Methicillin resistance and enterotoxigenicity of Staphylococci isolated from milk and white cheese in Iraq

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Abstractr

Two hundred staphylococcal isolates isolated from milk and white cheese samples, which were collected from local markets in Baghdad. The predominant species was *Staphylococcus aureus* 97 isolates (48.5%), followed by *S.chromogenes* 82 (41%) and 21 (10.5%) *S.epidermidis* isolates. The pattern of antibiotic susceptibility of Coagulase Positive Staphylococci (COPS) and Coagulase Negative Staphylococci (CONS) isolates to 3 antibiotics (Methicillin, Tetracyclin and Vancomycin) was determined using disc diffusion method; the results revealed that 80 *S. aureus* isolates (82.47%) found to be methicillin resistant (MRSA) while 8 isolates (8.24%) were vancomycin resistant (VRSA) and 18 *S. aureus* isolates (18.5%) resist tetracycline antibiotic. Sixty four CONS isolates (62.13%) were methicillin resistant, 28 CONS isolates (27.18%) resist tetracycline, and 5 CONS isolates (4.85%) were vancomycin resistant. Suckling mouse bioassay was tested to investigate the staphylococcal enterotoxin biological activity. Results showed that 131 isolates which constitutes 65.5% of the examined isolates, gave a positive result. Both COPS and CONS isolates were shown to be enterotoxigenic, COPS represented by *S. aureus* species occupied the higher ratio of the enterotoxigenic staphylococci, 86 *S.aureus* isolates (65.64% of the enterotoxigenic staphylococci) gave the positive ratio of the intestine weight to the body weight which was ≥ 0.083 while 45 CONS isolates (34.35%) were enterotoxin producers. These toxins were thermostable staphylococcal enterotoxins which gave the same toxic effect after heating to 100°C for 30 minutes.

Keyword: Staphylococci, Methicillin resistance, Enterotoxin, Milk and cheese.

مقاومة المثيسيلين وانتاج السموم المعوية من المكورات العنقودية المعزولة من الحليب و الجبن الأبيض في العراق

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الخلاصة:

عزلت 200 عزلة من المكورات العنقودية من عينات الحليب والجبن الأبيض التي تم جمعها من الأسواق المحلية في بغداد، وكانت المكورات العنقودية الذهبية *Staphylococcus aureus* هي الأكثر شيوعا في العينات إذ بلغ مجموع العزلات 97 عزلة (48.5%)، تلاها النوع *S.chromogenes* 82 عزلة (41%) ثم النوع *S.epidermidis* 21 عزلة (10.5%). تم تحديد اختبار الحساسية لعزلات المكورات العنقودية الموجبة و المكورات العنقودية السالبة للإنزيم المخثر للبلازما لثلاث من مضادات الحياة (المثيسيلين، التتراسايكلين، والفانكومايسين) باستخدام طريقة الانتشار بالأقراص. أظهرت النتائج أن 80 عزلة *S. aureus* (82.47%) كانت مقاومة للمثيسيلين بينما كانت 8 عزلات مقاومة للفانكومايسين و 18 (18.5%) عزلة من *S. aureus* مقاومة لمضاد الحياة تتراسايكلين. بينما كانت 64 عزلة (62.13%) من المكورات العنقودية

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السالبة للإنزيم المخثر للبلازما مقاومة للمثيسيلين و 28 عزلة (27.18%) مقاومة للتتراسايكلين و 5 عزلات (4.85%) مقاومة للفانكومايسين. تم اختبار تجريع الفئران الرضيعة في التحري عن الفعالية الحيوية للسموم المعوية للمكورات العنقودية واظهرت النتائج ان 131(65.5%) عزلة انتجت سموما معوية فاعلة احيائيا. بينت النتائج أن كلا من المكورات العنقودية الموجبة و السالبة للإنزيم المخثر للبلازما كانت منتجة للسموم المعوية، وقد شكل النوع *S. aureus* 65.64% من المجموع الكلي للعزلات المنتجة بينما كانت المكورات العنقودية السالبة للإنزيم المخثر للبلازما منتجة للسموم المعوية بنسبة 34.35% ، وتم اختبار ثبات هذه السموم في ظروف الحرارة العالية وأكدت النتائج إن هذه السموم المعوية ثابتة بالحرارة إذ بقيت فعالة إحيائيا حتى بعد تعرضها للتسخين لمدة 30 دقيقة في درجة حرارة 100°C.

