Original Article

Evaluation of the toxic shock syndrome gene (TSSTI) of Staphylococcus aureus in deceased Neonates of Tehran Forensic Medicine Organization from October 2017 to October 2018

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Abstract

Introduction: Staphylococcus aureus due to the production of toxins such as enterotoxins and toxic shock syndrome toxin (TSST1) can be effective in causing sudden infant death syndrome (SIDS). The present study aimed to identify cases of sudden neonatal deaths and to evaluate the frequency of Staphylococcus aureus in different tissues of neonates referred to Tehran Forensic Medicine Organization during one year as well as quantification of TSST1 toxin and related gene trace in referred samples. **Materials and Methods**: During the time period from October 2017 to October 2018, 90 samples, including brain, kidney and spleen samples, from SIDS cases referred to forensic medicine organization were studied. A Part of each sample was used for culture and isolation of Staphylococcus aureus and the other part was used for quantitative and qualitative evaluation of TSST1 by ELISA and Real-time PCR. **Results and Discussion**: 57 cases (63%) out of 90 investigated neonatal deaths, were female and 33 ones (37%) were male. Mortality rate was higher in the 1-6 month age range than other age groups and season had no significant effect. Among the 90 examined samples, only 17 (about 19%) of S. aureus were isolated, all of which were renal. The TSST1 toxin was also detected in only two cases (about 2%), with 1.7 ng in one case and 1.5 ng in the other one. Overall, although the presence of TSST1 toxin in the sample can be considered noteworthy, there was no significant relationship between the presence of this toxin and the occurrence of sudden death syndrome in SIDS cases.

Keywords: Staphylococcus aureus, Sudden infant death syndrome (SIDS), TSST1 toxin

Introduction

Sudden Infant Death Syndrome (SIDS) is referred to sudden infant death less than two years. Despite extensive research, including biography review, site of death and complete autopsy, no reason has been explained.

Even after routine Forensic pathological and toxicological scrutiny, no specific findings are found in these individuals. It usually occurs in sleep between the ages of two and four months in a time interval between 12 midnight and 9 am and no clear pathophysiology has been defined for this issue.

In recent years, researchers have reported that bacteriological examination of infants' tissues died of SIDS, can be a good solution to find the correct etiology of different cases of SIDS in infants and subsequently to provide appropriate solutions to prevent SIDS deaths. It has been proven that investigating and identifying various bacterial toxins as well as the cellular components of bacteria that have super antigenic properties can determine the cause of many SIDS.

Various studies have so far proven that many of these toxins and bacterial super antigens cause toxic shock syndromes, fatal sepsis and SIDS by the induction of a cytokine storm in their host [1].

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What has been clarified from epidemiological studies is that sleeping in prone position is one of the most important risk factors and can be prevented.

Other important risk factors have also been reported as exposure to smoke and the use of soft bedding for the infant [1]. SIDS is the most common cause of neonatal mortality in developed countries and accounts for about 20% of all NICU mortality [2]. Pyrogenic Toxins was formally defined as a type of Staphylococcus aureus by Rosenbach in 1884. He observed that in the solid medium, Staphylococci form two types of white and yellow colonies and the yellow one was in fact Staphylococcus aureus [3].

Staphylococcus aureus can play a role in SIDS, the most important of which is TSST1 of Staphylococcus aureus, an optional Gram-positive and anaerobic cocci, as the most important species of Staphylococcus aureus in medicine [4]. Staphylococcus aureus causes a wide range of infections from simple skin infections to threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome and septicemia. It is one of the 5 most common reasons of nosocomial infections, especially post-operative wound infections.

Some phages can infect Staphylococcus aureus, thereby, they can transmit the penton-valentine toxin-producing gene to it and increase the pathogenicity of the strains [5]. In children, Staphylococcus aureus causes a severe infection called Staphylococal Scarlet Skin Syndrome [6]. The strains that produce exotoxin TSST1 may cause a potentially fatal and deadly disease called Toxic Shock Syndrome.

