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**ORIGINAL ARTICLE**

# Virulence factors and antibiotic susceptibility patterns of *Klebsiella pneumonia* strains Histamine producing bacteria isolated from sputum

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**ABSTRACT**

**Objective:** Histamine- producing bacteria(HPB) may be associated with much respiratory problem. *Klebsiella pneumoniae* is one of the most important pathogenic bacteria and is most frequently recovered from clinical specimens and can cause a classic form of primary pneumonia.

**Methods:** Fifty-one samples of sputum collected from patients suffering from respiratory problem in the advisory clinic for chest and respiratory disease Basra city. Isolates of *Klebsiella pneumoniae* were identified by their morphological and biochemical characteristics. And The colony was cultured on Niven's agar medium and. purple colony on Niven's medium is indicator of Histamine Producing Bacteria ( HBP) according to Niven's *et al*., 1981. All the isolates of *Klebsiella pneumoniae* identified of *hdc* gene by PCR and gene expression *hdc* detection by HPLC technique and identified were subjected to antibiotic sensitivity testing by MIC the Bio Meraux company according to16 antibiotics resistance profile and study Factors affecting Histamine production.

**Results:** Fifty one(51) samples were collected from the patients sputum suffering from respiratory problem in the advisory clinic for chest and respiratory disease and two hundred and fifty five (255) isolates were tested using phenotypic classification based on chemical properties of bacteria. (50)strains of *Klebsiella pneumoniae* were identified by their morphology and biochemical characteristics. Out of above only eleven (11, 21.56%)strain of *Klebsiella pneumoniae* were positive for histamine production depending on the Niven´s medium as a histamine producer indicator and has been confirmed in PCR for the presence of the gene histadine decarboxylase *hdc*. Histamine concentration was 46.06µg/ml that produced from *Klebsiella pneumoniae* was detected by HPLC technology. Majority of the strains HPB isolated were sensitive to Trimethoprim . The proportion concentration of histamine produced from *Klebsiella pneumoniae* in pH(6.5) was 29.04µg/ml and less at a NaCl 1mg was 2.75 µg/ml.

**Conclusion:** The *K. pneumonia* have a new virulence factor it can be cause or enhanced pathogenicity and high sensitive against many antibiotics Trimethoprim, while the overall resistance pattern high resistance Ampicilin, Ambicillin/Sulbactam.



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## INTRODUCTION

*Klebsiella pneumoniae* is one of the most important pathogenic bacteria. *K. pneumoniae* is a part of the normal flora of humans where they inhabit mucosal surfaces. *Klebsiella pneumoniae* is most frequently recovered from clinical specimens and can cause a classic form of primary pneumonia. *Klebsiella pneumonia*e can also cause a variety of extra pulmonary infections, including enteritis and meningitis in infants, urinary tract infections in children and adults and septicemia[1]and nosocomial infections by *K. pneumoniae* are still much more prevalent, and may be more dangerous due to the rapid development and spread of antimicrobial resistance in hospital settings[2,3]. Multidrug resistant bacteria cause serious nosocomial and community acquired infections that are hard to eradicate by using available antibiotics. Moreover, extensive use of broad-spectrum antibiotics in hospitalized patients has led to both increased carriage of *Klebsiella* and the development of multidrug-resistant strains that produce extended–spectrum beta-lactamase[4,5]. Studies have shown that antibiotic consumption leads to selective pressure increasing beta-lactam resistance in bacteria of the genus *Enterobacteriaceae*[6]. Pathogenicity of *K. pneumoniae* is due to the presence of many virulence genes which encode virulence factors that allow it to attack the immune system of mammalians and cause many kind of diseases. Some of these virulence factors are: biofilm formation, hypermucoviscosity, capsule synthesis, adhesions, iron uptake and lipopolysaccharides formation[7,8]. *K. pneumoniae* has been found capable to resist many antibiotics especially third generation cephalosporins like cefotaxime, ceftriaxone and Ceftazidime[9]. Many clinical features of *K. pneumoniae* infections are related with virulence gens according to number and mode of action of these genes[10]. A hazardous level of histamine is produced by the microbial decarboxylation and Enteric bacteria have been reported to be the dominant histamine-producing bacteria (HPB) *Klebsiella pneumonia*e as the primary histamine-forming microorganism[11].

## MATERIALS AND METHDS

**Sample collection and identification**

Fifty-one samples of sputum collected from patients suffering from respiratory problem in the advisory clinic for chest and respiratory disease Basra city. The samples were cultured by the streaking on blood agar and MacConkey agar and then incubated at a 37°C for 24 hours. The bacterial colonies was purified and identified according to colonies morphology , color and size .The colony was cultured on Niven's agar medium. The plate were incubated at 37°C for 48hrs. purple colony on Niven's medium is indicator of Histamine Producing Bacteria ( HBP) according toNiven's *et al*., 1981[12].

