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STUDY OF THE EFFECTS OF DIFFERENT WAYS OF INFECTION ON THE DENSITY OF *P. AERUGINOSA* BIOFILM IN MALE AND FEMALE WHITE MATURE RABBIT

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Abstract

Rabbits exposure to *P. aeruginosa* causes pathological lesions and biochemical changes. This was observed during the experiment in which seventy-five rabbits of both sexes were divided into five groups, first group was inoculated with 0.2 ml of distill water while the other four groups inoculated with 0.2 ml of 2×10^8 CFU/ml "by using 1ml disposable syringes" as following the 2nd group infect the pregnant by inoculation intrauterine, 3rd infect male and female by Intraperitoneal injection, 4th group infected males by inoculation (intra scrotum) then mixed with normal female and 5th group infected females by inoculation intrauterine then mixed with normal male. The results of SEM show difference in severity of biofilm varies from intensive to moderate and also mild. In groups that have been infected locally G2, G4 and G5 the biofilm is intensive in the affected organ such as (testis, uterus and fetus) and moderate to mild in other organs such as (liver, spleen, ovary) which had been infected by bacterial dissemination. As for the infected group in intraperitonum G3 the biofilm is intensive in the internal viscera such as the liver, spleen and ovaries but it was moderate or mild in the organs transferred to it venereally.

Key words: *P. aeruginosa*, fetus, liver and spleen.

Introduction

P. aeruginosa appeared as one of the most problematic Gram-negative nosocomial pathogens (Moradali *et al*., 2017). According to World Health Organization, (2017) *P. aeruginosa* have been classified by several healthcare organizations as serious threat to public health, considering its high prevalence with associated high mortality rates and limited treatment options, this pathogen has been identified as a critical research priority for the development of novel therapies. It can cause a wide array of hospital-associated diseases, including respiratory tract infections, urinary tract infections, wound infections and bacteraemia (Hossein *et al*., 2015; Bassetti *et al*., 2018), which is lead to cause of morbidity and mortality in cystic fibrosis (CF) patients and immunocompromised individuals or have underlying medical conditions such as urinary tract, respiratory tract and skin infections (Zheng *et al*., 2019; Hamzah and Hasso, 2019). Due to evading host defence mechanisms and persisting of *P. aeruginosa*, it is ultimately responsible for the morbidity and mortality of about 80% of CF patients worldwide (Das and Manos, 2016). While Bassetti *et al*., (2018) are mentioned that *P. aeruginosa* causes a variety of skin and soft tissue infections ranging from the benign e.g. (cellulitis, post-surgical infections) to the immediately life threatening. Its commonly recognized as a cause of endometritis in mares and is regarded as a venereally transmitted pathogen (Tiago *et al*., 2012; Satué and Gardon, 2016; Hasso and AL-Janabi, 2019). Various studies have shown *P. aeruginosa* involved in causing infertility in males and females to causing complete immobilization of mouse spermatozoa in vitro (Negi *et al*., 2018)

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Material and Method

Experimental animals and management

Seventy five white mature rabbits of both sex (♂ and ♀) in (40-48) weeks age and (1.900-2.200) gm of weight were housed at animal house in department of pathology, college of veterinary medicine, Baghdad university, Iraq. In sterilized polypropylene rabbit cages,

12 h. light and dark cycle. Tempture 21°C, drinking tap water and fed on special formula feed pellets (Europian comission, 2007).

All animal were under identical management protocol and guidelines of institutional animal ethics committee. Animal divided equally into five groups each group containing (15) rabbits in ratio and (♂ 1:2 ♀). 1st group (control group) contain mixed of 15 rabbit of both sex and diet on normal rabbit pellets. 2nd group contain mixed normal (10♀) with normal (5♂) for (10) days then separate (♀) from (♂) then infect the pregnant at first day of second trimester locally by inoculation intrauterine with 1/10 of LD50 of *P. aeruginosa*, 3rd group contain mixed normal (10♀) with normal (5♂) for (10) days and separate (♀) from (♂) then infect the males and females at first day of second trimester by Intraperitoneal injection with 1/10 of LD50 of *P. aeruginosa*, 4th group contain mixed normal (10♀) with infected (5♂) locally by inoculation (intrascrotum) with 1/10 of LD50 of *P. aeruginosa* for (10) days then separate (♂) from (♀), 5th group contain mixed normal (5♂) with infected (10♀) locally by inoculation intrauterine with 1/10 of LD50 of *P. aeruginosa* for (10) days then separate (♀) from (♂). According to Al-Awadi and Alwan, (2014) the experimental animals were injected with (0.2 ml of 2×10^8) CFU/ml of *P. aeuginosa* by using 1ml disposable syringes equal to 1/10 of LD50. At day 32 of experiment all animal in groups euthanized by longitudinal abdominal opening and take (testes, prostate, ovary, uterine and placenta) for scanning electron microscope (SEM).