Toxin can enter the bloodstream and spread throughout the body [7]. Staphylococcus aureus is the most common bacterial isolate in the nasopharynx of healthy infants, so that more than 60% of these isolates are capable of producing various toxins including super antigenic toxins. These toxins are usually produced and secreted at temperatures between 37 and 40 degrees of Celsius, which is much lower than this temperature range.

In cases of infants' sleeping in a prone state, the temperature of the nasopharynx rises to near 37 degrees of Celsius, thus providing conditions for the production of bacterial toxins. Since toxin-producing bacterial strains are often present in all healthy infants, provision of specific conditions for the production of toxin and other various factors are more important than the presence of bacteria in the infant's body.

Among these predisposing factors to toxin production, it can be referred to the increase in the infant's body temperature due to various factors such as fever, various changes in the infant's body condition that increase the their temperature, as well as viral respiratory infections and pulmonary obstruction that prevent air from circulating in the respiratory mucosa [8, 9].

Staphylococcus aureus causes gastroenteritis and food poisoning by producing enterotoxins such as enterotoxins. Symptoms of food poisoning include diarrhea, vomiting, and abdominal pain. TSST1 and enterotoxins are super antigens [10, 11]. TSST1 is one of the toxins of Staphylococcus aureus that may be involved in sudden infant death syndrome [12].

In the study of Alfeali et al., the role of infectious agents and microbial toxins in the development of SIDS has been investigated and it has been specified that seasonal peak of SIDS is in winter [13]. In a study which was conducted by Fushim et al and was published in the Journal of Clinical Microbiology, the presence of the TSST1 gene from Staphylococcus aureus and other genes responsible for developing methicillin resistance was investigated. Meanwhile, it was shown in the carried out real time multiplex that toxic shock toxin and several other genes are traceable in different methicillin-resistant types. Rapid recognition and detection of Staphylococcus aureus toxins is also a useful tool for the prevalence assessment [14]. In a study carried out by Azar Farahmand et al., the TSST1 gene from Staphylococcus aureus in a related sample was examined and it was found that 17.5% of the test samples, which were based on positive culture method in terms of Staphylococcus aureus, had the TSST1 gene [15]. Given the lack of clarity in exact cause of death in many cases of SIDS, it is important to consider any factors that may have an impact.

Accordingly, the aim of present study was to evaluate the frequency of TSST1-producing Staphylococcus aureus strains in neonates with sudden death in Tehran from October 2017 to October 2018. Although some studies about staphylococcus aureus toxins in infants with sudden death syndrome have been done worldwide, no studies have been performed in Iran so far.

Considering that, some deaths are reported in children under two years old in the country, who do not have a clear etiology and are reported as white autopsy,

The aim of this study was to evaluate the frequency of strains with TSST1 toxin from Staphylococcus aureus in SIDS cases to help identify the cause of death in white autopsy cases.

Toxic shock syndrome toxin (TSST)

Staphylococcus aureus produces a wide range of exoproteins and protein toxins that are effective in the pathogenesis of bacteria. Toxins produced by bacteria are one of the most common causes which can, along with other bacterial pathogens, lead to onset or exacerbation of disease. Among the diseases caused by the toxins of Staphylococcus aureus, toxic shock syndrome can be referred which was first introduced by Tomi et al. It is a systemic disease that is caused by fever, hypotension, muscle pain, cutaneous rash, and eventually scaling of the hands and feet and it is caused by a toxin called TSST1.

TSST1 is a protein with a molecular weight of approximately 2400 Daltons and is typically produced by numerous strains of Staphylococcus aureus. This toxin can affect the immune system by stimulating the release of various cytokines [16, 17].

TSST1 is a pyretic super antigen which can be produced by 25% of the strains. It is produced by a growing bacterium which is a heat-resistant toxin that remains active if it is boiled for 1 hour. Its difference with other pyretic super antigens is that it lacks a cysteine ring. It is also capable of passing through the mucous membrane

High rate of oxygen, low rate of magnesium, and neutral pH are required for its production by the *tsth* gene encoded in the pathogenic islets involved in SIDS - Kawasaki syndrome and toxic shock syndrome [18].

TSST1, which is encoded by the *tsth* gene, is located on the chromosome of bacteria within the motile genetic elements known as pathogenicity islets of Staphylococcus. It has a super antigenic role.

