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Genotypic Detection of Some Biofilm Formation Genes Among Staphylococcus Epidermidis Isolated from Patients Suffering from Otitis Media

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Abstract

In the present study, a total 150 samples of patients suffering from patients with severe inflammation of otitis media, have been collected and tested during period from March 2021 to June 2021. The results showed that, out of 150 samples, 133(88.6%) give positive culture, while 17(11.4%) samples were negative culture. Out of 133 positive culture on different types of growth media, and the bacterial was identified according to gram stain, the results showed that, 65(48.8%) was classified as gram positive and 68(51.2%) as gram negative. Only 22 bacterial isolates are given growth for *Staphylococcus epidermidis* this was about 33.8% of patients, while 43(66.2%) isolates were related to other types of bacteria were causes inflammation of otitis media. To confirm the isolates of bacteria was used automated Compact Vitek-2 system use GP-ID cards which contained 64 biochemical tests. The results demonstrated that all 22 isolates were confirmed with ID massage confidence level ranging excellent (probability percentage from 94 to 99.7%, this technique was characterized by fast detection of bacteria. it was found that, *Staphylococcus epidermidis* was found in all isolates that identified by biochemical test (100%). Trypticase SoyBroth mixed with (1 percent) glucose was used in a biofilm test (quantitative biofilm formation) to conduct quantitative biofilm formation studies. This test was performed three times, thereby increasing the accuracy of the analysis. The results were graded as "none", "moderate", and "strong" biofilm former when the mean of OD value was less than 0.120, 0.120-0.240, and more than 0.240. Eighty-one percent of all Staphylococcus epidermidis isolates were found to be biofilm former, and this category accounted for 18 percent of the total isolates, while isolates that had moderate biofilm formation were four percent (18.2 percent). Specific sequence will be collected in billions of copies over the course of the PCR reaction, which is initiated by DNA polymerase that synthesizes a new strand of DNA complementary to the supplied template strand (Amplicon). In this study, *Staphylococcus epidermidis* were detected by *16SrRNA* genes by PCR technique, all 22(100%) *Staphylococcus epidermidis* were identified by Vitek 2 system give positive results for specific *16srRNA* gene at 176 bp. Molecular detection of biofilm formation genes (aae, *aap, atlE and embP genes*) was done for 22 isolates that previously detected as *Staphylococcus epidermidis*. The result presented 18(81.8% of all test results were positive, demonstrating the existence of the aae gene. The findings were found to be positive due to the existence of (858 bp) when compared to an allelic ladder, and on top of that, there was a result of 19 (86.3 percent) which indicated that the aap gene was active. It was determined that the findings were positive because of a presence of (400 bp) in the data set compared to an allelic ladder. 22(100 percent) of the samples yielded positive results for the atlE gene. When comparing allelic ladder and 17 percent provided positive findings for embP gene, the results were found to be positive. When allele ladder data was used, favorable findings were seen because of (455 bp) in relation to it.

Keywords

Genotypic Detection of Some Biofilm Formation Genes Among Staphylococcus Epidermidis

