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ANTIBIOTIC SUSCEPTIBILITY PATTERN AND BIOFILM-FORMING POTENTIAL OF GRAM-NEGATIVE CLINICAL BACTERIAL ISOLATES

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Abstract:
Bacteria can shift between planktonic forms (living as single cells) or establishing communities in the form of biofilms, rising on hard surfaces or rooted in a layer of exopolysaccharides (EPS). Biofilm formation by pathogens reduces vulnerability to antimicrobial treatments by making them more virulent and it may result in the development of persistent infections; hence, microbial biofilm can add to its pathogenesis. This study was designed to determine the biofilm forming potential of clinically isolated Gram-negative bacteria, and its relationship with antibiotic resistance. A total of 150 Gram-negative clinical samples were collected from Memon Medical Institute (MMI) Hospital in Karachi, Pakistan. To screen bacteria for the production of biofilm, Congo Red Agar (CRA) method was used. Quantitative analysis for No/Weak/Moderate and Strong biofilm producers was done with microtiter plate method. Furthermore, the crystal violet staining was also used by taking optical density (OD) at 595nm. Antibiogram pattern of strong biofilm forming isolates was done using clinical laboratory standards institute (CLSI) guidelines and the bacteria was classified as resistant to one, two and multi drug resistant (MDR). Most of the strong biofilm forming bacteria were observed as MDR (84.61%) which clearly reveals the involvement of biofilm in increased antimicrobial drug resistance. Monoplex PCR (polymerase chain reaction) expressed the presences of papC biofilm associated gene in five strong biofilm forming isolates.

Keywords: Gram-negative; Biofilm; Virulence; Antimicrobial resistance, MDR

1. Introduction

Gram-negative microorganisms are important infection causing bacteria in both society and health care settings. Wide extend antibiotic resistance is amongst one of the important health issues internationally[1]. With others; biofilm production, is also among the other virulence factors to escape antimicrobial treatments. Microbial biofilms are composite structure of cells which are not similar to a tissue, rather to an association may be defined as a city of microorganisms[2,3]. Biofilms are covered with extracellular polymeric substances (EPS), an exopolysaccharide which is a central protecting covering. The groups of bacteria are adherent to a surface or rooted in a matrix consist proteins, polysaccharide, as well as Environmental DNA (eDNA)[4,5]. Several genes are also associated with the ability of *E.coli* to form biofilm like pap C P-fimbriae (fim), coded by the fim gene cluster: the P-fimbriae (pap), coded by the pap (pyelonephritis-associated pili) gene; and α-hemolysin (hly), which comes from RTX toxin group on the basis of a common nonapeptide repeat in the C-terminal of protein [46].

With the protection by the matrix, bacteria in biofilms have strategy to evade the host protection system. Drug resistance consists of multiple phenomena like modification of the antibiotic target, altered permeability, genetical changes, along with biofilm formation[6]. Anton van Leeuwenhoek noticed first extinction of biofilm after the examination of his own dental plaque and investigations about biofilms have started since the 1970s. Since then, different researchers defined it as; Biofilms are communities of bacteria stuck onto surfaces sheathed in a glycocalyx matrix. Community of microbial cells permeable by water channels allow efficient biomass transportation between the population and the environment[7,8]. Microbial communities contain a large quantity of different cells living together encased in a self-produced extracellular polymeric matrix. Production of biofilm in bacteria is controlled by the process of Quorum sensing (QS). Through QS, signaling molecules or autoinducers, are produced and secreted by the bacterial cells. These autoinducer molecules enables the cell to sense the adequate population of bacteria (a quorum) has been produced[9]. Regulation by QS is highly sealed in bacteria and molecular progression, and chemical nature of the autoinducers is significantly different in both Gram positive and negative bacteria[10,11].

