococcal enterotoxins, foodborne illness, immunoenzyme assays

INTRODUCTION

Raw milk, as one of the basic foodstuffs, must be hygienically suitable as it can have an important negative effect on human health. The content of nutritive ingredients in milk, a pH of 6.6 and udder temperature of 38°C create the ideal conditions for the development of bacteria. Milk products, unpasteurized cheeses in particular, are very good media for the development of *S. aureus*. Cheese can be contaminated with this pathogenic bacterium through milk during the production process or by subsequent contamination (Samaržija et al., 2007). Compared with other pathogenic bacteria, the number of infective *S. aureus* necessary to cause an illness is relatively high and amounts to 10^5cfu/mL/g. The reason for this lies in the fact that the cause of illness is not the vegetative body of the bacterium but the enterotoxins that are produced (Lindquist et al., 2002). A great number of *S. aureus* strains produce extracellular thermostable staphylococci enterotoxins, which retain their biological activity even after thermal treatment (Fox et al., 2000). A direct threat to human health from the staphylococcal enterotoxins is their thermal resistance, and resistance to most proteolytic enzymes such as trypsin and pepsin, which enable their passage through the digestive tract in an intact form (Bennett, 2001). Based on epidemiological studies, it was concluded that the infective dose of staphylococcal enterotoxins for humans is from <1 μg to 40 μg (Ikeda et al., 2005). All staphylococcal enterotoxins are proteins with a relatively small molecular mass, from 26,900 to 29,600 Da. "Classical" enterotoxins include five main types of staphylococcal enterotoxins, A, B, C, D and E (SEA – SEE), which are thought to be re-

sponsible for 95% of all staphylococcal poisoning (Bergdoll and Wong, 2006). To date, 20 types have been identified and described: SEA-SEE, SEG-SER and SEU (Jörgensen et al., 2005; Hennekinne et al., 2006). Depending on the pH value, SEC is classified into 3 subgroups – SEC1, SEC2 and SEC3 (Kérouanton et al., 2007). SEA is the most important enterotoxin in staphylococcal poisoning outbreaks (>75% of outbreaks), followed by SED, SEC and SEB. Outbreaks associated with SEE are very rarely reported, and only SEA, SEB, SEC, SED and SEE are actually detectable by currently available enzyme immunosorbent assay (EIA) kits.

MATERIALS AND METHODS

The TRANSIA PLATE Staphylococcal Enterotoxin (ST0796; Raisio, Biocontrol) is intended to be used for detection of staphylococcal enterotoxins A, B, C, D and E in food samples and in culture supernatants. The method is based on a sandwich-type ELISA. The solid support of the reaction is a microtiter plate with divisible strips coated with antibodies specific for staphylococcal enterotoxins. The optical density is the average of negative controls plus 0.20 : T =(NC1+NC2) / 2 +0.20. The sample is considered positive if its optical density is higher or equal to the threshold. The sample is considered negative if its optical density is lower than T-0.05. Between T-0.05 and T, the sample is considered doubtful. For extraction of staphylococcal enterotoxins, we used dialysis concentration procedures (polyethylene glycol, mol. wt 20,000, cellulose dialysis bag □ 6,000-8,000 Da). The detection limit is 0.25ng/ml.

The RIDASCREEN SET kit (Ridascreen set Total R4105, R-Biopharm GmbH, Darmstadt, Germany) is a commercial staphylococcal enterotoxin (SE) visual immunoassay kit. The kit utilizes monovalent capture antibodies against SE types A to E (SEA to SEE) and it simultaneously detects and identifies the enterotoxin type. A cut-off value for the evaluation of a result as negative or positive is calculated by adding 0.15 to the OD value of the negative control. The detection limit is 0.25ng/ml.

The VIDAS™ SET2, a new generation VIDAS™ staphylococcal enterotoxin test (SET2, 30701; bioMérieux) allows simultaneous detection of seven enterotoxin serotypes (SEA, SEB, SEC_1, SEC_2, SEC_3, SED and SEE). The method uses an enzyme-linked fluorescent assay (ELFA) with monoclonal anti-enterotoxin antibodies. After extraction (a protocol without concentration), the sample (500 μl) is placed in the sample well of a VIDAS™ SET reagent strip and detection is carried out using the VIDAS automated System: the results are given in a relative fluorescence value (RFV). The system automatically measures the fluorescence of the newly formed compound of 4-methyl umbelliferone at 450 nm, and then calculates and compares the result with the internal reference and interprets the result as positive or negative. The detection limit is 0.25ng/ml.

