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RESEARCH ARTICLE

INHIBITION OF *Acinetobacter baumannii* ADHESION BY ANTI-FIMBRIAL ANTIBODY: THE FIMBRIAL ANTIGEN EFFECTIVENESS

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ABSTRACT

Objective: Collecting samples of *Acinetobacter baumannii* taken from different clinical cases of wounds, septicemia, and urinary tract infections. That was accomplished by taking (296) samples from Baghdad educational hospital and Ibn-al-Baladi hospital. Samples were cultured on solid media (McConkey and blood agars), and according to microscopical, cultural, and biochemical identification, in addition to using API 20-E system, (21) isolates of *A. baumannii* were identified and in percentage of 47.619, 9.523, 14.285, and 28.571 for wound, blood, sputum, and urine samples, respectively. **Methods:** detection of fimbriated bacterial isolates among 21 isolates, and all those isolated were fimbriae forming isolates; isolate number (9) was selected as an effective isolate in formation of fimbriae. Non-forming fimbriae isolate of Shigella flexneri is used as negative control. **Results and Conclusion:** the average of adherence of fimbriated bacterial cell with human epithelial cells was reached (50) adherent bacterial cell per epithelial cell compared with the average of adherence of control isolate (12) adherent bacterial cell per epithelial cell, the inhibition processes are performed: Inhibition of bacterial adherence by specific antibodies of fimbriae antigen showed inhibition effect of adherence in respect to fimbriated isolate *A. baumannii* 9 also the subminimum inhibitory concentration for four antibiotics (Gentamicin, Tobramycin, Cefepime, and Amikacin) inhibit the adherence of fimbriated isolate. The isolates (used in the study) have the ability to agglutinate Saccharomyces cerevisiae and human red blood corpuscles (RBCs). The study of effect of different fimbriae extract concentrations (25, 50, 100 µg/ml) on immune cells; consequently, reached to the following results: Concentrations of (25, 50, 100) µg/ml showed a negative effect on lymphocyte and PMNs viability which increased significantly ($P \leq 0.05$) with increasing of fimbriae extract concentration. On the other hand, concentrations of (25, 50) µg/ml of fimbriae extract caused a significant increase ($P \leq 0.05$) noticed in the average of T-cells forming the active T-Rosette (41.3, 54.3)% and the total (62, 81)% compared with control which reached (28.3, 40.6)% respectively. The present study results revealed an increase in phagocytosis of killed yeast cells by phagocytes. To our knowledge this is the first preliminary report in Iraq showed the role of fimbriated *A. baumannii* in adherence on epithelial cell and inhibition of this adhesion by specific antibody, also this report investigated the role of fimbrial antigen on some immune cell.

Keywords: *A. baumannii*, Fimbriae antigen, Adhesion, Inhibition.

INTRODUCTION

Members of the genus Acinetobacter are non-motile, ubiquitous Gram-negative bacteria that can be recovered from a wide range of sources such as soil, water, food products and medical environments [1]. The latter source is of particular significance because *A. baumannii* is the Acinetobacter genomic species of greatest importance in human medicine. A large number of reports describe outbreaks of nosocomial infections that include urinary tract infections, secondary meningitis, wound and burn infections, and particularly nosocomial pneumonia [2, 1].

