Prevalence of enterotoxigenic *Staphylococcus aureus* with characterization of enterotoxins for subsequent detection

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1. Abstract

In this work 68 *S. aureus* isolated from mastitic cows (n=48), camel milk (n=5) and human samples (urine n = 4, abscesses aspiration n = 3, sputum n = 2, nasal swabs n = 2, vaginal swabs n = 1 and wound swabs n = 3) were investigated using phenotypic and molecular identification to detect the occurrence of enterotoxins. Eighteen out of 48 *S. aureus* isolated from mastitic cows and 5 out of 15 *S. aureus* isolated from human samples were enterotoxin producers. All toxigenic *S. aureus* isolated from clinical mastitic cows (13) were toxin type D producer while *seb* and *sed* were detected from subclinical mastitic cows. Among human isolates, 2 isolates produced mixed toxins A and E, 2 isolates produced mixed toxins C and D and one isolate produced type C toxin. No toxin was detected from camel isolates. All toxigenic *S. aureus* isolated from cows (13) were toxin type D producer. Among human isolates 2 isolates produced mixed toxins A and E, 2 isolates produced mixed toxins C & D and one isolate produced type C toxin. Five *S. aureus* crude enterotoxin type D were investigated by protein SDS-PAGE. It was clear that all isolates had from 9 to 7 bands. All the isolates had a band at 148-159 kDa, 35-42 kDa, 28-30 kDa, 23-26 kDa, 20-22 kDa and 18-19 kDa. 4 isolates had a band at 48-55 kDa, 3 isolates had a band at 39-42 kDa, 3 isolates had a band at 31-34 kDa and one isolate had a band at 17 kDa.

Key words: Enterotoxin, Multiplex PCR, Mastitic milk, *S. aureus*, SDS.

2. Introduction

Staphylococcus aureus is a major threatening bacterium which is incriminated for many clinical infections; besides, it is a foremost reason of bacteremia in industrialized nations **[1].** *S. aureus* causes a wide scope of diseases that usually including the skin, delicate tissue, joints, and contaminations related with inhabiting catheters or prosthetic gadgets **[2,3].** *It* is a common pathogen of the skin and mucosal tissues of humans and animals, with approximately 20–30% for persistent while 60% for intermittent colonization **[4].** Staphylococcal food poisoning (SFP) is a disease condition that has been resulted from the consuming of foods containing abundant quantities of one (or more) preformed enterotoxin **[5].** The monitoring of SFP disease is of a public and commercial importance as it causes loss of working days and productivity, hospital costs, and

economical misfortunes in food ventures, cooking organizations and eateries **[6].**

S. aureus enterotoxins (SEs) are powerful gastrointestinal exotoxins synthesized by *S. aureus* during the logarithmic stage of growth or in between the exponential and the stationary phase **[7].**SEs are in a big toxins family that contains about 20 different staphylococcal and streptococcal exotoxins, with a shared phylogenetic relations, structure, role, and sequence homology. Currently, there are about 23 enterotoxins having distinct serological entities, including SEA, SEB, SEC, SED, and SEE. DNA-DNA hybridization and PCR were considered the most common, effective and reliable techniques that applied for toxin genotypes identification. A multiplex PCR was used for the detection of different genes of staphylococcal enterotoxins A to E (*entA, entB, entC, entD,* and *entE*), toxic shock syndrome toxin 1 (tst), exfoliative toxins A and B (*etaA and etaB*), and intrinsic methicillin resistance (*mecA*) **[8]**.

Electrophoresis is a common method for proteins separation based on size, shape and/or charge. Because of the importance of *S.aureus* enterotoxins in the public health and food sectors, the aim of the present work was the characterization of *Staphylococcus aureus* enterotoxins. It was achieved by collection of *Staphylococcus* isolates from different sources, identification of *Staphylococcus aureus* isolates, detection of *Staphylococcus aureus* enterotoxins by PCR and characterization of the staphylococcal enterotoxin by SDS.

2. Materials and Methods

Samples:

A total of 210 samples (cattle milk *n* = 125, camel milk *n* = 55 and human samples *n* = 30) were collected for detection of *Staphylococcus* species within a duration from April 2017 to January 2018 **(Table 1)**. The cattle milk samples were collected from bovine's dairy farms in Giza, Egypt. The human samples were collected from farms workers, patients from Cairo University Hospitals (CUH) and different human laboratories and the camel milk samples were collected from Aswan governorate.

