Global Scientific JOURNALS

GSJ: Volume 10, Issue 4, April 2022, Online: ISSN 2320-9186
www.globalscientificjournal.com

EVALUATION OF DIFFERENT METHODS USED FOR DETECTION OF BIOFILM FORMATION BY ORAL *STAPHYLOCOCCI* AND ASSOCIATION OF THIS ABILITY WITH ENHANCED ANTIBIOTIC RESISTANCE

[1]Samiaa Anjum, [1,2] Bibi Khadija, [1]Rooh Ullah, [1*]Rani Faryal

[1]Departement of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan

[2]Department of Medical Laboratory Technology, National Skills University, Islamabad, Pakistan

[*]Corresponding author Email address: ranifaryal@qau.edu.pk

KeyWords
Congo red agar, *Ica* genes, Microtiter plate, *Staphylococci*

ABSTRACT

Staphylococcal species are one of the common causes of biofilm-associated infections. Several methods are available for the detection of this ability. In the present study, oral *Staphylococci* were checked for biofilm formation by different methods to compare their reliability and to find their association with antibiotic resistance. For isolation of *Staphylococcus* species, saliva samples were collected from 102 healthy individuals. Purified and confirmed isolates from samples were checked for antimicrobial susceptibility against recommended antibiotics according to CLSI guidelines and biofilm-forming ability by using Congo red agar (CRA), Microtiter plate (MTP) and amplification of intercellular adhesion (*Ica*) genes. The validity of methods and metrics for test performance was also determined. Among the *Staphylococcus* group, *S. aureus* was the prevalent species. By CRA, 62% and by MTP, 38% of *Staphylococcus* isolates were able to produce biofilms. *Ica A, B, C, D* genes were found in 77%, 38%, 27% and 57% of the isolates, respectively. About 15% and 25% of isolates were biofilm formers by MTP and CRA, respectively, despite of the absence of *Ica AD* genes. CRA and MTP exhibited a lower sensitivity in comparison to PCR method. Biofilm formers also showed high resistance against cephalothin, ceftriaxone, cefoxitin, penicillin, tetracycline and chloramphenicol. The PCR method remains a better tool to screen biofilm production in *Staphylococcus* species. Biofilm-forming species showed high antibiotic resistance compared to non-biofilm formers.

Introduction

Staphylococci are opportunistic, intrinsically multidrug-resistant organisms and were considered to be transient resident flora of the human oral cavity (Ohara-Nemoto *et al*., 2008). They are often isolated from tongue, saliva, mucosal surfaces, supragingival tooth surfaces and the periodontal pocket (Kim & Lee, 2015). The increased use of indwelling medical devices has had considerable impact on the role of *Staphylococci* in clinical medicine. The predominant species isolated in these infections are *Staphylococcus epidermidis* and *Staphylococcus aureus* (Gad *et al*., 2009). In oral cavity, *S. aureus* has been associated with dento-alveolar infections, oral mucosal lesions and exacerbating dental diseases by forming a biofilm with periodontal pathogens (Kim & Lee, 2015; Nourbakhsh & Namvar, 2016). In dental health care settings, there is an increasing chance of cross-infections by *S. aureus* during dental therapy (Kimmerle *et al*., 2012). Apart from *S. aureus, S. epidermidis* is a saprophyte which is a part of human skin and mucosa with a capacity to cause diseases in immunocompromised patients (Abu Taleb *et al*., 2012).

It was found that their major pathogenic factor is the ability to adhere to biotic as well as abiotic surfaces, where they subsequently form a complex biofilm (Kord *et al*., 2018; Oliveira & Cunha, 2010). Biofilm increases the severity of *Staphylococcus* related infections under defined conditions and results in an increased resistance of the organism to therapeutic antimicrobial agents (Boles & Horswill, 2008; Nourbakhsh & Namvar, 2016). Biofilm formation is mediated by polysaccharide intracellular adhesion (PIA) which is encoded by *Ica ADBC* operon and provide protection against host immune system and antimicrobial substances (Nourbakhsh & Namvar, 2016; Sheriff & Sheena, 2016). Among *Ica* genes, *Ica A* and *Ica D* have been reported to play a significant role in biofilm formation in *S. aureus* and *S. epidermidis*, as the *Ica A* gene encodes N-acetylglucosaminyltransferase, involved in the synthesis of N-acetyl glucosamine oligomers from UDP-N-acetyl glucosamine. Furthermore, *Ica D* increases the expression of N-acetylglucosaminyltransferase, hence leads to the phenotypic expression of the capsular polysaccharide. *Ica C* also act as a receptor for polysaccharides and the function of *Ica B* gene still remains undiscovered (Nourbakhsh & Namvar, 2016; Gowrishankar *et al*., 2016).

