The Role of Ica Operon and Biofilm Formation in Coagulase Negative Staphylococcal Infection

Ashwak M.F. Abu Taleb, Mervat S. Mohamed, Randa S. Abdel-Latif and Manar Gouda
Microbiology & Immunology Department, Faculty of Medicine, Zagazig University

ABSTRACT

Coagulase negative staphylococci are normal skin commensals and are frequently isolated from clinical specimens. CoNS are a major cause of sepsis in the neonatal intensive care unit. The virulence of these bacteria is mainly due to their ability to form biofilms on indwelling medical devices. One important element in this process is the ica operon (intercellular adhesion operon), a gene cluster encoding the production of polysaccharide intercellular adhesion (PIA), which mediates the intercellular adherence of bacteria and the accumulation of multilayer biofilm . This work aimed to evaluate that pathogenic CoNS isolates are more likely to be positive for the ica operon and to produce biofilm than isolates isolated randomly from healthy individuals. Also to compare between antibiotic sensitivity of biofilm producing CoNS isolates and non-biofilm producing CoNS isolates. Finally to detect source of infection in neonatal intensive care unit using biotyping, antibiogram and plasmid profile as epidemiological markers. This study was conducted from April 2010 to April 2011, at Medical Microbiology and Immunology Department, Faculty of Medicine Zagazig University. The study included 40 neonates admitted to NICU, with picture of bacteremia with the mean age 17.43± 7.2 days. From them 40 blood samples were taken from peripheral sites and 40 skin swabs were taken from axilla for culture on blood culture bottles and blood agar respectively. 40 age matched healthy neonates as control group and 25 health care workers from NICU to detect source of infection were enrolled in the study. The biofilm production was examined using congo red agar and the presence of genes icaA, icaD were determined by PCR. Biotyping, antibiogram and plasmid profiles were used as epidemiological markers to detect source of infection in NICU. The isolated CoNS were (32.5% , 37.5%, 20% and 24 from blood samples, skin swabs, control and health care workers respectively and the most common isolated organism was S. epidermidis followed by S. haemolyticus then S. hominis. Also the results of qualitative detection of biofilm formation were 69.2%, 33.3% and 33.3% from the studied specimens respectively but all control were non-biofilm forming. The icaA and icaD genes were 76.9% , 40% and 33.3% from studied specimens respectively but both genes were not found in any control isolates. We conclude that the isolates of CONS infections are more likely to be positive for ica operon and health care workers play a role in dissemination of CONS infection in hospital.

INTRODUCTION

Coagulase negative staphylococci (CoNS) are normal inhabitants of human skin and mucous membranes. They have long been dismissed as culture contaminants. Later on CoNS are recognized as etiologic agents of wide variety of infections. CoNS play a role in bacteremia, central nervous system shunt infection, endocarditis, urinary tract infection, surgical site infection, endophthalmitis, foreign body infection and many other infections. Patients with CoNS infections are usually immunocompromised, with indwelling or implanted foreign bodies [1].

CoNS are a major cause of sepsis in the neonatal intensive care unit (NICU) especially late onset sepsis in preterm infants [2].

Approximately 17% of very low birth weight (<1500gm) neonates develops an episode of CoNS bacteremia and this event is associated with a significant increase in morbidity, mortality, duration of hospital stay and overall cost of treatment [3].

Sepsis due to CoNS is common in the NICU. The incidence of CoNS sepsis varies between 1.3 and 19.9% depending on birth weight and gestational age. Most of these infections respond well to vancomycin, the first drug of choice. A minority of neonates develops a persistent staphylococcal bacteremia, which does not respond to vancomycin. For these neonates rifampin may be a safe and effective additive treatment to vancomycin[4].

The virulence of these bacteria is mainly due to their ability to form biofilms on indwelling medical devices. Biofilm related infections often fail to respond to antibiotic chemotherapy guided by conventional antibiotic susceptibility tests [5].

The mechanism by which CoNS attach to prosthetic material and elaborate biofilm is

being increasingly understood. This is a complex and multistep process [6].

One important element in this process is the ica operon (intercellular adhesion operon), a gene cluster encoding the production of polysaccharide intercellular adhesion (PIA), which mediates the intercellular adherence of bacteria and the accumulation of multilayer biofilm [7].

The operon is composed of five genes, icaA, icaD, icaB, icaC and icaR. IcaA and icaD were chosen to be tested in this study due to their importance in the operon, as they code for proteins (icaA,D) that together represents a novel enzyme combination that is responsible for the production of PIA [8].

CoNS have become the leading cause of infections due to its biofilm formation. Numerous studies have shown that coagulase negative staphylococci biofilm formation is a two step process, in which bacteria first adhere to the surface (initial attachment phase), and subsequently form cell to cell aggregates and a multilayered architecture (accumulative phase).In the accumulative phase the PIA, encoded by the ica ADBC locus is the major component mediating intercellular adhesion [9].

Multiresistant staphylococci frequently cause nosocomial infections. Also many staphylococcal antibiotic resistance determinants are plasmid encoded. The wide variety of plasmids present in staphylococci has made the use of plasmid profiles convenient for studying outbreaks caused by these bacteria [10].

The aim of this study was to evaluate that pathogenic CoNS isolates are more likely to be positive for the ica operon and to produce biofilm than isolates isolated randomly from healthy individuals. Also to compare between antibiotic sensitivity of biofilm producing CoNS isolates and non-biofilm producing CoNS isolates. To detect source of infection in neonatal intensive care unit using biotyping, antibiogram and plasmid profile as epidemiological markers.

SUBJECTS, MATERIAL & METHODS

This work was carried out in the Microbiology & Immunology Department and neonatal intensive care unit (NICU), Faculty of medicine, Zagazig University in the period from April 2010 to April 2011.

Subjects:

1. **Patients** included 40 neonates admitted to NICU, Pediatric Department, Zagazig University Hospitals (22 males and 18 females, with mean age 17.43± 7.2 days and age range 1-30 days) with picture of suspected bacteremia (as suspected clinically by NICU doctor). 40 blood samples were taken from peripheral sites and 40 skin swabs were taken from axilla.
2. **Control** included 40 healthy neonates who had no features of sepsis chosen randomly (24 males and 16 females, with mean age 16.8± 7.6 days and age range 1-30 days). From them 40 skin swabs were taken from axilla.
3. **Health care workers** included 25 health care workers from NICU (19 females and 6 males, with mean age 28.5±6 years and age range from 20 to 35 years). They were 5 doctors, 15 nurses and 5 workers (skin swab were taken to detect source of infection).