Super antigens stimulate T cells by activating the VB variable region on receptors of the MHC class 2 and TCR cells. Activated T cells release cytokines such as interleukin-1 and TNF-alpha. These factors can cause shock and tissue damage [19].

Enterotoxin C is transmitted horizontally through the islets of pathogenicity causing food poisoning. Staphylococcus aureus is capable of producing various toxins such as alpha, beta, gamma, delta and leukocidin toxins.it can also secrete numerous cytolytic toxins, including alpha-toxin, leucosidine, and pentone valentine.

Alpha-toxin, encoded by the *hla* gene, is a pore- making toxin and has cytolytic effects on a wide range of host cells including erythrocytes, epithelial and endothelial cells, monocytes and macrophages. It is also associated with soft tissue and severe skin infections, necrotic pneumonia and even sepsis. [20].

Materials and Methods

As the current research was a descriptive cross-sectional study, the related population included all infants under two years old who died without specific etiology. The studied population were referred to Forensic Medicine Organization for the cause of death from October 2017 until the end of October 2018.

Target group selection criteria included the following points:

Type of studied population: deceased Infants who were referred to the Forensic organization of Tehran Province

Gender: Baby boys and girls

Age range: Infants younger than 2 years

Exclusion criteria for the target group consisted of heart disease, Neonates with pathologic findings and congenital defects, neonates with Positive results in terms of toxins studied in the forensic ward.

The samples of studied neonates were divided into three sections. Some of them were used for pathological and toxicological studies and a part was considered for microbiological studies.

Microbiological specimens, including spleen, brain and kidney of died neonates, were sent to the laboratory in sterile and buffered media and they were cultured in Mannitol Salt Agar. Catalase, coagulase, and DNAse tests were then performed for specifying positive results in terms of Staphylococcus aureus. Then,toxic shock syndrome was evaluated using ELISA method by identifying SIDS cases and presence of tsst1 gene was determined by real time PCR.

Results and Findings

Results of epidemiological studies

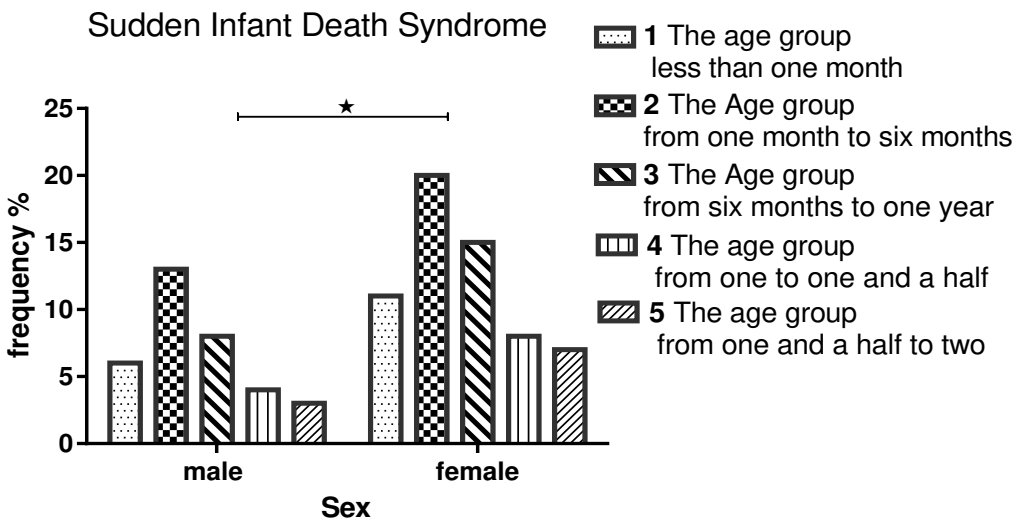
Due to investigation of frequency of deaths in neonates with TSST1 toxin of Staphylococcus aureus who were referred to forensic medicine organization during a period of one year, 307 deceased infants were referred to the noted center in Tehran province. In the present study, out of 307 deaths under two years who were sent to Tehran Forensic Medicine Organization, 90 cases of sudden death in children who were 2 years and under 2 years were referred to the center from October 2017 to October 2018.