The biofilm formation make the bacteria difficult to treat because the sessile bacteria embedded in the biofilm have decreased antibiotic sensitivity, and antibiotics have a reduced ability to penetrate the extracellular matrix, which is made up of extracellular polymeric (EPS) substances and other materials produced by the bacteria[12,13]. Infections in humans are thought to be caused by 65–80% of biofilm-forming bacteria.The EPS is essential for bacterial adhesion because it serves as a barrier and border between the microbial population and the outside environment. Enzymes that can break down EPS polymers, which are crucial to the life cycle of biofilms[14]. When starved, they provide carbon and energy, and when detached and dispersed, they degrade biofilms. While the bacteria in the deeper levels of the biofilm, where hypoxia is more prevalent, show decreased metabolism and have gone into a sessile, inactive state, the bacteria in the outer biofilm layers exhibit active metabolism[14,15]. One of the primary factors contributing to the persistence of pathogenic bacteria linked to serious illnesses and outbreaks in hospitals is biofilm production[16]. It is a widespread occurrence among microorganisms and a significant virulence factor that contributes to the colonization of living tissues or medical devices[17,18]. It also results in treatment failure because it reduces susceptibility to antimicrobial drugs and builds host defense mechanisms' resistance, which both result in treatment failure[1]. Patients suffering from severe infectious diseases may now be saved due to the discovery of chemicals with antibacterial properties. However, the fast emergence of bacterial strains that are resistant to antibiotics has often resulted in treatment failure. Another medical issue is the biofilm-associated bacterial infections,

which are frequently challenging to treat. The current study was designed to see the prevalence of biofilm-producing bacterial isolates and their antibiotic susceptibility patterns.

2. Results

2.1. Sample Collection and biofilm Screening

A total of 150 Gram-negative clinically isolated bacterial isolates were tested for their biofilm producing potential by Congo red agar (CRA) procedure. Among the tested bacterial isolates, *Escherichiacoli*(*E. coli*) was 54.6%, *Klebsiella* Spp. 41.6%, *Salmonella* Spp.30.7%, and *Pseudomonas* Spp.24.0%. (Table 1)

Table 1. Sample collection and biofilm screening.

Isolates	**Total**	**Biofilm Formation**	**Percentage (%) of Biofilm formation**
E. coli	75	41	54.66
*Klebsiella*Spp.	12	5	41.66
*Salmonella*Spp.	13	4	30.76
*Proteus*Spp.	22	4	18.18
*Pseudomonas*Spp.	25	6	24.00
*Acinetobacter*Spp.	3	0	00
Total	150	60	40.0

All of the biofilm forming isolates were classified as No, Weak, Moderate, and Strong, by calculating their OD average of triplet observation and for each category, cut off values were set between the lowest 0.114 to highest 3.373 OD. In overall isolates 13 isolates of *E.coli* were strong biofilm former (table 1 and 2). No biofilm production was noted in *Acinetobacter* Spp. isolates. From all of the tested isolates, the biofilm forming potentials was observed in 40.0% of the isolates as shown in table 2.

Table 2. Biofilm forming categories of isolates.

Biofilm formation	**OD range**	**Isolates (*n*)**	**Percentage (%)**
No	0.114 to 0.196	10	16.67
Weak	0.209 to 0.410	27	45.00
Moderate	0.816 to 0.440	10	16.67
Strong	0.892 to 3.373	13	21.66
Total		60	100

2.2. Antibiogram profiles

Overall resistance was classified into three categories as resistant from one category, resistant from two categories, and multidrug resistant (MDR). Mostly the isolates were observed to be MDR.The highest antibiotic resistance was seen against Ampicillin, and Nitrofurantoin. (Table 3)

2.3. PCR for papC gene

Five strong bifilm isolates were tested for the presence of papC gene all five contained papC gene which is among the different virulance genes found in *E.coli* (figure 2).

Table 3.*The Antibiogram profiles of tested bacteria.*

Antibiotics	*E. coli* (*n*=75) (*n*/%)	*KlebsiellaSpp.* (*n*=12) (*n*/%)	*PseudomonasSpp.* (*n*=25) (*n*/%)	*Salmonella Spp.*(*n*=13) (*n*/%)	*Proteus Spp.* (*n*=22) (*n*/%)
AMC	71 (94.6)	12 (100)	NT	NT	14 (63.6)
AK	41 (54.6)	7 (58.3)	3 (12.0)	5 (38.4)	5 (22.7)
N	56 (74.6)	9 (74.0)	NT	NT	NT
ST	58 (77.3)	9 (74.0)	NT	NT	NT
CN	37 (49.3)	7 (58.3)	8 (32.0)	10 (76.92	4 (18.1)
C	50 (66.6)	10 (83.3)	NT	11 (92.3)	16 (72.7)