Methods:

Blood samples were cultured on blood culture bottles, and skin samples were plated on blood agar and incubated at 37°C for 24 h [2].

1- Identification of CoNS (according to Barid[11]).

Species identification was performed by using the API Staph [2].

2- Antibiotic susceptibility testing: as recommended by (NCCLS) was performed by using disk diffusion method: using antibiotic discs (Penicillin (P) 10 units. Ampicillin (AMP) 10 µg. Amoxicillin-clavulanate (AMC) 20 µg+10 µg. Methicillin (ME) 5 µg. Cephradine (CE) 30 µg. Vancomycin (VA) 30 µg. Gentamycin (CN) 10 µg. Tetracycline (TE) 30 µg. Chloramphenicol (C) 30 µg. Erythromycin (E) 15 µg. Ciprofloxacin (CIP) 5 µg. Rifampin (RA) 5µg).

3- Detection of biofilm production: Qualitative detection was done by using congo red agar (CRA). The medium was composed of brain heart infusion broth (Oxoid) (37 gm/l), sucrose (Sigma) (5 gm/l), agar number 1(Oxoid) (10 gm/l) and congo red dye (Sigma) (0.8 gm/l). Black colonies with a dry crystalline consistency indicated biofilm production while red colonies indicate no biofilm production[12,13].

4 - Detection of the icaA and icaD operon:

DNA extraction:

- Chromosomal DNA was extracted from isolated colonies by using QIAamp® DNA Mini kit (Qiagen GmbH, Hilden, Germany).

DNA amplification:

PCR-GOLD Master-Mix Beads (Bioron, The ENZYME Company, Germany).Each bead contains all of the necessary reagents, except primer and templet for performing a 20 ul PCR amplification reaction.

Primers (Qiagen): for PCR amplification
For detection of icaA,
Forward primer sequence is:
5'-TCTCTTGCAGGAGCAATCAA -3
Reverse primer sequence is:
5'-TCAGGCACTAACATCCAGCA-3
For detection of icaD,
Forward primer sequence is:
5'-ATGGTCAAGCCCAGACAGAG-3
Reverse primer sequence is:
5'-CGTGTTTTCAACATTTAATGCAA-3
Primers were designed to give a predicted product size of 188- bp for icaA gene and 198-bp for caD gene[13] (Bioron, The ENZYME Company, Germany). 4 µl (25 pmol) of each primer, 2 µl of template DNA and sterile distilled water to a total volume of 20 µl. The amplification was performed in a DNA thermal cycler (DNA thermal cycle – Elmer, Cetus, Norwalk, CT, USA), for 30 cycles, Denaturation at 94°C for 1min, annealing at 60 °C for 1min for icaA and at 59°C for 1 min for icaD, extension at 72°C for 2.5 min, then final extension at 72°C for 10 min [2].
Detection of the amplified product were detected by gel electrophoresis in parallel with a molecular weight marker.

5– plasmid DNA analysis : Plasmid was extracted from the clinical isolates by using QIAprep® Spin Miniprep kit (Qiagen GmbH, Hilden, Germany). The QIAprep miniprep procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt. The unique silica membrane used in QIAprep Miniprep Kits completely replaces glass for plasmid minipreps. The QIAprep Miniprep procedure was analyzed using agarose gel electrophoresis. Ten µl of the plasmid DNA preparation were removed to a clean microcentrifuge tube and 5 µl of the loading buffer was added to the microcentrifuge tube. The samples were loaded to 0.8% agarose gel and molecular weight marker (1000-20000) was run in parallel. The gel was visualized by UV transilluminator at 320 nm wavelength and photographed.

RESULTS

Table (1) Shows that neonate group included patients (40 neonates with picture of suspected bacteremia from them 40 blood samples and 40 skin swabs were taken), control (40 healthy neonates from them 40 skin swabs were taken) and health care workers (25 health care workers from NICU). 13 CoNS isolates (32.5%) were isolated from 40 blood samples, 15 CoNS isolates (37.5%) were isolated from 40 skin swabs, eight CoNS isolates (20%) were isolated from 40 control and 6 CoNS isolates (24%) were isolated from 25 health care workers, 5 isolates were isolated from nurses and only 1 isolate from workers.

Table (2) shows that *S. epidermidis* was the most commonly isolated organism. There was no statistically significant difference between patients, control and health care workers as regarding species.

Table (3) shows that there was statistically significant difference between patients, control and health care workers as regarding their ability to produce biofilm, presence icaA gene and icaD gene.

There was complete agreement between the results obtained from detection of both icaA and icaD genes. All positive icaA were also positive for icaD and all negative icaA were also negative for icaD.

Table (4) shows that there was very good agreement between two tests regarding Kappa factor.

All isolates were 100% sensitive to vancomycin and rifampicin. The resistance to different antibiotics is higher in biofilm forming isolates than in non-biofilm forming isolates. There was statistically significant difference between them. Table (5) 34 CoNS isolated from patients and HCW has been classified into 14 groups.

Table (6) shows that the isolated CoNS strains from patients and HCW (34 isolates) were found to have 9 different plasmid profiles; it was found that 21 strains (61.8%) harbored plasmid of different sizes. The molecular weight of plasmids ranged from 1.3 to 14 MDa and the number of plasmids ranged from 1 to 4. Plasmid profiles of the isolated CoNS showed that 13 strains were plasmidless, 10 strains had a single plasmid, 5 strains had 2 plasmids, 5 strains had 3 plasmids, while 1 strain had 4 plasmids.

Health care workers play a role in dissemination of CoNS infections in NICU. Only two CoNS isolates which were isolated from hands of nurses were proved to be the source in 3 cases of neonatal septicemia. These 2 nurse strains were multidrug resistance, contain plasmids, biofilm producer and contain ica genes. One strain was *S. epidermidis*, the other was *S. haemolyticus*.

Also there were 2 strains which were biofilm non producer but ica gene positive, one was isolated from blood and the other strain was isolated from skin, both were plasmidless and non multidrug resistance.

Table (1): Demographic data of the studied groups and Numbers, percentages of CoNS isolates from different groups.

