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Immunological Study of *Klebsiella Pneumoniae* Isolated from *Pneumoniae* Infection Patients

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ABSTRACT

This study aimed for isolation and identification of *Klebsiella pneumoniae* was an opportunistic pathogen responsible for a wide range of clinical syndromes such as pneumonia in both hospital and community settings. Assessment some immune parameter this study was carried out in Al-Diwaniya Teaching Hospital in Al-Diwaniya Province during the period from December 2018 to February 2019. A total of 272 individuals in both sex: 139 males and 133 females, including 210 sputum and blood specimens were collected from patients with pneumonia and 62 blood specimens were collected from healthy persons as a control group. The result of Microbiological tests of sputum was found 120(57.2%) specimens as Gram negative bacteria. From these specimens found 62(51.7%) have been appeared as positive result for K. *pneumoniae* and represented a major cause for pneumonia in this study, 37(59.7%) of them were males and 25(40.3%) female. 59(95.16%) of K. *pneumoniae* isolates have capsule when stained negatively with Indian ink. While, 60 (96.77%) isolates were appeared hypermucoviscosity (HVM) phenotype. The level of interferon gamma (IFN-γ) and toll like receptor (TLR4) concentration was measured by using enzyme-linked immunosorbent assay (ELIZA) and was found that IFN-γ is significantly ($p < 0.05$) raised in all age groups of patients in comparison to the healthy control groups. The level of IFN-γ in age group (1-10) years recorded high percentage (293.123) compared with other age groups.. On the other hand, the level of TLR4 concentration was found that is significantly ($p<0.05$) raised in all age groups of patients in comparison to the healthy control group. The level of TLR4 in age group (51-60) years recorded high percentage (12.993) compared to other age groups.

1. Introduction

Pneumonia can be transmitted by airborne germs from an infected individual are inhaled by someone else. Anyway, most instances of pneumonia are attributable to self-infection with one or more types of microbes that originate in the nose and mouth. In healthy people, typical upper airway bacterial residents such as "Streptococcus *pneumoniae*" and "Haemophilus influenze" are the most common bacteria causing community-acquired pneumonia (CAP) (Hanada et al., 2018).

Otherwise, hospital acquired pneumonia (HAP) is usually caused by more resistant bacteria, such as "K. *pneumoniae*", "Staphylococcus aureus", "Pseudomonas aeruginosa", and "Escherichia coli". Individuals with a serious impairment of their immune system become susceptible to pneumonia caused by so-called

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opportunistic germs, such as certain viruses, fungi, and bacterial related to tuberculosis (mycobacteria), which wouldn't ordinarily cause illness in normal individuals. To beat with its constant exposure to potentially infectious microbes, the lung depends on a hierarchy of defense mechanisms (Paczosa & Mecsas, 2016).

Klebsiella pneumoniae considered as one of the most important pathogens but the mechanism by which this bacterium causes diseases is still not fully understood and approximately most studies have limited facts about this mechanism because of not wide ranges of virulence factors investigated, and few studies exist at the present time to study the relationship between virulence factors and resistance of K. *pneumoniae* to antibiotics (Paczosa & Mecsas, 2016).

Successful defense against infections requires a coordinated action of multiple immune cell subsets. In this context, it is widely appreciated that interferon γ (IFNγ) and toll like receptors 4 TLR4 decisively coordinate immune responses by modulating cell-autonomous immunity and inflammatory responses, and by dictating immune cell-to-cell communications. While type I IFNγ are the major effector cytokines of the host defense response against bacterial infections. Type I IFNγ protect against the progression of a localized K. *pneumoniae* lung infection to invasive disease (Ivin et al., 2017).

Toll-like receptors 4 recognize pathogens, resulting in onset of the inflammatory response. TLR4 are expressed in both cells of hematopoietic origin and stromal cells e.g. lung epithelium. When K. *pneumoniae* enters the lung, bacterium specific TLR4 are activated, triggering the release of cytokine and chemokine that attract and activate neutrophils. TLR4 the most important TLR for the recognition of K. *pneumoniae* by virtue of its capacity to sense lipopolysaccharide (LPS) present in the outer membrane of this Gram-negative pathogen (Sender & Stamme, 2014).