For the detection of biofilm production by *Staphylococcal* species various simple and cost effective tests are available (Sheriff & Sheena, 2016). Moreover, DNA-based techniques like PCR methods have been used for molecular detection of biofilm formation (Darwish & Asfour, 2013). For the assessment of biofilm, a controversy still exists among scientists regarding what is the most reliable method for biofilm detection(Kord et al., 2018; Oliveira & Cunha, 2010). In this regard, the present study was designed to determine biofilm formation in clinical *Staphylococcus* species isolates to evaluate the reliability of phenotypic and molecular methods for detection of a multidrug resistant biofilms.

Material and methods

Bacterial isolates

The present study was approved from Bio-Ethical Committee (BEC) of Quaid-I-Azam University (QAU), Islamabad. For isolation of bacteria, 102 saliva samples (2-3 mL) were collected from healthy students and staff members (51 males and 51 females) of QAU, Islamabad, by passive drooling method after taking written informed consent. Samples were processed within 2 h after collection in Molecular Medicine Laboratory, QAU. All specimens were cultured on nutrient agar (Oxoid) and incubated at 37 $^{\circ}$C for 24 h. colonies that had grown were further purified and subjected to standard microbiological and biochemical tests for identification of isolates. For final confirmation of *Staphylococcus* species, API-10E-Staph system kits (Bio-Merieux, France) were used.

Antibiotic susceptibility testing

Antimicrobial susceptibility testing was done by Kirby-Bauer disc diffusion method recommended by Clinical and Laboratory Standards Institute (CLSI) guidelines. Results were observed according to break point of inhibition recommended by CLSI-2018-M100 (CLSI, 2018).

Methods used for detection of biofilm formation in Staphylococcal isolates

Biofilm formation in *Staphylococcus* isolates was carried out by using two phenotypic methods (Congo red assay and Microtiter plate method) and genotypically by amplification of *Ica* operon genes.

Congo red assay (CRA)

For qualitative analysis of biofilm formation, CRA method was used as described previously (Freeman *et al*., 1989). All isolates were inoculated on CRA plates and incubated at 37°C for 18-24 h. Results were recorded according to a 5-colourimetric scale method (Oliveira and Cunha, 2010).

Microtiter plate assay (MTP)

Biofilm forming ability of all isolates was quantified by performing MTP assay as described previously (Hasannejad Bibalan, Javid, Samet, Shakeri, & Ghaemi, 2014). Cut off optical density was calculated from control and results were interpreted using criteria as described by Abou El-Khier *et al.,* (2015).

PCR for amplification of Ica operon genes

For molecular identification of biofilm forming genes in *Staphylococcus* isolates, amplification of *Ica ADBC* operon genes was carried out. DNA was isolated by Phenol-Chloroform method (Mehri *et al*., 2017). The *Ica A, Ica B, Ica C* and *Ica D* genes were amplified by using primers given in Table 1. A reaction mixture of 10.0 µL was prepared, composed of master mix 5.0 µL (Thermoscientific 2X), 0.2 µL of each primer, PCR water 3.6 µL and 1.0 µL of template DNA. Thermal cycler conditions include; initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 45 seconds, annealing for 45 sec at different temperatures, extension at 72°C for 45 seconds, and final extension at 72°C for 7 min (Table 1).

Table 1. Primer sequences used in PCR for amplification of *Ica ADBC* operon genes

Primers	Sequence 5' → 3'	Product size (bp)	Tm (°C)
Ica A	F: 5'-ACACTTGCTGGCGCAGTCAA-3'	188	52
	R: 5'-TCTGGAACCAACATCCAACA-3'		
Ica B	F: 5'-AGAATCGTGAAGTATAGAAAATT-3'	880	52
	R: 5'-TCTAATCTTTTTCATGGAATCCGT-3'		
Ica C	F: 5'-ATGGGACGGATTCCATGAAAAAGA-3'	1056	39
	R: 5'-TAATAAGCATTAATGTTCAATT-3'		
Ica D	F: 5'-ATGGTCAAGCCCAGACAGAG-3'	198	42
	R: 5'-AGTATTTTCAATGTTTAAAGCAA-3'		

Comparative analysis of different methods used for detection of biofilm formation in Staphylococcal isolates

For the comparative analysis of biofilm detection methods, the validity of methods which includes sensitivity, specificity and accuracy was carried out. Furthermore, the metrics for test performance such as positive and negative predictive values (PPV/NPV), and positive and negative likelihood ratio (PLR/NLR) for the methods were also compared against the PCR gold standard method. Moreover, for the analysis of data IBM SPSS version 21.0 software was used.