Isolation and identification of *Staphylococcus aureus* from clinical samples:

A loopful from each sample was cultured on mannitol salt agar (Oxoid), 5% sheep blood agar and Baird Parker medium (Oxoid). The plates were incubated at 37^{o}C for 18–24 h and investigated for culture characters. The colonies were examined for: hemolysis on sheep blood agar, lecithinase activity on Baird Parker medium which contains egg

yolk tellurite emulsion **[9]** and mannitol fermentation on mannitol salt agar. Moreover, API ® Staph Kit (bioMe´rieux, France) was used for confirmation of the isolates following the instructions of manufacturer.

Molecular identification of toxin encoding genes of *S. aureus* [8]:

Polymerase chain reaction was conducted in Biotechnology Centre for Services and Research (BCSR) - Faculty of Veterinary Medicine, Cairo University. DNA of *S. aureus* isolates was extracted using Qiagen extraction kit according to manufacturer manual of Qiagen, Germany**.** The used primers were showed in **Table (2).** The specific amplified DNA bands were detected using transilluminator and compared to size marker ladder (Amersco Cleveland ,USA). Cowan, I strain of *S. aureus* was used as positive control **[10]**.

Characterization of enterotoxins extracted from *S. aureus* isolates by Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS PAGE)

Each *S. aureus* isolate was refreshed into tryptone soy broth and incubated at 37°C for 18 to 24 h with shaking. Cells were removed by centrifugation at 24,000 x g at 4°C for 20 min, and the supematants were sterilized by filtration through a 0.2-um filter (Schleicher & Schull, Dassel, Federal Republic of Germany). The supernatant was used for enterotoxin detection **[11].** The SDS PAGE was performed as described by **[12]**, SDS-polyacrylamide gel and performed according to the method described by **[13].**

4. Results

Identification of *S. aureus* among different used samples

S. aureus isolates appeared as yellow colonies on mannitol salt agar and beta hemolytic on blood agar. Biochemical reaction showed positive catalase and coagulase and microscopically appeared as Gram positive cocci arranged in clusters. Also, they showed positive reaction for staphytect test **(Figure1)**. All isolates were identified using API **(Figure 2)**.

As shown in **Table 3,** among the examined samples (210), 68 *Staphylococcus aureus* isolates were identified from mastitic cow (48), camel milk (5) and 15 human samples (urine *n* = 4), abscesses aspiration *n* = 3, sputum *n* = 2, nasal swabs *n* = 2, vaginal swabs *n* = 1 and wound swabs *n* = 3) with an incidence of 32.4%.

Prevalence of *S. aureus* enterotoxin producers among the isolates

Table 4 and Figure 3 illustrate that 18 out of 48 *S. aureus* isolated from mastitic cows and 5 out of 15 *S. aureus* isolated from human samples were enterotoxin producers. All toxigenic *S. aureus* isolated from clinical mastitic cows (13) were producer of toxin type D while *seb* and *sed*

were detected from subclinical mastitic cows. Among human isolates, 2 isolates produced mixed toxins A and E, 2 isolates produced mixed toxins C and D and one isolate produced type C toxin. No toxin was detected from camel isolates

Results of SDS - PAGE among some isolates.

Five *S. aureus* crude enterotoxin type D were investigated by protein SDS-PAGE and Coomassie blue staining **(Table 5 and Figure 4)**. Protein concentration was determined by using a protein assay reagent with bovine serum albumin protein standards (Sigma) buffered identically to the assayed proteins. It is clear that all isolates had from 9 to 7 bands. All the isolates had a band at 148-159 kDa, 35-42 kDa, 28-30 kDa, 23-26 kDa, 20-22 kDa and 18-19 kDa. 4 isolates had a band at 48-55 kDa. 3 isolates had a band at 39-42 kDa. 3 isolates had a band at 31-34 kDa. One isolate had a band at 17 kDa.