Introduction:

Food is an important factor for the transfer of antibiotic resistance. Such transfer can occur by means of antibiotic residues in food, through the transfer of resistant food-borne pathogens or through the ingestion of resistant strains of the original food microflora and resistance transfer to pathogenic microorganisms, *S. aureus* strains are known to be frequently resistant to antibiotic therapy due to their capacity to produce an exopolysaccharide barrier and because of their location within microabscesses, which limit the action of drugs [1,2].

As previously related, it is well known that determinants of resistance to antibiotics and other toxic substances in staphylococci, as in other pathogens, are largely carried by accessory genetic elements, especially plasmids, transposons and their relatives, a particularly important resistance determinant in staphylococci is that for methicillin and other β -lactam compounds, giving rise to the notorious MRSA acronym.

Staphylococcal Food Poisoning (SFP) leads to inflammatory changes throughout the gastrointestinal tract with severe lesions in the jejunum and ileum. The direct inhibitory effect of purified Staphylococcal Enterotoxins (SEs) on intestinal tone, contractility and colonic transit has been noted in the laboratory animals, ingestion of SEs within food cause food poisoning, which is characterized by severe vomiting and diarrhea, those symptoms occur within hours after eating of SE-contaminated food [3,4]. Despite the significant progress in the understanding of staphylococcal enterotoxin associated inflammation of gastrointestinal tract, it is still unclear how this inflammation is initiated *in vivo* and what is the exact role of each of the immune and non immune cells that contribute to the progression of the disease [5].

Suckling Mouse Bioassay was used in order to detect the ability of staphylococcal isolates to secrete extracellular heat stable enterotoxins in their broth culture supernates, by testing the enteotoxins' biological activity; 1 mouse unit of SE activity was defined arbitrarily as the amount of toxin producing a ratio of intestinal weight to carcass weight of 0.083.

Regarding the facts above this research aimed:

- Isolate and assessment of the spread of staphylococci in milk and white cheese.
- Detection of the presence of MRSA among the staphylococcal isolates isolated from milk and white cheese.
- Testing the suckling mouse bioassay to detect the staphylococcal enterotoxigenicity.

Methods

I- Staphylococcal Isolation and Identification

The Iraqi Standard Criterion No.3/2270 in Isolation, Enumeration and Identification of Microbiological Groups in Foods, [6] was depended in staphylococcal isolation and identification.

II- Antibiotic Susceptibility Test

The modified Kirby-Bauer method was used. The diameter of inhibition zone for individual antimicrobial agent was translated in terms of sensitive, intermediate and resistant categories by comparison with the standard inhibition zone [7,8], table 1.

Table 1- Diameter interpretive standards of inhibition zones according to NCCLs [7-9]

ID	Antimicrobial Agent	Disc Potency	Diameter of inhibition zone (mm)		
			Resistant	Intermediate	Sensitive
1	Methicillin	5 µg	≤ 9	10-13	≥ 14
2	Tetracyclin	30 µg	≤ 14	15-18	≥ 19
3	Vancomycin	30 µg	≤ 12	14-16	≥ 17

III- Detection of Thermostable Enterotoxins by Suckling Mouse Bioassay

This bioassay was illustrated by: Newborn Swiss albino suckling mice (2-4 days old) were separated from their mothers immediately before use. A 0.1ml of broth culture supernatant had been administered orally to each mouse, and then the mouse was left at 25°C for 2 hrs. After administration, the mice were killed by decapitation, the abdomen was opened, and the entire intestines (not including the stomach), was removed with forceps. The intestines from each group of three mice were pooled and weighted, and the ratio of gut weight to remaining carcass weight was calculated [10,11] .

❖ Preparation of crude culture filtrates

Staphylococcal isolates were inoculated in a Trypticase Soy Broth (TSB) containing 0.6% yeast extract for 48 h at 37°C in a shaker incubator. Then the culture was centrifuged for 10 min in an eppendorf centrifuge with a speed of 3000 rpm, the obtained supernatant was transported in the amount ranging from 1 to 1.5 ml to a test tube, and prepared to be administered orally to the suckling mice directly or after heated at different temperatures (65 and 100°C) for various periods of time (15, 30 min).