RESULTS AND DISCUSSION

The results of testing are shown in Tables 1, 2, 3, 4, 5 and 6.

Based on the results of testing 60 cheese samples for the presence of coagulase-positive staphylococci, presence was detected in 30 (50%) samples (Table 1). Cheese samples taken during the summer period showed contamination higher than 70% (21 samples) in contrast to the winter period when the presence of coagulase-positive staphylococci was detected in 9 samples (30%). The total number ranged from 3.079±0.17 to 6.41±0.03 (MS ± Sd, $\log_{10}$cfu/g). During the summer period, the lowest frequency was detected in sample 18 (3.079±0.17), and the highest in sample 27 (6.41±0.03). During the winter period, the lowest value detected was for sample 50 (3.38±0.21), and the highest for sample 39 (6.07±0.32). Samaržija (2007) obtained similar results, stating that out of 89 samples of autochthonous cheese types produced in Croatia, 54% were positive for the presence of *S. aureus*. In France, during 2009, six household staphylococcal food poisoning outbreaks were recorded. Cheese samples were available from six outbreaks and the staphylococcal food-poisoning diagnosis was confirmed through the high count of coagulase-positive staphylococci (Ostyn et al., 2011).

Table 1. Total number of coagulase-positive staphylococci in samples of domestic cheese (MS ± Sd, $\log_{10}$cfu/g).

	SUMMER PERIOD		WINTER PERIOD
Sample	Total number (MS ± Sd, $\log_{10}$cfu/g)	Sample	Total number (MS ± Sd, $\log_{10}$cfu/g)
1	5.86±0.06	31.	ND
2	ND	32.	5.86±0.02
3	4.92±0.12	33.	ND
4	6.11±0.16	34.	ND
5	ND	45.	ND
6	ND	36.	5.79±0.16
7	5.96±0.09	37.	5.92±0.11
8	5.97±0.03	38.	ND
9	4.20±0.21	39.	6.07±0.32
10	5.79±0.17	40.	ND
11	6.20±0.04	41.	ND
12	ND	42.	ND
13	ND	43.	ND
14	ND	44.	5.97±0.04
15	ND	45.	4.64±0.12
16	5.47±0.06	46.	5.85±0.03
17	6.30±0.03	47.	ND
18	3.079±0.17	48.	ND
19	ND	49.	ND
20	5.90±0.21	50.	3.38±0.21
21	6.30±0.33	51.	ND
22	6.34±0.30	52.	ND
23	5.71±0.09	53.	5.96±0.07
24	ND	54.	ND
25	5.87±0.23	55.	ND
26	5.53±0.16	56.	ND
27	6.41±0.03	57.	ND
28	5.68±0.09	58.	ND
29	4.69±0.26	59.	ND
30	5.95±0.32	60.	ND

ND – presence not detected; MS- mean value; Sd – standard deviation.

The production of domestic cheese is often accompanied by poor hygienic conditions. However, for an objective assessment of the real microbiological risk for human health, because of the possible presence of *S. aureus* and/or SEs in cheese, the only relevant one is the selection of appropriate analytical methods that can provide the relevant data on hygienic suitability from start to finish of the technological process in cheese production.

Verification of the Transia kit showed traceability of parameters (Table 2). The absorbance of positive and negative controls was ≥ 0.5 and ≤ 0.3, respectively. The absorbance of doubtful results ranged from 0.287 to 0.337. The obtained results complied with the kit performances. The external standards of 0.5 ng/g and 1 ng/g (negative samples spiked with the enterotoxin A, S9399, Sigma – Aldrich), were considered positive for staphylo-

Table 2. Verification of TRANSIA assay kit.

Parameters	Absorbance		Average	SD
Positive control	0.789	0.771	0.780	0.013
Negative control	0.137	0.141	0.139	0.003
Negative control	0.132	0.139	0.136	0.005
T	T = (NC1+NC2)/2+0.2 = 0.337			
T-0.05	0.287			
External standard 0.5ng/g	1.723	1.869	1.796	0.103
External standard 1ng/g	☐2.200	1.824	2.012	0.266

Table 3. Verification of the RIDASCREEN assay kit.