**Identification of *hdc* gene**

Bromophenol blue 3ml was added to the DNA solution. The bands of DNA examined under UV.

The fragment of the *hdc* gene (709 bp) was amplified by Primers *f -hdc* (5´- TCH ATY ARY AAC TGY GGTGAC TGG RG -3´) and *r -hdc* (5´- CCC ACA KCA TBA RWG GDG TRT GRC C -3´)[13,14]. Twenty five micro litter of master mix PCR (50U/ml) *Taq* DNA polymerase , 400 µM of each of nucleotides (promega). Twenty nanogram of DNA and 75 Peg mole of reface and forward primers. The PCR processes were run for 40 cycles at 94°C for 1 min , 54 °C for 1 min and 72°C for 1 min ( Theromcycal).Agarose 1% in 1ˣ TBE buffer (89 mMtris-borate , 2 mM EDTA, (PH 8.3).The PCR products were run at 86 V for 1 hr . Ethidium bromide (0.3µg/ml) were added to gels to be visualized bunds and UV transit- laminator were used for gene bands imaging.

**Gene expression *hdc* detection by HPLC technique**

Histamine was estimated by High performance liquid chromatography technique HPLC that produced by bacterial isolates the concentration of histamine based on absorption and wavelength[15].

**Antibiotic Susceptibility Test**

Antibiotic resistance of bacteria of *Klebsiella pneumonia* was determined by MIC the Bio Meraux company according to16 antibiotics resistance profile.

**Factors affecting Histamine production**

**PH effect:** was tested by using two different Niven´s medium pH( 6 and 6.5) . Each one inoculate with loopful of culture and Incubate at 37°C for 24 hrs. and then measured histamine production by HPLC system[15].

**NaCl affect:** was tested by using two different Niven´s medium NaCl(0.25% and 1mg) . Each one inoculate with loopful of culture and Incubate at 37°C for 24 hrs then measured histamine production by HPLC system[15].

**Temperature affect:** was tested by using two different Niven´s medium temperature (35°C and 40°C) each one inoculate with loopful of culture and Incubate at 37°C for 24 hrs then measured histamine production by HPLC system[15].

## RESULTS

Fifty-one samples of sputum collected from patients suffering from respiratory problem in the advisory clinic for chest and respiratory disease and 255 isolates, fifty (50) isolates were identified by their morphology and biochemical characteristics as *Klebsiella pneumoniae*. Morphology of *Klebsiella pneumoniae* identified were large, dome-shaped, mucoid colonies on blood agar and lactose fermenting colonies on MacConkey agar. Microscopically appear as gram-negative, short, plump, straight rods were seen. The biochemical characters identified were negative indole test, negative methyl red test and positive for Voges-Proskauer test, citrate utilization test, urease test, acid and abundant gas production from glucose, lactose, sucrose, maltose and mannitol sugar fermentation tests and only 11 of *Klebsiella pneumoniae* were positive histamine production and by 21.56%. Histamine producing bacteria HPB were detected according to colorimetric

change from green to violet color by Niven´s medium as shown in Figure 1. The number of *Klebsiella pneumoniae* isolates as non- HPB 40 out of 51 isolates (78.43%).