The highest incidence of mortality was in female infants and age group between one month to six months. In addition, the lowest mortality rate was related to female infants, in the age range between one year and a half to two years old.

In the study of frequency of the incidences of sudden infant death with TSST1 toxin of Staphylococcus aureus in the dead bodies referred to Tehran Forensic Medicine organization over a period of one year, 90 cases of sudden death of children less than two years old were referred to the noted organization. Among the 307 deaths of children under 2 years referred to forensic medicine organization in Tehran province, sudden deaths were reported in 90 cases in the performed study comprising 29% of all referrals. 16 cases of SIDS out of the 307 cases in the below the age of one month, included 0.052% of all referrals.

There were 30 deaths in the age range of one month to six months, which included 0.098% of all referrals. There were also 22 deaths in the age range of six months to one year that accounted for 0.071% of all referrals. There were 12 deaths at age range between twelve to eighteen months that accounted for 0.039% of all referrals, and finally there were

10 deaths in the age range of 18 to 24 months, consisting of 0.032% of all referrals.



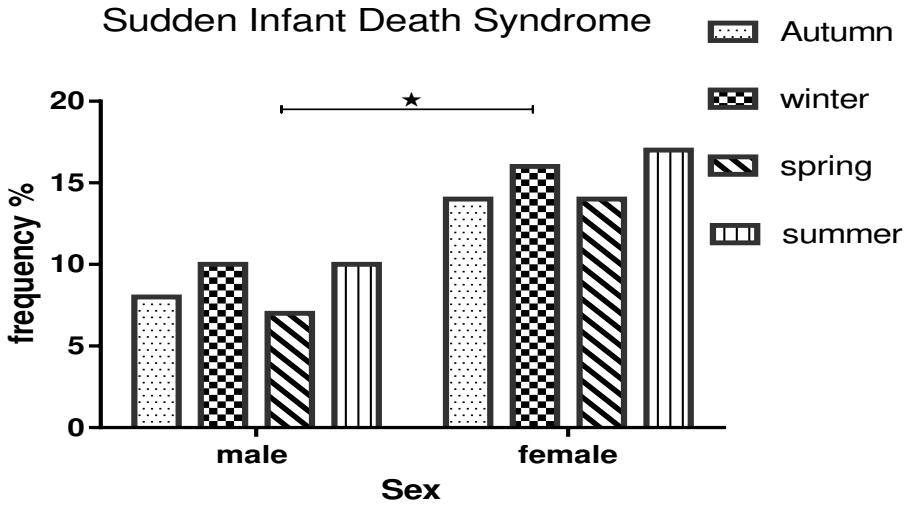
Graph 1) Frequency of mortality in neonates referred to Tehran Forensic Medicine organization due to sex and age parameters

As it is shown in Figure 1, boys and girls between 1 and 6 months had the highest incidence of SIDS in this study.

In the study conducted from October 2017 to the end of october 2018, 90 cases of sudden death were referred to forensic medicine organization in Tehran, including 20 cases of death in spring, 24 cases in summer, 21 cases in autumn and 24 cases in winter. However, the time of death in one case was unknown.

The investigations revealed that the highest frequency of death among female infants were related to summer and winter seasons including 13 cases in spring, 16 in summer, 13 in autumn and 15 in winter. In comparison, among male infants, 7 cases died in spring, 9 cases in summer, 8 cases in autumn, and 9 in winter (Figure 2).The results of this study showed that season had no effect on mortality rate and the frequency of mortality was similar in each season. Thus, no statistical difference was observed.

In addition, there was no significant relationship between sex and sudden infant deaths based on statistical indices.



Graph 2) Frequency of deaths in neonates referred to Tehran forensic science organization due to sex and season parameters

The results of present study showed that among the 90 cases of sudden death referred to forensics, Staphylococcus aureus was isolated from 17 cases. In the age group up to one month, results of five samples were positive in terms of culture of Staphylococcus aureus in Mannitol Salt Agar medium.

Among a total of 5 positive samples for the presence of Staphylococcus aureus, 3 cases were baby girls and two were baby boys.In addition, Staphylococcus aureus was not obtained from culture of brain and spleen transport of these neonates in mannitol salt agar medium.

