

Pathogenicity of *Klebsiella pneumoniae* isolated from diarrheal cases among children in Kirkuk city

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Abstract

The isolation and identification of 61 *Klebsiella pneumoniae* from 433 children suffering from diarrhea have been made. The study also included determination of some virulence factors of *K. pneumoniae* and the results showed that all the isolates (100%) have capsule, urease, and siderophore producers. Detection of virulent isolates by Congo red binding was also studied, 14/61 (22.9%) of the isolates were binding to Congo red. Adherence test was done for 13 isolates, and all *K. pneumoniae* (100%) showed its ability for adherence to human buccal cavity epithelial cells. In order to study the invasion character, the mice (Balb/C) infants were sucked with the suspension of the selected virulent (according to in vitro tests) isolates of *K. pneumoniae*, and the histological sections were performed to study the mode of the entry of bacteria into the enterocytes, and the histological changes in the liver. Microscopical examination for histological sections showed pathological effect on two organs when compared with the control organs.

Introduction:

Classical diarrheagenic bacteria such as: *Salmonella*, *Shigella* and *Yersinia pestis*. possess one or more mechanism for causing diarrhea, still others, are normal flora associated with certain syndrome based on the presence of virulence factors that are not possessed by all strains or even by the same strains at all times, the later are commensals of human and animal intestinal tract. Many studies were conducted to evaluate the role of agent of gastroenteritis previously thought to be mere commensals of the gastrointestinal tract as an opportunistic pathogen [1]. In recent years, many studies were conducted to evaluate the role of agent of gastroenteritis previously thought to be mere commensals of the gastrointestinal tract, several cases of diarrhea due to *Hafnia alvei* [1, 2, 3], *Citrobacter freundii* [4, 5], *Enterobacter aerogenes* [6], *Morganella morganii* [7], *Providencia alcalifaciens* [8, 9], *Proteus mirabilis* [10], *Pseudomonas aeruginosa* [11, 12] and *Klebsiella spp.* [13, 14] have been reported. The pathogenic mechanisms of *Klebsiella* infections have identified a number of bacterial factors contributing to pathogenesis of the bacterium. Both *in vitro* and *in vivo* models have been established to investigate the interaction between bacterial cells and the host [15]. Several bacterial factors were known to contribute to the pathogenic mechanisms of *Klebsiella* infections. The virulence factors of *Klebsiella spp.* focuses on some major bacterial factors: capsule, fimbriae (pili), lipopolysaccharide serum resistances, siderophores and toxins [15, 16]. The aim of this study is to evaluate the ability of *K. pneumoniae* isolates to express some virulence factors *in vivo* and *in vitro*.

Materials and Methods:

Sample collection:

The study was carried out on children (out and inpatient suffering from diarrhoea) attending Azadi teaching hospital and Pediatric hospital in Kirkuk city, from June 2009 to June 2011, where a total of (454) children submitted to the study under physician supervision. Stool specimens were collected in

disposable, clean screw-capped, commercially available containers used for this purpose. All the specimens were processed immediately or used Carry Blair transport media if delayed for 1-2 hours after their collection and then cultured [17].

Bacterial isolation and identification:

Collected samples were cultured directly on MacConkey agar; XLD agar, SS agar and EMB agar for primary isolation of the Enterobacteriaceae and blood agar to detect beta hemolytic isolate [17]. All isolates incubated aerobically at 35 °C for 24 hours, and select suspicious colonies for definitive microscopic examination, culture characteristics, biochemical testing and the usage of API 20E System (BioMérieux/France) for identifying Enterobacteriaceae and other gram negative rods [18].

Detection of some virulence factors:

1. Capsule production:

- ❖ Wet mount Indian ink staining: [19].
- ❖ Modified (1%) Congo red staining: The bacterial culture is mixed with a drop of 1% Congo red and smear was prepared on a slide to a circle of 2 cm in diameter after drying, examined under microscope. Capsulated bacteria appear as hallow zone [20].