CTX	67 (89.3)	11 (91.6)	NT	NT	NT
CAZ	56 (74.6)	11 (91.6)	11 (44.0)	8 (61.5)	NT
FEP	52 (69.3)	11 (91.6)	13 (52.0)	8 (69.2)	NT
MEM	13 (17.3)	2 (16.6)	13 (52.0)	0	0
IPM	14 (18.6)	2 (16.6)	13 (52.0)	0	0
TZP	49 (65.3)	9 (74.0)	14 (64.0)	NT	2 (9.0)

AMC: Amoxicillin–clavulanic acid.AK: Amikacin. N: Nitrofurantoin. ST: Sulfamethaxazole-trimethoprin. CN: Gentamicin. C: Chloramphenicol. CTX: Cefotaxime. CAZ: Ceftazidime. FEP: Cefepime. MEM: Meropenem. IPM: Imipenem. TZP: Piperacillin-tazobactam. NT: Not tested.

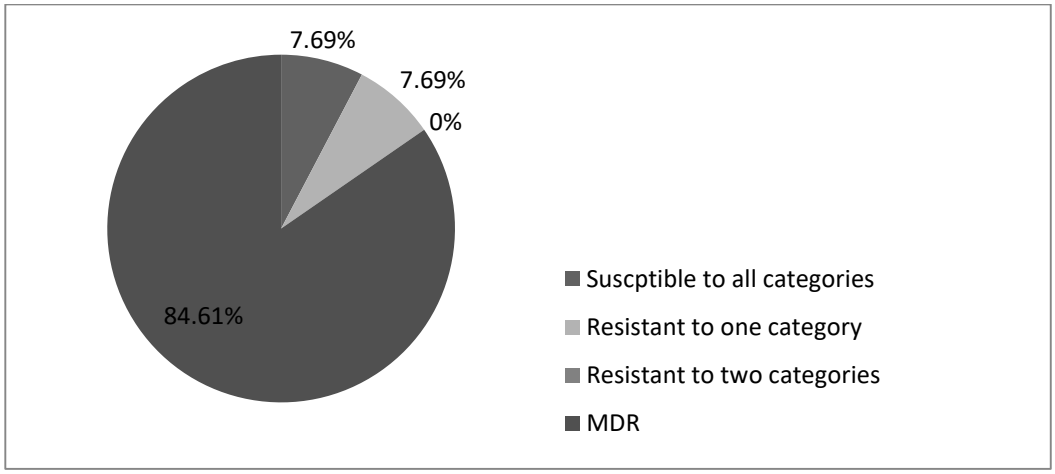
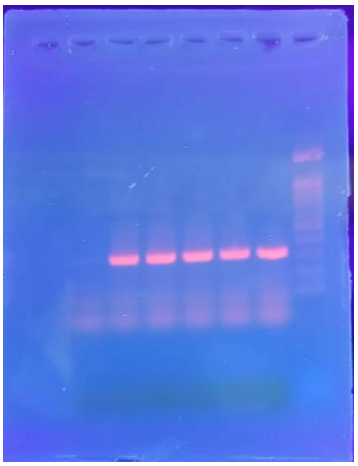


Figure 1. Categories of antibiotic resistance.



Figur 2. PCR for papC Gene

3. Discussion

With the time, antibiotic resistance has become a threatening challenge to all health care concerns[19]. Several studies have shown significant relationship between the biofilm related increased antimicrobial resistances [15,20]. Connection between the MDR and biofilm production is the key needed to better investigate the resistance profiles of biofilm forming strains[20].