Biofilm formation, genes, , Otitis Media, , Inflammation, PCR

Otitis media is a set of inflammatory sicknesses of the center ear (Seppanen *et al.*, 2019) One of the 2 most important kinds is acute otitis media (AOM), an contamination of fast onset that typically provides with ear pain (Ali *et al.*, 2020). In younger kids this will bring about pulling on the ear, accelerated crying, and terrible sleep. Decreased consuming and a fever will also be gift (Meherali *et al.*, 2019). The different most important kind is otitis media with effusion (OME), usually now no longer related to symptoms, despite the fact that sometimes a sense of fullness is described; it's far described because the presence of non-infectious fluid with inside the center ear for extra than 3 months (Yadav, 2019). Chronic suppurative otitis media (CSOM) is center ear infection that effects in discharge from the ear for extra than 3 months (Xu *et al.*, 2020). It can be a difficulty of acute otitis media. Pain is not often gift (Unisa *et al.*, 2020). All 3 styles of otitis media can be related to listening to loss. The maximum not unusual place bacterial pathogen in AOM is *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, accompanied through nontypeable *Haemophilus influenzae* and *Moraxella catarrhalis* (Fagö-Olsen *et al.*, 2019). These 3 organisms are accountable for extra than 95% of all AOM instances with a bacterial etiology (Cleary & Clarke, 2017). *Staphylococcus epidermidis* are observed in continual otitis media with effusion (Enoksson *et al.*, 2020). Chronic otitis media describes a few long-time period troubles with the center ear, along with a whole (perforation with inside the eardrum that doesn't heal) or a center ear contamination (otitis media) that doesn't enhance or continues returning is characterized through recurrent or continual ear discharge (otorrhea) over (2-6 weeks), thru a perforation of the TM (Coleman *et al.*, 2018). COM takes place while the ET will become bloked again and again because of allergies, a couple of infections, ear trauma or swelling of the adeniodes (Mlynski *et al.*, 2021). COM can be suppurative or non suppurative continual, continual suppurative otitis media (CSOM) includes a perforation (hole) with inside the tympanic membrane and lively bacterial contamination with inside the center ear area for numerous weeks or extra (Khomtchouk *et al.*, 2020). *S. epidermidis* Seen to have an extremely tiny white colony, this kind of Staphylococcus figure prominently in human body flora, spreading nosocomial infections. S. epidermidis is a pathogen that is opportunistic and is often seen in patients with weakened or damaged health, as well as those who have ongoing OM (da Silva et al., 2020). Staphylococcus colonization and pathogenicity are linked to the virulence properties of coagulase-negative staphylococci. This bacteria is a hazardous contaminant that lives on medical facility tools and spreads quickly from one individual to another, rapidly spreading a contagion. Protein –rich and warm, typically present in surgical incisions, arterial lines, and venous catheters (Neoh et al., 2017). The biofilm is a very complex collection of bacteria that is found on a highly resistant surface. These types of biofilms usually occur on surfaces which are buried in or coated by an aqueous solution (Kurzbaum et al., 2019). Micro-colonies are embedded in a hydrated matrix of microbially generated proteins, nucleic acids, and polysaccharides that creates a feature form on the surface. Bacterial biofilms are surface-associated, sessile colonies. Mature biofilms begin with cells that are planktonic at first; these cells, which include unicellular and multicellular forms, adhere to a surface, and then together and/or in combination form into multicellular colonies and embed themselves in an exopolysaccharide matrix (Pandit et al., 2020). This biofilm network is made up of cells, however the cells function more as collective groups that share residence space and have channels that provide water and vitamins to the cells located inside the biofilm. Planktonic cells are less resistant to environmental stressors or microbial toxins than biofilm organisms. Even in inflamed tissues or clinical devices, biofilm cellular donation is far less likely to trigger an immune response than planktonic cells (Sakarikou *et al.*, 2020). Microbes shape a biofilm in reaction to many elements, which may also encompass cell popularity of particular attachment sites on a surface, dietary cue, or in a few instances, through publicity of planktonic to sub-inhibitory concentrations of antibiotics (Omar *et al.*,

2017).

Materials and Methods

Patients and collection of samples

For a period of three months from March 2021 to June 2021, the Cross Sectional research was performed (2021). A total of 150 individuals from the age range of 20 to 55 from the city of Hilla attendet to Hilla general teaching hospital with acute ear infection. Following conventional technique for microscopic inspection and isolation of bacteria, sterile cotton swabs were used to collect samples from each patient. In order to prevent any contamination, specimens were gathered carefully. One aliquot of the obtained material was inoculated onto a Blood agar media tray that was set up on site for aerobic cultivation, and the remaining two were taken directly to the laboratory. The remaining sample was then sent to the Department of Microbiology ,Faculty Dentistry babylon university where it was inoculated into Blood agar, MacConKey agar, Mannitol agar, and Nutrient agar medium. After incubation at (37oC) for (24) hours, the remaining sample was moved to an anaerobic chamber to be further investigated. Gram stain, colony morphology, biochemical test, Compact VITEK-2 System, and 16SrRNA method identified a staphylococcus epidermidis aerobic bacterial isolate.