SEM protocol was completely done in a biosafety cabinet (class-2), also all samples were passed *via* etch chambers from types (SPI-pore) polycarbonate track, with (0.08 μm) pore size filters were used specially for bacterial sample preparation. according to (Fischer *et al*., 2012).

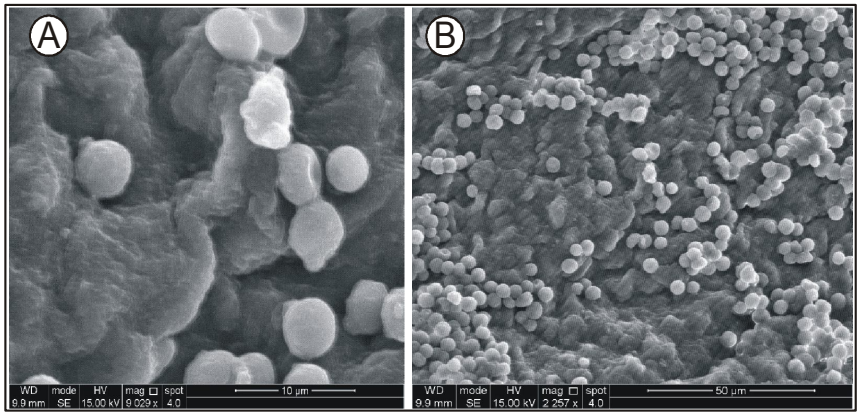

Fig. 1: SEMof G2 uterus in for *P.aeruginosa* biofilm showed: A- intensive biofilm in the section of uterus (WD9.9mm, HV15.00kv, mag9029).B- intensive biofilm in the section of uterus (WD9.9mm, HV15.00kv, mag2257).

P. aeruginosa isolation

Isolate of *P. aeruginosa* was provided by media diagnosis center in Erbil city then transported to Baghdad university - Veterinary medicine collage in suitable condition in universal tube by using ice box. It was isolated from a case of human. A loop full of bacteria was transferred from the original universal tube and cultured on Pseudomonas agar, incubated at 37°C/24 hrs in order to examine their morphology and pigment production. Later the bacteria subcultured on blood agar and incubated at 37°C/24 hrs, then subcultured on nutrient broth and incubated at 37°C/24 hrs. The broth was centrifuged and washed 2 times by Phosphate buffer saline (PBS) and then 0.3 ml of the bacterial suspension was injected intraperitoneal (I/P) in mouse and the bacteria was re-isolated from the internal organs on Pseudomonas agar at 37°C/24 hrs. This process was repeated 3 times and the process of preparation redone after each isolation in order to maintain the virulence factors of the bacteria (Al-Awadi, 2014). Finally, Diagnosis confirmed by culturing the bacteria on the selective media (Pseudomonas agar) with special antibiotic supplements (Centrinix®) and the physical properties were examined including the characteristic blue green color produced by the bacterial colony and the thermal test in which *P. aeruginosa* was the only member of *Pseudomonas* genus that can growth at 42°C (Weihui and Shouguang, 2015). According to Al-Awadi and Alwan, (2014) the experimental animals were injected with 1/10 of LD_{50} is a challenge dose equal to (0.2 ml of 2×10^8) CFU/ml by using 1ml disposable syringes, determination the number of colony forming units in a bacterial suspension or homogenate through a technique used in Microbiology is "surface viable count" (Miles *et al*.,1938).

Result

Scanning electron microscope(SEM)

Group 1: All of internal organs were normal.

Group 2: SEM image showed intensive biofilm in parenchymal tissue of fetus and uterus fig. (1A & 1B), other SEM images showed light biofilm in (spleen, liver, kidney and ovary)

Group 3: SEM image showed intensive biofilm in liver fig. (2A), kidney fig. (2B), ovary fig. (2C). spleen fig. (2D), other SEM images showed light biofilm in oviduct fig. (2E), testis and uterus.