2. Siderophore production: [21].

3. Gelatin liquefaction test: [22].

4. Hemolysin production: [23].

5. Invasiveness (Binding to Congo red): [24].

6. Lecithinase production: [23].

7. Extracellular protease: [25].

8. DNase production: [17].

9. Adherence Tests

❖ Buccal Epithelial Cells Isolation: Epithelial cells were prepared according to [26].

❖ Bacterial suspension preparation: Previous to the adhesion assay, the bacterial suspensions were standardized as follows: the bacterial growth harvested from CFA medium, and suspended in PBS.

Before obtain a final concentration of (10^8 bacteria/ml) washing twice resuspended in PBS.

❖ Adherence to Human Buccal Epithelial cells: Five milliliters of bacterial suspension (10^8 bacteria/ml) was added to 3 cover slips (covered with epithelial cells) in a petri dish for each isolates (the control was prepared without adding bacteria), and incubated at 37°C for 1 hour with moderate agitation every 10 minutes. In order to remove non adherent bacteria, washing the cover slips with PBS, the epithelial cells were fixed by methanol for 15 minutes, stained with giemsa stain 30% for 20 minutes, lifted to dry at room temperature after washing with giemsa buffer, irreversibly putted on the slide, the bacterial binding to the epithelial cells was examined by light microscope under oil immersion lens. The results were expressed as the total number of bacteria attached to cells was divided by the number of epithelial cells with bacteria adhered [27].

10. Pathogenicity of *Klebsiella spp.* to suckling mice:

❖ Suckling mouse test: Mouse infection was done by culturing bacterial isolates in BHI broth for 24 hours at 35°C, (3) suckling Balb/C mice aged 4-6 days old were gavaged with 0.1ml of 2×10^6 cell/ml through a feeding needle for each isolate and (3) mice ingested with 0.1ml of sterile normal saline (used as diluent's) and left as control. After 24 hours post-infection, mice were sacrificed with chloroform; the liver and intestine were taken and fixed in 10% buffered formalin for histopathological study [9, 11].

❖ Histological study: The preparation of histological sections depended on standard method of [28]. The sections were examined by light microscope under magnification power 100X and 400X. Photographs were taken by digital-camera.

Results and discussion:

Isolation and identification:

The results of identification were showed that among the 454 cultured stool sample 433 sample gave positive culture and 767 bacteria isolated from children with diarrhea, *Klebsiella spp.* isolated were 93 isolates with 12.13%. 61 bacterial isolates belonged to *K. pneumoniae*.

Virulence factors of *K. pneumoniae*

1. Capsule production:

The results in present study indicated that all *Klebsiella* isolates were able to produce capsule (Table-1) and (Figure-1). Two methods were used to stain capsule; India ink and 1% Congo red, the later one is simpler and cheaper than the first one. Capsules probably help bacteria to resist phagocytosis and prevent killing by bactericidal serum factors [29].

2. Urease activity:

As observed in Table-1 when the isolates diagnosed by conventional method and API 20E System, and all 61 (100%) isolates of *K. pneumoniae* gave positive

result for urease test. The importance of urease enzyme was in its ability to utilize urea in the urine as substrate for its activity which result in liberation of CO₂ and production of ammonia which increase the pH of the urine towards alkalinity which facilitates the precipitation of crystals and formation of stones, besides it decreased the antibiotics effect during the treatment of UTIs [30]. However, in *Yersinia enterocolitica*, urease activity contributes to acid tolerance and may promote bacterial survival prior to infection [31].

3. Hemolysin and siderophore production:

The results of this study revealed that all isolates of *K. pneumoniae* were unable to produce hemolysin on blood agar, and 61 (100%) isolates were able to produce siderophore (in presence of dipyrilidil). These results are similar to that reported by Pyanne and others [32], they found that *K. pneumoniae* have the ability to provide iron and produce siderophore without hemolysin production. Production of siderophore by *Klebsiella* was also confirmed by Nassif and Sansonetti who found that *Klebsiella* isolates have an ability to produce siderophore in presence of dipyrilidil [33].