Results

Isolation of oral Staphylococci

A total of 102 saliva samples were collected from healthy individuals of mean age 26 years±4.3 (male to female ratio was 1:1). Out of

102 saliva samples screened, 95 samples were culture positive, from which 97 isolates were produced. Among the 97 isolates, 52 were confirmed as *Staphylococcal* spp. among which the most prevalent species were *S. aureus* (*n*=36) followed by *S. epidermidis* (*n*=16).

Antibiotic susceptibility pattern

Highest sensitivity was observed against amikacin (95%) followed by clindamycin (89%), nitrofurantoin and linezolid (80%), ciprofloxacin, sulfonamides, tobramycin and ofloxacin (77%), tetracycline and chloramphenicol (64%). Lowest sensitivity was observed against amoxicillin (45%). High resistance was observed against cephalosporin of first and third generation i.e. 57% resistance was observed against cephalothin and 52% was against ceftriaxone although about 50% resistance was also observed against methicillin (Table 2).

Table 2. Antibiotic susceptibility pattern of oral *S. aureus* and *S. epidermidis* isolates

Antibiotics		**Sensitive** *n* **(%)**	**Intermediate** *n* **(%)**	**Resistant** *n* **(%)**
Aminoglycosides	TOB 30µg	34 (77.0)	02 (5.00)	08 (18.0)
	AK 30µg	42 (95.0)	01 (2.00)	01 (2.00)
Fluoroquinolones	CIP 5µg	34 (77.0)	04 (9.00)	06 (14.0)
	OFX 5µg	34 (77.0)	00(0.00)	10 (23.0)
Cephalosporins	KF 30µg	04 (9.00)	15 (34.0)	25 (57.0)
	CRO 30µg	18 (41.0)	03 (7.00)	23 (52.0)
Sulfonamides	TMP/SMX 25µg	34 (77.0)	03 (7.00)	07 (16.0)
Cefoxitin	FOX 30µg	18 (41.0)	04 (9.00)	22 (50.0)
Tetracycline	TE 30µg	28 (64.0)	06 (14.0)	10 (23.0)
Penicillin	AML 10µg	20 (45.0)	05 (11.0)	10 (23.0)
Lincosamide	DA 10µg	39 (89.0)	01 (2.00)	04 (9.00)
Nitrofurantoin	F 300µg	35 (80.0)	01 (2.00)	08 (18.0)
Chloramphenicol	C 30µg	28 (64.0)	02 (5.00)	14 (32.0)
Linezolid	LZD 30µg	35 (80.0)	00(0.00)	09 (20.0)

TOB; Tobramycin, AK; Amikacin, CIP; Ciprofloxacin, OFX; Ofloxacin, KF; Cephalothin CRO; ceftriaxone, AML; Amoxicillin.

Detection of the biofilm forming ability by CRA and MTP method

By MTP and CRA method, 38% (20/51) and 62% (32/52) isolates were confirmed as biofilm producers, respectively. Bright black colonies were produces by 46% (24/52) of isolates and were confirmed as strong biofilm producers by CRA assay while no strong biofilm former isolate was detected by using MTP method. By CRA method, 15% (8/52) isolates produced dry opaque colonies and were identified as weak biofilm producers while by MTP, 38% (20/52) isolates showed weak biofilm forming ability. Among all isolates 63% (32/52) were confirmed as non-biofilm formers by MTP method, whereas 39% (20/52) isolates were identified as non-biofilm former by CRA method. Results of both methods did not correlate with each other.