Ethical Approval

Each patient consented before to their participation in the research.

Identification of Staphylococcus epidermidis by gram stain, biochemical tests, Compact VITEK-2 System

Identification of Staphylococcus epidermidis isolates by gram stain and biochemical tests

The identification tests were conducted on all of the isolates, including cultural, morphological, and biochemical features according to (Baron et al., 1994; MacFadden, 2000).

Identification of Staphylococcus epidermidis isolates with Compact VITEK-2 System

The Compact VITEK-2 System was used to test and identify all Staphylococcus epidermidis isolates (BioMerieux). The isolates are identified via biochemical reactions called phenotypic reactions. Each well of the Vitek-2 card has a separate fluorescent biochemical test included inside it. Ten out of the 64 glucose assimilation tests were for the following: phosphatase, urea, nitrate, and actidione. The Vitek-2 machine, which was integrated into the card-filling and -sealing machine, then automated the whole card-handling process, including filling, sealing, and transferring to the incubator. Each report has a decoding mechanism for it. ID-GP (identification of Gram-positive bacteria) databank was used to discover the obtained findings. The resulting ID comes from the appropriate supporting program, which determines these automatically. For data analysis, the tests were only repeated if the original results showed "poor discrimination" or "no ID." Strain inoculation was place in a medium incubator set to 37°C overnight. the VITEK-2 Systems technique, as instructed by the manufacturer (BioMerieux).

Biofilm Production

The standard biofilm assay, developed by Merritt et al. (2006), used in tissue culture plate methods is termed the semi quantitative micro titer plate test (biofilm assay).

1. Agar plates were infected with isolate from new plates and incubated anaerobically at 37°C for 72 hours. Then, the sample was diluted 1:100 with TSB.
2. Two separate microplate wells were prepared. They contained 200 microliters of the diluted culture, while broth served as the control to make sure there was no binding of the media to the plates. The three injections were given to all of the isolates.
3. Incubated in a humidified 37°C environment for 24 hours. After incubation, each well was emptied using a small volume of water. To get rid of free-floating bacteria, the wells were cleaned four times with phosphate buffer saline (pH7.2).
4. After the adhering sessile organisms established a biofilm in the plate, it was fixed in an oven at 37 degrees Celsius for 30 minutes.
5. Crystal violet (0.1 percent v/v) was used to color all the wells in the experiment. Deionized water was used to remove the excess stain, and the plates were allowed to dry to avoid further discoloration.
6. The crystal violet solution was first dissolved by adding 175 µl of an acetone/ethanol (20:80, v/v) mixture. This process involves measuring the optical

density (O.D.) at 570 nm, then analyzing the results in the table below (1).

Table (1) Classification of bacterial biofilm development by TCB method

Biofilm formation	Mean of O.D. value at 630 nm
Non	<0.120
Moderate	0.120-0.240
High	>0.240

Identification of Staphylococcus epidermidis by specific 16srRNA gene

The primer sequence and PCR conditions that used in study are listed in Table (1).

DNA extraction form bacterial culture

The isolates were sequenced and genomic DNA was taken from each, the DNA extracted according to manufacturer instructions of Genaid company . The isolated DNA was kept at a temperature of 16 degrees Celsius. Staphylococcus epidermidis and its virulence factors were identified using extracted DNA from infected skin scrapings that was tested for molecular identification.

Primers Sequences

The primers sequences and PCR conditions that used in study are listed in Table (2).

Table (2): 16SrRNA gene and biofilm formation genes primers sequences with their amplicon size Base pair (bp) and their condition.

