Role of Interleukin-36 in Response to *Pseudomonas Aeruginosa* Infection

Muslim Idan Mohsin[1], Mohammed Jasim Al-Shamarti[1], Rusul Idan Mohsin[3], Sarmad Al-Sahaf[2]

[1]Department of Laboratory Investigations, Faculty of Science, University of Kufa, Najaf, Iraq. [2]Baghdad College of Dentistry, University of Baghdad. [3]Department pathological analysis, collage of Al-Tossi University, Najaf, Iraq.

Abstract

Background: One of the causative agents of the lower respiratory tract (LRT) is *Pseudomonas aeruginosa* which can lead to severe infection associated with a lung infection. Many cytokines are secreted in response to bacterial infection, in particular, interleukin IL-36 cytokine in response to *P. aeruginosa* infection. IL36 promotes primary immune response via binding to the IL-36 receptor (IL-36R). Indeed, an over activity of IL-36 might be an initiating factor for many immunopathologic sceneries in pneumonia.

The aims of Study: the involvement of IL-36 in the *P. aeruginosa* infection could be a clue to find a specific way for treatments of different inflammatory and degenerative lung diseases.

Methodology and Results: we used manual and VITEK2 system to isolate and purify all the species, also using qPCR real-time to demonstrate that the involvement of IL-36 cytokine in response *P. aeruginosa* infection which is isolated from (LRT). We found that one of the most common pathogens is *P. aeruginosa* after *Klebsiella pneumonia*, and more resistance bacterium against antibiotics is *P. aeruginosa* (P4). Furthermore, we found that IL-36 cytokine increased in response to *P. aeruginosa* infection.

Conclusion: *P. aeruginosa* is one of the main causative agents in LRT in many hospitals in Iraq, and the contamination and antibiotics resistance for these nosocomial pathogens is increased. Also, IL-36 expression significantly upregulated in human lung epithelial (A549 cells) after infected by *P. aeruginosa* at the mRNA level.

Key words: *IL36, Pseudomonas aeruginosa, LRT infection and A549 cells*

Introduction

One of Gram-negative bacterium is *Pseudomonas aeruginosa,* which is the main causative agent of LRT infection. It can lead to nosocomial infection and chronic infection in immunocompromised patients. The most serious cases are sepsis and pneumonia [(1)]. Due to virulence factors of *P. aeruginosa* lead to increase the proportion of multidrug resistance (MDR) in *P. aeruginosa* [(2, 3)].

In recent publication, IL-36 is classified as one of members of IL-1 family [(4)]. IL-36 binds with IL36 receptor complex and IL-1 receptor accessory protein (IL-1RAcP). Receptor of IL-36 could bind to the IL-33 and the IL-1 receptor [(5)]. The IL-36 induces primary immune response and contributes to neutrophil accumulation or/ and dendritic cell activation [(4-6)]. During early stage of *P. aeruginosa* infection, many immune cells are activated [(7, 8)]. Interestingly, it has been reported that IL-36 mRNA is unregulated during *P. aeruginosa* infection in human bronchial cells [(9)].

Taken together, these clarifications suggest that IL-36 are involved in host defense against *P. aeruginosa*. This involvement could be because inflammatory cells recruitment and/or activation. The involvement of IL-36 in response to *P. aeruginosa* infection is still unclear. We hypothesized that IL-36 is involved in acute *P. aeruginosa* lung infection. In this study, we also demonstrate that IL-36 produced by A549 cells during *P. aeruginosa* infection which is isolated from LRT.

Methodology

Bacterial strains:

P. aeruginosa (P4) was chosen based on the antibiotics resistance and virulence factors. *P. aeruginosa* (P4) was used for all experiments, and grown in lysogeny broth medium or agar. *P. aeruginosa* were grown an overnight culture and were cultured at 37 °C to reach OD_{600} 0.4. It was equal $1x10^8$. *P. aeruginosa* was 2x washed and diluted by DMEM medium with 10% heat-inactivated foetal calf serum to achieve an appropriate MOI.

Culturing of human lung cell line

The incubation conditions for A549 cells was 37ºC and 5% CO_2. Trypsinisation was used to harvest A549 cells. Briefly, A549 cells were washed with 5ml HBSS without Ca^{++} and Mg^{++}. Then 2.5ml of 1x trypsin (Lonza) was added and keep the flask in incubator for 8min. 10ml of DMEM medium was added to pellet. The pellet was spent down at 300xg for 5min and then re-suspended with 5ml DMEM. An appropriate number of A549 cells were counted for seeding to next experiments.