Neonate group	No. of persons	Clinical specimens	No. of specimens	Gender		Age (Range)	CoNS isolates	
				M	F		No.	%
Patients	40	Blood samples	40	22	18	1-30 days	13	32.5
		Skin swabs	40	22	18	1-30 days	15	37.5
Control	40	Skin swabs	40	24	16	1-30 days	8	20.0
Health care workers	25	Skin swabs	25	6	19	20-35 years	6	24.0

Table (2): Species identification by API Staph of CoNS isolated from neonate group.

Specimens	Patient isolates				Control isolates (8)		Health care worker isolates (6)		X^2	P
	Blood (13)		Skin (15)		No.	%	No.	%		
	N0.	%	No.	%						
S. epidermidis	7	53.8	10	66.7	6	75.0	3	50.0	1.44	0.69
S. haemolyticus	4	30.8	4	26.7	1	12.5	2	33.3	1.0	0.78
S. hominis	1	7.7	1	6.6	1	12.5	1	16.7	---	---
S. xylosus	1	7.7	0	0.0	0	0.0	0	0.0	---	---

Table (3): PCR for icaA , icaD and Biofilm formation of CoNS isolates.

Neonatal group	Patient isolates		Control isolates (8)	Health care worker isolates (6)	X^2	P
	Blood (13)	Skin (15)				
Biofilm						
Positive strains	9 (69.2%)	5 (33.3%)	0 (0.0%)	2 (33.3%)	10.5	0.01
Negative strains	4 (30.8%)	10 (66.7%)	8 (100%)	4 (66.7%)		
icaA						
Positive strains	10 (76.9%)	6 (40.0%)	0 (0.0%)	2 (33.3%)	12.43	0.00
Negative strains	3 (23.1%)	9 (60.0%)	8 (100%)	4 (66.7%)		
icaD						
Positive strains	10 (76.9%)	6 (40.0%)	0 (0.0%)	2 (33.3%)	12.43	0.00
Negative strains	3 (23.1%)	9 (60.0%)	8 (100%)	4 (66.7%)		

Table (4): Relation between slime production and presence of ica genes by PCR among 40 patients.

		PCR		Total & percentage	Kappa	P
		Positive	Negative			
Biofilm	**Positive**	14	0	14 (50%)	0.85	0.00
	Negative	2	12	14 (50%)		
Total & percentage		16 (57.1%)	12 (42.9%)	28		

Table (5): Antibiogram of CoNS isolated from patients and HCW .

Group	Resistant pattern (12 discs)	No. of isolates (34)	Sources		
			Patient isolates		Health care worker isolates (6)
			Blood isolates	Skin isolates	
A	P,AMP,AMC,ME,CE,CN,TE,C,E,CIP	8	4	3	1
B	P,AMP,AMC,ME,CE,CN,TE,E,CIP	8	2	4	2
C	P,AMP,AMC,ME,CE,CN,TE,C,E	5	4	0	1
D	P,AMP,AMC,ME,CE,CN,C,CIP	1	0	1	0
E	P,AMP,AMC,ME,CE,CN,C,E	1	0	1	0
F	P,AMP,AMC,ME,CE,TE,C,E	1	0	1	0
G	P,AMP,AMC,ME,CN,C,CIP	1	0	1	0
H	P,AMP,AMC,CE,CN,TE,E	1	1	0	0
I	P,AMP,AMC,CN,TE,E	1	0	1	0
J	P,AMP,AMC,CE,E,CIP	1	1	0	0
K	P,AMP,AMC,E	1	0	1	0
L	P,AMP,CN,E	1	0	0	1
M	P,AMP,AMC	2	1	1	0
N	---------	2	0	1	1

Table (6): Plasmid profile of CoNS isolated from patients and HCW.

Pattern Number	M.W of detected plasmid in kpb	No. of isolates (34)	Sources		
			Patient isolates		Health care worker isolates (6)
			Blood isolates (13)	Skin isolates (15)	
-	plasmidless	13	3	8	2
I	2.465 kpb (1.6 MDa)	2	0	1	1
II	2.589 kpb (1.7 MDa)	2	2	0	0
III	10.000 kpb (6.6 MDa)	3	1	2	0
IV	2.787 kpb (1.8 MDa) 15.874 kpb (10.5 MDa)	2	1	1	0
V	2.405 kpb (1.6 MDa) 21.14 kpb (14 MDa)	3	1	1	1
VI	1.948 kpb (1.3 MDa) 3.224 kpb (2.1 MDa) 9.147 kpb (6.1 MDa)	2	1	1	0
VII	2.265 kpb (1.5 MDa) 8.305 kpb (5.5 MDa) 21.14 kpb (14 MDa)	3	2	0	1
VIII	1.948 kpb (1.3 MDa) 2.265 kpb (1.5 MDa) 7.248 kpb (4.8 MDa) 21.14 kpb (14 MDa)	1	1	0	0
IX	21.14 kpb (14 MDa)	3	1	1	1

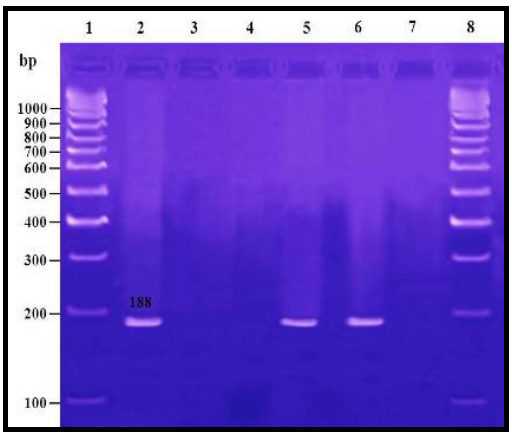

Fig. (1): PCR detection of icaA gene
Lane 1, 8 DNA molecular weight markers
Lane 7 negative control
Lane 3, 4 negative strains
Lane 2, 5, 6 positive strains (188-bp)

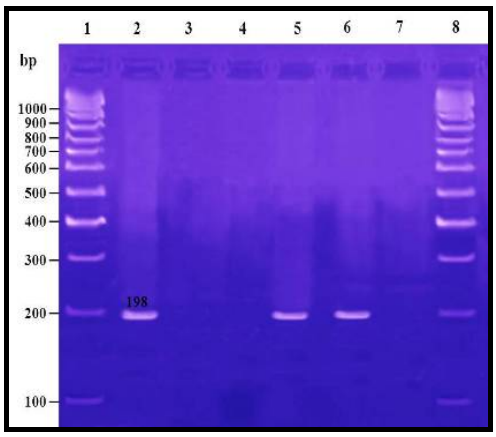

Fig. (2): PCR detection of icaD gene
Lane 1, 8 DNA molecular weight markers
Lane 7 negative control
Lane 3, 4 negative strains
Lane 2, 5, 6 positive strains (198-bp)