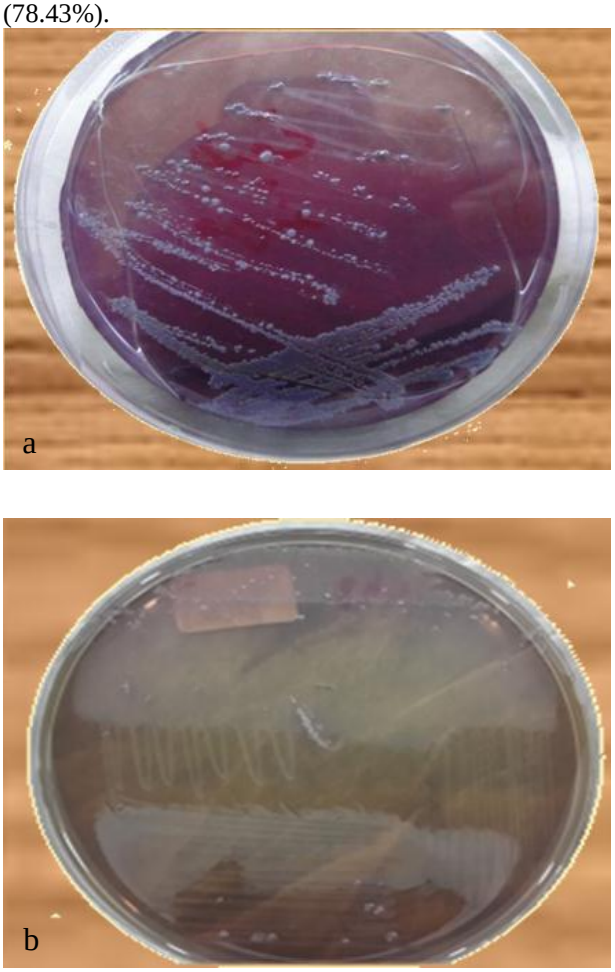



Figure 1. ***Klebsiella pneumoniae*** **isolated grown on Niven's media (a): Positive bacteria HPB, (b):Negative bacteria Non HPB.**

The presence of histamine producing bacteria can be recognized by the formation of purple halo colonies on Niven's agar and has been confirmed in PCR for the presence of the gene histadine decarboxylase *hdc* as shown Figure 2 which has the ability to convert histidine to histamine.

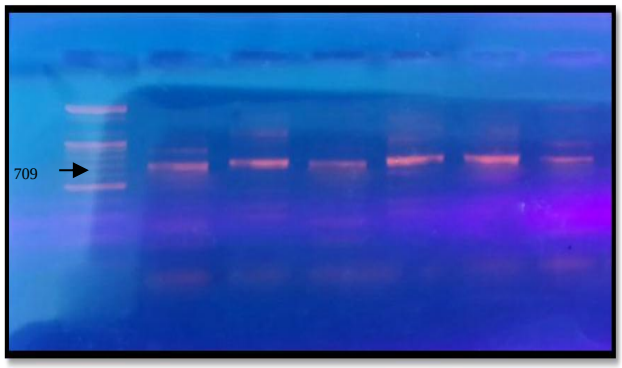



Figure 2. **Positive Histamine producing bacteria isolates of *Klebsiella pneumoniae* for 709pb gene bands.**

The level of histamine was estimated by HPLC technique the concentration of histamine in the *Klebsiella pneumoniae* was 46.06 µg/ml as shown in Figure 3.

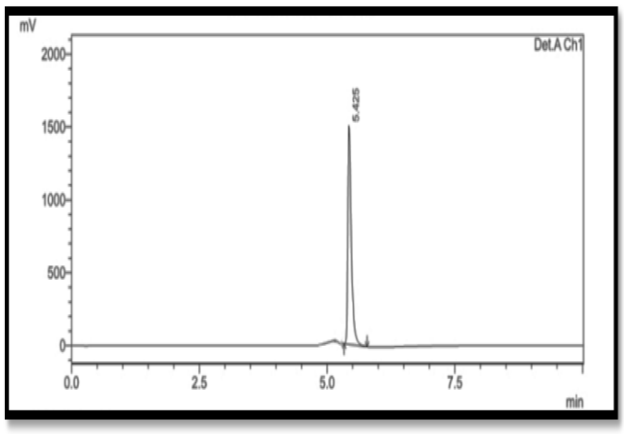



Figure 3. **HPLC analysis for histamine concentration detection of *K. pneumonia*.**

## Antibiotic Susceptibility Test by MIC

Results are commonly reported as the Minimal Inhibitory Concentration (MIC), which is the lowest concentration of drug that inhibits the growth of the organism. An antibiotic is a kind of ubiquitous contaminant in the aquatic environment with industrial effluents and sewage discharge. The bacterial isolates were exposed to 16 antibiotics for susceptibility testing.