Results:

Staphylococcal prevalence

Two hundred colonies were identified morphologically as gram positive cocci arranged in grape-like irregular clusters which are characteristics to *Staphylococcus spp*. [12]. Growth and characteristcs of the isolates on Staph No 110, mannitol salt agar, Baird-Parker agar, milk agar and blood agar in addition to the results obtained by applying biochemical tests: catalase, oxidaes, coagulase, DNase, urease, gelatinase, and growth in different salt concentrations showed that 97 (48%) of the staphylococcal isolates isolated from milk and white cheese samples were *S.aureus*, followed by 82 (41%) *S.chromogenes* and 21 (11%) *S.epidermidis*, figure 1.

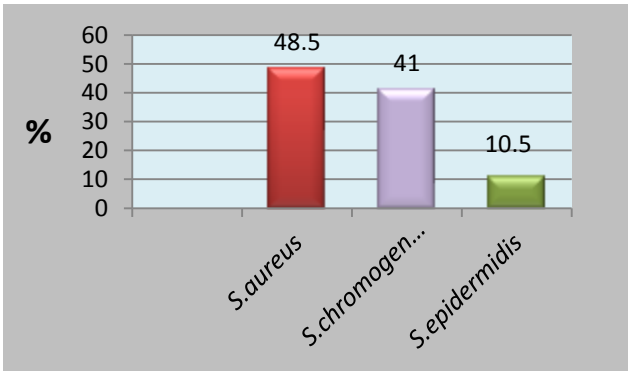

Figure 1- The prevalence of *Staphylococcus aureus* according to all the Staphylococcal isolates isolated from milk and cheese samples

Contamination of food products with *S. aureus* pathogens may result from their presence in the basic raw material - milk, this is of great importance especially in countries with large production of dairy products such as cheeses [13].

Coagulase negative staphylococci (CONS) species most commonly isolated from mastitic milk were *S.chromogenes* and *S. simulans*. *S. chromogenes* was the major CONS species in subclinical mastitis, the majority of CONS isolates in milk samples in a research in USA, were *S. chromogenes* [14].

Antibiotic Susceptibility

The pattern of antibiotic susceptibility of COPS and CONS isolates to 3 antibiotics (Methicillin, Tetracyclin and Vancomycin) was determined using disc diffusion method according to the guidelines recommended by the National Committee for Clinical Laboratory Standards (NCCLs)[9]. Figure 2- depicted the results of antibiotic susceptibility of COPS and CONS isolates isolated from food samples.

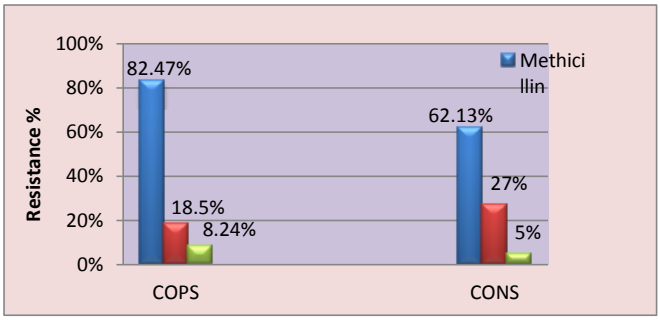

Figure 2- Antibiotic Susceptibility of COPS (*S. aureus*) and CONS isolates

Results of antibiotic susceptibility obtained by this study confirmed that 80 *S. aureus* isolates (82.47%) found to be methicillin resistant (MRSA), while 8 isolates (8.24%) were vancomycin resistant (VRSA). Eighteen *S. aureus* isolates (19%) resist tetracycline antibiotic. Resistance to tetracycline occurs by three mechanisms efflux, ribosomal protection, and chemical modification [15]. In an investigation done by [16], a high sensitivity (98%) was recorded to methicillin in the examined *S. aureus* isolates isolated from buffaloes.

It was found that *S. aureus* isolates were highly sensitive to tetracycline and vancomycin as 100% by [17], vancomycin sensitive *S. aureus* were also reported by [18]. Fourty one percent of *S. aureus* isolates showed to be tetracycline resistant in a study done by [19], regarding vancomycin, high sensitivity was reported as 100%.

Results obtained from a study done in Iran by [20] showed that 54% of *S. aureus* isolates isolated from raw and pasteurized milk and ice cream were MRSA, while only 7% of them were VRSA, and 23% were tetracycline resistant and the rest were sensitive.

Antibiotic resistance is a major public health concern since resistant bacteria can persist and circulate in the environment with possible transmission to humans via contaminated food and water, consequently MRSA is currently the most commonly identified antibiotic-resistant pathogen in many countries worldwide [21, 22].