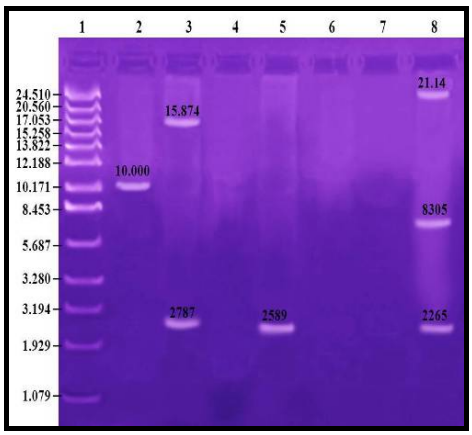

Fig. (3): Plasmid profile of some of the studied CoNS isolates.
Lane 1 shows molecular weight marker
Lane 2 shows 1 plasmid (10.000 bp)
Lane 3 shows 2 plasmids (2787, 15874 bp)
Lane 4, 6, 7 show plasmidless strains
Lane 5 shows 1 plasmid (2589 bp)
Lane 8 shows 3 plasmids (2265, 8305, 21140 bp)

DISCUSSION

Coagulase negative staphylococci are normal inhabitants of human skin and mucous membranes. They have long been dismissed as culture contaminants, but now CoNS are a major cause of nosocomial and health care related infections. CoNS are the major cause of sepsis in NICU [14].

The pathogenic potential of this commensal organism is not clearly known and may be due to ability to adhere to biomaterial and production of extracellular slime, a mechanism by which it can cause severe and irreducible infections [15].

Slime production is under genetic control and is mediated by the ica operon, a polysaccharide intercellular adhesion (PIA) is synthesized, this supports cell to cell bacterial contact by means of a multilayered biofilm [16]. Biofilm impair the penetration of antibiotics, normal immune responses and increase difficulty of eradicating biofilm infections [17].

Our results show that out of 40 patients,13 CoNS isolates (32.5%) were isolated from 40 blood samples, 15 CoNS isolates (37.5%) were

isolated from 40 skin swabs samples, eight CoNS isolates (20%) were isolated from 40 control and 6 CoNS isolates (24%) were isolated from 25 health care workers.

These results agree with the results of de silva et al [2] who found that CoNS are a major cause of sepsis in NICU. Also these results go well with results of Dobbins [18] who found that 37% of infections were caused by CoNS.

This study shows that *S. epidermidis* was the predominant CoNS among blood samples, skin swabs of patients, control and health care workers (53.8%, 66.7%, 75%, 50% respectively), followed by *S. haemolyticus* then *S. hominis* and *S. xylosus*.

These results agree with results of Koura et al [19] who reported that *S. epidermidis* was the predominant CoNS (79.0%), *S. haemolyticus* (4.6%), *S. hominis* (2.3%) and *S. xylosus* (2.3%).

We aimed to evaluate that ica operon and biofilm production are associated with CoNS diseases. The biofilm production was examined using qualitative congo red agar (CRA), and the presence of genes icaA, icaD was determined by PCR.

Congo red agar method has significant clinical applicability; it can be used in routine diagnostic bacteriology laboratory. Because of its rapidity, the results of the test could be provided along with the final culture and sensitivity report on the second post inoculation day thus, ascribing the isolate as having a pathogenic potential and not as a mere commensal [19].

Our results show that out of 13 CoNS isolated from blood samples , 9 isolates (69.2%) were able to form biofilm as detected by CRA, while out of 15 CoNS isolated from skin swabs , 5 isolates (33.3%) were biofilm forming.

These results are matched with the results obtained by Arciola et al [13] who found that the percentage of biofilm formation among CoNS was 49%, and Muller et al [20] found that 46% of CoNS are biofilm producer by CRA. Ziebuhr et al [21] had reported higher incidence, they found that 87% of CoNS isolates are biofilm forming while de silva et al [2] reported that only 25% of the tested CoNS were biofilm positive by CRA. Nayak et al [22] found that 57% of CoNS strains were slime forming on CRA.

We found that CoNS isolated from skin swabs from neonate with neonatal septicemia are more than CoNS isolated from healthy neonate and also 33.3% of them have the ability to form biofilm however non of control isolates have the ability to form biofilm, this suggested that neonate in NICU replace their own commensal flora by hospital strains. Widerstrom et al [23] stated that colonization of patients with CoNS precedes infection with these organisms.

The difference in these results may be explained by the difference in locality and environmental conditions, taking in mind the fact that the ability of biofilm production although controlled by a chromosomal gene, this gene can be transferred from one strain to another by conjugation and so can be more predominant or less predominant in different localities [24].

None of the isolates of control group (commensals) included in our study shows the ability to produce biofilm. These results are the same results obtained by Arciola et al [13] and de silva et al [2] . This can be explained by the fact that biofilm production is a virulent factor and the control isolates which is comprised from commensal strains are not virulent [24].

In our study for the genetic basis of biofilm production the ica operon was chosen. This choice depends on the fact that, although the genetic basis of biofilm formation in CoNS is multifactorial, synthesis of a polysaccharide adhesion by ica ADBC-encoded enzymes is the best understood mechanism of CoNS biofilm development [25].

Our results show that 10 isolates (76.9%) out of 13 CoNS isolated from blood samples and 6 isolates (40%) out of 15 CoNS isolated from skin samples contain icaA gene. The gene was not found in any of the control isolates. Among 6 CoNS isolated from health care workers, only 2 isolates (33.3%) contain icaA gene.

In another study, PCR method used for detection of ica operon was very sensitive among CoNS species isolated from blood of infected neonates and from the skin of infected and healthy ones. They reported that 40% of CoNS strains were positive for ica operon [2].

Our PCR results had shown that both icaA and icaD gene are either present in certain strain or absent, and no single strain had shown the presence of one gene. Our results regarding this point agree with results of Arciola et al [13] and de silva et al[2].

These results confirm the fact that both genes are part of one operon and so, either the entire operon is present or absent [24].

In this study, both the icaA and icaD genes are present in all biofilm producing strains; this indicates that the presence of both genes is essential for biofilm production. This result is supported by the results of a study done by Mack et al[26], who had inactivate each gene

separately by insertion of different transposone and both insertions had lead to complete inactivation of PIA synthesis.