2. Materials & Methods

Table 1
Commercial kits in the current study

Kit	Company	Origin
API-20 E	bioMerieux®	USA
Human IFNγ ELIZA	Elabscience	European
Human TLR4 ELIZA		

Table 2
Culture media in the current study

Medium	Abbreviation	Company	Origin
Blood agar	BA	Himedia	India
CHROM agar	CHROM agar	Becto and Dickinson	France
Eosin methylene blue agar	EMB agar		
MacConkey agar	MCA agar	Himedia	India
Xylose lysine deoxycholate agar	XDL agar		

2.1. Patients and Clinical Samples

A total of 272 individuals in different gender and age groups (139 males and 133 females), 210 clinical samples of patients which from respiratory tract and 62 samples from healthy as a control group, between first December 2018 and first February 2019 in Al-Diwaniya Teaching Hospital in Al-Diwaniya Province clinical specimens (blood and sputum) were collected at the same day of consultation as in follow:

2.2. Samples Collection

Samples were collected by using swabs from sputum. The sterile cotton swabs were immersed in BHI agar tubes and transferred to the laboratory (Collee et al., 1996) , and three ml of fresh blood were collected. Blood was obtained by vein puncture after disinfect the skin with 70% alcohol to avoid contamination. The blood samples allowed to clot for about 1 hour at room temperature, the clot loosed gently by a wooden stick. Then, samples were centrifuged for 10 minutes at 3000 revolutions per minute (rpm), the serum separated and transferred to other tubes for storage at -20 ° C (Lewis et al., 2001).

2.3. Identification of *Klebsiella Pneumoniae* Isolates

Isolates were identified depending on the morphological features on culture medium and biochemical tests according to the classification of MacFaddin (2000).

2.4. Cultural Characteristics

All samples were inoculated on to BA, MCA, EMB, XLD and CHROM Agar and incubated at 37˚C for 24 hours under aerobic condition. Colonies were purified by sub-culturing for further identification. Color, shape, size, edge, type of haemolysis and lactose fermenter were observed (Harley & Prescott, 1996).

2.5. Virulence Factors Detection

2.5.1. Capsule

The negative staining bacteria of Indian ink was inquired to detect the presence of bacterial capsule, according to Atlas et al., (1995).

2.5.2. Hypermucoviscosity Test (HMV)

Mucoid isolates of K. *pneumoniae* were identified by inoculating bacteria on MCA and incubated at 37 °C for 24 hours, after incubation the colony touched by a loop and lifted vertically away from surface of MCA, colonies formed a string ≥ 5 mm in length show HMV isolates (Macfaddin, 2000).

2.6. ELIZA for Determination of IFN-γ and TLR4 Concentration

2.6.1. Principle of ELZA Assay

The assay of ELIZA was performed according to the method mentioned by the manufacturing company. The OD was measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of human IFN-γ was calculated in the samples by comparing the OD of the samples to the standard curve.

3. Results & Discussion

3.1. Clinical samples Collection

The result of Microbiological tests of 210 sputum was found 74(35.2%) specimens as Gram positive bacteria and 120(57.2%) specimens as Gram negative bacteria, while 16(7.6%) of rest specimens wasn't show any growth. The results of isolation were summarized in Figure 1.

The most general results in this study evidenced that the majority of patients with pneumonia showed the presence of Gram-negative bacteria more than Gram positive bacteria. Similar observations have been made by other investigators in the world. For instance, in the United States, Barazile and Nepal infection with Gram negative bacteria account for about 50-70% of nosocomial infections, and similar data have been reported from other parts of the world (Parajuli et al., 2017).

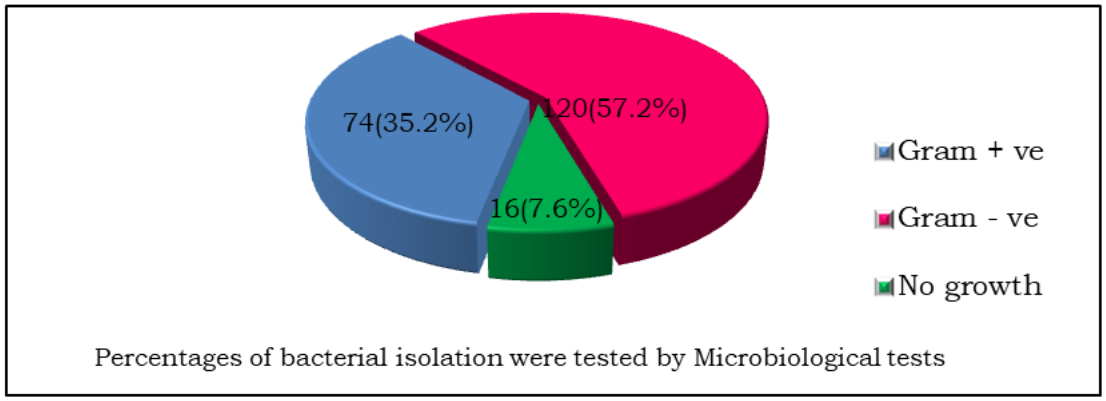

Fig. 1. The percentage of bacterial isolation from the lower respiratory tract infections