Group 4: SEM images showed

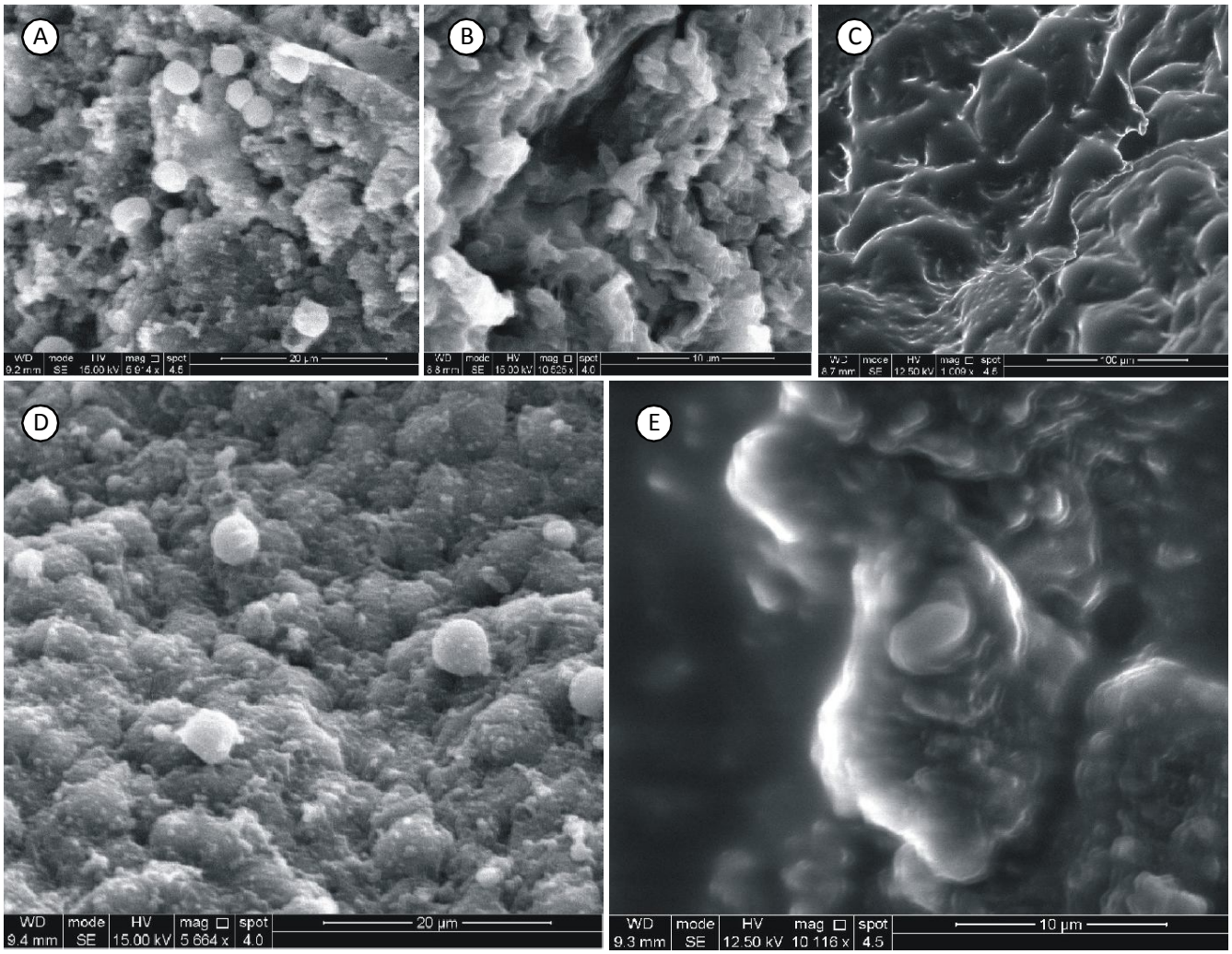

Fig. 2: SEM image in G3 for *P. aeruginosa* biofilm showed A-intensive biofilm in liver (WD9.2mm, HV1500kv, mag5914) B-biofilm in kidney (WD8.8mm, HV15.00, mag10.525).C- biofilm in ovary (WD8.7mm, HV12.50kv, mag1009). D-biofilm in Spleen (WD9.4, HV15.00kv, mag5664).E-biofilm in oviduct (WD9.3mm, HV12.50kv, mag10116).

intensive biofilm in testis, other SEM images showed light biofilm in uterus, fetus, liver, spleen, kidney and ovary.

Group 5: SEM image showed intensive biofilm in uterus fig. (3A) and fetus fig. (3B), other SEM images showed light biofilm in (testis, spleen, liver, kidney and ovary).