Gerke et al [16] found that inactivation of icaD lead to the loss of cell aggregation and PIA production indicating that icaD plays an essential role in intercellular adhesion and PIA production in vivo. Møretrø et al [27] had shown the importance of icaA gene for the production of PIA.

Ica locus is a virulence marker for clinically significant CoNS isolates. Its presence in a high percentage of clinical isolates and its association with the strains ability to produce slime strongly suggests a role of icaA and icaD in the pathogenic mechanism of CoNS infections. The genotype characterization by PCR represents a highly sensitive method for the detection of ica locus [19].

Our results indicate that 50% of CoNS isolated from patients were positive for slime production where 57.1% were positive for icaA, icaD genes. There was a positive correlation between slime production by CRA plate and the presence of ica genes by PCR.

PCR can detect ica operon in strains that appear to be non-biofilm producers with CRA. We found 2 strains which are biofilm non producer but ica gene positive, one was isolated from blood, the other strain was isolated from skin, both were plasmidless and non multidrug resistance.

These results agree with the result of Handke et al [28] who had reported the presence of biofilm negative staphylococci that contain the ica operon. The presence of ica operon among the biofilm negative strains had been extensively explained by many authors. Some authors consider point mutation in the icaA gene of biofilm negative strains as an important explanation and this also was suggested by de silva et al [2].

Another explanation had been made by Møretrø et al [27] and Ziebuhr et al [21] who found that 1.332 bp sequence element, known as IS 256, causes inactivation of icaA gene and leads to biofilm negative phenotype forming.

Also Mack et al [29] had genetically engineered 9 biofilm negative strains that contain ica operon, this had resulted from transposon insertion in different chromosomal loci, four loci within the ica operon and five loci outside the operon.

Handke et al [28] stated that the finding of the prevalence of the ica locus among biofilm negative strains might be explained by low level of expression of the ica locus in those strains due to strict gene regulatory mechanism.

However these results are different from the results obtained by Arciola et al [13] who found that biofilm negative staphylococci lake these two genes and thus had suggested that the phenotypic change may be caused by a deletion of the ica operon rather than an insertion event which inactivates the ica genes.

Nofal et al [24] stated that, CRA and PCR are both valid tests in the detection of biofilm formation. The choice should depend on the health condition of the patient and the availability of different reagents, also an economic background must be put in mind. PCR is a rapid tool of diagnosis especially if we consider that a large proportion of patients are critically ill and rapid diagnosis is required. On the other hand for cases which can tolerate delay in diagnosis and also for research work it is more important to differentiate between virulent and avirulent strain by CRA.

In our study show that CoNS isolates from blood samples were resistant to penicillin (100%), ampicillin (100%), amoxicillin/clavulonic acid (100%), methicillin (76.7%), cephradin (92.3%), gentamycin (84.6%), tetracyclin (84.6%), chloramphenicol (61.5%), erythromycin (92.3%) and ciprofloxacin (53.8%) whereas all isolates were sensitive to vancomycin and rifampicin.

This finding is close to results of Koura et al [19] who found that most isolates of CoNS were resistant to penicillin (95%), ampicillin (85%), gentamyvin (80%), cefotaxime (80%), tetracyclin (75%), amoxicillic (75%) and oxacillin (74%) whereas resistance to vancomycin and teicoplanin was found in only two strains (5%).

Our results coincided with others who found that most strains were resistant to penicillin and tetracycline and 5.1% of CoNS were resistant to vancomycin [30] and others showed that 100% of CoNS were sensitive to vancomycin [31].

The difference in susceptibility tests would be due to difference in the study duration, population and hospital care patterns of the antimicrobial prescription.

We observed that resistance to different antibiotics is higher in biofilm forming isolates than in non-biofilm forming isolates. This is in agreement with Koura et al [19] who found that slime positive isolates were multidrug resistance as compared to slime negative isolates.

Also Koksal et al [32] reported that methicillin resistance was significantly higher in slime positive isolates (81%) than in slime negative isolates (57%).

This can be explained by the fact that biofilm is the most important virulence factor of CoNS because it enables attachment and persistence of bacteria and enhances bacterial resistance to antibiotics and host defense mechanisms [33].

In this study antibiogram have been used for phenotyping of CoNS isolates. According to antibiogram the neonatal cases and HCW isolates (34 isolates) have been classified into 14 groups indicating that antibiogram could be a relatively sensitive marker.

A lot of workers have also successfully used antibiogram for typing their nosocomial isolates[34]. This was contraindicated by other workers who reported limited usefulness and low discriminatory power of antibiotyping in comparing different isolates in epidemiologic studies [35].

Antibiogram might be non reproducible in many instances due to the probable exchange of R factor among isolates [36].

To overcome limitation of phenotypic analysis, genotypic methods have been used for strain typing of CoNS. Plasmid profile has been used widely in this field as it is simple rapid molecular technique.

Plasmid profile analysis was done among neonate cases and HCW in NICU. In our study it was found that 21 isolates (61.8%) out of 34 isolates harbored plasmids of different sizes. The molecular weight of plasmids ranged from 1.3 to 14 MDa and the number of plasmids ranged from 1 to 4.

In our study, the detected plasmid sizes were 1.3, 1.5, 1.6, 1.7, 1.8, 2.1, 4.8, 5.5, 6.1, 6.6, 10.5, 14 MDa. Plasmid profiles of the isolated CoNS showed that 13 strains (38.2%) were plasmidless, 10 strains had a single plasmid, 5 strains had 2 plasmid, 5 strains had 3 plasmids, while 1 strain had 4 plasmids. The isolated CoNS strains were found to have 9 different plasmid profiles. Thus, possession of a different class of plasmids by the isolate might reflect different degree of virulence and differing pathogenicity [37].

Plasmid profiles were first used to distinguish different strains of CoNS by Parisi and Hecht [38] who were able to demonstrate the existence of a common strain of *S. epidermidis* causing infection among infants in a neonatal unit. These findings suggested that the infective strain was being passed from infant to infant.

Abo El-Ela and Al-Essa [39] suggested that plasmid subtyping method is discriminative and sensitive. Other workers found plasmid analysis was of no value in epidemiologic studies because of instability of plasmids, which might be lost during storage[40]. Moreover genetic unrelated strains may harbor the same plasmid[41]. Additionally fragile plasmid or plasmids found in low copy number are difficult to extract [35].