COPS isolates derived from raw milk and cheese exhibited resistance to vancomycin, this probably because MRSA isolates that are resistant to beta lactam drugs may develop induced resistance to vancomycin [23,24].

Regarding CONS isolates, they were methicillin resistant in 62.13 percent (64 isolates), 28 CONS isolates (27.18%) resist tetracycline, and only 5 CONS isolates (4.85%) were vancomycin resistant [1]. Results by local investigators showed higher methicillin resistance rates among CONS isolates of 84% and 86.6% respectively in the studies of [25 and 26]

It was reported that 30.8% of CONS were tetracycline resistant, but none of them resist vancomycin [19]. Vancomycin has been the major drug used for treatment of methicillin resistant staphylococci, but recovery of isolates with intermediate resistance and the reports of several cases of high level resistance to vancomycin have spurred the search for newer agents [15].

CONS isolatesin the study of [27] were multi-resistant in a percent 57.1%, these results provided evidence that carrying antimicrobial resistance genes has became reasonably widespread in milk and cheese samples.

The resistance to penicillin G is explained by the production of β-lactamase [28], while resistance to methicillin is encoded by *mecA* gene which is located on *Staphylococcus* cassette chromosome (SCC), the SCC can be transferred horizontally between various staphylococcal species which suggested that CONS acts as reservoir for the dissemination of resistance genes to COPS [29, 30].

Resistance to methicillin in CONS of dairy products was recorded worldwide [31-33].

The consumption of dairy products containing CONS is considered to be safe, no available reports of CONS associated health hazards following the ingestion of dairy products [33,34]. Nevertheless, the occurrence of multidrug-resistant CONS in dairy products and the possible transfer of drug resistance between microorganisms in food matrices, suggests that only specifically selected antibiotic susceptible isolates of CONS should be used in starter cultures [33,35].

Depending on the fact that SEs inducing diarrhea and causes inflammatory changes in the gastrointestinal tract [5, 36, 37], so that the suckling mouse bioassay was tested to investigate the staphylococcal enterotoxin biological activity.

Among the 200 staphylococcal isolates isolated from milk and cheese samples, examined with suckling mouse bioassay, 131 isolates which constitutes 65.5% of the examined isolates, gave a positive result as depicted by the tables 2 and 3.

Table 2- The biological activity of the COPS enterotoxins in suckling mouse bioassay represented by the intestine weight /body weight ratio

	Isolate	IW/BW		Isolate	IW/BW
1	S 14	0.083	8	S 31	0.083
2	S 15	0.084	9	S 40	0.112
3	S 16	0.087	10	S 41	0.085
4	S 17	0.113	11	S 50	0.087
5	S 21	0.083	12	S 51	0.033
6	S 27	0.112	13	S 55	0.111
7	S 30	0.083	14	S 56	0.089
	Isolate	IW/BW		Isolate	IW/BW
15	S 57	0.093	57	S 150	0.088
16	S 58	0.086	58	S 155	0.112
17	S 59	0.114	59	S 156	0.063
18	S 60	0.087	60	S 157	0.086
19	S 63	0.103	61	S 161	0.099
20	S 64	0.085	62	S 163	0.083
21	S 65	0.113	63	S 164	0.096
22	S 68	0.084	64	S 165	0.115
23	S 70	0.091	65	S 168	0.112
24	S 73	0.116	66	S 171	0.024
25	S 77	0.076	67	S 173	0.083
26	S 80	0.085	68	S 174	0.084
27	S 81	0.037	69	S 177	0.094
28	S 83	0.096	70	S 180	0.087
29	S 85	0.049	71	S 185	0.096
30	S 91	0.084	72	S 191	0.086
31	S 93	0.095	73	S 194	0.106
32	S 94	0.083	74	S 195	0.113
33	S 95	0.117	75	S 196	0.085
34	S 97	0.083	76	S 198	0.109
35	S 98	0.094	77	S 199	0.083
36	S 100	0.082	78	S 200	0.094
37	S 102	0.115	79	S 203	0.088
38	S 103	0.085	80	S 204	0.091
39	S 108	0.116	81	S 205	0.115
40	S 109	0.113	82	S 206	0.087
41	S 111	0.068	83	S 207	0.109
42	S 112	0.044	84	S 208	0.085
43	S 113	0.037	85	S 214	0.115
44	S 117	0.088	86	S 215	0.091
45	S 119	0.110	87	S 217	0.110
46	S 120	0.092	88	S 219	0.089
47	S 121	0.083	89	S 220	0.084
48	S 125	0.086	90	S 223	0.105
49	S 130	0.085	91	S 227	0.084
50	S 132	0.099	92	S 228	0.083
51	S 133	0.090	93	S 230	0.094
52	S 136	0.084	94	S 232	0.093
53	S 140	0.100	95	S 233	0.086
54	S 144	0.116	96	S 234	0.090
55	S 145	0.105	97	S 235	0.087
56	S 147	0.054			