Detection of the biofilm forming genes (Ica genes) by PCR

Ica A was detected in 77% (40/52) of *Staphylococcus* isolates and 57% (30/52) isolates were positive for *Ica D* gene. *Ica B* gene was present among 38% (20/52) isolates and *Ica C* was observed in only 27% (14/52) isolates. Regarding correlation of CRA and MTP me-

thod, with the presence of *Ica AD* genes, 46% (24/52) isolates were non-biofilm forming when assessed by MTP and 39% (20/52) isolates were observed as non-biofilm formers by CRA method but were positive for *Ica A* or *Ica D* genes. Similarly, 31% (16/52) and 13% (9/52) isolates were non-biofilm formers by MTP and CRA method, respectively, but were positive for *Ica B* or *Ica C* genes. About 15% (8/52) and 25% (13/52) of isolates were biofilm formers by MTP method and CRA, respectively, despite the absence of *Ica AD* genes. Similarly, 33% (17/52) and 52% (27/52) of isolates were observed as biofilm formers on MTP and CRA but were negative for *Ica BC* genes. But only 6% (3/52) of biofilm formers by MTP and CRA method were positive for whole *Ica* operon while 10% (5/52) and 8% (4/52) non-biofilm isolates by MTP and CRA method were observed as positive for complete *Ica* operon.

Association of antibiotic resistance with biofilm forming ability of Staphylococci

Biofilm former strains showed higher resistance than the non-former isolates except for tobramycin, amikacin and ciprofloxacin. Highest resistance among the biofilm formers was observed for cephalothin, ceftriaxone, cefoxitin, penicillin, tetracycline and chloramphenicol from 33.33-82% (Table 3).

Table 3. Antimicrobial resistance pattern exhibited by biofilm former and non-biofilm former oral *Staphylococci*

Antibiotic		**Biofilm formers** *n* **(%)**	**Non-biofilm formers** *n* **(%)**
Aminoglycosides	TOB 30µg	06 (18.18)	04 (21.0)
	AK 30µg	01 (3.03)	01 (5.26)
Fluoroquinolones	CIP 5µg	05 (15.15)	05 (26.31)
	OFX 5µg	07 (21.21)	02 (11.0)
Cephalosporins	KF 30µg	27 (82.0)	12 (63.0)
	CRO 30µg	19 (58.0)	06 (32.0)
Sulfonamides	TMP/SMX µg	07 (21.21)	03 (16.0)
Cefoxitin	FOX µg	19 (58.0)	06 (32.0)
Tetracycline	TE 30µg	11 (33.33)	05 (26.31)
Penicillin	AML 10µg	17 (52.0)	06(32.0)
Lincosamide	DA 10µg	03 (9.09)	01 (5.26)
Nitrofurantoin	F 300µg	07 (21.21)	01 (5.26)
Chloramphenicol	C 30µg	11 (33.33)	04 (21.0)
Linezolid	LZD 30µg	07(21.21)	01 (5.26)

Comparative analysis of detection methods used for biofilm formation

Sensitivity and specificity of PCR was estimated to be 100% and 86% respectively, with 94% accuracy. CRA and MTP had a relatively acceptable sensitivity (69% and 65%) and low specificity (45% and 38% respectively) to identify biofilm phenotype, when compared to PCR (Table 4).

Table 4. Comparison of different diagnostic parameters for testing validity of CRA, MTP and PCR methods for *Staphylococci* biofilm detection

Statistical	**SN**	**SP**	**PPV**	**NPV**	**PLR**	**NLR**	**Accuracy**

parameters	(%)	(%)	(%)	(%)	(%)	(%)	(%)
CRA	69	45	67	47	1.56	1.51	60
MTP	65	38	40	63	1.75	1.68	48
PCR	100	86	91	100	1.17	1.15	94

SN; Sensitivity, **SP**; Specificity, **PPV**; Positive Predictive Value, **NPV**; Negative Predictive Value, **PLR**; Positive Likelihood Ratio, **NLR**; Negative Likelihood Ratio.