3.2. Identification of *Klebsiella Pneumoniae* Isolates

In the current study, from 120 sputum specimens that found as Gram negative bacteria, 62(51.7%) specimens have been appeared as positive result for K. *pneumoniae* and represented a major cause for pneumonia while 58(48.3%) of specimens were represented other Gram-negative bacteria (Figure 2). Many different results of nosocomial K. *pneumoniae* isolations were detected in all over the world such as: in Iraq, Ali & Ismael (2017) were isolated K. *pneumoniae* in low percentage (8.1%) from sputum in Erbil City.

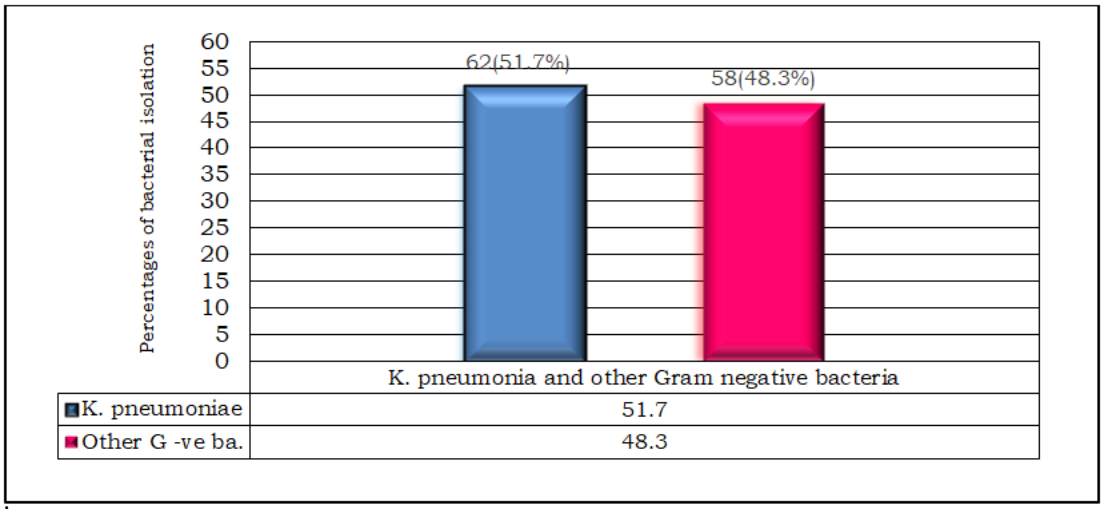

Fig. 2. Percentage of isolation of 120 isolates of K. pneumonia and other Gram-negative bacteria depending on Microbiological tests

Sixty tow isolates were belonging to K. *pneumoniae* positively from respiratory tract infections, 37(59.7%) of them were males and 25(40 .3%) female (Table 3).

Table 3
Distribution of K. *pneumoniae* isolates among both sex

Type of sex	K. *pneumoniae*	Type of sex
Male	37	59.7
Female	25	40.3

Most of the K. *pneumoniae* isolates in this study were collected from male patients and this result similar to Nirwati et al. (2019) study who found that K. *pneumoniae* isolation rate was 64.07% in male and was 35.93% in female patients.

3.3. Cultural and Morphological Characteristics

Isolates of K. *pneumoniae* were detected based on cultural, Morphological, microscopical and biochemical characteristics, according to MacFaddin (2000). Colonies of K. *pneumoniae* were observed, rounded, smooth, convex and Gamma hemolytic when cultured and streaked on BAB. While the colonies on MCA and EMB agar which appeared typically large, mucoid, with pink to red pigment, usually diffusing into the surrounding agar, indicating fermentation of lactose and acid reduction. On the other hand, colonies found with yellow color on XLD agar as indicator for saccharide fermentation (Figure 3). The confirmatory cultured tests were performed by growing all 62 isolates of K. *pneumoniae* given greenish blue colonies on Chrome agar media (Figure 3)

