Int. j. adv. multidisc. res. stud. 2023; 3(5):749-755

Received: 14-08-2023
Accepted: 24-09-2023

International Journal of Advanced Multidisciplinary Research and Studies

ISSN: 2583-049X

Determination of Some Virulence Factors in ESKAPE Group Associated with Bacterial Ulcers Infections in Al-Najaf-Iraq

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Abstract
ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) are among the most common opportunistic pathogens in nosocomial infection. Colonization of the ulcers by potentially ESKAPE group bacteria constitutes the threat of their transmission to various human tissues and organs. Thus, there is an increased risk of the development of general infections, which would be particularly serious for persons with impaired immunity; such a health risk should be taken into consideration. A total of 104 specimens were collected from patients suffering from different types of ulcers infections included 77 (74%) diabetic foot ulcers, 15 (14.5%) bed ulcer, and 12 (11.5%) Varicose dermatitis infections for each sex with age groups from 23-67 years. Identification of bacterial isolates was first made by the bacteriological methods including colonial morphology, Gram stain, and other biochemical tests. Identification of all suspected bacterial isolates was confirmed by the automated vitek-2 compact system using GP and GN-ID cards. The ability of ESKAPE group to form biofilm was detected by using microtiter plates (MTP). The results showed that all isolates were produced of biofilm (100%).
Also, the results indicated that 2 *P. aeruginosa* isolates were the best of isolates form biofilms, its absorbance value was 3.3995, while K4 and K7 showed the highest biofilm formation where the absorbance value was (3.3995).

Keywords: ESKAPE, Biofilm, Congo Red, MDR, Adherence, Ulcer Infections

Introduction
Microorganisms (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* species) are part of the so-called 'ESKAPE' pathogens to point out that they are the cause of most cases of hospital infections and express mechanisms that allow them to effectively 'escape' from the action of antibacterial drugs (Rice, 2008) [28].
Virulence factors of nosocomial ESKAPE bacteria represent paradigms of resistance, pathogenesis, and disease transmission. There is a range of antimicrobial resistance mechanisms used by the nosocomial ESKAPE pathogens, such as enzymatic inactivation, modification of drug targets, changing cell permeability through porin loss or increase in expression of efflux pumps, and mechanical protection provided by biofilm formation (Santajit and Indrawattana, 2016) [34].
Biofilms are complex microbial communities living as a thin layer on biotic or abiotic surfaces, implanted in a matrix of extracellular polymeric substances created by the biofilms themselves. Microorganisms within the biofilm can interact with each other, as well as the environment. The major component of the matrix is secreted extracellular polymeric substances, mainly consisting of polysaccharides, proteins, lipids, and extracellular DNA from the microbes (Sharma *et al*., 2014) [35].
The emergence of high-level resistance to antimicrobials is an increasing threat to global health (Levy and Marshall, 2004), and even a small increase in antibiotic-refractory bacterial subpopulations or MIC could herald the emergence of higher-level resistance (Balaban *et al*., 2004). Therefore, any factor contributing to an increase of antibiotic resistance is critically important. Antibiotic efflux pumps represent one of the most important antimicrobial resistance mechanisms used by *K. pneumoniae* and *E. cloacae* clinical isolates (Peleg *et al*., 2010; Zavascki *et al*., 2010) [26, 41]. The increased efflux of the antimicrobial agent leads to the reduction of its intracellular concentration, which can enhance bacterial survival (Piddock, 2006) [27]. This study was suggested and designed to study the correlation between biofilm formation and multi drug resistance ESKAPE Group isolated from different ulcer types disease as well as the antibiotic resistant for certain types of antibiotic especially to β-lactam.

Methods

The research was carried out at the Bacteriology and Molecular Laboratories, Department of Biology, Faculty of Sciences, Kufa University, Iraq.

Clinical Specimens and Patients

During the period of study 104 specimens were taken from patients suffering from different types of ulcers infections at AL-Sadder Medical City /Al-Najaf-Iraq for three months, from September 2021 to November 2021.