The growth of bacteria in host tissues is limited not only by host defense mechanisms but also by its supply of available iron. The supply of free iron in the host milieu may be extremely low; many bacteria attempt to secure their supply of iron in the host by secreting high-affinity iron chelators called siderophores. Aerobactin is a hydroxamate-type siderophore occasionally found in *Klebsiella* strains. *K. pneumoniae* strains that produce aerobactin were more virulent in mouse, whereas strains not producing this siderophore were less likely to be virulent [34].

4. DNase, lecithinase, lipase and extracellular protease production:

The results expressed in Table-1 showed that all isolates of *K. pneumoniae* were unable to produce extracellular protease, lecithinase, lipase, and DNase. The extracellular enzymes of bacteria like protease, Lecithinase, lipases, and nuclease are not shown to have a direct role in invasion or pathogenesis, but these enzymes presumably may associate in bacterial nutrition or metabolism [35].

5. Invasiveness (binding to Congo red)

Congo red (CR) dye agar test was first used by Surgalla and Beasly for differentiation of virulent and a virulent *Yersinia pestis* [36]. Subsequently, it was used other researchers as phenotypic marker to differentiated invasive and non- invasive *E. coli* and *Shigella spp.* [37].

In the present study, the results showed that from 61 *K. pneumoniae* 14 (22.9%) gave positive (red colony) (Figure-1), while 47 (77%) did not bind to the dye even after 72 hours and were thereafter declared negative (Table 4-4). Berkhoff and Vinal (1986) found that about half of the *E. coli* was CR positive, which were obtained from environmental origin.

Likewise, Panigarhy and Yushen also found 13/21 (61.9 %) *E. coli* were CR positive. While Al-Maisary, 2002 found that 37 (33.3%) *E. coli* were CR positive from 111 *E. coli* isolated from stool collected from children suffering from diarrhea [38]. While Al-Saleem found that 15 out of 18 *Shigella spp.* isolated from stool and rectal swabs collected from children suffering from acute diarrhea, were binding to Congo red, also she noted kerato-conjunctivitis in the eyes of experimental animals in sereny test [39]. However, the sensitivity of the test was proved 58.69%. It was also revealed that from 51 isolates of *E. coli*, which were CR negative, were also negative with the Sereny test. The test proved 100% specific without showing any false negative. Therefore, this test can be used as a primary screening test to screen non-invasive *E. coli* from the potentially invasive *E. coli*. Binding of CR dye was also found different according to their serovars. It was observed that not all strains of same serovars were negative or positive.

The same result was obtained with the Sereny test [36].

Table-1: virulence factors of *K. pneumoniae*

Isolates Virulence factors	<i>K. pneumoniae</i>
Capsule	61(100%)
Siderophore	61(100%)
Urease	61(100%)
Beta-lactamase	28 (45.9%)
Invasiveness	14 (23%)
Gelatin liquefaction	0 (0%)
Lipase	0 (0%)
Protease	0 (0%)
Lecithinase	0 (0%)
Haemolysin	0 (0%)
Deoxyribonuclease	0 (0%)
Total	61