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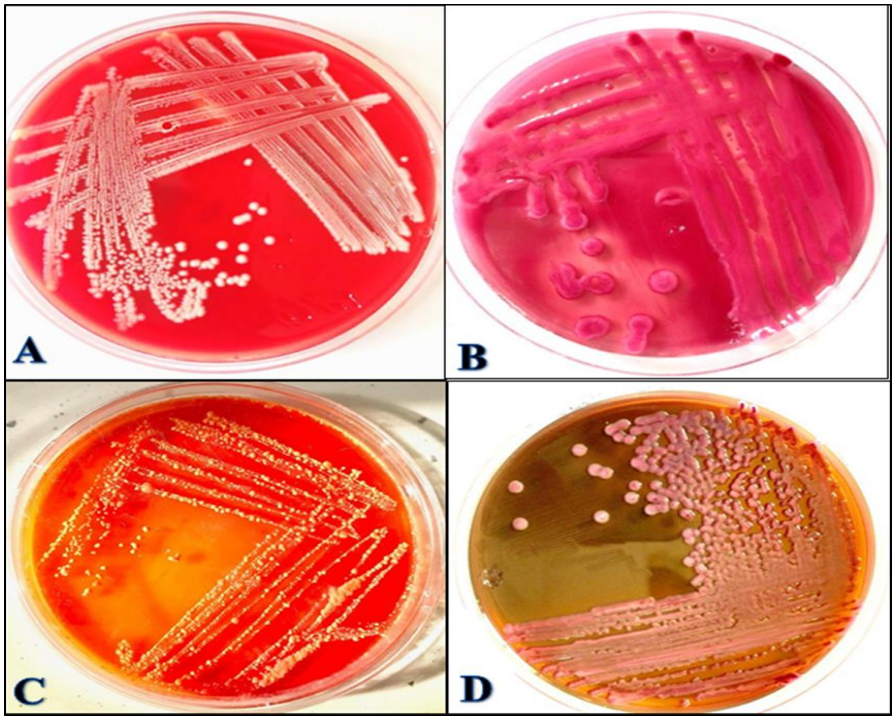

Fig.3. Colonies of K. *pneumoniae* growing on different culture media after 24 hrs of incubation under aerobic condition .

(A): Blood agar,; (B): MacConkey (C): Xylose lysine deoxycholate agar, (D): Eosin methylene blue agar

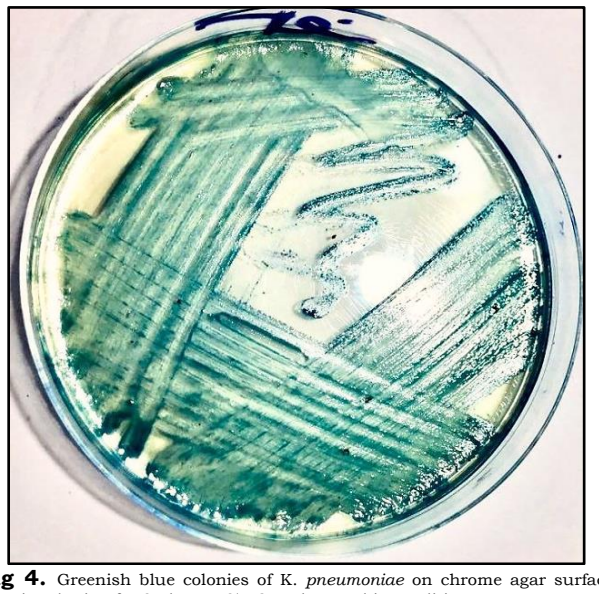

Fig 4. Greenish blue colonies of K. *pneumoniae* on chrome agar surface after incubation for 24 hru at 37oC under aerobic condition

3.4. Microscopically Diagnosis

The results of light microscopic examination under oil immersion lens and the magnification power (1000 X). Cells of K. *pneumoniae* isolates were appeared Gram negative rod and non-spore forming.

3.5. Biochemical Tests

All Gram-negative isolates that grown on MCA, EMB agar, XLD agar undergo biochemical tests in order to distinguish K. *pneumoniae* isolates from other members of related lactose and sucrose fermented bacteria. All isolates of K. *pneumoniae* were appeared lactose and sucrose fermenters. The biochemical tests that mentioned in Table (4) have been carried out for all (62) K. *pneumoniae* isolates under study according to MacFaddin (2000). Isolates were showed positive results for catalase test, while negative results for oxidase, lipase and protease test.

Table 4
Biochemical tests for detecting K. *pneumoniae* isolates under study

Biochemical Test	Result
Lactose	+ve
Sucrose	+ve
Catalase	+ve
Oxidase	-ve
Lipase	-ve
Protease	-ve

+ve: Positive result, - ve: Negative result.

