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Molecular identification and antimicrobial susceptibility of *Staphylococcus aureus* isolated from children with Nasolacrimal Duct Obstruction

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ABSTRACT

Nasolacrimal duct obstruction (Dacryocystitis) is an inflammation of the lacrimal sac and duct. It is an important cause of ocular morbidity, both in children and in adults. Clinical features of this disease are pain, swelling, redness over the lacrimal sac at medial canthus, tearing, crusting, fever, digital pressure over the lacrimal sac may extrude pus by the punctum. It is notable that, in chronic cases, tearing may be the only symptom. Aim of this study was Isolation and molecular identification of staphylococci from children with Nasolacrimal Duct Obstruction. Twenty five swabs were obtained from the conjunctival sac of children with nasolacrimal duct obstructions after applying pressure over the lacrimal sac and allowing the purulent material to reflux through the lacrimal punctum , then cultured on blood agar, MacConkey agar, and Mannitol Salt agar media. The samples collected from patients who attend Al-Nahrain eye specialty center. Vitek 2 system was performed based on manufacturer's protocol to confirm *Staphylococcus aureus* identification on mannitol agar, (49%) of all samples were *Staphylococcus aureus,* (16%) of all samples were *Staphylococcus epidermis*, also the results showed that 5% of samples were *Ralstonia mannitolilytica* and the rest of samples distributed between Pantoea spp(5%) and Citerobacter freundii (10%), partial *rpoB* gene (1457383..1458229) was determined for 12 cultures by PCR technique with specific primer pair and electrophoresed on Agarose gel. Most of the cases gave culture positive,
demonstrating the importance of this clinical disorder for ophthalmologists to explicitly examine the occurrence of nasolacrimal obstruction symptoms before any intraocular procedures were scheduled

Keywords: Molecular Identification, Nasolacrimal Duct Obstruction.

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INTRODUCTION

The infection in the tear drainage system is a nasolacrimal duct obstruction (NLDO), tears flow from each eye through narrow canals, a tear sac (lacrimal sac) and a tear duct (nasolacrimal duct) (1). The primary cause is Idiopathic inflammatory obstruction, the secondary one involve trauma, infection, inflammation, neoplasia, and mechanical obstruction (2). The inflammation and fibrosis may be secondary to coexisting infectious colonization inside the lumen of the lacrimal sac (3). Clinical features of this disease is pain, swelling, redness of the lacrimal sac at medial canthus, tearing, crusting, fever, digital pressure on the lacrimal sac may extrude pus through the punctum. It is notable that, tearing may be the only symptom in chronic cases, (4). In most cases, NLDO is congenital in children. The most common cause is the non-canalization of the inferior caudal end of the duct. Spontaneous resolution of the obstruction happens in 96% of affected children in the first year in the natural course and conservative management is the main factor(5). Microorganisms usually accomplished with the acute type of dacryocystitis, physicians consider gram-negative bacteria in diabetes and immunosuppressive conditions, gram-positive bacteria was the predominant microorganisms include *Staphylococcus aureus*, *Staphylococcus haemolyticus*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* that may be accompanied by both aerobic and anaerobic gram-negative pathogens have been identified as common microorganisms in children (6). The genus Staphylococcus is divided into thirty six species and twenty one subspecies(7). The use of highly sensitive and precise genomic targets can provide an alternative means of accurately identifying Staphylococcus species, so the aim of this study is the isolation of staphylococcus on a selective media and using polymerase chain reaction technique for the molecular identification.

MATERIALS AND METHODS

Isolation and Identification of *Staphylococcus aureus*

Only children aged under 2 years were involved in this research. This study included 25 patients with infection of the lacrimal sac, dacryocystitis in Al-Anbar, Iraq in 2019. Demographic data were collected from each patient. The samples collection was carried out by applying pressure on the lacrimal sac and allowing reflux of the purulent material through the lacrimal punctum. Then, sterilized swabs were used to obtain the samples from the pus of lacrimal sacs of patients. After recording the samples, they were cultured on different media. The culture media included blood agar, MacConkey agar, and Mannitol Salt agar. Following this, Nutrient broth and Brian heart infusion agar were used to store the samples. All media were incubated at 37oC for 24 h.