Detection of Biofilm Formation in ESKAPE Group

1. Detection by Tissue Culture Plate Method

Using the techniques of Lizcano *et al.*, (2010) [21], semi-quantitative assessments of biofilm formation were measured using tissue culture-treated, 96-well polystyrene plates. The adhesion's absorbance (A 630), stained biofilms was measured using a microtitre plate reader. A biofilm-positive phenotype was defined as having a value of ≥ 0.120 at absorbance of 630 nm. Biofilm formation considered high, moderate, or weak (OD630nm) as shown in Table 1.

Table 1: Classification of bacterial adherence by TCP (Tissue Culture Plate) method

Biofilm formation	Adherence	Mean OD Values at 630 nm.
Non/Weak	Non/Weak	< 0.120
Moderate	Moderately	0.120-0.240
High	Strong	> 0.240

2. Congo-Red Agar Method (CRA)

It was prepared by adding 52 gms of Brain heart infusion agar media to a liter of distilled water was sterilized by autoclaving at 121°C/15 psi for 15 min. On the other hand, Congo red stain (0.8 g/L) was prepared as a concentrated aqueous solution and autoclaved at 121°C for15 min, sugar (sucrose 50 g/L) was sterilized by filtration. Both dye and sugar were added to the agar after its cooling till 55°C. Plates were then inoculated and incubated aerobically at 37◦C for 24 hrs, this media used to detect on the biofilm production (Freeman *et al*., 1989) [10]. Positive result was indicated by black colonies with a dry crystalline consistency. Weak slime producers usually remained pink, though occasional darkening at the centers of colonies was observed. A darkening of the colonies with the absence of a dry crystalline colonial morphology indicated an indeterminate result.

Antibiotic Susceptibility Test

In this study used 20 types of commonly used antibiotics include: Ampicilin10, Ceftazidime 30, Cefotaxime 10, Ceftriaxone 30, Cefepime 30, Imipenem 10, Meropen 10, Gentamycin 30, Rifampin 5, Trimethoprim sulfamethoxazole 25, Erythromycin 60, Azithromycin 15, Chloramphenicol 30, Doxycycline 30, Tetracycline 30, Ciprofloxacin 5, Levofloxacin 5, colistin 10, Rifampin 5, Vancomycin 10, Tetracycline 30.

The antibiotic sensitivity report was performed according to Kirby-Bauer disc diffusion fashion on Mueller-Hinton agar (Morello *et al*., 2006) [23]. It Briefly, the investigated isolates allowed to multiplication for overnight at 37ºC in BHI broth referred to 0.5 McFarland turbidity standard equal to $1.5X10^8$ CFU/ml, the MH agar plates were fully spreading with 0.1ml of growth suspension and then fixed antibiotics disks on the surface. The applied plats left for 10-15 minutes and then incubated for 24 hrs at 37ºC as a standard cultural condition. The fixed antibiotics were classified as sensitive (S), Intermediate (I), or resistant (R) according to diameters of halo zone in millimeters (mm) around the individual disk, the results were compared with clarifying list of (CLSI, 2021) [9].

Results and Discussion

ESKAPE Group Isolation and Identification in Ulcer Infections

The results showed that 88(%) specimens revealed positive culture on MacConky agar and/or blood agar, while 16(%) specimens where no growth appear on blood agar and MacConky agar. The results of culture showed that 135/152(%) isolates belonged to Gram-negative bacteria, 17/135 (30.3%) isolates belong to Gram-positive bacteria (Fig 4-1). The results of culture showed 44 samples are mix growth, on blood agar showed 15/17 isolates belong to *S. aureus*. Opportunistic and pathogenic Gram-negative bacteria strains isolated from the patients suffering bed ulcer, varicose dermatitis and diabetic foot ulcers infections are *E. coli* showed that 34, *P. aeruginosa* reached to 24, *K. pneumonia* showed that 24, *Acinetobacter* showed that 2, *Burkurdria* showed that 30, *Enterococcus* showed that 2, *Providncia regetti* showed that 2, *Proteus* showed that 8, *Enterobacter* showed that 11. This pathogen is well adapted to the hospital environments due to biofilm formation that provides long survival advantages for the pathogen, and effectively prevent eradication by the host immune system or antimicrobial drug treatment (Groenewold *et al*., 2018) [12].