5. Discussion

S. aureus causes different pathologies in human and animals. It causes food borne illness epidemics and it is one of the most important reasons of mastitis. It is associated with intramammary contaminations in cows causing financial misfortunes and milk-security issues. *Staphylococcus aureus* is mainly detected in finished milk products and the direct identification of the bacteria from food is possible with consideration to sample processing **[14,15].**

In the present study 210 samples were collected from 125 cattle milk, 55 camel milk and 30 human samples (urine n=8, abscesses aspiration n=4, kidney abscess n=1, nipple discharge n=1, sputum n=3, nasal swabs n=3, vaginal swabs n=3 and wound swabs n=7) . They were investigated for the occurrence of enterotoxigenic *Staphylococcus aureus*. The usual routine of isolation and identification of pathogenic *Staphylococcus* strains includes: collection and subsequent isolation of colonies on selective culture medium such as mannitol salt agar and Baird–Parker agar for 24–48 h at 37 °C, followed by biochemical identification using coagulase test for suspected colonies and further confirmation by other biochemical tests and molecular techniques **[16].**

The selectivity of mannitol salt agar is based on its high concentration of salt; staphylococci are able to tolerate this high concentration (up to 10 %). **Faller and Schleifer ,1981 [17]** mentioned that mannitol salt agar and Baird-parker medium are specifically selective for staphylococci and used mainly in food Microbiology.

It is clear that 48 *S. aureus* isolates (38.4%) were detected from mastitic cow

milk and identified according to **[18]**. For milk and dairy items, egg items and infant food, data about *S. aureus* limits have been actualized in current enactment and just low quantities of *S. aureus* target cells are endured **[19].** Although milk is an essential and nutritive food, it is the most common susceptible food to staphylococcal food poisoning because *S. aureus* is a principal contaminant of raw milk **[20].** Milk is a good medium for *S. aureus* propagation and enterotoxin production. Besides, enterotoxins can maintain their biological activity even after heat treatment like pasteurization **[21].**

Among human staphylococci 15 out of 30 isolates were identified as *S. aureus* from urine (4), abscesses aspiration (3), kidney abscesses (0), nipple discharge (0), sputum (2), nasal swabs (2), vaginal swabs (1) and wound swabs (3) with a total incidence of 50%. The coagulase positive *Staphylococcus aureus* was frequently involved in suppurative infection and it is a major human pathogen that causes a variety of manifestations, around 30% of the human is colonized with *S. aureus* **[3].** Recently, **Hamdy *et al.* 2019 [22]** investigated the risk factors of *Staphylococcus aureus* bacteremia in children, including methicillin resistance.

Also 5 *S. aureus* isolates were detected from camel milk (9.1%). **Mahran *et al.* 2020 [23]** recorded that 112 out of 165 she camels milk samples (67.9%), 59 out of 125 camel nasal samples (47.2%), 3 out of 5 worker nasal swabs (60%) and 16 out of 25 abscesses' swabs from human infected wounds (64%) were positive for staphylococci, only 34 isolates were *S. aureus* (17.9%).

Ingestion of food contaminated with staphylococcal enterotoxins (SEs) is the leading cause of Staphylococcal food borne poisoning. The classical antigenic-based classification of SEs includes five classical types: SEA, SEB, SEC, SED, and SEE. In latest years, new types of SEs (SEG, SEH, SEI, SEJ, SEK, SEL, SEM, SEN, SEO, SEP, SEQ, SER, and SEU) have been detected by **[24].**

In the present study, multiplex PCR was used for molecular detection of genes encoding enterotoxins of *S. aureus*. Molecular identification of SEs production using multiplex PCR is more sensitive than immunological methods **[25,26].** It is clear that 18 out of 48 *S. aureus* isolated from mastitic cows and 5 out of 15 *S. aureus* isolated from human samples were enterotoxin producers. All toxigenic *S. aureus* isolated from clinical mastitic cows (13) have produced toxin type D while *seb* and *sed* were detected from subclinical mastitic cows. Among human isolates 2 isolates produced mixed toxins A and E, 2 isolates produced mixed toxins C and D and one isolate produced type C toxin.

SED was detected from milk isolates. Among human isolates, 2 isolates produced mixed toxins A & E, 2 isolates produced mixed toxins C & D, and 1 isolate produced type C toxin. It was assumed by **[27]** that not only the classical enterotoxins have been involved in SFP, but also many molecular researches have been suggested the prominent role of other newer SEs.

El Jakee *et al.* 2010[28] recorded that RPLA results showed high incidence of type C enterotoxin followed by type A and type B with incidence of 34 (32.1%), 19 (17.9%) and 15 (14.2%) respectively among *S. aureus* isolated from bovine milk samples. Also using RPLA, 10 out of the 25 *S. aureus* isolated from cattle, buffaloes and human were found to be toxigenic and enterotoxin C (50%), A (20%), A& B, A& C and enterotoxins A, B, C and D (10 % each) were detected **[29].**

Classical enterotoxin genes have been detected in a variety of cheese and milk, but little is known about the prevalence of newly described enterotoxin genes in these products **[30]**. The most common enterotoxins in milk and milk products are enterotoxins A and B. However, these toxins are less common in strains isolated from animals and more common in those of human origin, it assumed that may occur during processing and food manipulation **[31].**

In our search the result of multiplex PCR showed also mixed gene of enterotoxin of *S. aureus*. **Fisher *et al.* 2018[27]** recorded that some *S. aureus* enterotoxin genes are occasionally found alone, but more commonly in groups, carried on large mobile segments of DNA called mobile genetic elements (MGEs).