Antibiotics sensitivity test

The Kirby-Bauer standardized single disk method was performed according to Kirby method [10]. Briefly, Mueller Hinton medium plates were prepared. Using an L-shape sterile spreader, *P. aeruginosa* is evenly seeded throughout the plates. The bacterial cells density was approximately 1×10^8 cell/mL. This density was compared with OD_{600} about 0.4. The antibiotic disks were used with standard concentration. The antibiotics disks are evenly dispensed and placed onto the medium and then incubated at 37 ºC 24h in an incubator. Using a ruler, inhibition zone was measured to the nearest millimeter (mm) Therefore, the susceptibility of bacteria to an antimicrobial agent was detected as compared to the zones of inhibition determined by formerly NCCLs (2014) [11].

Infection assay

To count number of *P. aeruginosa* per cells, it was used a kanamycin-protection assay. 1ml of overnight culture of *P. aeruginosa* was centrifuged and re-suspended in DMEM to prepare different MOI 10, 50 and 100. The A549 cells were infected at 37°C at 5% CO_2 for 2hr, and then 2x washed twice with HBSS. All wells were 3x washed with HBSS, and added 0.2% (v/v) Triton X-100 added for 15min. Serial dilutions were plated onto LB agar or nutrient media and incubated at 37°C for 36hr. CFU equation were used to count the bacterial colonies. The CFU equation as: CFU/mL = (no. of colonies x dilution factor) / volume of culture plate.

Real time RT-qPCR

Mini Kits of QIAGEN RNeasy was used to extract mRNA from infected and uninfected A549 cells. Complementary DNA (cDNA) was made for the qPCR by using a Reverse Transcription Kit. 2x qPCR SYBR green Master Mix from Primer Design Precision company was used to perform PCR. Primers and probe sets in plate for our interested gene and internal house keeping control. Then the plate of qPCR was placed into the machine which is 7900HT AbiPrism system and run by 40 cycles. qPCR technique allows the cycling point identification where the product is visible by means of fluorescence emission. The C_t value correlates to the quantity of target Interleukin 36 (IL36). IL-36 primers are used f: GGACCGTATGTCTCCAGTCAC, r: GTCCCCGACTTTAGCACACA, and β-actin primers are used f:CCTTTGCCGATCCGCCG and r:GATATCATCATCCATGGTGAGCTGG. These primers were obtained from Thermofisher Company. The level of relative expression was normalized against suitable housekeeping genes. The $\Delta\Delta C_t$ method was used for comparing relative fold expression differences.

Statistical Analyses

GraphPadd prism.8 was used to make all graphs and analyse the data statistically. The significant differences between samples were determined using one and two-way ANOVA for multiple comparisons. Results are shown as mean ± SEM.

Results

Isolation of bacteria

The aim was to examine which species are the most virulent pathogen in lower respiratory tract infection. We collected 50 specimens from different hospitals in alnajaf province. We diagnosed form all samples depending on phenotyping, biochemical tests, morphology shape and selective media. Findings showed that only 21 specimens have bacteria but other samples have no bacteria. The highest number is *Klebsiella pneumonia 9* (42.86%). The lower number is *Staphylococcus aureus 3* (14.28%). However, our interest pathogen *P. aeruginosa* 5 (23.81)

% and other bacteria were about (19.05), as demonstrated in **Fig 1**. It seems that the main our interested bacterium from the main pathogen in lower respiratory tract infection.

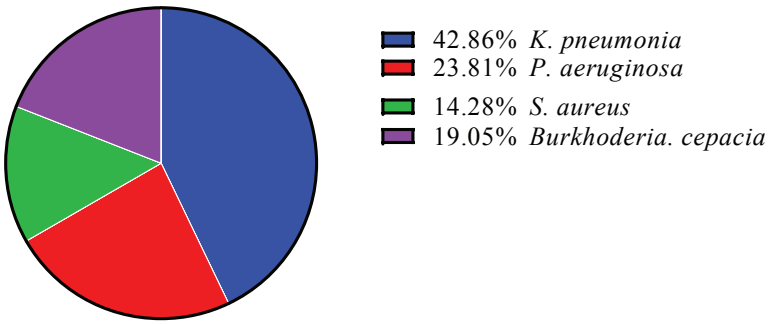

Figure.1 Percentage of different types of bacteria isolated from lower respiratory tract infection (LRT). Data shows number and percentage of isolates from 50 sample of LRT infection from different hospitals in Alnajaf province (Alsadar Medical city and Alforat hospital). Pure colonies were isolated and placed in VITEK2 microbial identification. To more validation, manual diagnostic methods have been performed included biochemical and culturing tests.