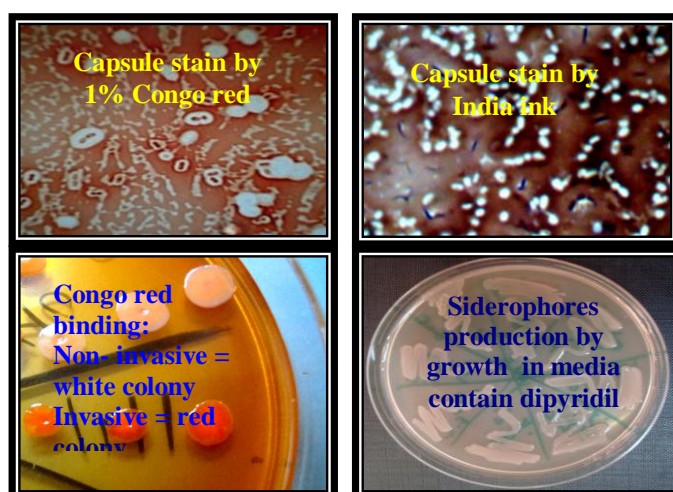


Figure-1: virulence factors produced by *Klebsiella* isolates

6. Adherence

Adherence ability of (13) *K. pneumoniae* and one isolate of *Escherichia coli* that used as control were tested for adherence ability to human buccal epithelial cells. The results showed that all *K. pneumoniae* have the adherence ability and different letters represent differences in adherence ability. *K. pneumoniae* number (287) possessed highest ability for adhesion, while *K. terrigena* number 256 possessed lowest ability for adhesion, while Al-Sa'doun found that all *Klebsiella spp.* isolates showed adherence ability to epithelial cell isolated from urine of healthy women [40].

An often necessary step in the infection process is the adhesion of pathogenic bacteria to host tissues, bacterial adhesion to the oral mucosa has been demonstrated by in vitro studies [41], as well as by scanning electron microscopy (SEM) analysis of in vivo mucosal samples [42]. The molecular substrates of bacterial adhesion are adhesins located on the

bacterial surface. Adhesins exist in fimbrial and non-fimbrial forms [43].

The first form of adhesins is composed of proteins and carbohydrates in the form of fimbriae, which are semi-selectively stainable by ruthenium red. The entirety of fimbriae forms an additional but not obligatory polysaccharide- rich surface layer denoted as glycocalyx [44]. The glycocalyx is peripherally located to the outer membrane in Gram-negative bacteria and to the peptidoglycan in Gram-positive ones. Bacteria can easily be promoted to form fimbriae in vitro by addition of a high concentration of carbohydrates [45].

The colonization by pathogens causing enteritis, of the mucous surfaces of the host requires special factors encoded by the microorganisms that specifically bind to host epithelial cells [46]. Many studies showed that several adhesins were involved in the interactions between *K. pneumoniae* and human intestinal cell lines [47], while Maroncle *et al.*, reported that intestinal cell adhesion may not be

essential for colonization of the GI tract or represents only one late stage in the process: bacteria have to counteract several barriers such as the mucous layer and peristalsis and metabolically adapted to the environment [48].

7. Histopathological changes on intestine and liver of suckling mice:

Isolates of *K. pneumoniae* numbers 89, 280, 486; were possessed different virulence characters (Table-2) were selected for studying the histopathological changes. Suckling mice infection was done through a feeding needle with 0.1ml of 2×10^6 cell/ml *Klebsiella* spp. suspension, after 24 hours post-infection, the liver and intestine of tested mice and control (mice sucked normal saline that used in preparation of bacterial suspension) were investigated for pathologic changes.

Pathologic changes induced in the intestine of suckling mice by *K. pneumoniae* infection were studied by conventional histology, sections. Many polymorphonuclear leukocytes were seen in the lamina propria. There was villous atrophy, with considerable degeneration and vascular congestion. On the other hand, tissue damage of the mucosa with necrotic and entire sheets of epithelial cells had sloughed off in some parts. The control mice's intestine histology section showed normal villous architecture with uniform microvillus brush border and no inflammatory changes. Also the control mice's liver histology section showed normal liver architecture with normal cell and no inflammatory changes. The pathologic changes induced in the liver of suckling mice by *K. pneumoniae* included swelling hepatocytes and distribution of polymorphonuclear. There was hemorrhage, with considerable fatty degeneration and vascular congestion, also tissue damage with necrotic and vaculation was seen in the liver tissues of some mice that received *K. pneumoniae* suspension. Although there are similar inducible changes by all selected *K. pneumoniae*

(Figure-2 to Figure-9) in liver and the intestine of suckling mice included hemorrhage, lymphocytes infiltration and necrosis. The two most important were *K. pneumoniae* numbers 280 and 486 that induced severe changes according to the specialized histopathologist diagnosis.