Catalase test for *staphylococcus* identification was examined by using the catalase reagent (70% distilled water: 30% H_2O_2). Several drops of this reagent were placed on the slide. Then, a single colony from mannitol salt agar was selected and taken by a wooden stick and mixed with the reagent drop to investigate if there are any bubbles that formed as explain in the equation below.

$2H_2O_2 \rightarrow 2H_2O + O_2$ (gas bubbles)

To confirm the presence of *Staphylococcus aureus*, coagulase test was performed. This reagent includes taken fresh blood samples and centrifuged. The plasma

was separated and diluted 1:10 with normal saline. Then, 500 of diluted plasma was added to the test tube and mixed with 100 of growth culture. The mixture was incubated at 37 °C and checked after 4 hrs to check if the fibrinogen will convert to fibrin because of coagulase enzyme produced by *Staphylococcus aureus*. Moreover, Vitek 2 system was performed based on manufacturer's protocol to confirm *Staphylococcus aureus* identification on mannitol agar.

Molecular study:

Twelve cultures out of twenty five were positive for coagulase and analyzed by VITEK system to prove these isolates belong to *Staphylococcus aureus,* the genome of *Staphylococcus aureus* isolates were extracted by using gDNA Bacteria kit Quick protocol (Geneaid Biotech Ltd), before that *Staphylococcus aureus* was cultured on mannitol salt agar for 24 hrs. Following this step, the bacteria were taken and incubated on nutrient broth for 24 hrs. An equal amount (1 ml) of each overnight incubated sample was centrifuged and the DNA was extracted manually from the isolated *Staphylococcus aureus*. The extracted genomic DNA was electrophoresed on agarose gel(1.5% : 50 V/hr) , DNA bands were checked under the UV light, and images were taken.

Each PCR mixture was prepared with 25 µl of Green Master Mix 2x (promega), 17µl of nuclease free water, 2µl of each primer at 10 pmol/µl, and 4µl of DNA (equaling 25 to 250 ng). The thermal cycling conditions to amplify the partial *rpoB* gene (899 bp) was included initial denaturation for 300 sec. , followed by 35 cycles of denaturation at 94°C for 45 sec. , annealing at 52°C for 60 sec. and extension 72°C for 90 sec., then the final extension step at 72 °C for 600 sec. Concluded the reaction program.

A specific primer pairs were used for amplification of *rpoB* gene after checking with specific softwares (data not shown), PCR amplified products (10µl) and DNA molecular – weight marker were electrophorized on 1% Agarose gel with ethidium bromide staining to verify the size of the amplicon, the remaining of PCR product were sequenced at (NICEM-USA , Apparatus : Applied Biosystem).

Results and Discussion

Dacryocystitis may be primary idiopathic stenosis (5) (12), usually in elder female and middle age(1).It may be secondary, associated with malformatio n of the tear duct, fracture, eye infection, neoplasm or tra uma. However, a substantial number of patients can toler ate simple stenosis
with epiphora for several years(1)(3).

A total of 25 children's patients with dacryocystitis were enrolled in this study, their ages arranged from 6 months to 2 years, the samples took by using swab from left and right eye which included males (69 %) and females (31%) who visited Al-Nahrain eye specialty center as shown in table (1).

Table (1): The percentage of patients and the position of infected eyes.

No.	Eye affected	Percentage of Patients		
		Males %	Females %	Total %
1	Right	37%	21%	58%
2	Left	32%	10%	42%
Total		69%	31%	100%

Table (2): Frequency of bacterial species (%).