Discussion

In present study, evaluation of the isolation frequency of *S. aureus* and *S. epidermidis* from oral cavity of healthy adults along with its antibiotic susceptibility profile and biofilm forming ability was determined. Furthermore, comparative analysis of methods which were used for the detection of biofilm formation by antibiotic resistant oral *Staphylococci* was also carried out**.** It was found from present work that *S. aureus* (36/52, 69.2%) was the commonly isolated organism from saliva samples followed by *S. epidermidis* (16/52, 30.7%). Antibiotic susceptibility testing of these isolates showed that majority of isolates were sensitive to the commonly used antibiotics. Resistance was seen against cephalosporin of first and third generation i.e. 57% against cephalothin and 52% against ceftriaxone. Results from the present study were in line with the study conducted by Bello *et al*., (2013) and Rahman *et al*., (2015) which reported that majority of oral bacteria showed susceptibility to most of the antibiotics and no single bacteria was resistant to amikacin, amoxicillin and imipenem. Contrary results were reported by Silva *et al*., (2016), where amoxicillin was an ineffective drugs because of its high resistance while in current work majority of isolates were susceptible to amoxicillin. The methicillin resistance rate for *S. aureus* observed in this study was 31% which was similar to the results of Bueris *et al*., (2005). Although most of the oral bacteria from present study showed antibiotic sensitivity, even low resistance can be a serious health risk and lead to the spread of antibiotic resistance. Furthermore, the detection of MRSA strains indicates that they are already defunded at the community, and thus these individuals may serve as a reservoir and source of multi-resistant pathogenic *S*. *aureus* strains.

In present work, results showed that 38% and 62% of *Staphylococcus* isolates exhibit biofilm production by MTP Rehman et awhere only 23% of isolates had a biofilm phenotype identified by both CRA and MTP. Kord et al., (2018) also reported no correlation between CRA and MTP method as only 9.7% of isolates were identified as biofilm formers by both methods. In our study, 38% isolates were biofilm formers by MTP method which was very close to the findings of Arciola *et al*., (2001) and Liduma *et al*., (2012). However, similar to our study, lower rates of biofilm phenotype by MTP was reported by Gad *et al*., (2009) and Saising *et al*., (2012) from Egyptian and Thailand population respectively. These discrepancies in results could be due to the use of different spectrophotometric procedures and sources of clinical specimen (Abou El-Khier et al., 2015), and also attributed to the fact that phenotypic expression of biofilm formation is highly sensitive to *in vitro* conditions and hence can be detected variably by different methods (Shrestha *et al*., 2018). CRA method results from the present study were in complete disagreement with the study carried by Kord *et al*., (2018) and Hassan et al., (2011). Similar to the present work, results reported by El-Mahallawy *et al*., (2009) and Abou El-Khier et al., (2015). PCR is used as a gold standard method for biofilm detection (Rampelotto *et al*., 2018). PCR amplification of *Ica* gene locus represents that *Ica A* and *Ica D* were most detected (77% and 57% respectively) while, *Ica B* and *Ica C* genes were least prevalent genes (38% and 27%, respectively) expressed by *Staphylococcus* isolates. Current findings were in accordance with the reports of Gad *et al*., (2009). There were some variants in present study which were non-biofilm formers by phenotypic methods but were *Ica* positive. Chromosomal point mutations, post-translational regulation and negative translational mechanisms may be the reasons of non-biofilm formation in strains in which *Ica* locus is present and thus affects the production of biofilm associated proteins (Abu Taleb *et al*., 2012; Darwish & Asfour, 2013; Los *et al*., 2010). Some isolates were observed as biofilm formers by phenotypic methods but were

detected negative for *Ica* genes suggesting that this may be due to an *Ica* gene-independent control of biofilm formation/adhesion process in *Staphylococci* (Kord *et al*., 2018). Biofilm forming bacteria are usually less susceptible to antibiotics than planktonic bacteria (Pinheiro *et al*., 2014; Shrestha *et al*., 2018). Biofilm former strains from the present work showed higher resistance than the non-former isolates except for tobramycin, amikacin, and ciprofloxacin. Our findings are somewhat similar to the work conducted by Shrestha *et al*., (2018).

Conclusion

The current study showed that PCR method is more appropriate, as it is less costly as well as less likely that results would be misinterpreted. In contrast, CRA, although easier and faster to perform but less sensitive and therefore, cannot be recommended as a screening test for identifying biofilm production by *Staphylococcus* species. Biofilm forming ability also enhanced antibiotic resistance. Moreover, the present study revealed that the presence of *Ica* genes alone does not lead to biofilm formation. On the other hand, the biofilm-forming ability of some isolates in the absence of *Ica* genes emphasizes the importance of *Ica*-independent mechanisms of biofilm formation.

Acknowledgment

We would like to acknowledge all the faculty members and laboratory staff of the Department of Microbiology, Quaid-I-Azam University, Islamabad for their cooperation during data and sample collection.

References

Abou El-Khier, N. T., El-Kazzaz, S. S., Elganainy, A. E. (2015). Phenotypic and genotypic detection of biofilm formation in Staphylococcus epidermidis isolates from retrieved orthopaedic implants and prostheses. *British Microbiology Research Journal International*, 9(4);1–10.