Some of the challenges in the prevention and treatment of the infections caused by this opportunistic pathogen are its remarkable widespread resistance to different antibiotics and its ability to persist in nosocomial environments and medical devices [1]. *A. baumannii* survives for several days on inanimate objects and surfaces found normally in medical environments, even in dry conditions on dust particles. These survival properties most likely play a significant role in the outbreaks caused by this pathogen. The potential ability of *A. baumannii* to form biofilms could explain its outstanding antibiotic resistance and survival properties [3]. Bacterial biofilms, arrangements in which the cells are morphologically, metabolically and physiologically different from their planktonic counterparts [4], have been found on the surface of medical devices such as intubation tubes, catheters, artificial heart valves, water lines and cleaning instruments [5]. The surfaces of all of these medical and dental devices are normal targets for colonization by complex microbial communities. While forming and establishing these multicellular structures, the cells composing them secrete exopolysaccharides which serve to fortify and maintain the structure of the biofilm. It is not well understood whether these steps and cell components are involved in the apparent ability of *A. baumannii* to form biofilm on abiotic surfaces. Furthermore, the mechanism by which this bacterium forms biofilm may pose a challenge because of its well established non-motile phenotype. Adherence of bacteria to epithelial cells is an essential step towards colonization and infection [6]. Bacterial adherence to host cells is mediated by fimbria or membrane components. Furthermore, many pathogenic bacteria are capable of invading non-phagocytic cells and evolve to survive within the host cells. The cellular invasion of bacteria contributes to evasion of humoral immunity, persistence in the host, and penetration into deep tissues. Bacterial pathogens gain entry to non-phagocytic cells via two mechanisms; a zipper-like mechanism and a trigger mechanism, which were initially classified based on morphological differences [7, 8]. The zipper-like mechanism (receptor-mediated entry) requires the direct interaction of bacterial ligands to the host's cell surface receptors and involves local cytoskeletal rearrangement at the invasion site. In contrast, the trigger mechanism is initiated by the injected bacterial effector proteins delivered by the type III secretion system. The effector proteins regulate cytoskeleton dynamics and induce dramatic cytoskeletal rearrangements such as membrane ruffles. The coal of this study is investigated the ability of inhibition the adhesion and infection of *A. baumannii* in vitro by specific antibody and antibiotic subMIC.

MATERIALS AND METHODS

Collection and identification of bacterial isolates

Twenty one isolates of *A. baumanni* were isolated from patients presented with different infections at Baghdad educational hospital and Ibn-al-Baladi hospital in Baghdad. They were obtained from midstream urine from patients suffering from urinary tract infections (6 isolates), wounds infections (10 isolates), bacteremia (2 isolates), nasal swabs (3 isolates). Bacterial identification included morphological and biochemical tests were done followed by the complementary API 20-E system. Acinetobacter Clinical isolates were cultured routinely at 30°C in LB medium or mineral medium [9]. Growth conditions were accomplished as described previously [10].

Adhesion to polystyrene

According to Gohl *et al.* [11], overnight cultures were washed twice with mineral medium and suspended in mineral medium to an optical density at 600 nm (OD_{600}) of 1.5. Then, 1 ml of cell suspension was dropped onto a petri dish and incubated at 30°C for 2 h. The petri dish was washed three times with 15 ml of

phosphate-buffered saline (PBS; pH 7.5) by gentle shaking for 1 min. Cells attached to the bottom of the petri dish were visualized with Light-microscopy. In addition, every test was done in duplicate. Polystyrene Petri dishes were also used to detect and characterize the structures formed by *A. baumannii* cells on plastic surfaces.

Biofilm assays

One millilitre of fresh medium in polystyrene sterile tubes was inoculated with 0.01 ml of an overnight culture. Duplicate cultures for each sample were incubated for 8 h either shaking at 200 r.p.m. in an orbital shaker or stagnant at 37ºC. The supernatant of the other tube was aspirated and rinsed thoroughly with distilled water. Afterward, the cells attached to the tube walls were visualized and quantified by staining with crystal violet and solubilization with ethanol–acetone as described by O'Toole *et al*. [12]. The OD_{580}/OD_{600} ratio was used to normalize the amount of biofilm formed to the total cell content of each sample tested to avoid variations due to differences in bacterial growth under different experimental conditions. All assays were done at least twice using fresh samples each time.

Microscopy experiments

Cells attached to the bottom, and the inner sides of Petri dish were visualized with regular light microscopy after staining with crystal violet as described by O'Toole *et al*. [12]. *A. baumannii 9* cells, the highest biofilm former, with a Canon digital camera (Diagnostic Instruments).

Agglutination assays on glass slides

The protocol of Gohl *et al*. [11] was followed. In brief, bacterial culture was applied to a glass slide and mixed with a freshly prepared solution of human RBCs (veterinary Serum Institute, Iraq), or with 1% *S. cerevisae* suspension. The slide was gently rotated until agglutination was visible under light microscope.

Bacterial adherence assay

In each well of a 24 well cell culture plates, *A. baumannii* was added to epithelial cells at a ratio of bacterial cells to epithelial cells of 100:1. The cells infected with bacteria were incubated in a 5% CO_2 at 37°C for 1 h. Finally, the cells were washed three times with PBS, fixed with methanol for 20 min, and stained with Safranin solution [13].