Infection is a dynamic process involving invasion of the body by pathogenic microorganisms and reactions of the tissues to microorganisms and their toxins. Pathogenic microorganisms isolated from clinical samples are of great threat to human health. The outcome of an infection depends on the virulence of the pathogen and the relative degree of resistance or susceptibility to antimicrobial chemotherapy [49].

A study were made by [9] on suckling mice sucked the whole bacterial suspension (2×10^6 cell/ml) of *Providencia. alcalifaciens* and histological section were performed to study the mode of entry of bacteria into the enterocytes, the histological section showed pathogenic lesions in the intestinal tissues such as necrosis, desquamation and severe mucinous degeneration have been observed in the epithelial lining of the intestinal villi accompanied by edema, hyperplasia, in payer's patches and infiltration of polymorph nuclear neutrophils and mononuclear lymphocytes and other changes.

When a pathogenic strain is ingested into a suitable host the first pathogenic step of the bacteria is taken when organisms breach the intestinal epithelium of lymphoid follicles and enter the lymphatic system, sequentially moving from the Payer's patch tissue to the lymph nodes and then to the spleen and liver. At each step of infection these pathogens respond to changing host environments by expressing appropriate factors that ensure their survival. Host cell responses to bacteria provide clues to the mechanisms used by the bacteria to infect the host and provide insight into possible mechanisms that the host might invoke to engage and eliminate the invading pathogens [50].

Table-2: virulence factors possessed by *K. pneumoniae*

Isolates number	Capsule	Urease	Siderophore	Adhesion	Mucoid colony	Invasiveness	Mouse pathogenicity
<i>K. pneumoniae</i> 22	+	+	+	+	+	-	+
<i>K. pneumoniae</i> 89	+	+	+	+	+	+	+
<i>K. pneumoniae</i> 280	+	+	+	+	++	-	+
<i>K. pneumoniae</i> 287	+	+	+	+	++	-	+
<i>K. pneumoniae</i> 292	+	+	+	+	+	-	+
<i>K. pneumoniae</i> 486	+	+	+	+	+++	-	+
<i>K. pneumoniae</i> 488	+	+	+	+	++	-	+

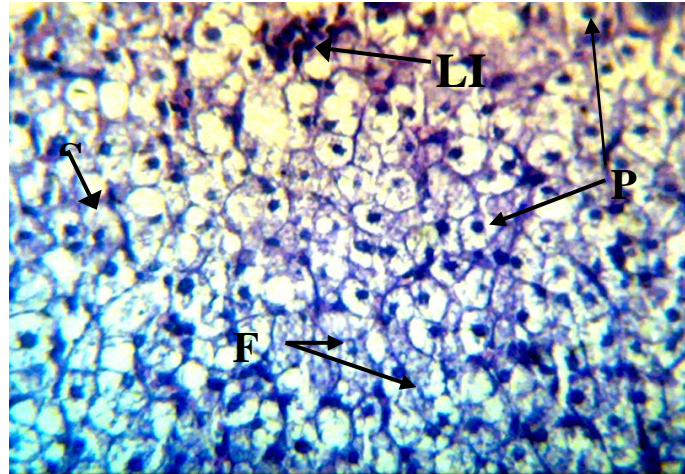


Figure 2: Liver section of treated suckling mouse with 2×10^6 of *K. pneumoniae* (NO. 280) suspension / ml, after 24 h. has lymphocyte infiltration (LI), focal degeneration in hepatic cell (FOD), swelling hepatocytes (SH), pyknotic nuclei (PN) (as labeled) (H & E, 400 X).