Five *S. aureus* crude enterotoxin type D were investigated in the present study by SDS-PAGE. It is clear that all investigated isolates' supernates had from 9 to 7 bands. All the isolates had a band at 28-30 kDa, 23-26 kDa and 20-22 kDa. Protein profile analysis of 8 enterotoxigenic *S. aureus* strains were analyzed by Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE), all enterotoxigenic isolates had a band at 26 to 29 kDa **[32]. Munson *et al*. 1998 [33]** recorded that the expected size for mature SEG from the derived amino acid sequence is 27,042 Da, corresponding well to the apparent sizes for mature SEG determined from both SDS-PAGE analysis (28,800 Da) and from a size exclusion chromatography column (approximately 30 kDa). SEs and SE*l*s constitute a family of structurally related exoproteins that range in size from ~22 to 28 kDa **[34].** Presently, 23 enterotoxins have been identified as distinct serological entities including SEA, SEB, SEC, SED, and SEE, these toxins are basic proteins made up of approximately 220–240 amino acids and have

similar molecular weights of 25–30 kDa **[35]. Mansour *et al.* 2017[36]** concluded that SEs are small proteins (MW 26.900 - 29.600 kDa), they resist the majority of proteolytic enzymes and thus remain their action in the gastrointestinal tract.

6. Conclusion

Our study was aiming to detect the enterotoxigenic strains of *S. aureus* from different animal and human samples. Multiplex PCR was applied as an applicable and rapid molecular technique for detection of different types of enterotoxins. Furthermore, types of enterotoxins were detected via SDS. Staphylococcal food poisoning is a threatening issue and needs more appropriate control measures and prompt diagnosis

7. References

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Table 1. The collected samples used in the study

Samples		Number
Cattle milk (125)	**Mastitic milk**	**40**
	Subclinical mastitis	**85**
Camel milk (55)	**Apparently healthy milk**	**55**
Human samples (30)	**Urine**	**8**
	Abscesses aspiration	**4**
	Kidney abscesses	**1**
	Nipple discharge	**1**
	Sputum	**3**
	Nasal swabs	**3**
	Vaginal swabs	**3**
	Wound swabs	**7**
	Total	**210**

Table 2. Primers for toxin genes of *S. aureus* used in multiplex PCR (Mehrotra *et al.*, 2000):

Primers	Nucleotide sequence	bp
***Sea*:** Forward	**5` GGTTATCAATGTGCGGGTGG 3`**	**102 bp**
Reverse	**5` CGGCACTTTTTTCTCTTCGG 3`**	
***Seb*:** Forward	**5` GTATGGTGGTGTAACTGAGC 3`**	**164 bp**
Reverse	**5` CCAAATAGTGACGAGTTAGG 3`**	
***sec*:** Forward	**5` AGATGAAGTAGTTGATGTGTATGG 3`**	**451 bp**
Reverse	**5` CACACTTTTAGAATCAACCG 3`**	
***sed*:** Forward	**5` CCAATAATAGGAGAAAATAAAAGG 3`**	**278 bp**
Reverse	**5` ATTGGTATTTTTTTTCGTTC 3`**	

Table 3. Prevalence of *S. aureus* among the examined samples

Samples		Number of investigated samples	*S. aureus*	
			No	%
Cow milk	Clinical mastitic	40	33	82.5
	Subclinical mastitis	85	15	17.6
Camel milk	Apparently healthy milk	55	5	9.1
Human samples	Urine	8	4	50
	Abscesses aspiration	4	3	75
	Kidney abscesses	1	0	0
	Nipple discharge	1	0	0
	Sputum culture	3	2	66.7
	Nasal swabs	3	2	66.7
	Vaginal swabs	3	1	33.3
	Wound swabs	7	3	42.9
	Total	210	68	32.4

Table 4. Occurrence of enterotoxigenic *S. aureus* isolated among the isolates.