Abu Taleb, A. M. F., Mohamed, M. S., Abdel-Latif, R. S., and Gouda, M. (2012). The role of ica operon and biofilm formation in coagulase negative staphylococcal infection. *The Egyptian Journal of Medical Microbiology*, 21(1):21–32.

Arciola, C. R., Baldassarri, L., and Montanaro, L. (2001). Presence of icaA and icaDGenes and slime production in a collection of Staphylococcal strains from catheter-associated infections. *Journal of Clinical Microbiology*, 39(6):2151–2156.

Bello, O., Osho, A., Bankole, S., and Bello, T. (2013). Antibiotic Susceptibility Profiles and Bacteriological Risks Associated With Used Toothbrushes: A Case Study of Some Apparently Healthy University Students in Southwestern Nigeria. *American International Journal of Biology*, 1(1):1–12.

Boles, B. R., and Horswill, A. R. (2008). Agr-mediated dispersal of Staphylococcus aureus biofilms. *PLoS Pathogens*, 4(4):e1000052.

Bueris, V., Pimenta, F. C., Ito, I. Y., and Marin, J. M. (2005). Oral incidence of Staphylococcus aureus and antimicrobials agents resistance. *Brazilian Journal of Oral Sciences*, 4(12):676–679.

CLSI. (2018). *Performance Standards for Antimicrobial Susceptibility Testing*.

Darwish, S. F., and Asfour, H. A. E. (2013). Investigation of biofilm forming ability in staphylococci causing bovine mastitis using phenotypic and genotypic assays. *The Scientific World Journal*, 2013:378492. https://doi.org/10.1155/2013/378492

El-Mahallawy, H. A., Loutfy, S. A., El-Wakil, M., El-Al, A. K. A., and Morcos, H. (2009). Clinical Implications of icaA and icaD Genes in Coagulase Negative Staphylococci and Staphylococcus aureus Bacteremia in Febrile Neutropenic Pediatric Cancer Patients. *Pediatric Blood & CancerPediatric Blood & Cancer*, 52:824–828. https://doi.org/10.1002/pbc

Freeman, D. J., Falkiner, F. R., and Keane, C. T. (1989). New method for detecting slime production by coagulase negative staphylococci. *Journal of Clinical Pathology*, 42(8):872–874.

Gad, G. F. M., El-feky, M. A., El-rehewy, M. S., Hassan, M. A., Abolella, H., and El-baky, R. M. A. (2009). Detection of icaA , icaD genes and biofilm production by Staphylococcus aureus and Staphylococcus epidermidis isolated from urinary tract catheterized patients. *The Journal of Infection in Developing Countries*, 3(5):342–351.

Hasannejad Bibalan, M., Javid, N., Samet, M., Shakeri, F., and Ghaemi, E. A. (2014). Biofilm formation in Staphylococcus aureus and its relation to phenotypic and genotypic criteria. *Medical Laboratory Journal*, 8(3):1–7.

Hassan, A., Usman, J., Kaleem, F., Omair, M., Khalid, A., and Iqbal, M. (2011). Evaluation of different detection methods of biofilm formation in the

clinical isolates. *Brazilian Journal of Infectious Diseases*, 15(4):305–311. https://doi.org/10.1590/S1413-86702011000400002

Kim, G. Y., and Lee, C. H. (2015). Antimicrobial susceptibility and pathogenic genes of Staphylococcus aureus isolated from the oral cavity of patients with periodontitis. *Journal of Periodontal and Implant Science*, 45(6):223–228. https://doi.org/10.5051/jpis.2015.45.6.223

Kimmerle, H., Wiedmann-Al-Ahmad, M., Pelz, K., Wittmer, A., Hellwig, E., and Al-Ahmad, A. (2012). Airborne microbes in different dental environments in comparison to a public area. *Archives of Oral Biology*, 57(6):689–696. https://doi.org/10.1016/j.archoralbio.2011.11.012

Kord, M., Ardebili, A., Jamalan, M., Jahanbakhsh, R., Behnampour, N., and Ghaemi, E. A. (2018). Evaluation of Biofilm Formation and Presence of Ica Genes in Staphylococcus epidermidis Clinical Isolates Osong Public Health and Research Perspectives. *Osong Public Health and Research Perspectives*, 9(4):160–166. https://doi.org/10.24171/j.phrp.2018.9.4.04

Liduma, I., Tračevska, T., Bers, U., and Žileviča, A. (2012). Phenotypic and genetic analysis of biofilm formation by Staphylococcus epidermidis. *Medicina (Lithuania)*, 48(6):305–309. https://doi.org/10.3390/medicina48060045

Los, R., Sawicki, R., Juda, M., Stankevic, M., Rybojad, P., Sawicki, M., Malm, A., Ginalska, G. (2010). A comparative analysis of phenotypic and genotypic methods for the determination of the biofilm-forming abilities of Staphylococcus epidermidis. *FEMS Microbiology Letters*, 310(2):97–103.