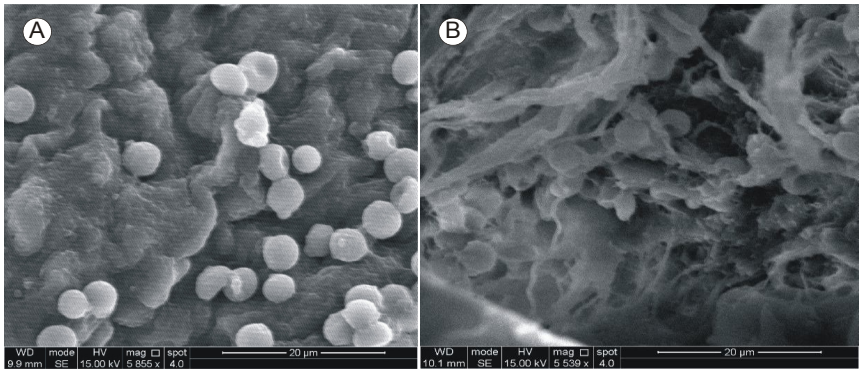

Fig. 3: SEM of G5 uterus for *P.aeruginosa* biofilm showed. A- intensive biofilm in the uterus (WD9.9mm, HV15.00kv, mag5855).B-intensive biofilm in reabsorbed fetus (10.1WD, kv1500HV, mag5539).

Discussion

The difference in severity of biofilm varies from intensive to moderat and also mild. In groups that have been infected locally G2, G3, G5 and G6 the biofilm is intensive in the affected organ such as (testis, uterus and fetus) and moderate to mild in other organs such as (liver, spleen, ovary) which had been infected by bacterial dissemination. As for the infected group in intraperitonum G4 the biofilm is intensive in the internal viscera such as the liver, spleen and ovarys but it was moderate or mild in the organs transferred to it venereally that's agree with Lee and Yoon, (2017) they said after bacteria attach to surfaces or each other, they undergo a series of changes to adapt to the new mode of life. As

surface-attached *P. aeruginosa* grow and form microcolonies, they start to build structures and water channels as the biofilm matures, Hirschfeld, (2014); Lenda *et al*., (2019) biofilm is a rich source of concentrated extracellular phagocyte-activating bacterial components, including DNA, LPS and EPS. Importantly, bacteria–neutrophil interactions has evolved since discoveries of the different immunostimulatory properties of planktonic and biofilm forms of pathogens, bacterial infections start with planktonic growth followed by a formation of biofilm. More importantly interactions of bacteria with neutrophils at planktonic stage of infections usually result in pathogen killing, controlled production of inflammatory mediators, neutrophil apoptosis and the resolution of acute inflammation, on the contrary, the biofilm stage of bacterial infection favors necrosis of neutrophils and causes chronic inflammation, It is worth mentioning according to Lee and Zhang, (2015); Yong *et al*., (2018) that *P. aeruginosa* coordinates infection via cell–cell communication termed quorum sensing (QS). Through production of QS signalling molecules, the expression of virulence-associated genes and the formation of biofilm are regulated in a cell density-dependent manner the *P. aeruginosa* QS systems are deeply interconnected and represent one of the most complex and best-studied bacterial signalling systems described, the major QS systems are the las (elastase) and rhl (rhamnolipid) systems which utilise acyl homoserine lactone (AHL), N-(3-oxododecanoyl)-l-homoserine lactone (3-oxoC12-HSL) and N-butanoyl-l-homoserine lactone (C4-HSL), respectively, for signalling, when the concentration of AHL reaches a threshold level, the LasR transcriptional regulator forms a complex with 3-oxo-C12-HSL that is synthesised by LasI (an autoinducer), which in turn binds to DNA, altering virulence gene expression, the rhl system, on the other hand, responds to C4-HSL produced by RhlI synthase. Similar to the las system, RhlR-a transcriptional regulator-induces virulence gene expression during times of high cell density.

But our results disagree with Bachta *et al.,* (2020) they said bacterial counts from infected organs, *P. aeruginosa* was detected in the liver, lungs and a minor proportion of gall blader, but no bacteria were detected in the intestinal tract, this was followed by a drastic six-logarithm expansion of bacteria in the gall bladder and similar four- to six-logarithm expansion in the intestinal tract, in contrast, total bacterial counts in the lung and liver by only one- to two logarithms over the same period, demonstrating that high levels of *P. aeruginosa* replication was not a universal phenomenon. Bacterial recovery in the gall bladder reached higher numbers than were originally in the inoculum, suggesting that the gall bladder may be a hospitable niche for *P. aeruginosa* replication. In support of this, also observed that *P. aeruginosa* growth in ex vivo bile preparations approached that of enriched medium, this dramatic expansion of bacteria had plateaued with only slight additional increases in all organs. Lumenal contents of the intestinal tract contained similar bacterial CFU as observed in whole intestine homogenates, most of the *P. aeruginosa* bacteria were in the lumen rather than in the bowel wall. These findings indicate that bacteremia is followed by a dramatic expansion of the bacterial population in the gall bladder and intestines, which is accompanied by *P. aeruginosa* excretion in the feces (Bachta *et al.,* 2020).

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