Samples		No of *S. aureus* isolates	Enterotoxigenic *S. aureus*		Type of Enterotoxin
			No	%	
Cow milk	Clinical mastitic cow	33	13	39.4	*sed*
	Subclinical mastitic cow	15	5	33.3	2 *seb* and 3 *sed*
Camel milk	Apparently healthy milk	5	0	0	-
Human samples	urine	4	1	25	*sec* & *sed*
	Abscesses	3	1	33.3	*sea* & *see*
	Sputum samples	2	1	50	*sea* & *see*
	Nasal swabs	2	1	50	*sec* & *sed*
	Vaginal swabs	1	0	0	-
	Wound swabs	3	1	33.3	*sec*
	Total	68	23	33.8%	

Table 5. SDS-PAGE profile analysis of 5 *S. aureus* enterotoxin type D.

No. of bands	Number of columns and % kDa											
	M	%	1	%	2	%	3	%	4	%	5	%
1	150	10.7	147	18.8	157	20.4	158	17.1	157	14.5	159	19.8
2	100	10.6	55	6.8	51	8.4	50	9.1	48	9.3	35	11.3
3	75	14.5	42	9.7	40	11.5	39	11.6	38	11.5	28	10.1
4	50	7.6	34	8	29	11.7	31	9.4	31	9.5	26	11.3
5	37	13.5	30	9.7	25	14.8	28	10	28	10.6	22	14.1
6	30	6.6	26	12.6	20	16.4	24	13.5	23	12.9		
7	25	16.6	21	14	19	16.9	20	14.3	20	15.2	18	16
8	20	10	20	15			18	15.1	18	16.6	17	17.4
9	15	7.7	18	5								
10	10	3										

M: protein marker

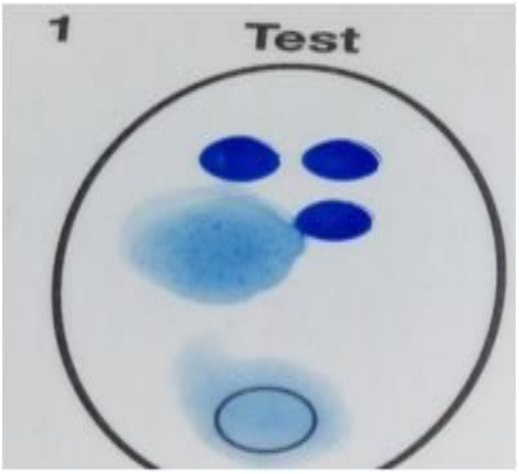


Fig .1. The latex slide agglutination test (staphytect test) showed positive reaction among *S. aureus* isolates

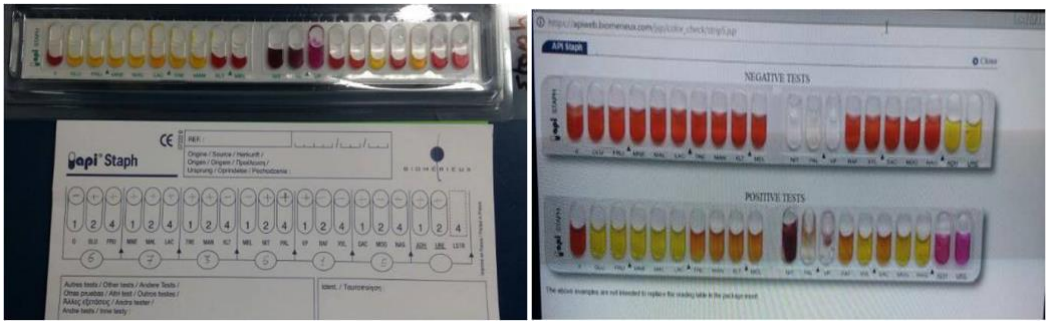
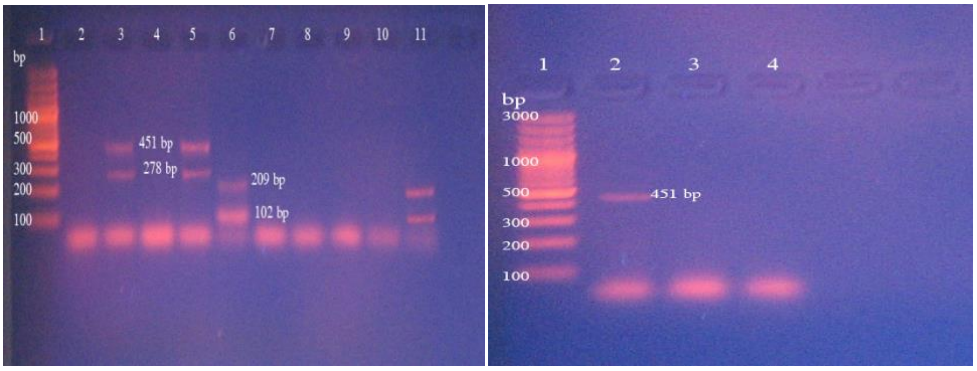
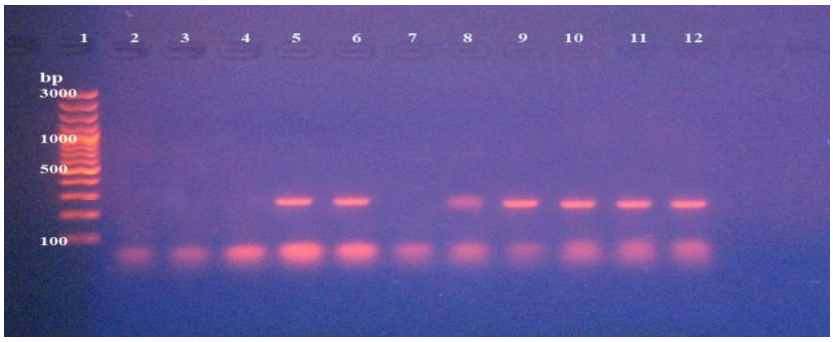