K. pneumoniae is an opportunistic pathogen which causes serious infections like, urinary tract infection, pneumonia, burn infection, and soft tissue infections in compromised and hospitalized patients. It has number of virulence factors such as a capsule that enable this pathogen to colonize and provides phagocytosis resistance (Hussein *et al*., 2009; Riquelme *et al*., 2018) [14, 29]. Negative results of specimens culture on MacConky agar and blood agar features can be attributed to the pathogen that responsible for illness maybe belong to anaerobic Gram-negative or/and positive bacteria or other atypical etiological agents such as viruses, fungi or parasites, etc. which need to special media, environmental conditions and techniques for growth (Jain And Barman, 2017) [15].

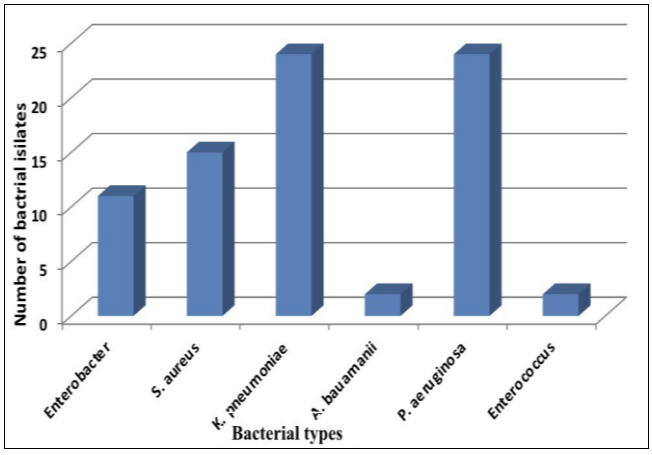

Fig 1: Distribution of bacterial types in ESKAPE groups

Table 2: Numbers and percentage of ESKAPE groups isolates in infections types

Infection Types	ESKAPE Group					
	E. cloacae	***S. aureus***	***K. pneumoniae***	***A. baumannii***	***P. aeruginosa***	***E. cloacae***
Diabetic foot ulcer	8	15	18	2	22	0
Bed ulcer	3	0	2	0	0	0
Varicose dermatitis	0	0	4	0	2	2
Total	11	15	24	2	24	2

This test was conducted to all isolates by commonly used antimicrobial agents by using the Kirby-Bauer disk diffusion method. All isolates test with fourteen antibiotics disc which included; carbapenem class (imipenem and meropenem); cephalosporins (ceftazidime); aminoglycoside (gentamycin); floroquniolone (ciproflaxcin, levofloxacin); tetracyclins (tetracyclin, doxycycline); ansamycin (rifampin); phenicols (chloramphenicol, pipracillin); folate pathway antagonists (trimethoprim sulfamethoxazole); macrolides (fusidic acid, erythromycin).

The phenotypic susceptibility of 24 K. pneumoniae, 24 Pseudomonas, 11 Enterobacter, 15 Staphylococcus, 2 Enterococcus and 2 Acenitobacter isolates used antimicrobial agents by using Kirby-Bauer disk diffusion method. The results were interpreted according to the diameter of inhibition zone and compared with stander zones of inhibition determined by CLSI, (2021) [9].

The results of this test revealed that Enterobcter and Klebsella and P. aeurogenosa have great resistance to most common antibiotics used in hospitals,The highest rate of resistance is seen with trimethoprim in Enterobacter and Klebsella and P. aeurginosa 100%, cefotaxime 92.3% in Pseudomonas while 100% Enterobacter and Klebsella and Acnitobacter Ampicillin high rate of resistance in P. aeurginosa 100%, and Ceftriaxone and Collistin and Gentamicin 84.61% while Doxycline and Levofloxacin and Cefepime with percentage76.92 %. This study shows decrease rate of resistance for Impinem and Tetracyclie a proximally 61.53%.

Development of antibiotic resistance is often related to the overuse, and misuse of the antibiotics prescribed. Resistance of K. pneumoniae and E. cloacae continues to be an important clinical therapeutic problem, such that which can be found in an increasing multidrug resistance in these bacteria.

The emergence of high-level resistance to antimicrobials is an increasing threat to global health (Levy and Marshall, 2004), and even a small increase in antibiotic-refractory bacterial subpopulations or MIC could herald the emergence of higher-level resistance (Balaban *et al*., 2004). Therefore, any factor contributing to an increase of antibiotic resistance is critically important.