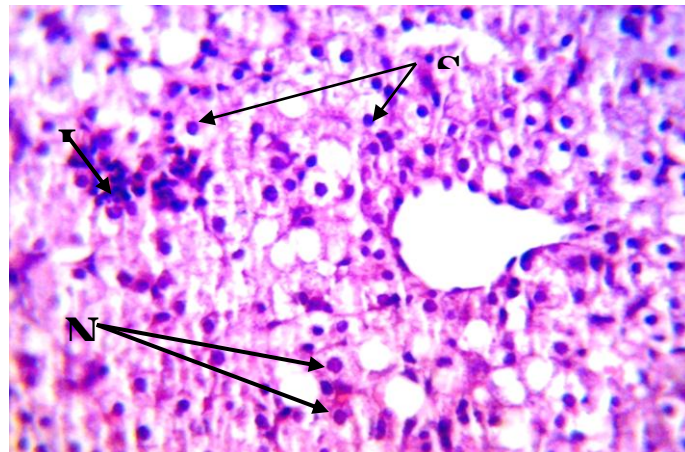


Figure 3: Liver section of treated suckling mouse with 2×10^6 of *K. pneumoniae* (NO. 287) suspension / ml, after 24 h. has Lymphocyte Infiltration (LI), swelling hepatocytes (SH), necrotic nuclei (NN) (as labeled) (H & E, 400 X).

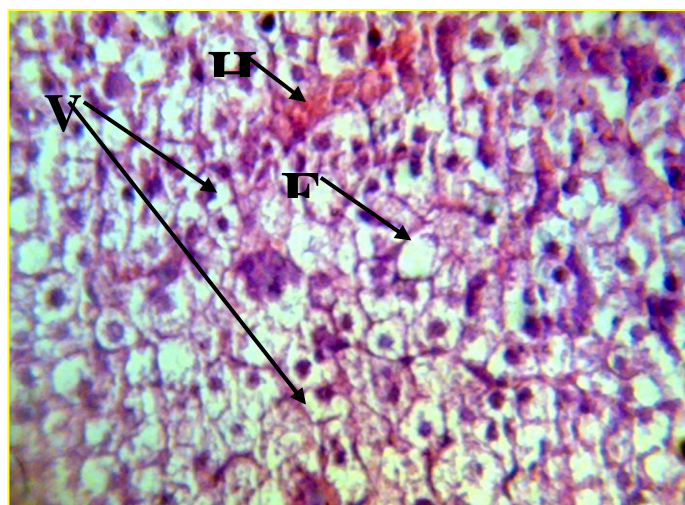


Figure 4: Liver section of treated suckling mouse with 2×10^6 of *K. pneumoniae* (NO. 486) suspension / ml, after 24 h. has vacuolation in the cytoplasm of hepatocytes (VC), fatty degeneration (FD) and hemorrhage (H) (as labeled) (H & E, 400 X).

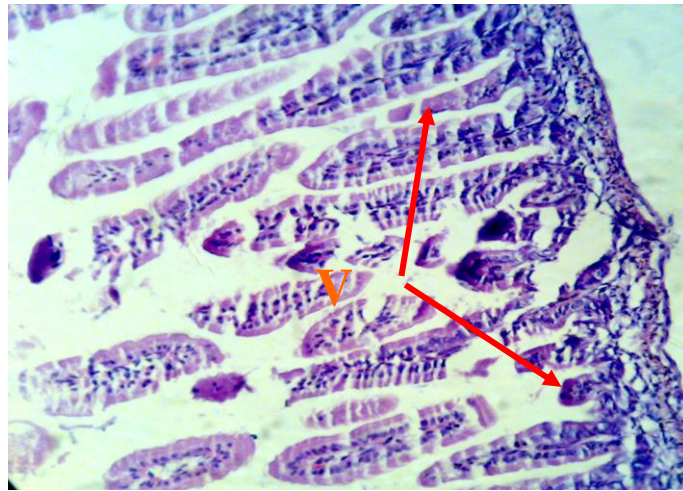


Figure 5: Intestine section of treated suckling mouse with 2×10^6 of *K. pneumoniae* (NO. 89) suspension/ml, after 24 h. has villous atrophy (as labeled) (H & E, 120 X).