Genes	Primer sequence (5'-3')	Size (bp)	PCR condition	Reference
16SrRNA	F: GGGCTACACACGTGCTACAA R: GTACAAGACCGGGAACGTA	176	Stage 1. 95°C, 2 min, Stage 2. 95°C, 30 sec. Stage 3. 58.0°C, 30 sec. Stage 4. 72°C, 20.0 sec. Stage 5. 4°C	Fredheim *et al.*, (2009)
aae	F: GAGGAGGATTTTAAAGTGC R: AACATGACCATAGTAACC	858	Stage 1. 95°C, 2 min, Stage 2. 95°C, 30 sec. Stage 3. 58.0°C, 30 sec. Stage 4. 72°C, 20.0 sec. Stage 5. 4°C	Fredheim *et al.*, (2009)
aap	F: ATACAACTGGTGCAGATGGTTG R: GTAGCCGTCCAAGTTTTACCAG	400	Stage 1. 95°C, 2 min, Stage 2. 95°C, 30 sec. Stage 3. 58.0°C, 30 sec. Stage 4. 72°C, 20.0 sec. Stage 5. 4°C	Fredheim *et al.*, (2009)
atlE	F: CAACTGCTCAACCGAGAACA R: TTTGTAGATGTTGTGCCCCA	682	Stage 1. 95°C, 2 min, Stage 2. 95°C, 30 sec. Stage 3. 58.0°C, 30 sec. Stage 4. 72°C, 20.0 sec. Stage 5. 4°C	Fredheim *et al.*, (2009)
embP	F: AGCGGTACAAATGTCAATATC R: AGAAGTGCTCTAGCATCATCC	455	Stage 1. 95°C, 2 min, Stage 2. 95°C, 30 sec. Stage 3. 58.0°C, 30 sec. Stage 4. 72°C, 20.0 sec. Stage 5. 4°C	Fredheim *et al.*, (2009)

Results

In the present study, a total 150 samples of patients suffering from patients with severe inflammation of otitis media, have been collected and tested during period from Maech 2021 to June 2021. The results showed that, out of 150 samples, 133(88.6%) give positive culture, while 17(11.4%) samples were negative culture as shown in Figure (1).

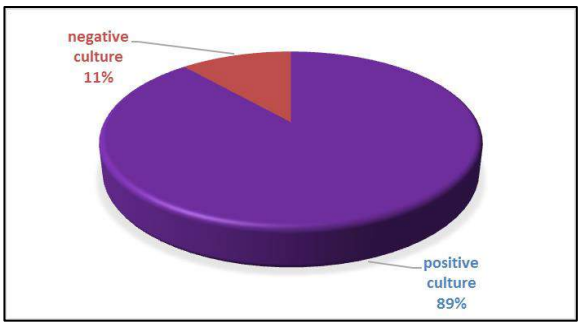

Figure (1): Positive and negative culture of all samples were collected from patients with severe inflammation of otitis media

Out of 133 positive culture on different types of growth media, and the bacterial was identified according to gram stain, the results showed that, 65(48.8%) was classified as gram positive and 68(51.2%) as gram negative. the results were shown in Figure (2)

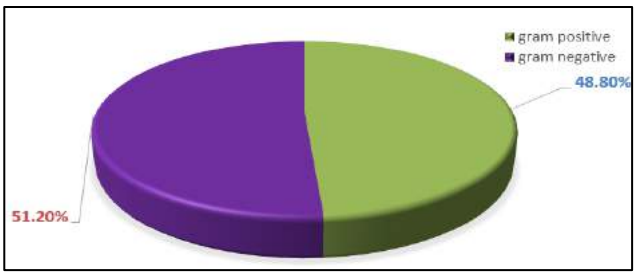

Figure (2): identification of bacteria according to by gram stain

Only 22 bacterial isolates are given growth for *Staphylococcus epidermidis* this was about 33.8% of patients as in Figure (3), while 43(66.2%) isolates were related to other types of bacteria were causes inflammation of otitis media. The biochemical test of identification *Staphylococcus epidermidis* were listed in table (3).