Antibiotic susceptibility tests

All isolates were tested for Minimum Inhibitory Concentration (MIC) according to the CLSI [14] using antibiotic including: Amikacin, Tobramycin, Gentamicin, and Cefepime. The growth appeared after 18 hrs and was compared with the control strains; *Escherichia coli* ATCC 35218.

Fimbriae extraction

The fimbriae were extracted from *A. baumannii* isolate number (9) by ammonium sulfate precipitation technique, then protein quantity was estimated in fimbriae sample using Lowery technique [15], it was (250) µgm/ml. The existence of specific antibodies of fimbrial antigen was determined by rabbit immunization and checked by Ouchtorlony gel diffusion test [16].

Inhibition of bacterial adherence by specific antibody

The procedure previously described by Ramphal *et al.* [17] was followed. Briefly, the immunized serum was diluted to 1:100, and then equal volumes of specific antibody (the final concentration) and bacterial suspension were mixed for 1hr in 37°C, and then the suspension was washed thrice with PBS, followed by bacterial adherence assay.

Phagocytosis of killed yeast cells test

0.25 ml fimbrial antigen, 0.25 ml of PMNS suspension, 0.25 ml yeast suspension, and 0.25 ml human blood were mixed, incubated for 30 min in 37°C, and then fixed on slid by Geimsa stain to measure Phagocytic Index (PI) [18].

Lymphocyte and polymorphonuclear cells (PMNs) viability test

Bacterial suspension was added to a mixture of lymphocytes (0.75 ml) and fimbrial antigen (0.5 ml). The tubes were incubated for 1hr then stained with trypan blue dye to measure the viability of cells [19].

T-Rosette test

According to Mendes *et al.* [20], 0.25 ml of T-cell suspension, 0.25 ml of SRBCs and 0.25 ml of fimbrial antigen were mixed and incubated at 4°C for 1hr.

RESULT AND DISCUSSION

Collection and identification of bacterial isolates. A total of 296 isolates were recovered from patients with different infection. They were obtained from midstream urine from patients suffering from urinary tract infections 6 (28.571%) isolates, wounds infections 10 (47.619%) isolates, bacteremia 2 (9.523%) isolates and nasal swabs 3 (14.285%) isolates, which show in figure (1).

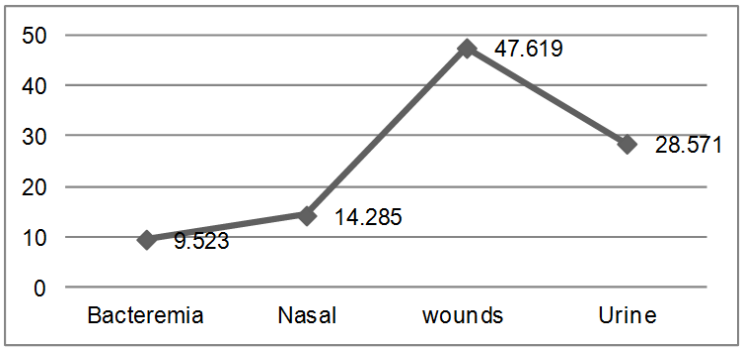

Figure 1: Distribution of *A. baumannii* from different infection

The primary pathogenic role of these bacteria is undoubtedly as a nosocomial pathogen, Healthcare-associated pneumonia, particularly ventilator-associated pneumonia in patients confined to hospital ICUs is the most common infection caused by this organism. However, infections including bacteremia, urinary tract infection and skin infections are often extremely difficult to treat because of wide spread resistance of this organism to a major group of antibiotics [1, 21, 22].

Acinetobacter spp. has been implicated as the cause of serious infections such as ventilator-associated pneumonia (VAP), urinary tract infection, wound infection, Bacteremia, mostly involving patients with impaired host defenses. However, the true frequency of nosocomial infection caused by *Acinetobacter* spp. is difficult to assess because its isolation in clinical specimens may reflect colonization rather than infection. Some clinicians believe that the recovery of *A. baumannii* in a hospitalized patient is an indicator of the severity of the underlying illness [23].