The results revealed that all isolates K. pneumoniae and E. cloacae have higher resistance to penicillins (amoxicillin, ampicillin, Penicillin G and Piperacillin), this may be due to the production of β–lactamases or failed antibiotics in reaches to the target penicillin binding protein (PBPs) (Harwood *et al*., 2000) [13]. Round *et al*., (2011) [31] founded that penicillin-triggered lysis could be partially prevented by heat shock pretreatment makes it clear that *in vivo* stresses, such as inflammation, respiratory bursts in phagocytes, and temperature upshift, may induce higher levels and increase resistance to penicillin. Moreover, the exchange of plasmids that carry antibiotics resistance genes between contact microbial cells may play role in increasing the ability of bacteria to resistance to several antibiotics (Angel *et al*., 2009; Juhas, 2015) [3, 16].

Regarded to third generation of cephalosporins (cefotaxime, ceftriaxone and ceftazidime), it was appeared that most isolates K. pneumoniae and E. cloacae were possess high resistant to cefotaxime, ceftriaxone and ceftazidime, its an important indicator for the presence of ESBLs. Resistance of K. pneumoniae and E. cloacae isolates to aztreonam also, may be results from production of ESBLs (CLSI, 2021) [9].

The most remarkable finding of this study is the high susceptibility level to imipenem and Meropenem (carbapenems), that most isolates of K. pneumoniae and E. cloacae were high resistance to imipenem and meropenem. These results are in disagreement with many previous studies Khan *et al*., (2014) and Rizwan *et al*., (2018) [30]. Also, these results are disagreement with previous study obtained by Vasconcelos *et al*., (2015) [38] found that 64/64 (100%) all isolates of K. pneumoniae and E. cloacae have high sensitive to these antibiotic.

Regarding susceptibility to tetracycline, K. pneumoniae and E. cloacae isolates were appeared high resistance to these antibiotic, these results are agreement with prior study obtained by Zhihui *et al*., (2003) [42]. pointed out all isolates of E. cloacae have high resistance to these antibiotic. This resistance is under the control of transmissible plasmids (Brooks *et al*., 2007) [7].

In the other hand the sensitivity of K. pneumoniae and E. cloacae isolates to Trimethoprim were low. These results are disagreement with preceding study obtained by Gamboa *et al*., (2013) [11] were 19 (65.5%). The difference in sensitivity and drug resistance in different geographic regions can be associated with different patterns of antibiotic use in different areas (Ruiz *et al*., 2013) [32].

In the last few years, Enterobacteriacae have been shown to be resistant to a variety of antimicrobial agents. This increase in resistance is primarily related to the frequent use of antimicrobials and to how easy it is for these microorganisms to build up resistance (Thomson, 2010) [37]. This profile has been particularly observed in the hospital environment, where outbreaks of infections of β-lactamases-producing enterobacteria are described (Vasques *et al*., 2011) [39].

Antibiotic efflux pumps represent one of the most important antimicrobial resistance mechanisms used by *K. pneumoniae* and *E. cloacae* clinical isolates (Peleg *et al*., 2010; Zavascki *et al*., 2010) [26, 41]. The increased efflux of the antimicrobial agent leads to the reduction of its intracellular concentration, which can enhance bacterial survival (Piddock, 2006) [27].

Table 3: Antimicrobials Sensitivity Test of *S. aureus* and *E. Faecium*

Antibiotic type	S. aureus			E. faecium		
	S	I	R	S	I	R
LEV	1(12.5)	0(0)	14(87.5)	0(0)	0(0)	2(100)
TE	0(0)	0(0)	15(100)	2(100)	0(0)	0(0)
VA	0(0)	1(2.5)	14(87.5)			
CN	3(25)	4(25)	8(50)	0(0)	0(0)	2(100)
C	3(25)	2(2.5)	10(62.5)			
TMB	3(25)	2(2.5)	10(62.5)	0(0)	0(0)	2(100)
DA	1(12.5)	0(0)	14(87.5)			
E	1.(12.5)	0(0)	14(87.5)			
AM	1(12.5)	0(0)	7(87.5)			
CIP	5(37.5)	2(2.5)	8(50)	0(0)	0(0)	2(100)
DO	3(25)	0(0)	12(75)	2(100)	0(0)	0(0)
RA	1(12.5)	0(0)	14(87.5)			
AZM	1(12.5)	0(0)	14(87.5)			