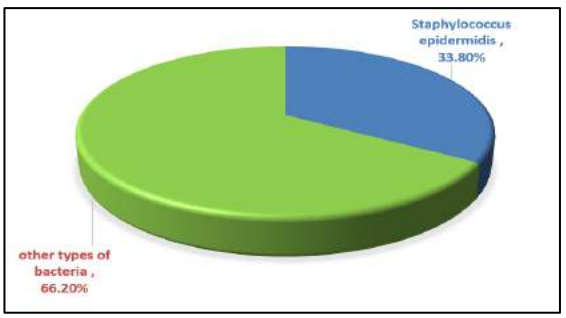

Figure (3): identification of Staphylococcus epidermidis from other types of bacteria

Table (3): Identification of Staphylococcus epidermidis by gram stain and biochemical tests

No.	test	result
•	Capsule	Mostly Capsulated
•	Catalase	Positive (+ve)
•	Citrate	Negative (-ve)
•	Coagulase	Negative (-ve)
•	Gas	Positive (+ve)
•	Gelatin Hydrolysis	Negative (-ve)
•	Gram Staining	Positive (+ve)
•	H_2S	Positive (+ve)
•	Hemolysis	Negative (-ve)
•	Motility	Negative (-ve)
•	MR (Methyl Red)	Negative (-ve)
•	Nitrate Reduction	Positive (+ve)
•	Oxidase	Negative (-ve)
•	Pigment	Negative (-ve)
•	Shape	Cocci
•	Urease	Positive (+ve)
•	VP (Voges Proskauer)	Positive (+ve)

To confirm the isolates of bacteria was used automated Compact Vitek-2 system use GP-ID cards which contained 64 biochemical tests. The results demonstrated that all 22 isolates were confirmed with ID massage confidence level ranging excellent (probability percentage from 94 to 99.7%, this technique was characterized by fast detection of bacteria. it was found that, *Staphylococcus epidermidis* was found in all isolates that identified by biochemical test (100%).

Trypticase SoyBroth mixed with (1 percent) glucose was used in a biofilm test (quantitative biofilm formation) to conduct quantitative biofilm formation studies. This test was performed three times, thereby increasing the accuracy of the analysis. When the mean of OD value were <0.120, 0.120-0.240, and >0.240, respectively, the findings were evaluated as "none", "moderate", and "strong" biofilm former. Eighty-one percent of all Staphylococcus epidermidis isolates were found to be biofilm former, and this category accounted for 18 percent of the total isolates, while isolates that had moderate biofilm formation were four percent (18.2 percent). This table explains the findings (4).

Table (4) Production of biofilm in Staphylococcus epidermidis

Bacterial isolate No.	Biofilm			
	Strong	Moderate	Weak	% of biofilm Formation
Staphylococcus epidermidis (22)	18(81.8%)	4(18.2%)	0(0%)	100

Identification of Staphylococcus epidermidis by PCR technique:

Polymerase chain reaction (PCR) was founded on the use of DNA polymerase to synthesize a new complementary strand of DNA, and the end of the PCR reaction; the newly synthesized strand would have a vast quantity of copies of the particular sequence (Amplicon). In this study, *Staphylococcus epidermidis* were detected by *16SrRNA* genes by PCR technique according to Table (2), all 22(100%) *Staphylococcus epidermidis* were identified by Vitek 2 system give positive results for specific *16srRNA* gene at 176 bp as shown in Figure (4).