Parameters	Absorbance		Average	SD
Positive control	1.206	1.230	1.218	0.017
Negative control	0.092	0.086	0.089	0.004
Cut-off	0.239			
External standard 0.5 ng/g	☐2.200	1.920	☐2.060	0.198
External standard 1 ng/g	☐2.200	☐2.200	☐2.200	0.000

Table 4. Verification of the Vidas Set 2.

Parameters	RFV		Average	SD
Negative control	39	47	43	5.65
Positive control	570	528	549	29.69
External standard 0.5 ng/g	6866	5920	6393	668.923
External standard 1 ng/g	11314	10390	10852	653.367

coccal enterotoxin because their optical density was higher than the threshold.

Verification of the Ridascreen assay comprised visual and photometric analyses. Namely, based on the kit performances, it was specified that the test is valid only if both criteria (photometric and visual) are met. Visual and photometric analyses complied with the kit performances. The kit showed compliance of the positive and negative controls in both repeated tests. The cut-off value for evaluation of the result was 0.239. The external standards of 0.5 ng/g and 1 ng/g (negative samples spiked with enterotoxin A, S9399, Sigma – Aldrich), were considered positive for staphylococcal enterotoxin because their optical density was higher than the cut-off value (Table 3).

Verification of the Vidas Set 2 comprised the analysis of positive and negative controls and spiked samples in concentrations of 0.5 ng/ml and 1 ng/ml

(Table 4). Results were interpreted in accordance with the threshold. Results with test values less than the low threshold ≤0.13 indicated a sample without a detectable enterotoxin (negative sample). Samples with test values greater than (or equal to) the high threshold ≥0.13 are reported as positive. The external standards of 0.5 ng/g and 1 ng/g (negative samples spiked with the enterotoxin A; S9399, Sigma-Aldrich), were considered positive for staphylococcal enterotoxin, according to the RFV values.

According to the test results, out of 60 samples 24 samples were tested for the presence of SEs (Table 5). Out of 30 samples positive for the coagulase-positive staphylococci, the samples 3, 9, 18, 29, 45 and 50 were not tested for the presence of SEs because the total number of coagulase-positive staphylococci was below 10^5 cfu/g. The results of testing for the presence of SEs using the Transia kit showed that 17 (23.61%) samples were positive, 5 (6.94%) were negative (4, 17,

Table 5. Presence of staphylococcal enterotoxins in cheese samples.

Sample	TRANSIA			RIDASCREEN			VIDAS set 2		
	OD	OD	MS±SD	OD	OD	MS±SD	RFV	RFV	MS±SD
1	1.697	1.731	1.714±0.024	1.968	1.875	1.922±0.066	11267	9843	10555±1006.92
4	0.087*	0.088*	0.088±0.001	0.131*	0.139*	0.135±0.006	132*	103*	117.5±20.51
7	0.361	0.339	0.35±0.016	0.764	0.864	0.814±0.071	1257	2016	1636.5±536.69
8	0.863	0.942	0.903±0.056	1.235	1.547	1.391±0.221	2567	4502	3534.5±1368.25
10	1.045	1.210	1.128±0.117	1.879	2.013	1.946±0.095	5339	5289	5314±35.36
11	1.848	1.736	1.792±0.079	1.322	1.542	1.432±0.156	5840	6016	5928±124.45
16	0.572	0.496	0.534±0.054	0.983	0.938	0.961±0.032	5280	5015	5147.5±187.38
17	0.127*	0.173*	0.15±0.033	0.022*	0.031*	0.027±0.006	210*	198*	204±8.49
20	0.922	0.964	0.943±0.03	1.649	1.724	1.687±0.053	7863	8012	7937.5±105.36
21	0.192*	0.132*	0.162±0.042	0.073*	0.042*	0.058±0.022	121*	90*	105.5±21.92
22	0.216*	0.206*	0.211±0.007	0.109*	0.164*	0.137±0.039	321*	230*	275.5±64.35
23	1.527	1.534	1.531±0.005	2.045	1.982	2.014±0.045	3946	4014	3980±48.08
25	0.221*	0.162*	0.192±0.042	0.093*	0.134*	0.114±0.029	145*	200*	172.5±38.89
26	2.283	2.102	2.193±0.128	1.769	1.467	1.618±0.214	5972	6480	6226±359.21
27	0.227	0.326	0.277±0.07	0.173*	0.224*	0.199±0.036	121*	239*	180±83.44
28	1.075	1.232	1.154±0.111	1.489	1.635	1.562±0.103	5389	6284	5836.5±632.86
30	0.786	0.824	0.805±0.027	0.987	1.264	1.126±0.196	4290	3324	3807±683.07
32	0.773	0.631	0.702±0.1	1.288	1.301	1.295±0.009	5690	6345	6017.5±463.15
36	1.783	1.824	1.804±0.029	1.775	1.870	1.823±0.067	11200	13000	12100±1272.79
37	1.434	1.536	1.485±0.072	1.784	1.339	1.562±0.315	9823	9902	9862.5±55.86
39	0.792	0.921	0.857±0.091	0.796	0.721	0.759±0.053	7345	9246	8295.5±1344.21
44	0.639	0.573	0.606±0.047	2.016	1.794	1.905±0.157	12657	11090	11873.5±1108.04
46	1.043	1.132	1.088±0.063	1.477	1.739	1.608±0.185	8765	7560	8162.5±852.06
53	0.432	0.449	0.441±0.012	0.749	0.499	0.624±0.177	720	1125	922.5±286.38