No.	Bacterial species	Male %	Female %	All Patients %
1	Staphylococcus aureus	30%	19%	49%
2	Staphylococcus epidermis	11%	5%	16%
3	Ralstonia mannitolilytica	5%	0%	5%
4	Pantoea spp	5%	0%	5%
5	Citerobacter freundii	10%	0%	10%
6	No growth	16%	0%	15%

Vitek 2 system was performed based on manufacturer's protocol to confirm *Staphylococcus aureus* identification on mannitol agar, (49%) of all samples were *Staphylococcus aureus,* (16%) of all samples were *Staphylococcus epidermis*, also the results showed that 5% of samples were *Ralstonia mannitolilytica* and the rest of samples distributed between Pantoea spp(5%) and Citerobacter freundii (10%), as shown in table(3).

Table (3): Vitek results for some samples in this study

No. of Sample	Catalase	Coagulase	Identification
1	Positive	Positive	Staphylococcus epidermis
2	Positive	Negative	Staphylococcus epidermis
3	Positive	Negative	-
4	Positive	Negative	Ralstonia mannitolilytica
5	-	-	Pantoea app
6	-	-	Citrobacter freundii
7	Positive	Positive	Staphylococcusaureus + Citrobacter freundii
8	Positive	Positive	Staphylococcus aureus
9	Positive	Positive	Staphylococcus aureus
10	Positive	Positive	Staphylococcus aureus
11	Positive	Positive	Staphylococcus aureus
12	Positive	Positive	Staphylococcus aureus
13	Positive	Positive	Staphylococcus aureus
14	Positive	Positive	Staphylococcus aureus
15	Positive	Positive	Staphylococcus aureus

Genomic DNA was extracted from 12 culture of *staph. aureus* isolates and electrophoresed to determine the presence the genomic DNA (figure1) after estimation of DNA concentration and purity by using Nanodrop, the concentration of DNA was ranged between 100 to 420 ng/µl and the purity of DNA (A260/A280) ranged between 1.4 to 1.8. *RpoB,* which encodes the highly conserved bacterial RNA polymerase subunit has previously been shown to be an effective and suitable target can be used the identification of *Staphyllococcus aureus* and other enteric bacteria (8). In this study , partial *rpoB* gene (1457383..1458229) was determined for 12 cultures by PCR technique with specific primer pair and electrophoresed on Agarose gel(1%) as shown in figure (2).

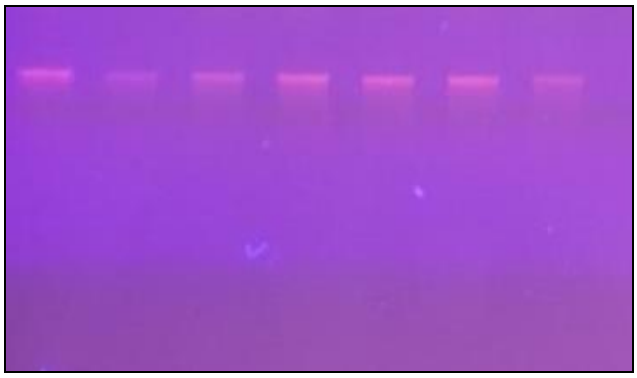

Fig.(1): Genomic DNA of *Staphylococcus aureus*,(1% Agarose).

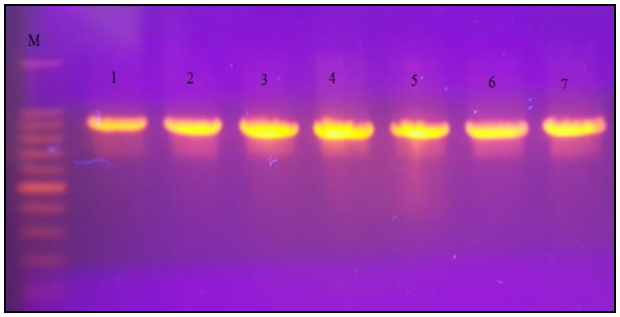

Fig.(2): Gel electrophoresis for PCR product of rpoB gene using (1% Agarose).