In biofilms, encasement cells in a surrounding substance enhances resistance to antimicrobials and cleaning agents by building bacteria not easy to eliminate and control[21]. Results of present study indicated that form 13 isolates of *E.coli* were classified into three categories as resistant from one or two categories, and MDR. The MDR pattern was observed in most of the isolates. The highest observed resistant was against Ampicillin, Co-trimoxazole and Nitrofurantoin, Ciprofloxacin/ Ofloxacin, and total resistant from different antibiotics was 31.8%. Our research findings are also very much relatable to previously done observations like, Qian W et al., study results showed that from 69 biofilm producers, 46 isolates were strong biofilm formers and their link analysis showed,

their populations were exhibiting more tough biofilm formation and they contained a larger magnitude of Extensively drug resistance (XDR)[15]. Another research by Bhandari S et al, reported bacterial growth from different clinical samples, at a hospital of tertiary-care. From all 5.1% have been *Pseudomonas aeruginosa* and a half were MDR. They were all biofilm-forming, and majority of them were strong producers[22]. Cepas V et al[23] presented the findings that Ceftazidime along with Gentamicin tolerance was associated to biofilm production, whereas in *E. coli*, Tazobactam/ Piperacillin, Colistin in *Klebsiella pneumoniae*, and Ciprofloxacin in *Pseudomonas aeruginosa*. Kamali et al, investigations resulted that 80 *P. aeruginosa* clinical isolates were resistance to all antibiotics at the rate of 12.5% against Amikacin and Piperacillin/Tazobactam to 23.75% to levofloxacin. MDR *P. aeruginosa* accounted for 20%, and 83.75% of isolates showed biofilm phenotype[24].

Gram-negative and positive, both organisms form biofilm which helps them to be protected from several eliminating agent and treatments[25]. Not only in Gram-negative but antibiotic resistance is also a problem with Gram-positive clinical isolates[14]. Results of a previous study showed that the drugs susceptibility analyzed Coagulase negative *Staphylococcus*(CoNS) showed highest resistance to Macrolides and Lincosamides and more sensitivity to Rifampicin and Linezolid and this indicated that the hospital environment can be occupied by biofilm producing CoNS with increased antibiotic resistance, and spread of these organisms can be a source of an increased risk of serious hospital acquired infections[14].

Apart from biofilm production, the number of ESBL producers among infected individuals in hospital settings has risen over time[26]. The therapeutic and clinical effects of the introduction of ESBL-producing and (carbapenem resistant) CR *Enterobacteriaceae* in this situation resulted in a sharp decrease in the range of available treatments[27,28]. It is widely known that other bacterial species may also provide *Enterobacteriaceae* with resistant plasmid. Therefore, the existence of a resistance gene offers a greater risk since it may spread to species that may be susceptible along with biofilm production. According to recent research from Rawalpindi, Pakistan, *E. coli* accounted for the majority of the CP-producing organisms (86%) [29]. According to another investigation, CP genes were found in 61 of the 72 isolates that were carbapenem-resistant[26]. In the current study, no carbapenemase-production was noted in *Proteus* and *Salmonella* isolates. While, 13.3% of the *E. coli* isolates showed carbapenemase-production potential, 8.3% in *Klebsiella* spp. and 36.0% in *Pseudomonas* spp.

4. Materials and Methods

4.1. Ethical concideration of the study

The current study was conducted by the Department of Microbiology, University of Karachi in collaboration with the Memon Medical Institute (MMI) Hospital Clinical Laboratory, Karachi, Pakistan. An ethical approval was obtained from the Department of Microbiology, University of Karachi before the start of study. As the current study did not have direct interaction with the patients or attendants, the patient consent forms were waived off. Hence, the ethical approval letter was also obtained from Memon Medical Hospital.

4.2. Collection of Samples

A total of 150 bacterial isolates were collected from the Memon Medical Institute (MMI) Hospital Clinical Laboratory, Karachi, Pakistan. The bacterial isolates were isolated from various clinical specimens like pus, urine, blood, high vaginal swab (HVS), and wound swabs. After receiving the isolated bacterial isolates, these were inoculated on MacConkey agar and Cysteine Lactose Deficient Medium (CLED) agarplates and were incubated at 37°C for the duration of 18 to 24 hours[30,31]. After the completion of incubation period, the plates were checked for bacterial colonies. Identification and differentiation of the bacterial isolates was made upon colonial, biochemical and morphological characteristics[32].