Our finding may indicate that health care workers play a role in dissemination of CoNS infections in the hospital. Only 2 CoNS isolates which were isolated from hands of nurses were proved to be the source in 3 cases of neonatal septicemia. These 2 nurse strains were multidrug resistance, contain plasmids, biofilm producer and contain ica genes. One strain was *S. epidermidis*, the other was *S. haemolyticus*.

These results suggesting horizontal transmission of epidemic strain from one patient to another through hospital staff. Hira et al.[42] stated that hospital personnel can be responsible for spreading multiresistant CoNS isolates. In conclusion isolates of CoNS infections are more likely to be positive for ica operon and to produce biofilm than isolates separated randomly from healthy individual. Both CRA and PCR are valid tests for detection of biofilm formation. Plasmid profile has a role in epidemiological typing of CoNS isolates. Health care workers play a role in dissemination of CoNS infections in the hospital. Presence of biofilm enhances resistance to different antibiotics. We recommended that infection control program to prevent spread of infections through hands of HCW. Increase concentration of antibiotics (if possible) to be effective against biofilm producing bacteria.

REFERENCES

1. **Piette A and Verschraegen G (2009):** Role of coagulase negative staphylococci in human disease. Veterinary Microbiol; 134(1-2): 45-54.
2. **de Silva GDI, Kantzanou M, Justice A, Massey RC, Wilkinson AR, Day NPJ and Peacock SJ (2002):** The ica operon and biofilm production in coagulase negative staphylococci associated with carriage and disease in a neonatal intensive care unit. J Clin Microbio; 40(2): 382-388.
3. **Stoll BJ, Gordon T, Korones SB, Shankaran S, Tyson JE, Bauer CR, Fanaroff AA, Lemons JA, Donovan EF, Oh W, Stevenson DK, Ehrenkranz RA, Papile LA, Verter J and Wright LL (1996):** Late onset sepsis in very low birth weight neonates: a report from the National Institute of Child Health and Human Development Neonatal Research Network. J Pediatr; 129(1): 63–71.

4. **Margreth N, van der Lugt, Sylke JS and Frans JW (2010):** Use of rifampin in persistent coagulase negative staphylococcal bacteremia in neonates, BMC Pediatrics; 10(1): 84-91.
5. **Yue Q, Andrew JD, Taghrid SI, Suzanne MG and Margaret AD (2010):** Antibiotic susceptibility of coagulase negative staphylococci isolated from very low birth weight babies: comprehensive comparisons of bacteria at different stages of biofilm formation. Annals of Clinical Microbiology and Antimicrobials; 9: 16-28.
6. ***M*ack D (1999):** Molecular mechanisms of *Staphylococcus epidermidis* biofilm formation. J Hosp Infect; 43: 113–125.
7. **Heilmann C, Schweitzer O, Gerke C, Vanittanakom N, Mack D and Götz F (1996):** Molecular basis of intercellular adhesion in the biofilm forming *Staphylococcus epidermidis*. Mol Microbiol; 20(5): 1083–1091.
8. **Götz F (2002):** Staphylococcus and biofilms. Mol Microbiol; 43(6): 1367–1378.
9. **Zhiqiang Q, Xiaomei Y, Lei Y, Juan J, Yuanzhu O, Soeren M and Di Q (2007):** Formation and properties of in vitro biofilms of ica negative *Staphylococcus epidermidis* clinical isolates. J Med Microbiol; 56: 83–93.
10. **Leonard WM (1988):** Use of plasmid profile in epidemiological surveillance of disease outbreaks and in tracing the transmission of antibiotic resistance. Clin Microbiol Rev; 1 (2): 228-243.
11. ***B*aird D (1996):** *Staphylococcus*: Cluster forming gram positive cocci. In: Mackie & McCarteny Practical Medical Microbiology. Collewe, J.G.; Marmion, B.P.; Fraser, A.G.; and Simmons, A. eds. Ch. 11. Churchill Livingstone, New York, pp. 245-274.
12. **-Mathur T, Singhal S, Khan S, Upadhyay DJ, Fatma T and Rattan A (2006):** Detection of biofilm formation among the clinical isolates of staphylococci: An evaluation of three different screening methods. Indian J Med Microbiol; 24 (1): 25-29.
13. **-Arciola CR, Baldassarri L and Montanaro L (2001):** Presence of icaA and icaD genes and slime production in a collection of staphylococcal strains from catheter associated infections. J Clin Microbiol; 39(6): 2151-2156.
14. **-Piette A and Verschraegen G (2009):** Role of coagulase negative staphylococci in human disease. Veterinary Microbiol; 134(1-2): 45-54.
15. **Greenwood WT, Cooper B, Hails J, Jones K, Kwaku F, Shaw S, Kibbler C, Sbulman R and Wilson PR (2004):** Linezolid versus teicoplanin in the treatment of gram positive infections in the critically ill: A randomized; double-blind, multicentre study. J Antimicrob Chemother; 53(2): 345-349.
16. **Gerke C, Kraft A, Süssmuth R, Schweitzer O and Götz F (1998):** Characterization of the N-acetylglucosaminyltransferase activity involved in the biosynthesis of the *Staphylococcus epidermidis* polysaccharide intercellular adhesion. J Biol Chem; 273(29): 18586-18593.
17. **Vuong C and Otto M (2002):** "*Staphylococcus epidermidis* infections" Microbes and Infection; 4(4): 481-489.
18. **Dobbins BM, Kiel P, Kindon A, McMahon MJ and Wilcox MH (2002):** DNA fingerprinting analysis of coagulase negative staphylococci implicated in catheter related bloodstream infections. J Clinic Patho; 55: 824-828.
19. **Koura BA, Badwy W, Zaghloul MH and El Mashad N (2007):** Plasmid pattern analysis in slime producing coagulase negative staphylococci in acute bacterial conjunctivitis, keratitis, chronic blepharitis and corneal ulcer. E J Med Microbiol; 16 (2): 338-349.
20. **Muller E, Takeda S, Shiro H, Goldmann D and Pier GB (1993):** Occurrence of capsular polysaccharide/adhesion among clinical isolates of coagulase negative staphylococci. J Infect Dis; 168: 1211-1218.
21. **Ziebuhr W, Heilmann C, Gtöz F, Meyer P, Wilmas K, Straube E and Hacker J (1997):** Detection of the intercellular adhesion gene cluster (ica) and phase variation in *Staphylococcus epidermidis* blood culture strains and mucosal isolates. Infect Immun; 65(3): 890-896.
22. ***N*ayak N, Satpathy G, Vajpayee RB and Pandey RM (2001):** A simple alternative method for rapid detection of slime produced by *Staphylococcus epidermidis* isolates in bacterial keratitis. Indian J Med Res; 114: 169-172.
23. **Widerstrom M, Monsen T, Karlsson C and Wiström J (2006):** Molecular epidemiology of methicillin resistant coagulase negative staphylococci in a Swedish county hospital: evidence of intra and interhospital clonal spread. J Hosp Infect; 64(2): 177–183.
24. **Nofal FA, Selim ES, El Behedy EM and El Shabrawy RM (2005):** Biofilm