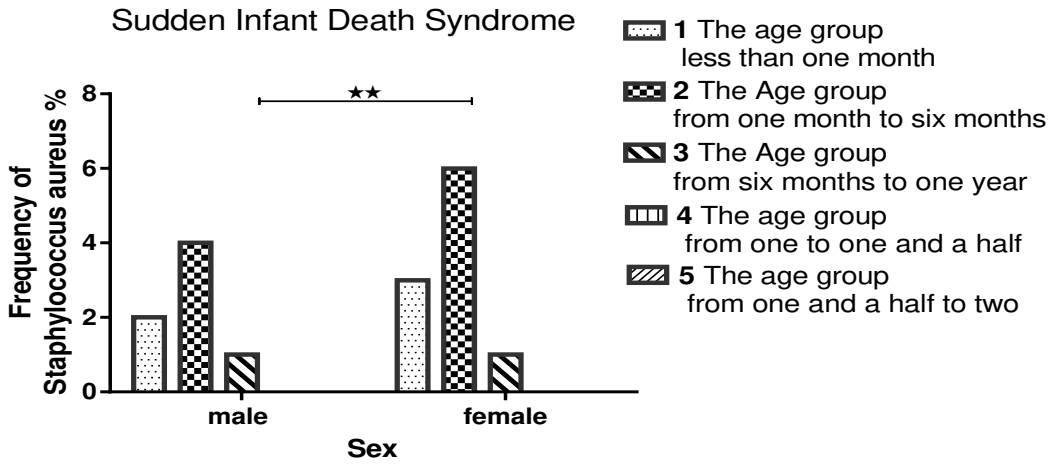
In the age group of one to six months, results of 10 cases were positive in terms of culture of kidney samples in mannitol Salt Agar and separation of Staphylococcus aureus .Six of them were female and four cases were male. In the age group of six months to 12 months, results of one female infant and one male infant were positive for culture in Staphylococcus aureus specific medium, therefore, these cases were isolated from all infants.

There were no positive result in terms of isolation of Staphylococcus aureus in culture medium in 12 to 18 months and also in 18-24 months of age.Most bacteria were isolated from infants between the ages of one month and six months (6 isolates), and no bacteria were isolated from one year to two-year-old infants.

The results also showed that all the bacteria were isolated from kidney tissue of neonates and no bacteria were isolated from brain and spleen. Table 1 shows the frequency of Staphylococcus aureus isolated from different age groups of neonates with sudden death referred to forensic medicine organization.

Table 1: Frequency of sudden death in infants under two years referred to Tehran Forensic Medicine organization

Age range	0-1 month	1 to 6 months	6 to 12 months	12 to 18 months	18 o 24 months
kidney	29.4% 5 cases	58.8% 10 cases	11.8% 2 cases	0% 0 cases	0% 0 cases
brain	0% 0 cases	0% 0 cases	0% 0 cases	0% 0 cases	0% 0 cases
Spleen	0% 0 cases	0% 0 cases	0% 0 cases	0% 0 cases	0% 0 cases



Graph 3) Frequency of Staphylococcus aureus in neonates with sudden death referred to forensic medicine organization from October 2017 to October 2018.

In the performed study, kidney, brain and spleen of deceased neonates referring to forensic medicine organization in Tehran were sampled in completely sterile conditions. In addition, considering the type of study and design carried out in the relevant organization, the necessary collaborations regarding the sterilization of space, beds and the associated surfaces after each autopsy were done.

ELISA results

Various dilutions of the existing standard in the kit were prepared in the present study. At first, 120 µl of standard solution with 120 µl diluent was used. By progressive dilution in eighth standard , it reached 6.25 ng / L and in the ninth dilution standard it reached 3.125 ng / L. Reading the test specimens in two extracts from kidney tissue of infants with SIDS , 1.7 and 1.5 ng TSST1 toxin was found.