Antibiotics sensitivity test

As shown in Fig 1, the percentage of *P. aeruginosa* seemed more correlate to be the main causative agent of LRT infection. These strains were selected to closely study to determine which strain has the highest antibiotics resistance. This section aimed to select the highest species to antibiotics resistance. All isolates were screened for antibiotics sensitivity. The finding shows that there is a variation of antibiotics resistance for isolates. *P. aeruginosa* (S-4) has highest antibiotics resistance than other samples, as demonstrated in Fig 2.

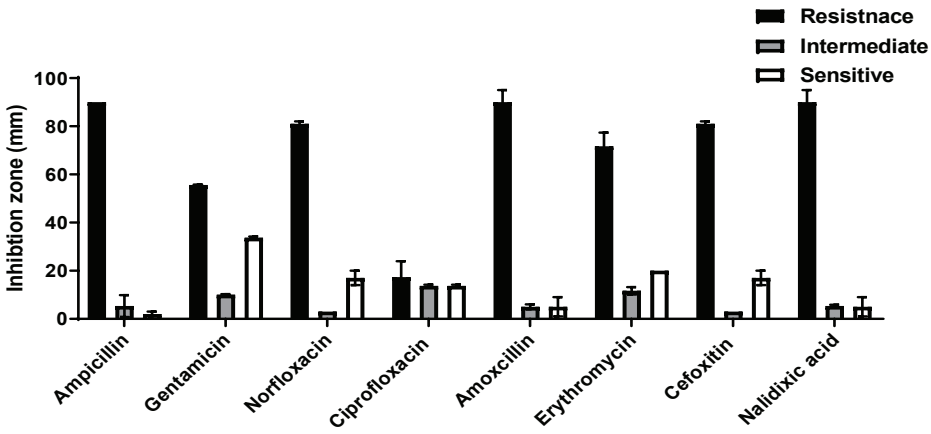

Figure.2 Antibiotic profiles of *P. aeruginosa* used in this study. *P. aeruginosa* **of bacteria are purified and plated on Muller Hinton agar using sterile swap, different types of antibiotics disk were placed on the plates, and bacteria were incubated for 24hr to measure the inhibition zone. The findings are mean of 3 independent experiments with duplicates.**

Infection efficiency of *P. aeruginosa* in A549 cells

This section was to test a different multiplicity of infection to determine which MOI can attach to A549 cells. This cell line was infected with ***P. aeruginosa*** to measure the total bacterial infection after 2hr, and to detect the best MOI to the further experiments. The CFU equation was used to count how many bacteria per host cells at 2hr. The MOI was used 10, 50, and 100 bacteria per each mammalian cell. The finding shows that the number of bacterial per host cells was increased related with MOIs (Fig 3). The MOI 100 was statistically significantly different than other MOI. It was suggest that this MOI can use to further experiments.

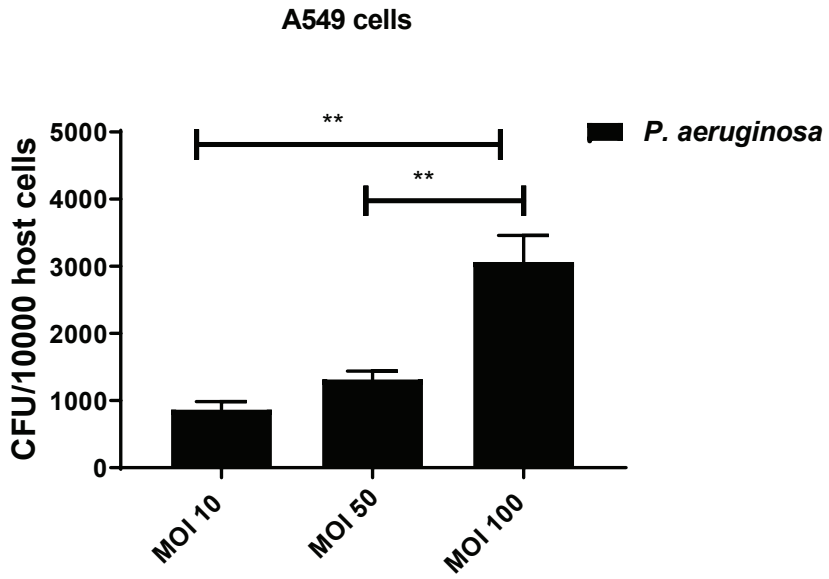

Figure .3 *P. aeruginosa* infection in A549 cells at different MOI of infection. A549 cells were infected at MOI 10, 50, 100. The total number of *P. aeruginosa* was counted after 2hr using CFU methods. Using one-way ANOVA to test the significant differences, where ** p<0.01 meant significant. The findings are the means of 3 experiments performed in 3 replicates (± S.E.M).