Finally, the last confirmatory step for detecting K. *pneumoniae* under study was done by using API 20 E. The API 20 E was one of accurate assay consists of a set of biochemical tests that were placed on a single strip. It's easy in preparation, low cost and rapid to diagnosis of bacterial species. For these reasons all bacterial isolates under study were examined via API 20 E (Figure 4.6). The results showed that 62(51.7%) belong to the K. *pneumoniae* of total Gram-negative bacterial isolates. Several studies have recommended the necessity of using (Api 20 E) to diagnose detect of *Klebsiella* spp. because this method has a high degree of accuracy, confidence. In addition, it is easy and fast, and it reduces effort and time (Sakkas et al., 2019).

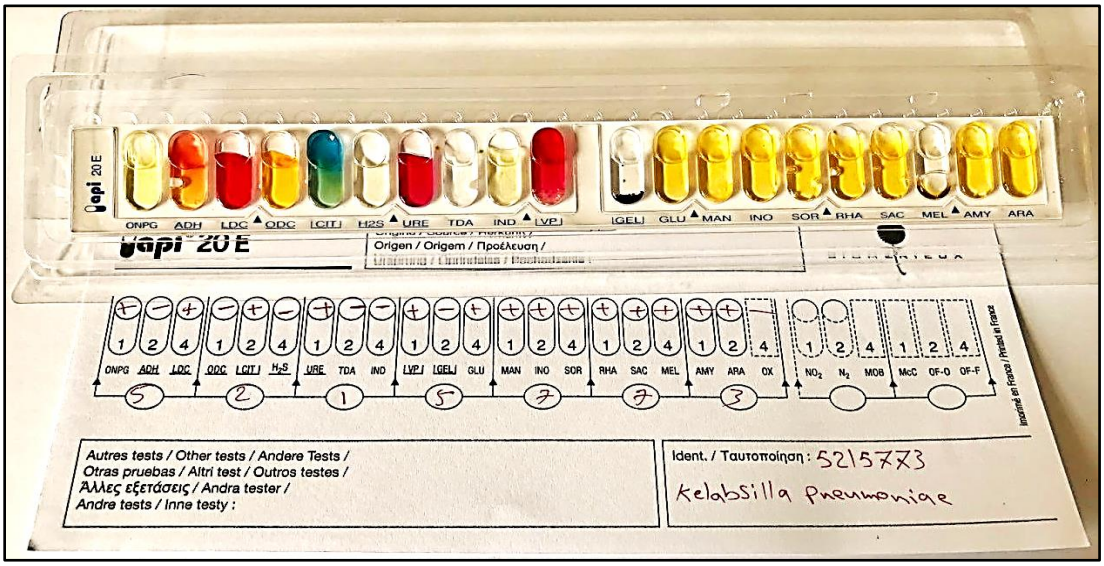

Fig. 6. The API 20 E positive result for K. *pneumoniae* isolate no. 45

3.6. Capsule and Hypermucoviscosity

All 62 K. *pneumoniae* isolates under study were examined to inquire a capsule, using negative staining of Indian ink and examined to detected HMV phenotype among these isolates by HMV test. Formation a mucoviscous line more than 5 mm between an inoculation loop and the colony of bacteria considered as a positive result for HMV production. The result of direct examination showing that 59(95.16%) of 62 K. *pneumoniae* isolates have capsule. While, 60 (96.77%) isolates were appear to produce HVM phenotype (Table 5).

Table 5
Virulence factors among 62 K. *pneumoniae* isolates

Virulence Factors	Result
Capsule	59(95.2%)
Hypermucoviscosity (HVM)	60(96.8%)

Previous reports have relationship between capsulated isolates and HMV phenotype (Gharrah et al., 2017). Confirmed that HMV production depend on the type of capsule. Vuotto et al. (2014), also summarized the relationship between many virulence factors like capsular polysaccharides, siderophores, aggregative adhesion, and fimbriae play a major role in the severity level of K. *pneumoniae* infections.

3.7. Interferon gamma (IFN-γ) Detection

Interferon gamma, the only member of type II interferon, it played an important role in immune systems. 62 patients positive pneumonia caused by K. *pneumoniae* and 62 controls were enrolled in the current study. Controls were matched with the cases in age and gender. The age groups of patients span from 1 to 60 years old.

The level of IFN-γ concentration in this study was measured by using ELIZA assay and was found that is significantly ($p < 0.05$) raised in all groups of patients in comparison to the healthy control group. The level of in age group (1-10) years recorded higher percentage (293.123) (pg/ml) IFN-γ compared with other age group followed by age group (<60), (11-20), (51-60), (41-50), (31-40) and (21-30) years (293.123, 278.234, 267.165, 256.234, 190.34, 167.234 and 134.234, respectively), while the level of IFN-γ concentration (pg/ml) among healthy control groups were arranged descending according to previous age groups in patients age group: (48.123, 42.134, 42.122, 41.234, 39.123, 35.123 and 25.122, respectively). All results of IFN-γ concentration levels are summarized in Figure (7).