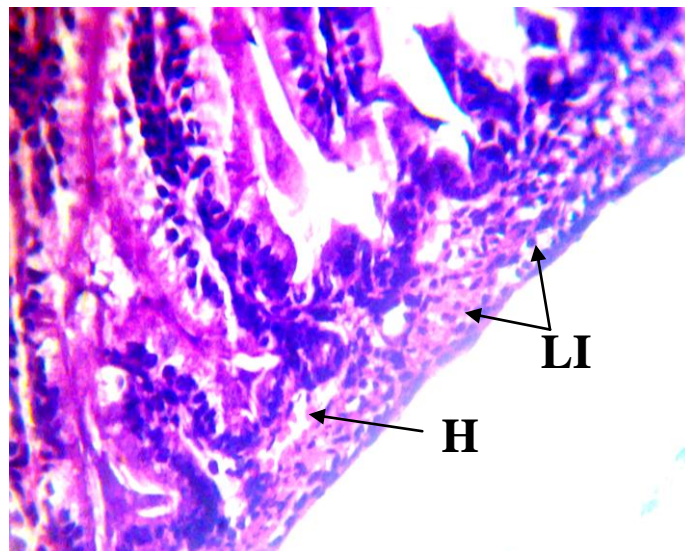


Figure 6: Intestine section of treated suckling mouse with 2×10^6 of *K. pneumoniae* (NO. 280) suspension/ml, after 24 h. has lymphocyte infiltration (LI), and necrosis (N) (as labeled) (H & E, 400 X).

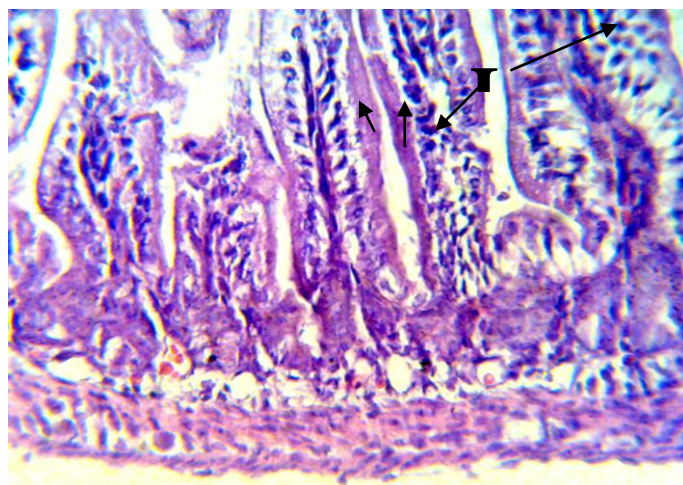


Figure 7: Intestine section of treated suckling mouse with 2×10^6 of *K. pneumoniae* (NO. 287) suspension/ml, after 24 h. has lymphocyte infiltration (LI), Focal necrosis in the villi (FN) (as labeled) (H & E, 400 X).