formation and the presence of intercellular adhesion locus (ica) among staphylococcal from catheter associated infections. New E J of Med; 33(6): 297-305.

25. **Fitzpatrick F, Humphreys H and O'Gara JP (2005):** Evidence for icaADBC-Independent biofilm development mechanism in methicillin resistant *Staphylococcus aureus* Clinical. J Clin Microbio; 43(4): 1973-1976.
26. **Mack D, Sabottke A, Dobinsky S, Rohde H, Horstkotte MA and Knobloch JK (2002):** Differential expression of methicillin resistance by different biofilm negative *Staphylococcus epidermidis* transposon mutant classes. Antimicrob Agents Chemother; 46(1): 178-183.
27. **Møretrø T, AL, Sidhu MS, Rudi K and Langsrud S (Hermansen L, Holck 2003):** Biofilm formation and the presence of the intercellular adhesion locus ica among staphylococci from food and food processing environments. Appl Environ Microbiol; 69(9): 5648-5655.
28. **Handke LD, Conlon KM, Slater SR, Elbaruni S, Fitzpatrick F, Humphreys H, Giles WP, Rupp ME, Fey PD and O'Gara JP (2004):** Genotypic and phenotypic analysis of biofilm phenotypic variation in multiple Staphylococcus *epidermidis* isolates. J Med Microbio; 53:367-374.
29. **Mack D, Rohde H, Dobinsky S, Riedewald J, Nedelmann M, Knobloch J, Elsner HA and Feucht HH (2000):** Identification of three essential regulatory gene loci governing expression of *Staphylococcus epidermidis* polysaccharide intercellular adhesin and biofilm formation. Infect Immun; 68(7): 3799-3807.
30. **Yasar N, Sengul D, Atahan A and Handan K (2005):** Investigation of staphylococcus to glycopeptides in a Turkish University Hospital. Clin Infect Dis; 5:31.
31. **Villari P, Sarnataro C and Iacuzio (2000):** Molecular epidemiology of *Staphylococcus epidermidis* in a neonatal intensive care unit over a 3 year period. J Clin Microbial; 38 (5) 1740-1746.
32. **Koksal F, Yasar H and Samasti M (2007):** Antibiotic resistance patterns of coagulase negative staphylococcus strains isolated from blood cultures of septicemic patients in Turkey. Microbiol Res; 16: 31-34.
33. **Sadovskaya I, Vinogradov E, Flahaut S, Kogan G and Jabbouri S (2005):** Extracellular carbohydrate containing polymers of a model biofilm-producing strain, *Staphylococcus epidermidis* RP62A. Infect and Immuni; 73(5): 3007-3017.
34. **Awad AS, Shaheeh MA and Fathy MM (2004):** Colonization rates and typing of *Staphylococcus aureus* isolated from the nose and the eczematous skin of patients with atopic dermatitis and their correlation with the severity of exzema. E J Med Microbiol; 13(2): 313-323.
35. **Eisn D, Russell E, Tymms M, Roper E, Grauson M and Turnidge J (1995):** Random amplified polymorphic DNA and plasmid analysis used in investigation of an outbreak of multiresistant *Klebsiella pneumonia*. J Clin Microbiol; 33(3):713-717.
36. **El Sweify MA, Khalifa K, Brenner DJ, Emad K, Mohammed FA and Heiba AA (1994):** Subtyping of nosocomial *Pseudomonas aeurginosa* isolates; phenotypic and genotypic markers. E J Med Microbiol; (3):500-505.
37. **Archer GL, Karchmer AW, Vishniavsky N and Johnston JL (1984):** Plasmid pattern analysis for differentiation of infecting from non-infecting *Staphylococcus epidermidis*. J Infect Dis; 149: 913-920.
38. **Parisi JT and Hecht DW (1980):** Plasmid profiles in epidemiologic studies of infections by *Staphylococcus epidermidis*. J Infect Dis; 141: 637-643.
39. ***Abou El-Ela M and Al-Essa S (2004):*** Molecular epidemiological study of *Enterobacter clocae* isolates from a neonatal intensive care unit. E J Med Microbiol; 13(4): 753-762.
40. **Dubois V, Aprin C, Ander C, Coulge L and Quentin C (2005):** Prolonged outbreak of infection due to TEM 21producing strains of *Pseudomonas aeurginosa* and Enterobacteria in a nursing home. J Clin Microbiol; 43(8): 4129-4138.
41. **Gori A, Esplimase F, Delpano A, Nonhoff C, Nicolas M and Struelens M (1996):** Comparison of pulsed field gel electrophoresis and randomly amplified DNA polymorphism analysis for typing extended spectrum B-lactamase producing *Klebsiella pneumonia*. J Clin Microbiol; 34 (10): 2448-2453.
42. **Hira V, Sluijter M, Estevä0 S, Horst-Kreft D, Ott A, de Groot R, Hermans PW and Kornelisse RF (2007):** Clinical and molecular epidemiologic characteristics of coagulase negative staphylococcal bloodstream infections in intensive care neonates. Pediatr Infect Dis J; 26(7): 607–612.

دور الموقع الجينى (إكا) وتكوين الغشاء الحيوى فى العدوى بالبكتريا العنقودية سالبة التجلط

أشواق محمد فؤاد ابو طالب – مرفت سليمان محمد – رندا صديق عبد اللطيف – منار جوده
قسم الميكروبيولوجيا الطبية والمناعة – كلية الطب - جامعةالزقازيق

تعتبر البكتريا العنقودية سالبة التجلط السبب الرئيسى للتسمم الدموي الوليدي فى وحدة العناية المركزة للاطفال.
البكتريا العنقودية سالبة التجلط تسبب العدوى نتيجة تكوين الغشاء الحيوى الذى يساعدها على الإلتصاق بأى جسم صناعي وكذلك مقاومة الجهاز المناعي والمضادات الحيوية المختلفة.