Mehri, H., Jahanbakhsh, R., Shakeri, F., Ardebili, A., Behnampour, N., Khodabakhshi, B., and Ghaemi, E. A. (2017). Investigation of glycopeptide susceptibility of coagulase-negative staphylococci (CoNS) from a tertiary care hospital in Gorgan, northern Iran. *Archives of Pediatric Infectious Diseases*, 5(1):e37264.

Nourbakhsh, F., and Namvar, A. E. (2016). Detection of genes involved in biofilm formation in Staphylococcus aureus isolates. *GMS Hygiene and Infection Control*, 11:Doc07. https://doi.org/10.3205/dgkh000267

Ohara-Nemoto, Y., Haraga, H., Kimura, S., and Nemoto, T. K. (2008). Occurrence of staphylococci in the oral cavities of healthy adults and nasal-oral trafficking of the bacteria. *Journal of Medical Microbiology*, 57(1):95–99. https://doi.org/10.1099/jmm.0.47561-0

Oliveira, A., and Cunha, M. D. L. R. S. (2010). Comparison of methods for the detection of biofilm production in coagulase-negative staphylococci. *BMC Research Notes*, 3(1):260. https://doi.org/10.1186/1756-0500-3-260

Pinheiro, L., Brito, C. I., Pereira, V. C., Oliveira, A. De, Camargo, C. H., and Cunha, M. D. L. R. D. S. da. (2014). Reduced susceptibility to vancomycin and biofilm formation in methicillin-resistant Staphylococcus epidermidis isolated from blood cultures. *Memórias Do Instituto Oswaldo Cruz*, 109(7):871–878. https://doi.org/10.1590/0074-0276140120

Rahman, M., Islam, M. N., Islam, M. N., and Hossain, M. S. (2015). Isolation and identification of oral bacteria and characterization for bacteriocin production and antimicrobial sensitivity. *Dhaka University Journal of Pharmaceutical Sciences*, 14(1):103–109.

Rampelotto, R. F., Lorenzoni, V. V., Silva, D. da C., Coelho, S. S., Wust, V., Garzon, L. R., Nunes, M. S., Meneghetti, B., Brites, P. C., Horner, M., Hörner, R. (2018). Assessment of different methods for the detection of biofilm production in coagulase-negative staphylococci isolated from blood cultures of newborns. *Revista Da Sociedade Brasileira de Medicina Tropical*, 51(6):761–767. https://doi.org/10.1590/0037-8682-0171-2018

Saising, J., Singdam, S., Ongsakul, M., and Voravuthikunchai, S. P. (2012). Lipase, protease, and biofilm as the major virulence factors in staphylococci isolated from acne lesions. *Bioscience Trends*, 6(4):160–164.

Sheriff, R., and Sheena, A. (2016). Assessment of Biofilm Production in Clinically Significant Isolates of Staphylococcus epidermidis and Comparison of Qualitative and Quantitative Methods of Biofilm Production in a Tertiary Care Hospital. *International Journal of Scientific Study*, 4(6):41–46. https://doi.org/10.17354/ijss/2016/482

Shrestha, L. B., Bhattarai, N. R., and Khanal, B. (2018). Comparative evaluation of methods for the detection of biofilm formation in coagulasenegative staphylococci and correlation with antibiogram. *Infection and Drug Resistance*, 11:607–613. https://doi.org/10.2147/IDR.S159764

Silva, S. S., Ribeiro, M. de O., Gomes, F. I. F., Chaves, H. V., Silva, A. A. R. E., Zanin, I. C. J., and Barbosa, F. C. B. (2016). Occurrence and antimicrobial susceptibility of enteric rods and pseudomonads isolated from the dental prostheses biofilm. *Journal of Applied Oral Science*, 24(5):462–471.