4.3. Screening of biofilm forming bacteria

Bacteria were tested for biofilm production with the help of Congo Red Agar (CRA) differentiating them as biofilm and non-biofilm forming. CRA was prepared with the standard protocol as mentioned by Freeman. Briefly, brain heart infusion broth (Oxoid) 37gm/l, agar technical (Oxoid) 2gm/l, sucrose 5 gm/l and Congo red dye 0.8 gm/l in aqueous solution (autoclaved) separately was mixed. All bacterial isolates were cultured on CRA incubated for 48hr at 37^{o}C. Organisms with grayish to black colour colonies were considered as biofilm former whereas non-biofilm formers with pink colour colonies[33].

4.4. Biofilm formation on 96 well microtiter plates

Microtiter-plate methodwas used for quantitative analysis of isolated cultures for biofilm formation (12). Shortly, cultures were inoculated in 3–5 ml of Trypticase Soya Broth (TSB) following 24 hours incubation. Afterwards, dilution of cultures was done as 1:100 in fresh broth, added with 0.2% glucose. 200 µl diluted culture was taken into each well of flat-bottom 96-well microtiter plate, incubated (covered) for 48 hours. Free floating filling were removed and wells were thrice washed with PBS. All tests were performed in triplicate[34].

4.4.1.Biofilm Quantification

Wells were stained for 10 min with 125 µl of 0.2% Crystal Violet solution. Subsequently, Plate was washed with clean water, and left to air dry. Subsequently, 200 µl of Ethanol (95%) was added to all tested wells and left for 10 to 15 minutes at room temperature. Control was kept as blank TSB. OD (Optical density) of wells was observed at 595nm using a 96well-plate reader (Diatek Dc-200Bc).

4.4.2. Statistical categorization of Biofilm

Organisms were characterized as No, Weak, Moderate and Strong biofilm formers by manually calculation of cutoff value according to the given formulations, (table 1).

4.5. Antibiogram Assay

The antibiotic susceptibility/resistance testing was done by Kirby-Bauer disk diffusion method.0.5% MacFarland standard was used to make the inoculum of bacterial isolates and to inoculate the muller Hinton agar (MHA)media plates. After the inoculation of MHAmedia plates, the panel of antibiotic discs was placed on each of the patri plate and then these plates were incubated at 37°C for more 18 to 24 hours. The panel of antibiotics was selected based on the recommendations from the Clinical Laboratory Standard Institute (CSLI) guidelines-2020. Each of the inoculated plate was check for the appearance of zone of inhibitions around the tested antibiotic discs. Based on these ZOIs, the tested bacterial strain was considered as sensitive, resistant of intermediate susceptible to that particular drug [35,36].

The CLSI recommended antibiotic panel includes the drugs such as: cefotaxime (30 µg), amoxicillin-clavulanic acid (20 µg), amoxicillin (10 µg), cefepime (30 µg), ceftazidime (30 µg), imipenem (10 µg), meropenem (10 µg), amikacin (10 µg), gentamicin (10 µg), piperacillin-tazobactam (10 µg), sulfamethaxazole-trimethoprin (30 µg), and nitrofurantoin (10 µg)[37,38].

4.6. PCR for papC gene Monoplex PCR (polymerase chain reaction) was performed for the detection of papC gene forward primer GACGGCTGTACTGCAGGGTGTGGCG, reverse primer TCCTTTCTGCAGGGATGC AATA (Amplified DNA (bp) 328) . DNA was obtained from five strong biofilm forming isolates and PCR mixture was prepared. 25µl volume was obtained in PCR tubes containing 12 µl mastermix, DNA free water, 1µl of reverse and forward primers, 3µl of DNA template, intitiating by heating at 94 ^{o}C followed by denaturation at 96^{o}C for 1min, annealing, 63^{o}C for 30sec,extension 72^{o}C for 3 min and final extension at 72^{o}C for 7 min for 30 complete cycles.

Analysis of PCR product was done by using 2% of agarose gel electrophoresis stained with ethidium bromide [46].

5. Conclusions

An indication of an approaching threat has been noted in the current study area in terms of incidences of biofilm-forming and multidrug-resistant MDR pathogens. Therefore, regular monitoring of biofilm and beta-lactamase production in the clinical labs is advised, along with thorough infection control and preventive practices.

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Institutional Review Board Statement:

Informed Consent Statement: As the current study did not have direct interaction with the patient or their attendants, the Informed consent from the patient were waived off.

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