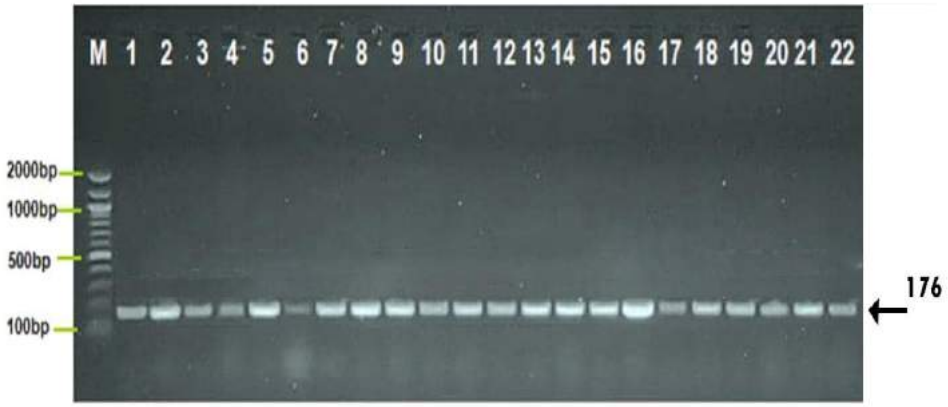

Figure (4): the ethidium bromide-stained 16SrRNA PCR products, the agarose gel electrophoresis procedure was done using voltage settings of 70 V for 50 minutes and U.V light of 301 nm. Approximately 2,000 bp ladder, running from lane (1-22) are Staphylococcus epidermidis gene that identifies the product size (176 bp).

The following 22 isolates previously identified as Staphylococcus epidermidis were screened for biofilm formation genes (aae, aap, atlE, and embP). 18(81.8% of all test results were positive, demonstrating the existence of the aae gene. While comparing the allelic ladder (Figure 5) with the amplified nucleic acid (Figure 19), the amplification success was determined by the presence of (858 bp) and the amplification failure was found by the absence of (858 bp). Using the allelic ladder from Figure 6, 22 (100 percent) produced positive findings for the atlE gene. Figure (7) and (17) demonstrate the existence of (682 bp) and positive findings for the embP gene respectively. According to the findings, the presence of (455 bp) demonstrated that the good results were identified (8).

Figure (5): Agarose gel electrophoresis at 70 volts for 50 minutes is used to visualise aae gene PCR products after they are stained with ethidium bromide in the presence of U.V light at 301 nm. 1200 base pairs of the ladder included the Staphylococcus epidermidis gene, and the size of the result is 1200 base pairs (858 bp).

Figure (6): Following ethidium bromide staining, the aap gene PCR products were observed using an agarose gel electrophoresis system set to run at 70 V for 50 minutes. -80 ng in lane (1,2,3,4,5,7,8,9,10,11,13,14,15,16,18, to...22) (400 bp).

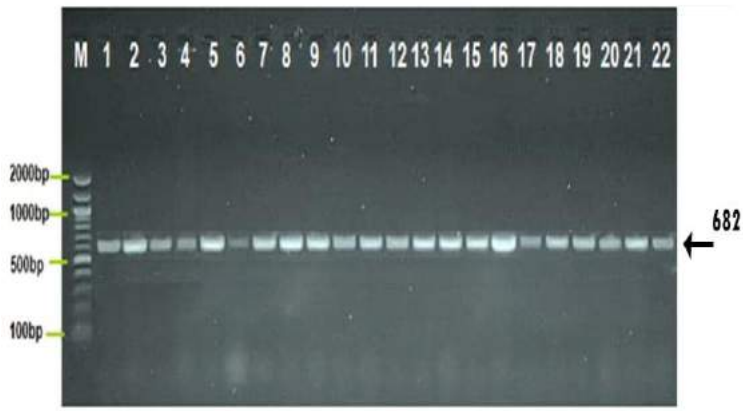

Figure (7): Agarose gel electrophores with voltage of 70 V for 50 minutes has been used for altE gene PCR products visualization in the presence of U.V light. Afterwards, the samples were stained with ethidium bromide. Approximately 2,000 bp ladder, running from lane (1-22) are Staphylococcus epidermidis gene that identifies the product size (682 bp).