الهدف من البحث:

-إثبات أن المعزولات المأخوذة من أمراض العدوى بالبكتريا العنقودية سالبة التجلط لها القدرة على تكوين الغشاء الحيوي وبها الموقع الجينى (إكا) أكثر من المعزولات المأخوذة عشوائيا من الأصحاء.
-المقارنة بين حساسية الميكروبات المكونة للغشاء الحيوي للمضادات الحيوية والميكروبات الغير مكونة للغشاء الحيوى.
-فصل البلازميدات من البكتريا العنقودية سالبة التجلط لإستخدامها فى تحديد مصادر العدوى في وحدة العناية المركزة للأطفال حديثي الولادة.

مواد وطرق البحث:

تم اخذالعينات من حالآت التسمم الدموي الوليدي :
العينات المرضية:٤٠ حاله تسمم وليدى بوحدة العناية المركزة للاطفال بمستشفى الزقازيق الجامعي (٤٠ عينة دم من الاورده الطرفية و ٤٠ مسحه من الجلد من تحت الإبط).
العينات الضابطة: ٤٠طفل من الأطفال حديثى الولادة الاصحاء يتم أخذها عشوائيا (٤٠ مسحه من الجلد من تحت الإبط).
25شخص من مقدمى الرعاية الصحية بوحدة العناية المركزة للاطفال بمستشفى الزقازيق الجامعي.
تم نقل العينات إلى معامل قسم الميكروبيولوجى والمناعة بكلية الطب البشرى بجامعة الزقازيق حيث تم التعامل معها كما يلى:
-زرع عينات الدم على الأجار المغذى فى زجاجات زراعة الدم و زرع مسحات الجلد علي الآجار المغذي وذلك لفصل البكتريا العنقودية ثم التعرف على البكتريا العنقودية سالبة التجلط وتحديد نوعها والتاكد منه عن طريق.API Staph
-تم إختبار حساسية الميكروب للمضادات الحيوية المختلفة.
-تحديد قدرة البكتريا العنقودية سالبة التجلط على تكوين الغشاء الحيوي باستخدام الأجار المضاف إليه صبغة الكونجو الحمراء، المعزولات الموجبة تظهر باللون الأسود والمعزولات السالبة تظهر باللون الأحمر.
-إستخراج الحامض النووى من البكتريا العنقودية سالبة التجلط وتحديد وجود الموقع الجينى (إكا) بإستخدام تفاعل إنزيم البلمرة المتسلسل.
-فصل البلازميدات من البكتريا العنقعودية سالبة التجلط وذلك لتتبع مصادر العدوى.

نتائج البحث

تم فصل ١٣ حالة من البكتريا العنقودية سالبة التجلط (٣٢.٥٪) من ٤٠عينه دم من الاطفال الذين يعانون من التسمم الدموي الوليدى بينما تم فصل ١٥ حالة (٣٧.٥٪) من ٤٠ مسحه من الجلد و كذلك تم فصل ٨ حالات (٢٠٪) من ٤٠ عينه ضابطة و٦ حالات (٢٤٪) من ٢٥ شخص من مقدمى الرعاية الصحية في وحدة العناية المركزة للأطفال بطب الزقازيق.
-كانت استاف إبى درميدس أكثر الأنواع التي تم فصلها من المجموعتين.
-بأستخدام الأجار المضارف إليه صبغة الكونجو الحمراء (مجموعه التسمم الدموي الوليدي) وجد أن ٩ حالات (٦٩.٢٪)من ١٣ عزلة بكتريا عنقودية سالبة التجلط من عينات الدم لها القدرة على تكوين الغشاء الحيوي و ٥ حالات (٣٣.٣٪) من ١٥ عزلة بكتريا عنقودية سالبة التجلط من مسحات الجلد لها القدرة على تكوين الغشاء الحيوي بينما لم تبد أى حالة من الحالات الضابطة قدرتها على تكوين الغشاء الحيوى. كذلك تم فصل حالتين (٣٣.٣٪) من ٦ عزلات من مقدمي الرعاية الصحية لها القدرة على تكوين الغشاء الحيوي.
-بعد البحث عن جينات "ica" تبين وجود الجينات فى جميع الحالات التى لها القدرة على تكوين الغشاء الحيوى وايضا تبين وجوده فى بعض الحالات التى لا تستطيع تكوين الغشاء الحيوى ولم يتواجد جينات "ica"فى اى بكتريا من العينات الضابطة.
كانت معظم العينات مقاومة لعقار البنسلين وحساسة لعقار فانكوميسين، ريفامبسين. و كانت مقاومة العزلات المكونة للغشاء الحيوي للمضادات الحيوية المختلفة أكثر من مقاومة العزلات الغير مكونة الغشاء الحيوى.
-على حسب حساسيه الميكروب للمضادات الحيوية المختلفة تم تقسمهم الى ١٤ مجموعة.
-يوجد به ٩ مجموعات من البلازميدات المختلفة و١٣ سلالة بدون بلازميدات.
-نتائج هذا البحث أوضحت أن مقدمي الرعاية الصحية لهم دور في نشر العدوي داخل وحدة العناية المركزة للأطفال. حيث وجد سلالتين تم عزلهم من أيدي الممرضات كانت هي مصدر العدوى لثلاثه حالات من التسمم الدموي. كلا من السلالتين كانت مقاومه لكثير من المضادات الحيوية و تحتوي علي بلازميدات و كذلك كانت مكونة للغشاء الحيوى و بها الموقع الجيني إكا .

-ومن هذاالبحث يتم استنتاج الآتى:

إن العزلات المرضية لها القدرة على تكوين الغشاء الحيوى وبها الموقع الجينى (إكا)
كلا من الاجار المضاف الية صبغة الكونجو الحمراء والبحث عن جينات (إكا) هي طرق صحيحة لتحديد قدرة البكتريا على تكوين الغشاء الحيوى.
البلازميدات لها دور فى تتبع مصادر العدوى.
مقدمى الرعاية الصحية لهم دور فى نشر العدوى.
وجود الغشاء الحيوى يساعد البكتريا على مقاومة المضادات الحيوية المختلفة.