Table 4: Antimicrobials Sensitivity Test of *P. aeruginosa* and *E. cloacae*

Antibiotic type	*P. aeruginosa*			*E. cloacae*		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Cefotaxime	0(0)	2(7.69)	22(92.3)	0(0)	0(0)	11(100)
Ciprofloxacin	6(30.7)	0(0)	18(69.23)	6(60)	0(0)	5(40)
Trimethoprim	0(0)	0(0)	24(100)	0(0)	0(0)	11(100)
Gentamicin	3(15.38)	0(0)	21(84.61)	0(0)	0(0)	11(100)
Ampicillin	0(0)	0(0)	24(100)	0(0)	0(0)	11(100)
Meropenem	7(30.7)	0(0)	17(69.23)	2(20)	0(0)	9(80)
Collistin	2(7.69)	1(7.69)	21(84.61)	0(0)	0(0)	11(100)
Cefepime	4(15.38)	1(7.69)	19(76.92)	0(0)	0(0)	11(100)
Ceftriaxone	3(15.38)	0(0)	21(84.61)	0(0)	0(0)	11(100)
Tetracycline	6(23.07)	2(7.69)	16(61.53)	3(20)	0(0)	9(80)
Levofloxacin	5(23.07)	0(0)	19(76.92)	0(0)	0(0)	11(100)
Imipinem	9(32.46)	0(0)	15(61.53)	2(20)	0(0)	9(80)
Doxycycline	5(23.07)	0(0)	19(76.92)	4(40)	0(0)	7(60)

Table 5: Antimicrobials Sensitivity Test of K. pneumoniae and A. baumannii

Antibiotic type	*K. pneumoniae*			*A. baumannii*		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
cefotaxime	0(0)	0(0)	24(100)	0(0)	0(0)	2(100)
Ciprofloxacin	3(21.42)	0(0)	21(78.57)	2(100)	0(0)	0(0)
trimethoprim	0(0)	0(0)	24(100)	0(0)	0(0)	2(100)
Gentamicin	2(14.28)	0(0)	22(85.71)	0(0)	0(0)	2(100)
Ampicillin	0(0)	0(0)	24(100)	0(0)	0(0)	2(100)
Meropenem	2(14.28)	0(0)	22(85.71)	2(100)	0(0)	0(0)
Collistin	0(0)	0(0)	24(100)	0(0)	0(0)	2(100)
Cefepime	0(0)	2(14.28)	22(85.71)	0(0)	0(0)	2(100)
Ceftriaxone	0(0)	1(7.14)	23(92.85)	0(0)	0(0)	2(100)
Tetracyclie	4(28.57)	0(0)	20(71.42)	0(0)	0(0)	2(100)
Levofloxacin	0(0)	2(14.28)	22(85.71)	2(100)	0(0)	0(0)
Impinem	2(14.28)	0(0)	21(85.71)	0(0)	0(0)	2(100)
Doxycline	3(21.42)	1(7.14)	20(71.42)	2(100)	0(0)	0(0)

Biofilm Formation in ESKAPE Group

The ability of 78 isolates of ESKAPE group to form biofilm was detected by using microtiter plates (MTP). Biofilms were quantified by measuring the absorbance of stained biofilms at 630 nm with a microtiter plate reader.

The results in this study were indicated according to Salwa *et al*., (2011) [33] in which 100% of K. pneumoniae and Staphylococcus and Enterobacter and Enterococcus isolates appeared high biofilm formation (strong positive adherence) appeared moderate biofilm formation (Table 6).

Also, the results indicated that Pseudomonas 2 isolates were the best of isolates form biofilms among other Pseudomonas isolates, its absorbance value was (3.3995), while the results pointed out that K4 and K7 were the higher biofilm formation among K. pneumoniae isolates that the absorbance value was (3.3995).

Mathur *et al*., (2006) [22] stated that the MTP method is an accurate and reproducible method for screening and determination of biofilm production. Therefore, in our study will used this method.

Microorganisms form biofilms that exist in the environment, whether upon inanimate objects or living animals, the bacteria attach to these surfaces, including soil and aquatic systems, and have been documented in the human literature developing on medical devices, within the middle ear, external ear, lungs, heart valves, surgical implants and tooth enamel (Singh *et al*., 2013) [36].