Attachment and formation of biofilms on abiotic surfaces

The initial assays showed that *A. baumannii* 9 cells which were chosen as the highest biofilm former compared with *S. flexneri* (Non fimbrial former) was used as a negative control, remained tightly adhered after washing with tap water and staining with crystal violet (figure 2A). Incubation of *A. baumannii* 9 cells overnight in LB broth without shaking at 37°C in polystyrene tubes resulted in their adherence to these surfaces. In contrast, no adherence was detected with S. *flexneri* cells cultured under similar conditions (figure 2B). These observations were confirmed further when cell attachment to plastic was quantified by relating the total cell mass to the stain retained by the attached cells as described previously. It is interesting to that the *A. baumannii* 9 cells form denser aggregates at the liquid–air interface than on the side surfaces of the tubes and plates. (figure 2B), Quantitative analysis showed that while the amount of *A. baumannii* 9 cells attached to polystyrene is significantly higher than *S. flexneri* when tested under the same experimental conditions (figure 2B). Schroll *et al.* [24] investigated the fimbriae of the clinical *K. pneumoniae* isolate were having the ability to form biofilm, this fimbrial adhesin may play a significant role in development of catheter associated *K. pneumoniae* infections . Adhesion to the surface is the first essential step in biofilm formation; but adhesins may also play a significant role in later steps of biofilm development, e.g. by promoting cell-cell contact. Indeed, various fimbrial adhesins have been shown to play a role in biofilm formation in different bacterial species including *E. coli, Pseudomonas aeruginosa, Vibrio cholerae* and *Vibrio parahaemolyticus* [25, 26].

Screening the ability of *A. baumannii* isolates to agglutinate human RBCs and Yeast

During investigation of a collection of 21 *A. baumannii* isolates, we observed that all isolates markedly agglutinated human RBCs, whereas S. flexneri negative control (clinical isolate was screened previously for loss of ability to agglutinate human RBCs) not showed agglutination phenomenon. We speculated the fimbriae of *A. baumannii* might also be responsible agglutination of yeast (figure 3B). Indeed, in addition to human RBCs, all *A. baumannii* were able to agglutinate yeast, indicating that agglutination of human RBCs and yeast was mediated by the same factor, fimbriae. Whereas *S. flexneri* was unable to agglutinate yeast (figure 3A). The fimbriae in bacterial pathogens are generally detected by agglutination assays.

Our results agreed with Iwahi *et al*. [27] and Müller *et al*. [28], as they showed the ability of fimbriae to agglutinate yeast, whereas Sepulveda *et al*. [29] showed *A. baumannii* able to agglutinate O and AB blood group. Also Braun & Vidotto [30] investigated the agglutination in O blood group was related to the fimbriae on *A. baumannii*. All the previous expriment indicated *A. baumannii* 9 was the efficient isolate in fimbrial production. Recently, Stahlhut *et al.* [31] observed a collection of *K. pneumoniae* isolates for fimbriae expression, strongly agglutinated yeast, in addition to yeast, they also observed agglutination of commercially sheep RBCs by the *K. pneumoniae* isolates.