Among the 17 neonates with sudden death that Staphylococcus aureus was isolated from them , only two

were positive for TSST1 toxin, and both were from 1 month to 6 months years old. The amount of TSST1 toxin in these two samples measured by ELISA was 1.7 and 1.5 ng. The results of this study also indicated that the production of this toxin from 17 isolates of Staphylococcus aureus was positive in only two isolates, which is consistent with the tissue positive cases mentioned above.

Results related to molecular section

Determination of extracted DNA concentration

After extraction for quantification, OD of samples of DNA were analyzed by Nano-Drop apparatus and samples were kept at a minimum concentration of 2 ng at -70 ° C and they were used for real time PCR.

Table 2) investigating Extraction of purity of DNA

row	Sample name	OD ng/µl	Purity 260/280
1	T1	78.2	1.73
2	T2	112.9	1.81
3	T3	120	1.78
4	T4	198	1.69
5	T5	210	1.87
6	T6	49	1.83
7	T7	139	1.66
8	T8	182	1.82
9	T9	163	1.75
10	T10	41	1.87
11	T11	56	1.92
12	T12	62	1.81
13	T13	45	1.82
14	T14	91	1.63
15	T15	45	1.68
16	T16	65	1.73
17	T17	73	1.78
18	T18	86	1.72
19	T19	74	1.64
20	T20	52	1.62
21	T21	75	1.61
22	T22	69	1.76
23	T23	58	1.74

Examination of Frequency of TSST1 gene of Staphylococcus aureus in neonates with SIDS

The presence of peak at 83 ° C indicates a specific reaction amplification. These peaks show amplification of a gene fragment of Staphylococcus aureus. Staphylococcus aureus with TSST1 gene (Staphylococcus aureus control strain ATCC 51650) was prepared from Pasteur Institute of Iran and the extracted genome was used as positive control after DNA extraction.

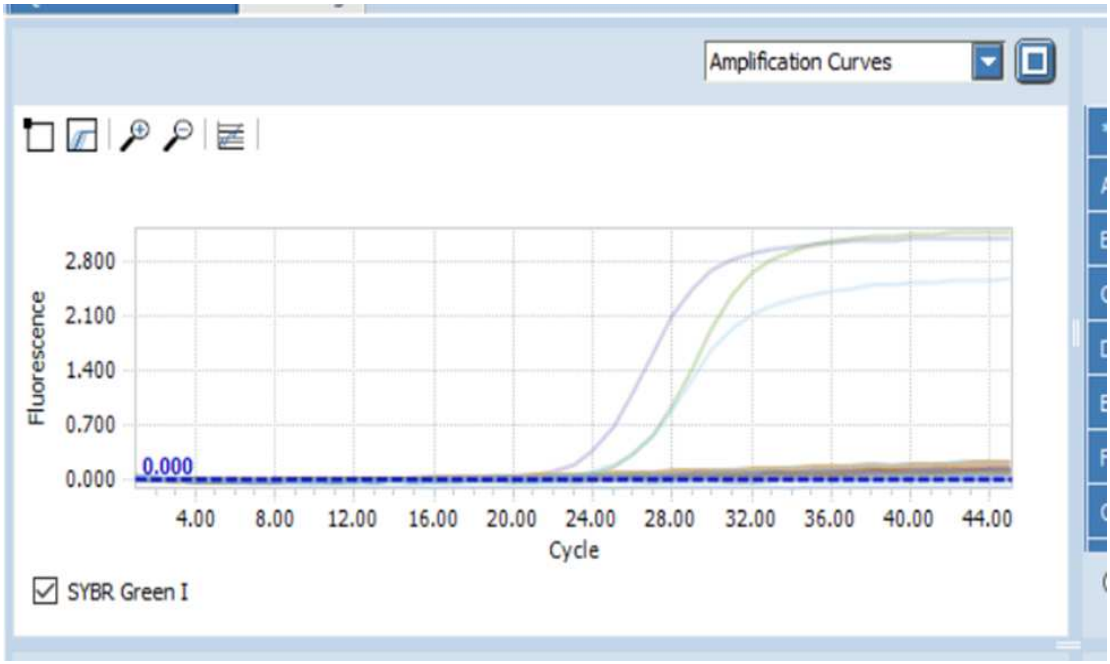


Figure 1) Real-time PCR results of TSST1 gene of Staphylococcus aureus with positive control of positive samples along with control of (Amplification Curves).