Biofilm formation in Gram negative bacteria occurs when bacterial cells first swim along a surface, using flagellar-mediated motility, until attachment occurs at a specific site

and their attachment is initially reversible (O'Toole *et al*., 2000) [25]. Yang *et al*., (2008) [40] conducted a study on biofilm formation by K. pneumoniae isolates found that 62.5% of the isolates generated biofilms which is less than the results obtained in our study (100%). This may be due to differences in geographical area and sample size.

Table 6: Biofilm Formation of *ESKAPE* Group

Stander range of OD	Biofilm	Adherence	*E. faecium* No.2 (%)	*S.aureus* No.15 (%)	*K. pneumoniae* No.24 (%)	*A. baumannii* No.2 (%)	P. aeruginosa No.24 (%)	*E. cloacae* No.11 (%)
< 0.120	weak	weak	0(0)	0 (0)	0(0)	0 (0)	0 (0)	0 (0)
0.120-0.240	moderate	moderate	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
>0.240	strong	strong	2(100)	15 (100)	24 (100)	2 (100)	24(100)	11 (100)
Total			2 (100)	15 (100)	24(100)	2 (100)	24 (100)	11 (100)

Congo-Red Agar Method (CRA)

This method was described by (Freeman *et al*., 1989) [10] to detect bacterial production of a slime layer using a specially prepared solid medium. The results showed that 16/42 (38%) isolates produced a strong slime layer indicative of the formation of black colonies with dry crystalline texture while 26 (61.9%) isolates did not produce a slime layer indicative of pink colony formation that the classification was based on (Khan *et al*., 2011) [17]. This result is in agreement with a study (Neela *et al*., 2009) [24] that found that about 57% of MRSA had the ability to form a clay film and that 43% of MRSA gave a negative result. It was noted that the results obtained are not in agreement with the study by Al-Hassani *et al* (2011) which showed that about 77.8% of the S. aureus isolates were clay-producing. Figure (-): Slime layer production by Congo red agar. The results showed that CRA assay is a good method to detect slime and biofilm production capacity and agreed with researcher Arciola *et al*. (2006) [4] who recommended that the CRA experiment is a reliable method for determining biofilm production, (Cabrera *et al*., 2013) [8]. The results of investigating slime layer production in MRSA isolates were consistent with several studies that confirmed the susceptibility of MRSA to slime production. The slime layer is formed by the ability of bacteria to produce Glycocalyx which surrounds the bacterial cell, Glycocalyx is an extracellular substance composed of a polysaccharide, a polypeptide, or both. Protecting the bacterial cell from drying out and losing nutrients, this layer encapsulates irregular glycocalyxes when they attach to the cell wall in a brittle manner known as the slime layer. While Glycocalyxis consists of a basic substance that is closely bound to the cell wall and hence is known as a Capsule (Kirisits *et al*., 2007) [18]. The slime layer encapsulates the bacterial cells, forms living thin films known as biofilms, and acts as a blocker against the effect of antibiotics within the bacteria cell, thus conferring resistance (Al-Khafaji, 2018) [2].

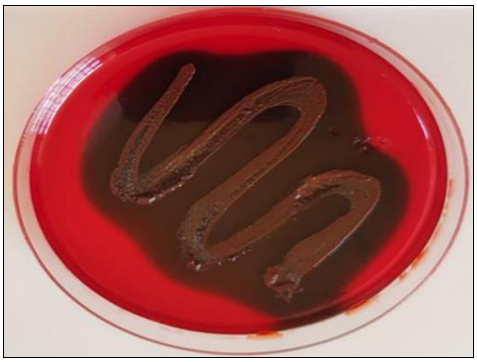

Fig 2: Slime layer production by Congo red agar

P. aeruginosa	*K. pneumoniae*	*E. cloacae*	*S.aureus*	*A. baumannii*	*E. faecium*
3/13(23%)	6/14(28.57%)	1/5(20%)	4/8(50%)	1/1(100%)	1/1(100%)

Conclusions

1. The study is designed to identify Correlation between Biofilm and Antibiotic Resistance.
2. The study is designed to identify Virulence factors and ulcer causes.

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