Fig .2. API among *S. aureus* isolates



a) b)



c)

Fig .3. Agarose gel electrophoresis showing the results of multiplex PCR for detection of enterotoxigenic *S. aureus* isolates.

a): Lane1: 100 base pair DNA molecular weight marker, lane 2: negative control, lane 3: positive amplicon 451 bp for enterotoxin type C and 278 bp for enterotoxin type D from urine sample, lane 4: wound swab, lane 5: positive amplicon of 451 bp for enterotoxin type C and 278 bp for enterotoxin type D from nasal swab, lane 6: positive amplicon of 209 bp for enterotoxin type E and 102 bp for enterotoxin type A from abscess aspiration from knee joint, lane 7: urine sample, lane 8: nasal swab sample, lane 9: abscess aspiration sample, lane 10; pus aspiration and lane 11: positive amplicon of 209 bp for enterotoxin type E and 102 bp for enterotoxin type A from sputum sample.

b): lane 1: DNA molecular weight marker, lane 2: positive amplicon of 451 for enterotoxin type C from wound swab, lane 3: nipple discharge and lane 4: vaginal swab.

C): lane 1: DNA molecular weight marker, lane 2, 3, 4 and 7: raw milk, lane 5, 6, 8, 9, 10, 11 and 12: positive amplicon of 278 for enterotoxin type D from raw milk.

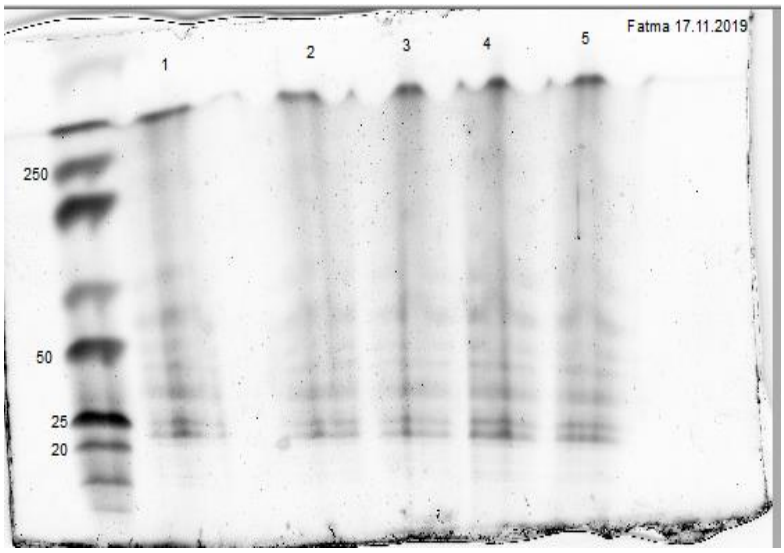


Fig .4. Determination of proteins among 5 *S. aureus* enterotoxin type D by SDS-PAGE.