Figure (8): Agarose gel electrophoresis at 70 volt for 50 min for embP gene PCR products visualized under U.V light at 301 nm after staining with ethidium bromide. M: 2000 bp ladder; lane (1,2,3,5,6,7,8,9,11,12,14,15,16,18,19,21,22) were positive for Staphylococcus epidermidis gene, the size of product is (455 bp).

Discussion

Common microorganisms (in this case, Staph epidermidis) were the most often isolated microorganism

species from ear infections. Prior to the emergence of these microorganisms, S. epidermidis was deemed non-pathogenic since they were considered pollutants from the skin (Hussain et al., 2021). According to Burmak et al. (2017), OM plays an important function in long-term pain management. Conjointly, antibiotics may be seen as contributing to the development of infections by bacteria not previously seen as infective, such S. epidermidis and Diphthroid bacilli. Antimicrobial chemicals, biocides, chemical stressors (such as pH scale and oxygen), and physical stresses (such pressure, heat, and freezing) are kept at bay because of the biofilm (Giacomett et al., 2021). A microorganism will undergo a microbial virulence issue composition change at intervals when it develops a biofilm, and the production of virulence issues and the ability to move is significantly decreased (Coleman et al., 2020). Microbes may use channels created inside biofilm, a protective environment, to carry nutrients and microbial waste products (Rooney et al., 2020). The biofilm possesses various host and environmental factors, which affects the overall growth pattern and behavior of the biofilm. These signals produced by bacteria in the biofilm can travel through the channels, and the resulting changes influence the overall growth pattern and behavior of the biofilm (Lee, 2020). Biofilm-associated infections are often caused by Staphylococci. Staphylococci are very common as commensals on the skin and secretory surfaces, and hence this unique position among biofilm-associated infections (Fontana & Favaro, 2018). When mature bacteria in biofilms have built up resistance to antimicrobial treatments, their level of ability to escape the host system is significantly elevated (Makabenta et al., 2021). A key human pathogen, S. epidermidis, utilizes a kind of in-vitro model systems to induce biofilm development (Le et al., 2019). The animate thing matrix that controls the microorganism cells in these biofilms is made up of DNA, proteins, and perhaps polysaccharides. Without those choline-binding proteins, the biofilm development would cease (Keren-Paz & Kolodkin-Gal, 2020). The S. epidermidis strains are found in most hospitalized patients, and this contributes to the ever-changing commensal bacterial community by facilitating the isolation of more virulent isolates. Nevertheless, the pathogenicity connection between this gene and the SesI macromolecule remains unclear, and more research is required to find out whether the SesI macromolecule increases the infective capacity of S. epidermidis (Salgueiro et al., 2019). S. epidermidis's ability to produce biofilms is unquestionable in animal models of biomaterial-related illnesses (Hofmans et al., 2018). however the discovery of distinct virulence factors, among which poly-γ-DL-glutamic acid is a prominent member, counters innate immune systems (Staudacher, 2017). S. epidermidis biofilm formation is usually conceived of as a four-step method: (1) adhesion, (2) accumulation, (3) maturity, and (4) dissemination (Balaure, & Grumezescu, 2020). Because adhesion and aggregation are both required for biofilm development, it was deduced that the aap macromolecule fulfills both essential roles. In the early stages of biofilm development, the bacterium attaches itself to a distant body or substrate. Early contacts have hydrophobic properties (Arciola et al., 2018). Binding to such abiotic surfaces may be facilitated by proteins that are unique to that particular abiotic surface (Gunaratnam et al., 2020). Both the adhesins/autolysins called atlE and aae are functional in the species known as S. epidermidis. While these proteins help to attach to vitronectin non-covalently, they also aid in the lifting of eDNA, which has recently been shown to be an important measure in S. epidermidis biofilm formation (Abbondio et al., 2019).

Conclusion

Staphylococcus epidermidis was important bacteria and it considered important etiological agents of severe inflammation of otitis media, and have biofilm formation genes responsible for causing inflammation of mucous layers lead to infection of otitis media.

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