Figure 2) Real-time PCR results of TSST1 gene of Staphylococcus aureus with positive control of positive samples along with control of (Melting CURVES).

The melting curve begins with a gradual increase in temperature from below the melting point of the product and continues to a temperature above their melting point.

The range of temperature variations in the drawing of the melting curve can be adjusted by the user. The amplified products melt at different temperatures based on the length of the amplified fragment and their amount of Guanine and Cytosine and when the products are melted, the amount of fluorescence is reduced and measured by the device.

The melting peaks can then be calculated through the separation and difference of the melting curve and they represent the amplified products during the reaction. These peaks are similar to the bands generated in the gel electrophoresis, thus, the melting curve data qualitatively result in the representation of amplified products at the end of the Cyber Green experiment.

The maximum fluorescence decrease was recorded at 83 ° C for TSST1 gene of Staphylococcus aureus in standard sample melting curve and positive test, (Figure 3).

Figure 3) Real-time PCR results of TSST1 gene of Staphylococcus aureus with positive control of positive samples along with control of (Melting Peaks)

According to examining the melt curve of the samples,the melting curve for each sample can be examined if fluorescent dyes such as cyber green are used in real time.

Since each gene has its own melting curve, therefore, the curves of one gene in all samples must match each other and all the curves must be single-peak. Due to the analysis of the real-time data of samples, the ones that their melting curves for both genes did not match the other, should be excluded. After removing unacceptable samples, it is time to analyze the data. At this point, since all the repetitions have been investigated in a real-time reaction at the same time, the resulting Ct for each sample should be averaged and it should be worked with the mean Ct. At the averaging stage, it should be noted that, in a real-time reaction, each sample is repeated twice simultaneously, therefore, it must be averaged for each sample but if the samples are of several types, for example one treatment that has two replicates, then the no average should not be taken anymore between repetitions of treatments.

Ct means the cycle in which Real Time products exceed a threshold. Some real-time software uses Cp instead of Ct.

Cp stands for crossing point and it is referred to a cycle in which the amount of Real Time products exceeds a crossing point.

In practice, there is no difference between analyzing data based on Ct or Cp.

In the studies performed by PCR Real Time, it was found that samples 11 and 16 were positive in terms of TSST1 gene and these are the numbers contained TSST1 toxin according to the ELISA test.in addition, there was some sort of correspondence between genomics and proteomics in the test samples and the genes were represented.

Frequency of positive cases of TSST1 gene of Staphylococcus aureus in samples of neonates referred to forensic medicine organization in Tehran province included 11% of all positive cases in terms of culture and 18% of all sudden deaths were positive for Staphylococcus aureus culture. Only 0.022 percent of neonates referred to forensic medicine center in Tehran had TSST1 gene and toxic shock syndrome toxin.