All isolates were grown in TSB-YE medium for 48 h in shaker incubator, mouse incubation period 2 h at 25^{o}C. Intestine weight/body weight ratios of the control group ranged from 0 to 0.003.

Table 3- The biological activity of the CONS enterotoxins in suckling mouse bioassay represented by the intestine weight /body weight ratio

	Isolate	IW/BW		Isolate	IW/BW
1	S 1	0.042	6	S 7	0.037
2	S 2	0.087	7	S 8	0.075
3	S 3	0.079	8	S 9	0.088
4	S 4	0.051	9	S 10	0.005
5	S 5	0.092	10	S 11	0.039
	Isolate	IW/BW		Isolate	IW/BW
11	S 12	0.046	58	S 89	0.109
12	S 13	0.099	59	S 90	0.080
13	S 18	0.048	60	S 92	0.028
14	S 19	0.089	61	S 96	0.035
15	S 20	0.079	62	S 99	0.043
16	S 22	0.013	63	S 101	0.087
17	S 23	0.097	64	S 104	0.032
18	S 24	0.089	65	S 105	0.108
19	S 25	0.084	66	S 107	0.089
20	S 26	0.057	67	S 114	0.093
21	S 28	0.082	68	S 115	0.089
22	S 29	0.057	69	S 122	0.054
23	S 33	0.088	70	S 123	0.087
24	S 34	0.028	71	S 124	0.095
25	S 35	0.046	72	S 126	0.069
26	S 36	0.085	73	S 127	0.111
27	S 37	0.106	74	S 128	0.085
28	S 38	0.017	75	S 129	0.091
29	S 39	0.083	76	S 131	0.044
30	S 42	0.091	77	S 134	0.088
31	S 43	0.040	78	S 135	0.070
32	S 44	0.100	79	S 160	0.048
33	S 45	0.026	80	S 162	0.083
34	S 46	0.039	81	S 175	0.035
35	S 47	0.098	82	S 178	0.056
36	S 48	0.067	83	S 179	0.032
37	S 49	0.083	84	S 183	0.089
38	S 52	0.013	85	S 190	0.032
39	S 53	0.065	86	S 192	0.026
40	S 54	0.086	87	S 193	0.084
41	S 61	0.038	88	S 197	0.092
42	S 62	0.085	89	S 201	0.085
43	S 66	0.021	90	S 202	0.082
44	S 67	0.034	91	S 210	0.054
45	S 69	0.028	92	S 211	0.092
46	S 71	0.053	93	S 212	0.094
47	S 72	0.047	94	S 213	0.039
48	S 74	0.096	95	S 216	0.085
49	S 75	0.082	96	S 218	0.056
50	S 76	0.091	97	S 221	0.020
51	S 78	0.093	98	S 222	0.084
52	S 79	0.089	99	S 224	0.032
53	S 82	0.017	100	S 225	0.085
54	S 84	0.087	101	S 226	0.036
55	S 86	0.050	102	S 229	0.045
56	S 87	0.083	103	S 231	0.093
57	S 88	0.056			

All isolates were grown in TSB-YE medium for 48 h in shaker incubator, mouse incubation period 2 h at 25°C. Intestine weight/body weight ratios of the control group ranged from 0 to 0.003.
Enterotoxin production results which were detected by the Suckling Mouse Bioassay revealed that 131 (65.5%) of the staphylococcal isolates were enterotoxigenic, while the rest were not (34.5%), figure 2.