Values reported are means of duplicates. OD – optical density; MS±SD – mean value, standard deviations; VIDASTM SET2 - RFV Relative Fluorescence Value; *- negative results; doubtful results are written in bold characters.

21, 22 and 25), whereas 2 (2.78%) samples (7 and 27) were defined as doubtful. Regarding the Ridascreen kit, 18 cheese samples were positive for the presence of SEs while 6 (4, 17, 21, 22, 25 and 27) were negative. There were no doubtful results. For the doubtful samples after testing by the Transia kit, the Ridascreen kit produced the following interpretation: sample 7 was positive for the presence of SEs while sample 27 was negative. Testing by the Vidas Set 2 gave results identical to those given by the Ridascreen kit: out of 24 samples, 18 (25%) were positive for the presence of SEs whereas 6 (4, 17, 21, 22, 25 and 27) samples were

negative (8.3%). The obtained test results correspond to the results of Vernozy et al. (2004), Hennekinne et al. (2006) and Ostyn et al. (2011). Results of verification complied with the performances of commercial kits. Spiked samples with external standards indicated a contamination with enterotoxin. The test results showed a strong correlation with the OD and RFV values. Results obtained with the Ridascreen and Vidas methods were identical, whereas the doubtful results obtained by the Transia method can be associated with the procedure of sample preparation. Namely, in order to increase the method sensitivity,

a dialysis concentration step of the water extract towards PEG (over night incubation) could cause false positive or doubtful results, decreasing the overall method specificity.

As a result of testing the performances of immunoenzymatic methods, Vernozy et al. (2004) came to the conclusion that VIDAS ™ SET2 had a greater specificity (100%) and sensitivity than TRANSIA PLATE Staphylococcal Enterotoxins. The high performances of VIDAS™ SET2 might be, at least in part, because of the use of new monoclonal antibodies and polyclonal antibodies directed against different antigenic sites. The high affinity of these antibodies could lead to a greater sensitivity. The VIDAS ™ SET2 test without the extraction step needs only 80 min to perform. Unlike the TRANSIA PLATE, they are automated detection tests, 'user friendly' and can be incorporated into a Hazard Critical Control Point (HACCP) program (Su and Wong, 1997). For these reasons, they can be used for large-scale enterotoxin screening of food.

CONCLUSION

A conclusive staphylococcal food-poisoning diagnosis is mainly based on the detection of staphylococcal enterotoxins in food. Therefore, there is a need for specific and sensitive methods for detecting these enterotoxins. Our test results suggest that all three immunoenzymatic methods are equally effective in the detection of staphylococcal enterotoxins in cheeses from domestic production. However, matrix preparation, the duration of analysis, specificity and sensitivity, give an advantage to the Ridascreen Total Set and the Vidas Set 2 Automated System.

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