The study groups in the current study, included 37 males and 25 females, it was observed that there were significant differences between males and females at a significant level ($p < 0.05$), when measuring the IFN-γ concentration, it was observed that the level of concentration IFN-γ in female patients and controls was 295.234 pg/ml and 46.234 pg/ml, respectively which is higher than the level of IFN-γ concentration in the male

patients groups, it was 245.342 and in the control groups it was 42.345. The results are summarized in the Figure (8).

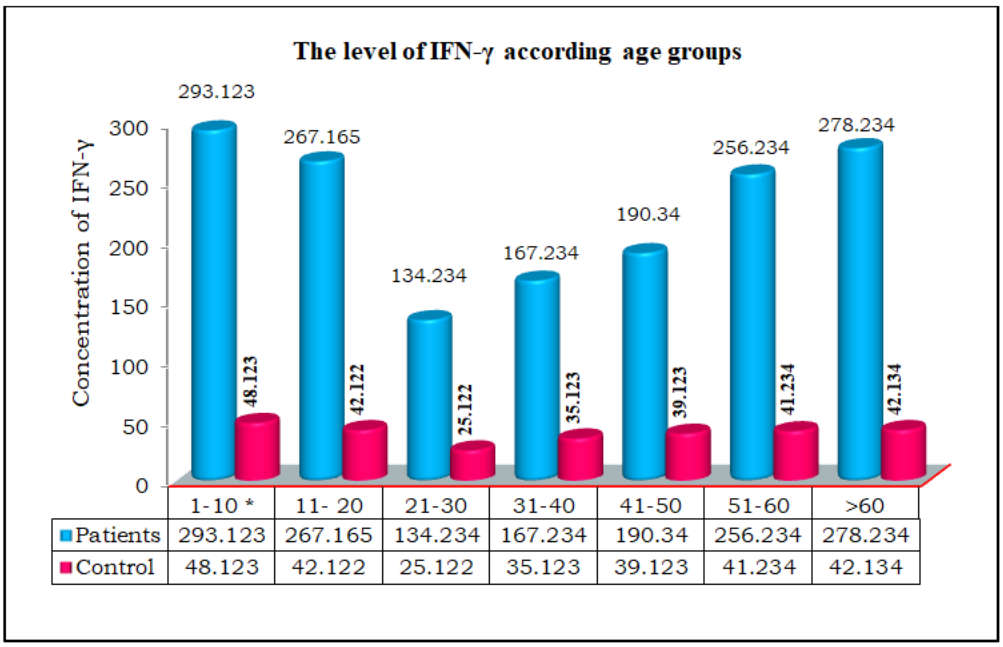

Fig. 7. Interferon gamma concentration (pg/ml) distributed according to the age groups for both patients and control groups

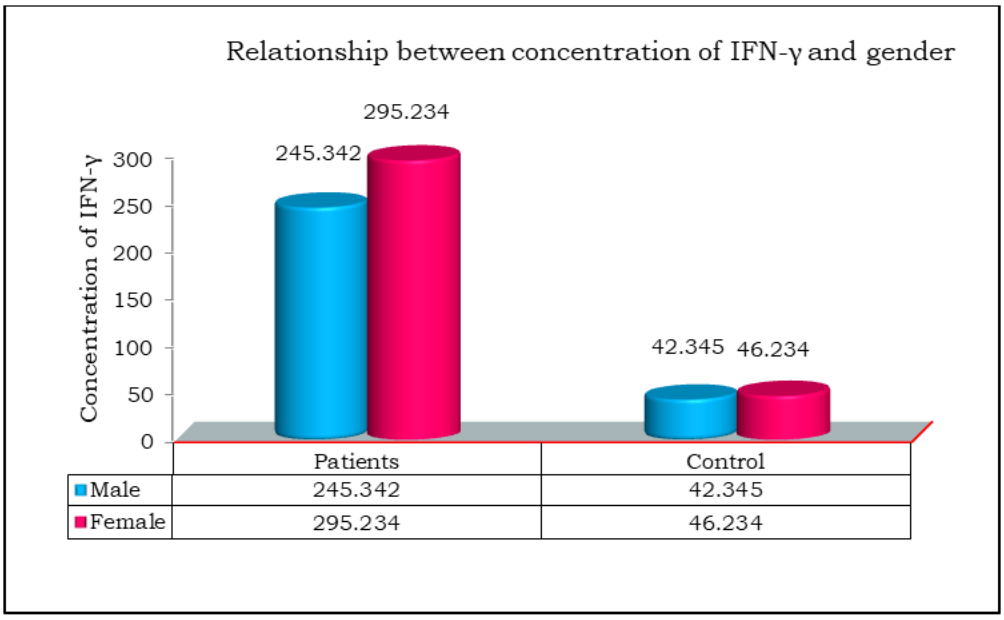

Fig. 8. The levels of IFN-γ (pg/ml) concentration according to patients with pneumonia and control sex groups