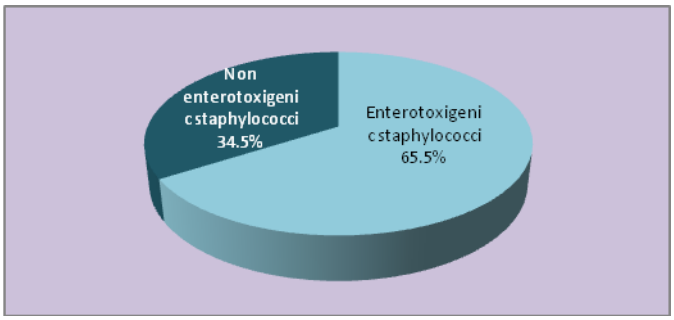

Figure 2- Enterotoxigenicity of the staphylococcal isolates isolated from milk and cheese samples, as assessed by the Suckling Mouse Bioassay

Both COPS and CONS isolates were shown to be enterotoxigenic, COPS represented by *S. aureus* species occupied the higher ratio of the enterotoxigenic staphylococci, 86 *S.aureus* isolates (65.64% of the enterotoxigenic staphylococci) showed positive ratio which was ≥ 0.083 Table 3-, while 45 CONS isolates (34.35%), table 2- were enterotoxin producers; figure 3.

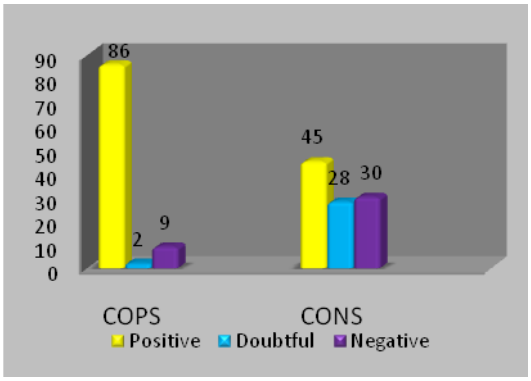

Figure 3- Diversity of Results in the biological test between coagulase-positive and coagulase-negative staphylococcal isolates

The staphylococcal enterotoxins, not the bacterium, settles in the small intestine and cause inflammation and swelling, this in turn can cause abdominal pain, cramping, dehydration, diarrhea and fever, SEs act as superantigens, binding to MHC II molecules and stimulating T cells to divide and produce lymphokines such as IL-2 and TNF-alpha, which induces diarrhea [5,38]. Assessment of staphylococcal isolates ability to produce enterotoxins was conducted by the use of suckling mouse bioassay, this test was devised initially by [39] and modified by [10] it was meant to assess the ability to produce enterotoxins by *E. coli* isolates, and after slight modifications it is still used as done by [11] to assess the ability of *Y.enterocolitica* isolates to produce enterotoxins.

It had been reported that the orally and intragastrically introduction of SEA and SEB to laboratory animals induced emetic responces, diarrhea and GI inflammatory changes [40].
The effect of heat on staphylococcal enterotoxins activity in suckling mice was detected; it had been applied on 4 Staphylococcal isolates.

Among all the examined crude preparations of ST, none were affected by heating at 65°C for 15 min and the same result was observed when the preparations of ST heated to 100°C for 30 min, table 4.

Table 4- Effect of heat on staphylococcal enterotoxin activity

Staphylococ--cal isolates	Toxin Activity IW/BW No heat mean ± SE	Toxin Activity IW/BW 65C^o, 15 min mean ± SE	X^2-Test value	Toxin Activity IW/BW 100C^o, 30 min mean ± SE	X^2-Test value
S 95	(0.117 ± 0.0003)	(0.117 ± 0.0012)	0.229 [a]	(0.116± 0.0012)	0.918 [a]
S 14	(0.083 ± 0.0015)	(0.085 ± 0.0035)	0.423 [a]	(0.084± 0.0010)	0.866 [a]
S 84	(0.087 ± 0.0058)	(0.091 ± 0.0064)	0.326 [a]	(0.091± 0.0081)	0.293 [a]
S 37	(0.106 ± 0.0025)	(0.104 ± 0.0055)	0.569 [a]	(0.104 ± 0.0015)	0.622 [a]

All isolates were grown in TSB-YE medium for 48 h in shaker incubator, mouse incubation period 2 h at 25^{o}C all values are mean.

a: statically non significant.

Statically analysis showed that there were no significant differences among the tested groups; the toxic activity has not been affected by applying heat even at 100^{o}C for 30 min. these results confirmed that these toxins are thermostable staphylococcal enterotoxins.

It was revealed that there was no influence of high temperature on the activity of the examined enterotoxins produced by *Y. enterocolitica*; even after heating to 65^{o}C for 15 min [11].

The detection of *S. aureus* and SEs in food is often difficult because food processing may kill the bacteria without destroying SEs, which are stable to high temperature and to inactivation by gastrointestinal proteases such as pepsin [40].

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