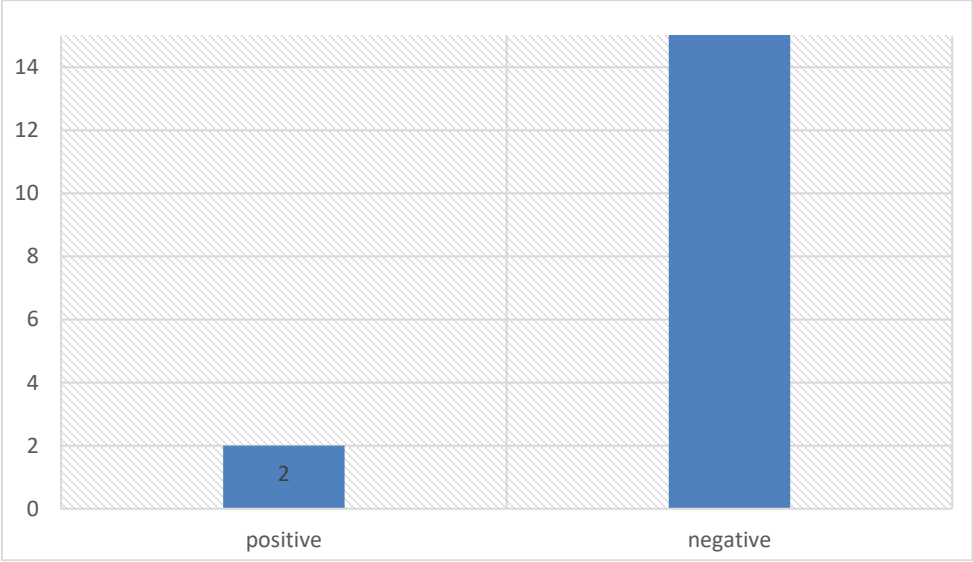


Figure 4) Frequency distribution of Staphylococcus aureus gene in neonates with SIDS

Totally, among 17 analyzed samples were positive in terms of the presence of tsst1 gene of Staphylococcus aureus using PCR.

Discussion and Conclusion

One of the most important etiologic agents of SIDS is the pathogenic and toxic strains of Staphylococcus aureus, and according to reports from five countries, more than half of all cases of SIDS are reported in these regions [8].

Therefore, the colonization of Staphylococcus aureus, then the identification of TSST1 toxin in neonates who died due to SIDS, as well as the searching for TSST1 toxin gene in Tehran was investigated in the current study.17 cases out of 90 neonatal deaths due to SIDS were isolated from Staphylococcus aureus in the present research. However, statistical analysis showed no significant relationship between the presence of Staphylococcus aureus and the occurrence of SIDS in neonates.

There are no adequate studies to determine the direct role of TSST1-producing Staphylococcus aureus in the development

of SIDS in neonates and only this bacterium is expressed as a risk factor for this deadly phenomenon.

In this study, all isolates of Staphylococcus aureus from deceased infants were separated from kidney tissue, and no specimens of spleen or brain tissue were positive.

In one study by Malam et al. ,it was proven that post-mortem kidney tissue is the most likely tissue to identify TSST1 in the infant's body with SIDS. These findings are consistent with the present study, which confirmed the presence of toxic shock syndrome of Staphylococcus aureus in only kidney tissue [21].

It was also specified in the noted study that SIDS was more common in the age group of one month to six months. Therefore, this age group should be considered the most sensitive age group for SIDS and preventive measures in this age group should be considered with greater emphasis.

In the present study, the presence of TSST1 toxin in kidney tissue was detected by ELISA in two of the 17 cases of Staphylococcus aureus.

Similar studies have been conducted worldwide to investigate the presence of different Staphylococcus aureus toxins in different tissues of neonates who have died of SIDS through antigen-based methods including ELISA.

According to recent epidemiologic studies, there is no significant relationship between sex and Staphylococcus aureus infections. The results also showed that the most bacterial isolation were in winter and summer, while it was specified based on the study by Alefali et al. that most cases of isolates were related to winter [22].

Therefore, a combination of genetic factors and the ability of the bacterium to establish and develop in the neonate can be hypothesized as the cause of SIDS. In this regard, the present study indicated that some neonates are infected with Staphylococcus aureus, but prevalence of infection with this bacterium is not high in neonates with SIDS.

Since the exothermic toxins produced by this bacterium occur only between 37 ° C and 40 ° C and the nasopharyngeal temperature is usually caused by air passing at 25 ° C in the mucosal surfaces, these toxins and bacteria cannot normally produce pathogenic activity.

Factors that can increase the temperature of mucosal surfaces up to the temperature of the toxins' stimulant may include upper respiratory tract infection and infant's sleeping in a prone position.

Current findings suggest that further research about the role of these toxins in neonatal death is essential. Such studies could provide clues to better understand these mechanisms of death and possibly to develop new strategies to reduce the incidence of SIDS [23].

Based on the comparison of the number of neonates with SIDS that were positive for TSST1 Staphylococcus aureus toxin with ELISA method and the number of positive samples in terms of TSST1 gene of Staphylococcus aureus, it was shown that the outcomes are completely consistent.

In the present study, after examining the kidney, brain, and spleen tissues of neonates who died due to SIDS, kidney tissues of only two positive samples in terms of TSST1 toxin of Staphylococcus aureus were isolated. No significant association was found between the presence of TSST1 and the development of SIDS in the present study.

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