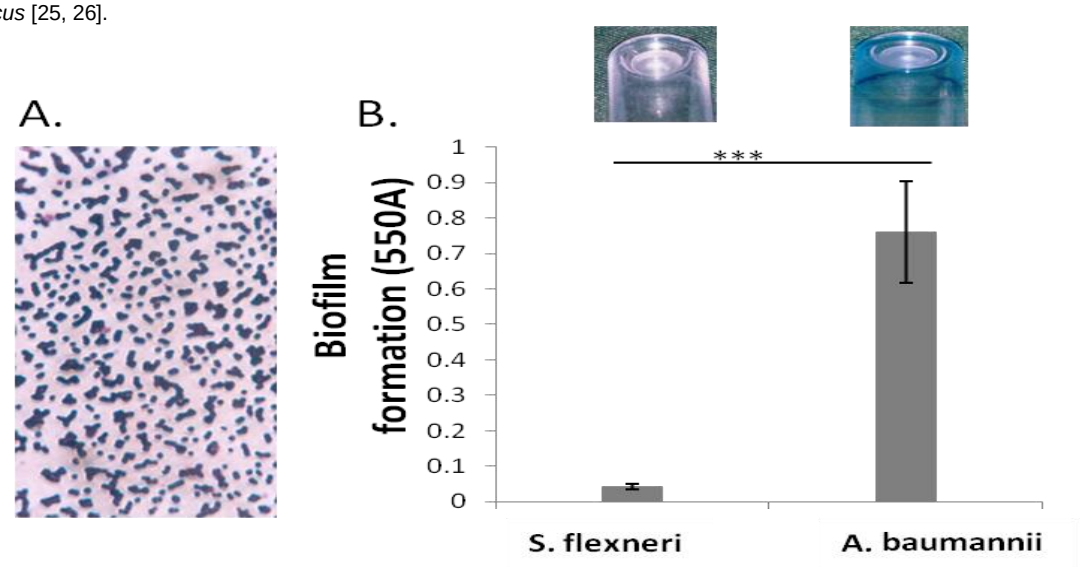

Figure 2: Quantification of biofilm formation for the indicated strains: *A. baumannii 9* attachment to polystyrene was visualized with regular light microscopy B. *A. baumannii* 9 and S. flexneri. Cultured in LB broth in polystyrene tubes without shaking either at 37 ºC. OD600. Strains were grown in LB medium for 24 hr at 37°C prior to crystal violet staining. Attached cells were detected by staining with crystal violet after washing with tap water. The biofilm was detected by crystal violet (CV) staining. To quantify the biofilm, CV was solubilized with ethanol-aceton and the absorbance was measured at 550 nm. Error bars represent standard deviations of means from two separate experiments; **, P < 0.01.

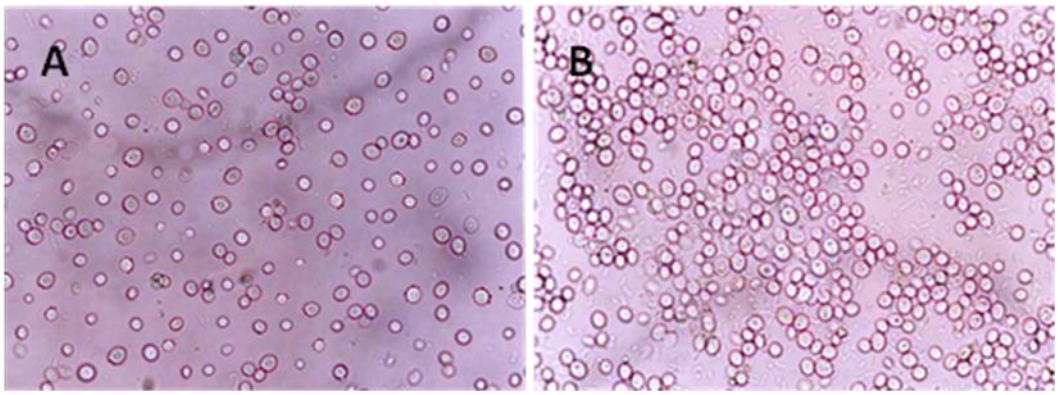

Figure 3: Agglutination patterns of Yeast under light microscope (40)X: A. Agglutination (-) of yeast with *S. flexneri* B. the agglutination (+) of yeast with *A. baumannii* 9.