IL-36 induced in A549 cells during *P. aeruginosa* infection

The aim of this section was to test whether IL-36 is changed in the A549 cells infected *P. aeruginosa* clinical strain. In brief, we tested a mRNA expression during 3 time points during bacterial infection. To do this, mRNA from A549 cells is extracted before and after infected by *P. aeruginosa*. Complementary DNA (cDNA) were made for all samples during 3-time points. The PCR products were quantified by using the SYBR green pigment. The C_t was used to quantify the findings. Also, $\Delta\Delta C_t$ method to calculated the fold change using the $2^{-\Delta\Delta C}{}_T$ equation. IL-36α mRNA level was significantly up regulated in response to *P. aeruginosa* infected A549 cells during all the time points included: 4, 6 and 24hrs post infection (**Fig 2**).

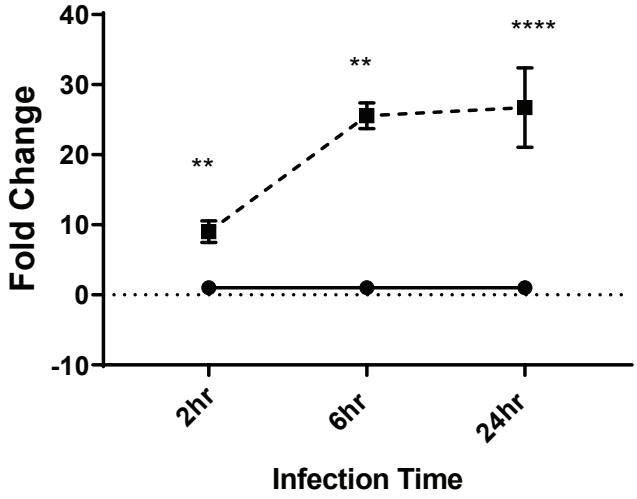

Figure.4 IL36 expression during *P. aeruginosa* infection in A549 cells over-time.

Both infected and non-infected A549 cells were harvested at 2, 6 and 24hr post-infection. Using qPCR to determine the mRNA level post infection. The significance of differences were analysed using two-way ANOVA, where ** $p<0.01$; **** $p<0.0001$ significant compared to non-infected A549 cells. The results are the means (± S.E.M) of 3 experiments in 2 replicates.

Discussion

The aims were to select the highest bacterial antibiotics resistance and to determine that IL-36 plays a role in *P. aeruginosa* infection. We found that *K. pneumonia* and *P. aeruginosa* are the main causative agents in LRT infection (Fig 1). This finding is in agreements with the previous findings of [12, 13]. Zhou et al have found that *P. aeruginosa* percentage was about 25.2% and *K. pneumonia* was 31% [14]. Ramana et al, also found that *K. pneumonia* is the most common pathogens in LRT infection [12]. This does not support our findings that revealed *P. aeruginosa* has a sensitivity to Ampicillin. Another study supported our result, which demonstrated *P. aeruginosa* to have resistance to erythromycin and sensitive to gentamicin (Fig 2) [15]. Taken together, it seems that *P. aeruginosa* is one of main the causative agents of LRT infection, and it must be taken seriously. It is probably that the mechanism of antibiotics resistant is upgraded and that are not likely updated in Iraq, in particular, alnajaf hospitals. The reason for that could be the clinician staff have a lack of awareness to avoid contamination. We strongly suggested that using antibiotic randomLy must be avoided; otherwise, the problem will be increased. In the **(Figs 3 and 4)**, we demonstrated that the bacterial efficiency in A549 cells and the role of IL-36 in *P. aeruginosa* infection. Interestingly, we showed that IL-36 mRNA level appeared to increase in A549 cells. Significant induction of IL-36 mRNA level was observed between 2 and 6h ($p \leq 0.001$) in A549 cells, while IL-36 mRNA was highly increased at 24h ($p \leq 0.0001$) relative to uninfected controls, as demonstrated in Fig 4. Some studies report that IL-36 plays a positive role in bacterial infection. In particular, it has a positive role in *P. aeruginosa* infection [12]. This observation was in agreement with our data that shows IL-36 increased during *P. aeruginosa* infection. Interestingly, Vos et al have reported that IL-36 mRNA is unregulated in human bronchial cells during *P. aeruginosa* infection (Vos, van Sterkenburg *et al.* 2005). Ultimately, this explanation suggests IL-36 induces as primary immune response of host defense against *P. aeruginosa*.