Five samples of PCR products for rpoB gene were sequenced with sanger dideoxy method and deposited at DDBJ/ Nucleotide database with Accession No.(**LC557034, LC571752, LC571989, LC571990, LC571991**) and strain name (**ZSA2020, ZSA2020_2, ZSA2020_3, ZSA2020_4, ZSA2020_5**), these sequences BLASTed at E-value 0.05 using BLASTn program , and using Nucleotide Collection (nt/nr) database the sequences were aligned with other deposited sequences in GenBank database at NCBI to show the identity, the percent identity was (100%) with the Accession number(MF679089.1) belongs to Staphylococcus aureus polymerase subunit beta (*rpoB*) gene isolated from Netherlands(data not shown). The distance tree of alignment results showed in figure (3). For ZSA2020 sequence , the max score (which represents the highest alignment score of a set of aligned segments from the same subject sequence) 1953 and equal to total score (which represents the alignment score of the whole sequence), E-value equal zero and identity 100 % .

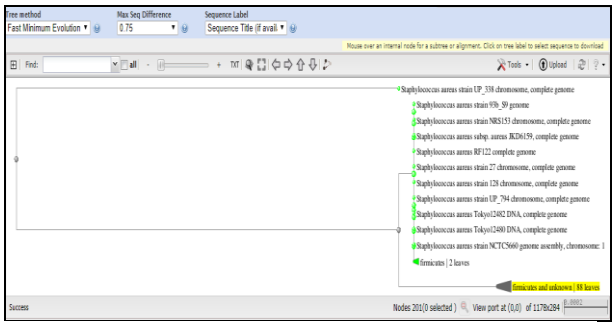

Fig.(3): Distance tree of BLASTing results of ZSA2020 sequence .

The DNA sequences were translated using EMBOSS software (http://www.ebi.ac.uk/Tools/emboss/) and the resulted protein sequences were subjected for confirmation.

Translated sequences were checked for identity using BLASTp software (results not shown). The protein sequences were used to find out their interactions with other proteins using STRING database / using sequence function, *rpoB* protein (appears in red node) interacts functionally with about 10 important proteins as shown in (Figure 4) and this relationship represented by colored lines, short lines represented closely relationship.

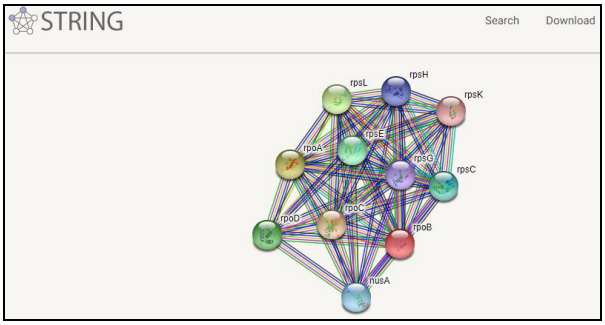

Fig. (4): Distance tree of BLASTing results of ZSA2020 sequence .

More than one research study proved occurrence of multiple mutations within *rpoB* for *S. aureus*.The *rpoB* gene, which codes the βsubunit of RNA p olymerase, has emerged as acore key gene candidate for p hylogenetic analysis and bacterial identification, in partic ular when studying closely related isolates (10,11), it has been shown that gene is more discriminatory than 16S ribosomal DNA gene(11) which has also been used for identifying Staphylococci species.

CONCLUSIONS

Most of the cases gave positive culture which highlighted the importance of this clinical condition for ophthalmolog ists to investigate specifically for the existence of nasolacr imeal obstruction symptoms before giving any intraocula r procedures. From sequencing results, *rpoB* gene has been shown to be more characteristic than the 16S ribosomal DNA gene, which has also been used for identification of enterobacteriaceae.

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