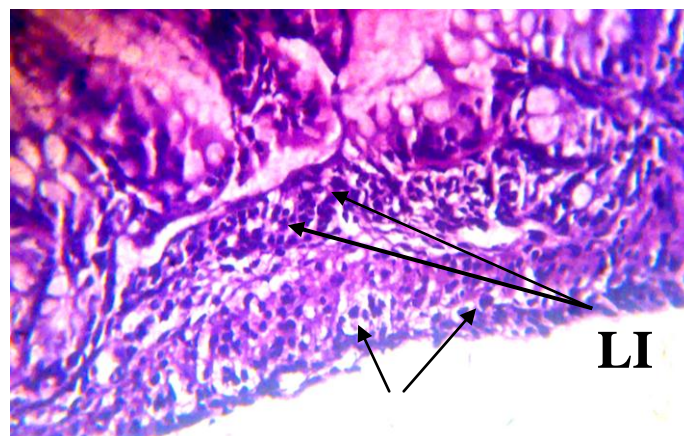


Figure 8: Intestine section of treated suckling mouse with 2×10^6 of *K. pneumoniae* (NO. 486) suspension/ml, after 24 h. has lymphocyte infiltration (LI) and degeneration in muscle fiber (DM) (as labeled) (H & E, 400 X).

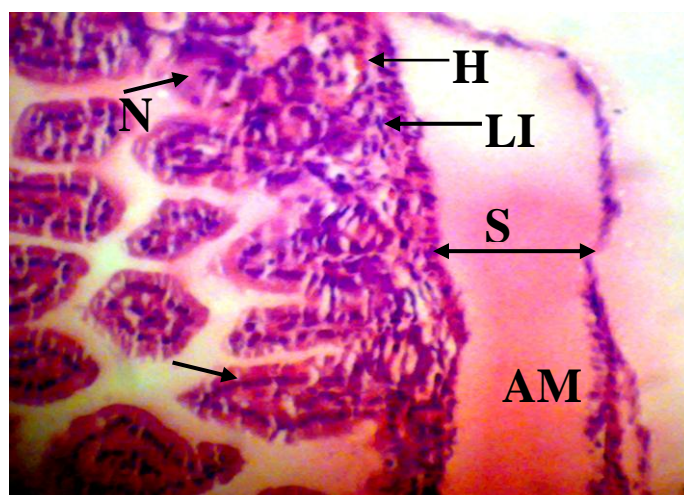


Figure 9: Intestine section of treated suckling mouse with 2×10^6 of *K. pneumoniae* (NO. 486) suspension/ml, after 24 h. has lymphocyte infiltration (LI), hemorrhage (H), epithelial necrosis (N), separating of adventitia (SA), and absence of muscularis (AM) (as labeled) (H & E, 400 X).

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إمراضية الكليبيسيلا الرئوية *Klebsiella pneumoniae* المعزولة من حالات الاسهال عند الاطفال

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الملخص:

أظهرت الفحوصات من بين 767 عزلة مشخصة، ان 93 عزلة بكتيرية تنتمي الى جنس الكليبيسيلا *Klebsiella spp.* ، 61 عزلة تعود الى النوع الكليبيسيلا الرئوية *K. pneumoniae*. أخضعت جميع عزلات الكليبيسيلا الرئوية لاختبارات التحري عن عوامل الضراوة فيها وظهرت النتائج ان جميعها تملك قابلية إنتاج المحفظة، انزيم اليوريز والسيدروفورات ولكن 61/14 (22.9%) عزلة كانت لها قابلية الارتباط بصبغة الكونغو الحمراء. اختبرت قابلية 13 عزلة لجراثومة الكليبيسيلا الرئوية للالتصاق، وظهرت النتائج ان جميعها (100%) لها قابلية الالتصاق على الخلايا الظهارية الفموية للانسان. ولدراسة خاصية الغزو والامراضية تم تجريع صغار الفئران بمعلق جرثومة الكليبيسيلا الرئوية والتي تملك صفات ضراوة حسب الاختبارات اعلاه التي اجريت في الزجاج، وحضرت مقاطع نسجية لمعرفة طريقة اختراق هذه الجراثيم للخلايا المعوية والتغيرات النسجية في الامعاء والكبد. بين نتائج الفحص المجهرى لهذه المقاطع النسجية وجود آفات مرضية في نسيج الامعاء الكبد للفئران المجرعة بمعلق العزلات المختارة مقارنة مع الاعضاء السليمة.