It was also observed that the mean for the concentration of IFN-γ in female patients groups was 295.234, and in the control groups it was 46.234, which is higher than the mean for the concentration of IFN-γ in the male patients groups, as it was 245.342 and in the control groups it was 42.345. The means and the standard deviations were calculated at a significant level $p < 0.05$ .IFN-γ is capable of organizing many protective functions to heighten immune responses in infections. It can exhibit its immunomodulatory effects by enhancing Ags and presentation and processing, increasing leukocyte trafficking, boosting the antimicrobial functions and affecting cellular proliferation and apoptosis.

This study, was found relationship between action of type I IFN and lung infection with K. *pneumoniae*, a pathogen with one of the highest emergences of antibiotic resistant strains, MDR and have more sever virulence factors such as (capsulated and mucoid phenotype). The results of the current study confirm the role of IFN-γ in fighting bacteria, especially K. *pneumoniae*, and defending the body through many mechanisms. There are many studies that show how IFN-γ works, such as the Xiong et al. (2016) found that research over the last twenty years demonstrates that activation of early inflammatory responses such as IFN-γ, is essential to clear K. *pneumoniae* infections.

In this study, significant differences were found for levels of IFN-γ concentration between age groups, and it is interesting that the age group (1-10) years showed the highest level for this IFN-γ and was followed by the age group (<60) years followed by (11-20), (51-60) years and the rest of the groups were somewhat close to each other. The reason for these results may be due to that the level of IFN-γ rises to counter infections caused by K. *pneumoniae*, especially in age groups that have a somewhat weak immunity such as infants, children and the elderly.

Schüller et al. (2013) explained the high levels of IFN-γ concentration in both adults and children and believed that IFN-γ levels may be close despite varying age groups and explained that plasmacytoid dendritic cells (pDC) release high concentrations of type IFN-γ in response to TLR7 and TLR9 stimulation in adults. However, newborn pDC are severely limited in secreting interferon α/β upon exposure to different viruses, despite expressing levels of TLR7 and TLR9 that are similar to adults.

3.8. Toll Like Receptor (TLR4) Detection

Toll like receptor dependent signaling, triggers numerous immune pathways, leading to neutrophil recruitment, antimicrobial peptide production. These pathways are required for the host to sense a bacterial pathogen and to mount a protective immune response (van Lieshout et al., 2015). In the present study, the same groups of patients and control groups that were subjected to the measurement of TLR4, again subject for measuring TLR4, were as follows: 62 patients with pneumonia and 62 controls groups. Controls were matched with the cases in age and gender. Also, the age groups of patients ranges from 1 to 60 years old.

The level of TLR4 concentration in this study was measured by using ELIZA assay and was found that is significantly ($p<0.05$) raised in all groups of patients in comparison to the healthy control group. The level of in age group (51-60) years recorded higher percentage (12.993) TLR4 (pg/ml) compared to other age groups followed by age group (<60), (1-10), (11-20), (41-50), (31-40) and (21-30) years (12.993, 12.559, 12.244, 11.455, 11.011, 10.111, 9.124, respectively), while the level of TLR4 concentration among healthy control groups were arranged descending according to age groups in patients age group: (1.012, 1.001, 1.222, 1.667, 1.899, 1.011 and 1.993, respectively) (Figure 9).