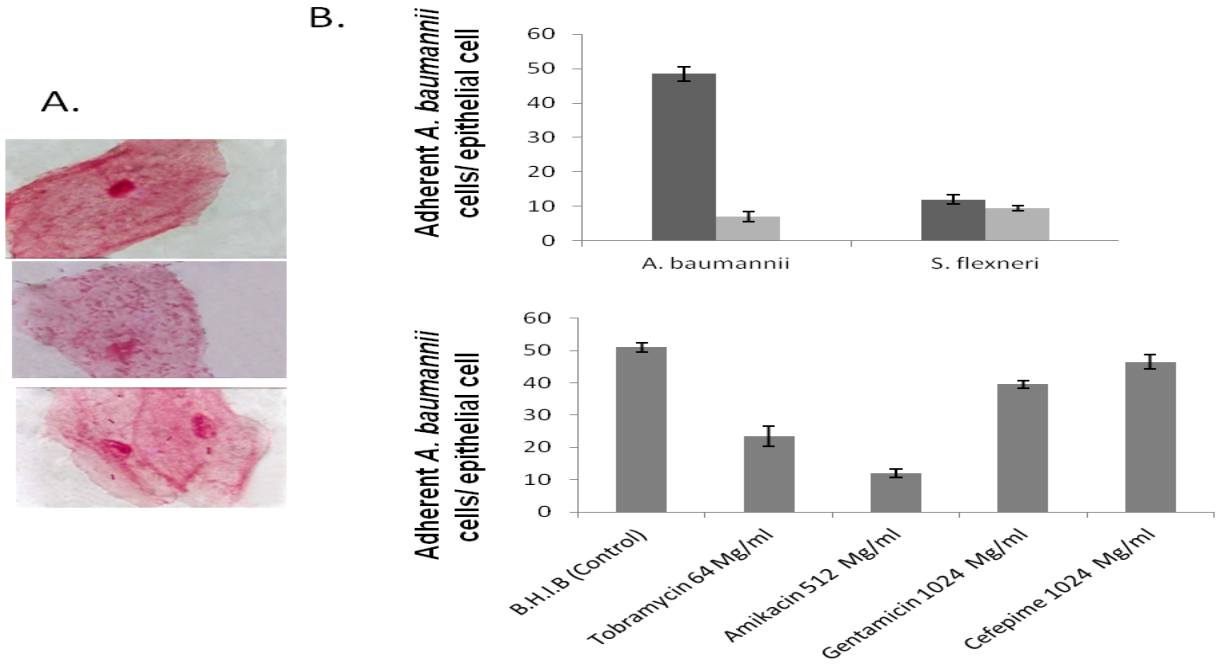

Figure 4: Adhesion of *A. baumannii* 9 on epithelial cells: A. showed the epithelial cells under light microscopy (100)X, it is control, A. baumannii 9 adherence and inhibition of adherence by specific antibody respectively, B. the inhibition of *A. baumannii* 9 adherence by antifimbrial antibody and subMIC.

Fimbriae plays a role in *A. baumannii* adherence on epithelial cells in vitro.

A. baumannii 9 was tested for adhesion to human epithelial cell and compared with *S. flexneri* as a negative control, the adhesion average for *A. b* 9 was (50) adherent bacterial cell/ epithelial cell, this indicate, there is significant differences (P≤0.05) in average adherence when compared with control (12) adherent bacterial cell/ epithelial cell. The role of fimbriae very important to adhere on human epithelial cell, this significant differences in adherence average depend on *A. baumannii* 9 fimbriae , there are receptors on epithelial cells interacts with fimbriae on *A. baumannii* [32], AL-Samaraay [33], showed the adherence average on human epithelial cell more stronger than on tissue culture that related to specific receptors to bacterial adhesion factor, there is high affinity between adhesion factor and their receptors on epithelial cells.

Fleischer & Prozondo-mordarsha [34] demonstrated that the adhesin proteins on bacterial surface facilitated the invasion of *A. baumannii* that isolated from UTI and naris. The inhibition of *A. baumannii* 9 on epithelial cell by anti-fambrial antibody, there is significant differences (P≤0.05) between *A. baumannii* 9 when react with serum before immunization (48.3) bacterial cell/ epithelial cell (adherence average) and adherence average of the same isolate (7) bacterial cell/ epithelial cell when react with serum contained anti-fimbrial antibody Figure (4A,B). Also there is no significant differences (P>0.05) between the adherence average before and after immunization (9.5 ،12) respectively, with *S. flexneri*.

We investigated the serum contained specific antibody has high effect on inhibition of bacterial adherence, adhesion consider the first step in invasion, colonization and infection. Our work revealed the inhibition of adhesion by specific antibody; the antibody will react with epitopes of fimbriae then lead to prevent the receptor on epithelial cell. Shubar [35] and Al-Salim [36] showed that the anti-fimbrial antibody from *Proteus mirabilis* and *S. flexneri,* respectively, has an effective role in inhibition of bacterial adherence on human epithelial cell.

Inhibition of *A. baumannii* 9 by antibiotic subinhibitory concentration

Some low concentrations of antibiotics lead to impair some virulence factors that important in the first step of infection. We inhibited the adhesion of *A. baumannii* 9 on epithelial cell by sub MIC for Amikacin, Tobramycin, Gentamycin, and Cefepime. The cultures were incubated at 37°C for 24 hr. There are significant differences (P≤0.05) in adherence average of *A. baumannii* 9 on human epithelial cells between that grew in BHIB broth and BHIB broth with Subinhibitory concentration antibiotics (Gentamicin, Tobramycin, Cefepime, Amikacin). The antibiotics showed different activity in inhibition of adherence of fimbriated isolate when they added antibiotic to growth medium, (Tobramycin, Amikacin) showed high activity that the average of adherence was (23, 12) adherent bacterial cell /epithelial cell and with significant differences (P≤0.05) respectively, compared with control treatment which was (51) adherent bacterial cell/ epithelial cell, whereas using (Gentamicin, Cefepime) in adherence inhibition process, although were significant, their averages were (39.5, 46.5).