Conclusions: IL-36 could contribute in early stage of immune response to *P. aeruginosa* infection. It is likely that the bacterium induced the inflammatory cell recruitment, and induced the cells to activate during infection. Our observation could approve our understanding for the mechanisms of bacterial pathogenesis in the epithelial cells, and also could identify a new therapeutic target in lung diseases using antibodies, or block material to this cytokine.

Acknowledgement: Research facilities provided by different hospitals in alnajaf city acknowledged. My thanks also to Professor Monk and Dr. Partridge from University of Sheffield, for providing me with cell line.

Declaration: The authors have no conflict of interest to declare.

Funding: The source of funding is by myself.

Ethics Clearance: This article does not contain any studies with human participants or animals performed by any of the authors.

References

1. Weinstein RA, Gaynes R, Edwards JR, System NNIS. Overview of nosocomial infections caused by gram-negative bacilli. Clinical infectious diseases. 2005;41(6):848-54.
2. Guillamet CV, Vazquez R, Noe J, Micek ST, Kollef MH. A cohort study of bacteremic pneumonia: The importance of antibiotic resistance and appropriate initial therapy? Medicine. 2016;95(35).
3. Mehrad B, Clark NM, Zhanel GG, Lynch III JP. Antimicrobial resistance in hospital-acquired gram-negative bacterial infections. Chest. 2015;147(5):1413-21.
4. Kwak A, Lee Y, Kim H, Kim S. Intracellular interleukin (IL)-1 family cytokine processing enzyme. Archives of pharmacal research. 2016;39(11):1556-64.
5. Gabay C, Towne JE. Regulation and function of interleukin-36 cytokines in homeostasis and pathological conditions. Journal of leukocyte biology. 2015;97(4):645-52.
6. Carrier Y, Ma H-L, Ramon HE, Napierata L, Small C, O'toole M, et al. Inter-regulation of Th17 cytokines and the IL-36 cytokines in vitro and in vivo: implications in psoriasis pathogenesis. Journal of Investigative Dermatology. 2011;131(12):2428-

37.

7. Aoyagi T, Newstead MW, Zeng X, Nanjo Y, Peters-Golden M, Kaku M, et al. Interleukin-36γ and IL-36 receptor signaling mediate impaired host immunity and lung injury in cytotoxic Pseudomonas aeruginosa pulmonary infection: Role of prostaglandin E2. PLoS pathogens. 2017;13(11):e1006737.
8. Gao N, Me R, Dai C, Seyoum B, Fu-shin XY. Opposing Effects of IL-1Ra and IL-36Ra on innate immune response to Pseudomonas aeruginosa infection in C57BL/6 mouse corneas. The Journal of Immunology. 2018;201(2):688-99.
9. Vos JB, van Sterkenburg MA, Rabe KF, Schalkwijk J, Hiemstra PS, Datson NA. Transcriptional response of bronchial epithelial cells to Pseudomonas aeruginosa: identification of early mediators of host defense. Physiological genomics. 2005;21(3):324-36.
10. Bauer A, Kirby W, Sherris JC, Turck MJAjocp. Antibiotic susceptibility testing by a standardized single disk method. 1966;45(4_ts):493-6.
11. Sader HS, Biedenbach DJ, Jones RNJDm, disease i. Global patterns of susceptibility for 21 commonly utilized antimicrobial agents tested against 48,440 Enterobacteriaceae in the SENTRY Antimicrobial Surveillance Program (1997-2001). 2003;47(1):361-4.
12. Ramana K, Kalaskar A, Rao M, Rao SD. Aetiology and Antimicrobial Susceptibility Patterns of Lower Respiratory Tract Infections (LRTI's) in a Rural Tertiary Care Teaching Hospital at Karimnagar, South India. American Journal of Infectious Diseases. 2013;1(5):101-5.
13. Egbe CA, Ndiokwere C, Omoregie R. Microbiology of lower respiratory tract infections in Benin City, Nigeria. The Malaysian journal of medical sciences: MJMS. 2011;18(2):27.
14. Zhou Y, Lin P, Li Q, Han L, Zheng H, Wei Y, et al. Analysis of the microbiota of sputum samples from patients with lower respiratory tract infections. Acta Biochim Biophys Sin. 2010;42(10):754-61.
15. Brown AN, Smith K, Samuels TA, Lu J, Obare SO, Scott MEJAEM. Nanoparticles functionalized with ampicillin destroy multiple-antibiotic-resistant isolates of Pseudomonas aeruginosa and Enterobacter aerogenes and methicillin-resistant Staphylococcus aureus. 2012;78(8):2768-74.