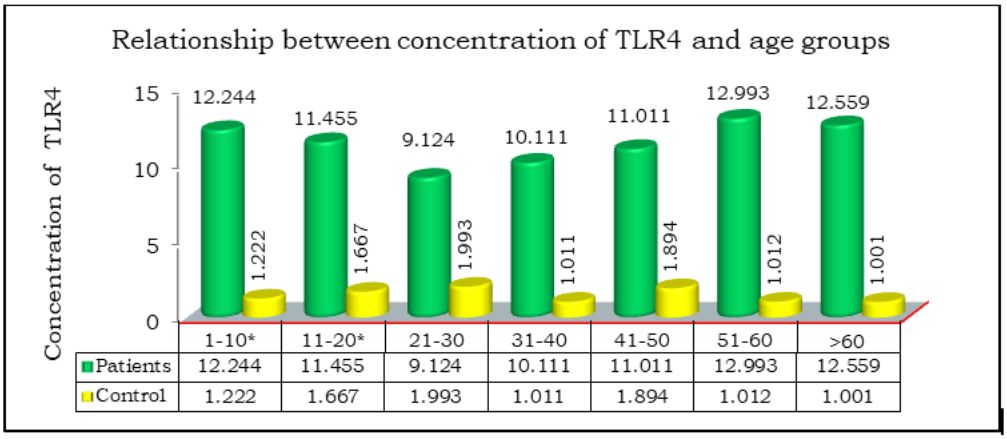

Fig. 9. The levels of TLR4 concentration according to patients with pneumonia and control age groups

The sex groups in this study, included 62 patients: 37 males and 25 females infected with K. *pneumoniae*, it was observed that there were significant differences between males and females at a significant level ($p<0.05$), when measuring the TLR4 concentration by ELIZA assay and it was observed that the level of TLR4 concentration in male patients was 11.786 and 1.034 was in control, respectively which were close to the level of TLR4 concentration in the female patients groups 12.043, and in the control groups was 1.996. (Figure 10).

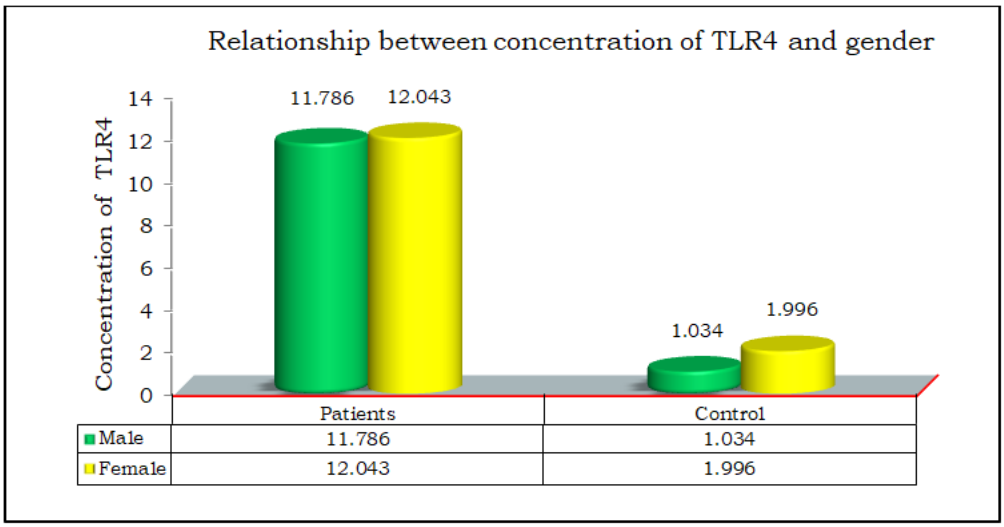

Fig. 10. The levels of TLR4 concentration according to patients with pneumonia and control sex groups

In the current study, it was also observed that the mean for the concentration of TLR4 in male patients' groups was 11.786, and in the control groups it was 1.034, which is higher than the mean for the concentration of TLR4 in the female patients' groups, as it was 12.043 and in the control groups it was 1.996. The means and the standard deviations were calculated at a significant level $p < 0.05$.

Toll-like receptors are transmembrane proteins that recognize infection and damaged host cells, which lead to the consequent inflammation reactions. TLR4 might influence the progress of pneumonia (Cai et al., 2015). Jeon et al. (2017) suggest that TLR4 is more important in the immune response against K. *pneumoniae* infection than TLR2. Therefore, TLR4 play cooperative roles in innate immune responses in the lungs that induces systemic inflammation during K. *pneumoniae* infection.

There is a strong relationship between the production of TLR4 and HMV + capsulated K. *pneumoniae*, and since all the isolates under study are capsulated and HMV phenotype, this justifies the production of TLR4 at high levels compared to the control groups. This interpretation is based on a study conducted by Hunt et al. (2014) in which confirmed that the strains of K. *pneumoniae* produces LPS, which can be sensed by the innate immune TLR4. In disease models, TLR4 deficiency renders the host less able to respond to infection by many pathogens. However, it has in the last ten years been reported that HMV + capsulated K. *pneumoniae* are masked from TLR4 recognition by their capsule.

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