Adherence average for the same isolate when grew in BHIB without subMIC was (51) adherent bacterial cell/ epithelial cell (figure 4). These findings may suggest that subMIC have impaired adherence factors.

The effectiveness of fimbrial antigen on immune cell

The phagocytosis process is the first defence step in the body preventing the foreign body from entering to human body (Ageler & werb, 1982). (25, 50) µg/ ml showed induce the phagocytosis process of the killed yeast figure (5), (25, 50) µg/ ml increase phagocytosis index PI (56, 65)% respectively, compared with the control 40% there is significant differences (P≤0.05)between them. Whereas significant decrease in phagocytosis process with 100 µg/ml, the concentrations of (100) µgm/ml caused a decrease in this percentage that the average reached (23)%. That means the fimbriae act as Immunomodulant. These results revealed the ability of fimbriae in increase the phagocytosis process. PMNS have the ability to distinguish yeast or surface virulence factors. Perhaps there are interaction sites between the carbohydrate compound on yeast surface and glycoprotein in phagocytes plasma membrane and this interaction encourages this process. This work agreed with Silver blatt *et al.,* [37] they showed there is correlation between number of fimbriae and phagocytosis process.

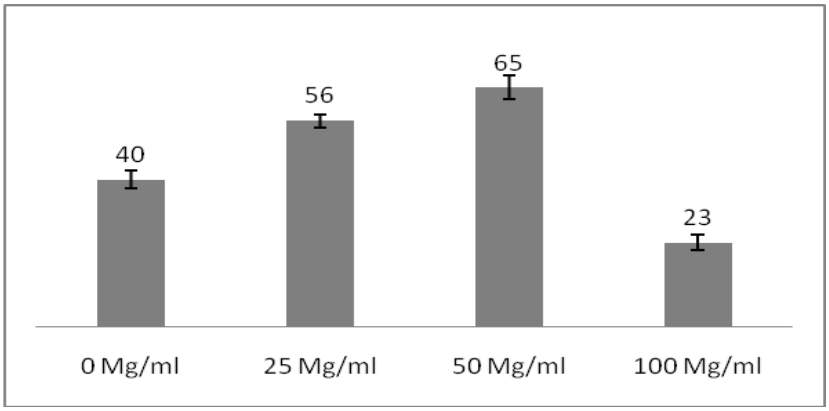

Figure 5: phagocytosis index: Phagocytosis for killed *saccharomyces cerevisiae* by phagocytes

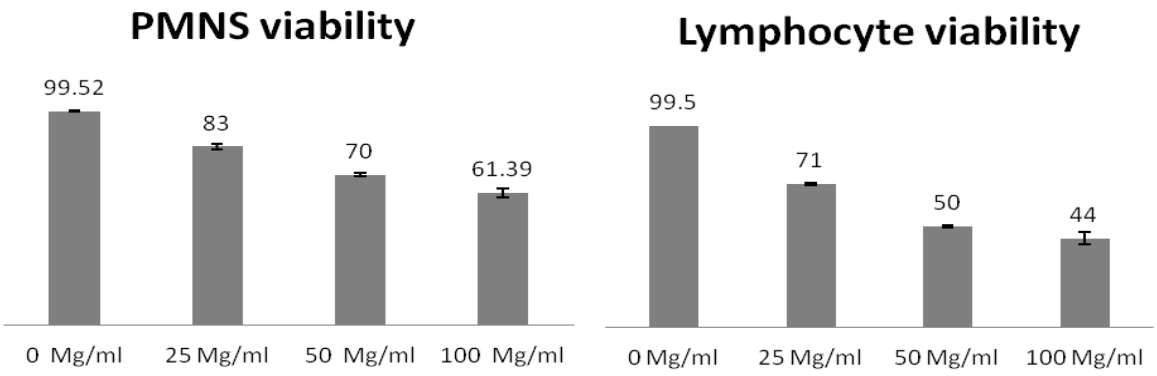

Figure 6: The effectiveness of fimbrial antigen on polymorph nuclear cells (PMNs) and lymphocyte viability

In the effectiveness of fimbrial antigen on PMNs and lymphocyte viability, the results showed the viability of PMNS and lymphocyte that isolates from healthy people was (99.5 ،99.52)% respectively . Whereas the viability of lymphocytes were (73, 60, 51.38)% with concentration (25, 50, 100) µg/ml of fimbrial antigen, and the viability of PMNS were (71, 50, 44)% respectively, with the same previous concentration.

These results clarified significant differences between lymphocytes, PMNS and the control, there is an inverse relationship between the fimbrial antigen concentration and the viability, these results agreed with Al-Salim, [36] she showed the high fimbrial antigen concentration of *S. flex*neri decrease the viability of lymphocyte and PMNS. The concentration of fimbrial antigen may cause osmotic shock and that lead to kill the cell.

The effectiveness of fimbrial antigen on T-Rosset, The surface marker CD2 on T- lymphocyte act as a receptor for sheep RBC, this CD2 help the T- lymphocyte to interact with SRBCs forming T-Rosette [38]. The results showed the active T-Rosette percentage in fimbriae antigen concentrations (25, 50, 100) µg/ml were (41.333, 54.33, 20.33)% respectively, in comparison with control (28.33)%, whereas the percentage for the total T-Rosette was (62, 81, 25)% respectively, in comparison with the control (40.66)%.

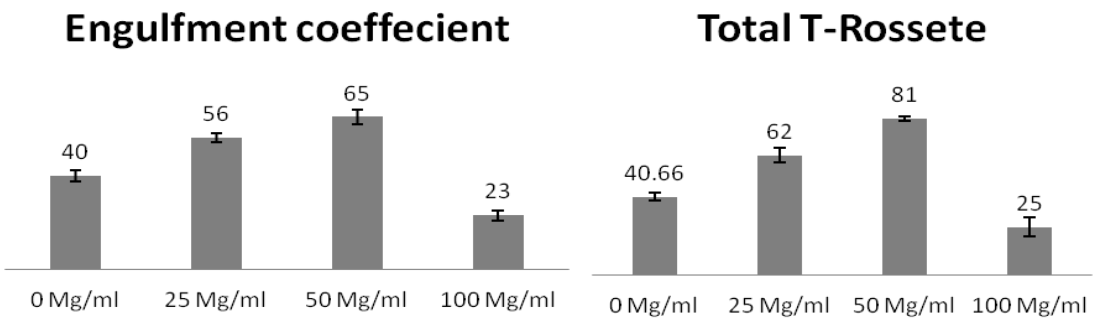

Figure 7: The effectiveness of fimbrial antigen on T-Rosset

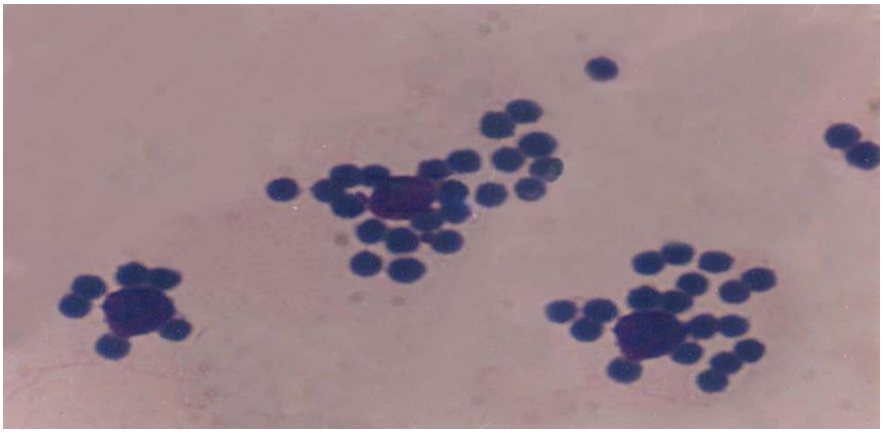

Figure 8: The T-Rosette (under light microscope 100X) in the presence of fimbrial antigen

The results showed there are significant differences between the cell coefficient and the control for each total and active T-Rosette and between the same cells coefficient. The T-Rosette increase in (25, 50)µg/ml that may be because of the interaction between the fimbriae antigen and lymphocyte stimulating attracting SRBCs for bind around the lymphocytes figure (7).

But the results showed decrease the percentage with 100 µg/ml concentration that may be because fimbrial antigen has the ability to affect the CD2 activity lead to effect the interaction with SRBCs.

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