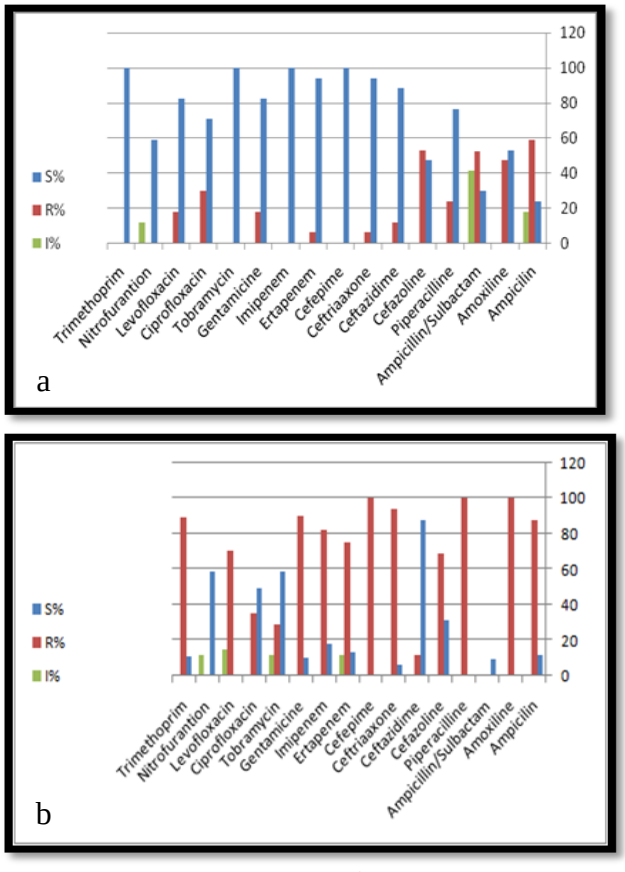



Figure 4. **Sensitivity and resistance of the *Klebsiella pneumonia* towards various antibiotic, a: Normal flora HPB b: Normal flora non- HPB.**

Antibiotic sensitivity and resistance test of 16 confirmed *K. pneumoniae.* This study showed that *Klebsiella pneumonia* histamine production highest degree of sensitivity against to Cefepime, Imipenem, Tobramycin and Trimethoprim 100%, while the isolates had been multidrug resistance Ampicilin 58.82%, Ciprofloxacin and Nitrofurantion(29.41%); respectively; as shown in Figure 4 a .*Klebsiella pneumonia* Non- histamine production, all of these antibiotics were categorized into three categories on the basis of their sensitivity. Results of one group had strains which were susceptible to Ceftazidime 88.23% and Aminoglycosides 58.82%. The second group had strains which were intermediate to antibiotics (Tobramycin and Levofloxacin). The third group contained strains which were resistant to synthetic penicillins(Amoxicillin, piperacillin) to 100% shown as Figure 4 b.

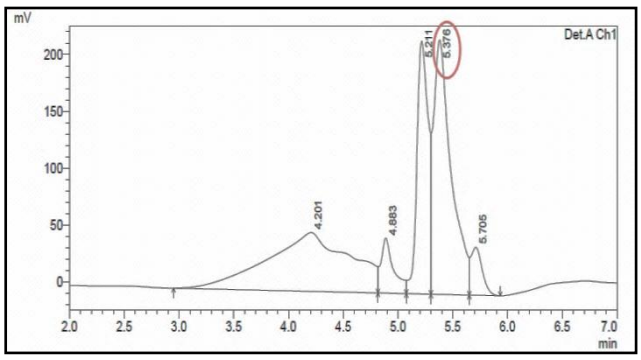
**Factors affecting Histamine production**

There are factors that affect the ability to produce histamine ( pH, NaCl and Temperature).

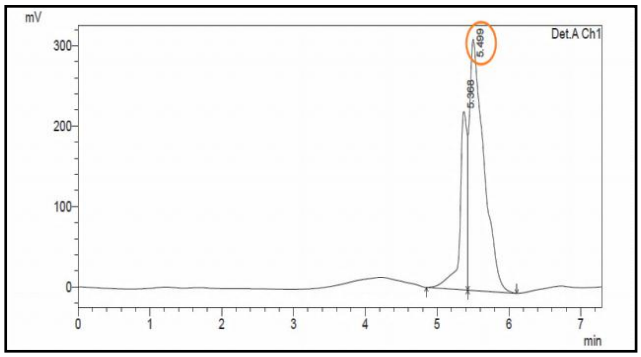
Table 1. **Factors affecting the concentration of histamine in histamine-producing bacteria**

| Isolates | PH | | NaCl mg | | Temperature | |
|---|---|---|---|---|---|---|
| HPB µg/ml | 6 | 6.5 | 1 | 0.25 | 35°C | 40°C |
| *K.pneumoniae* | 16.14% | 29.04% | 2.7% | 21.5% | 20.4% | 16.19% |

The results showed in the Table 1 factors affecting the production of histamine in HPB. *K .pneumoniae* had been the highest histamine concentration 29.04% µg/ml in the pH6.5 and , while in the NaCl 1 mg had been lower 2.75% µg/ml shown as Figure 5.




A

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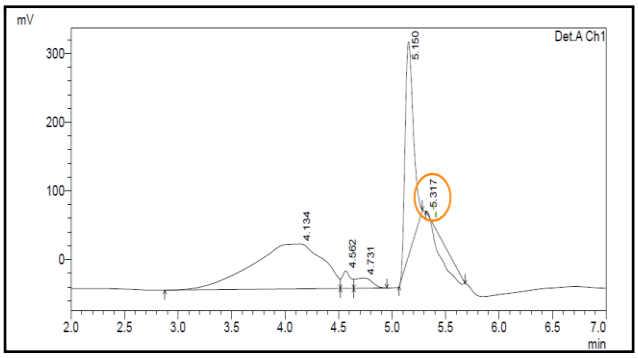


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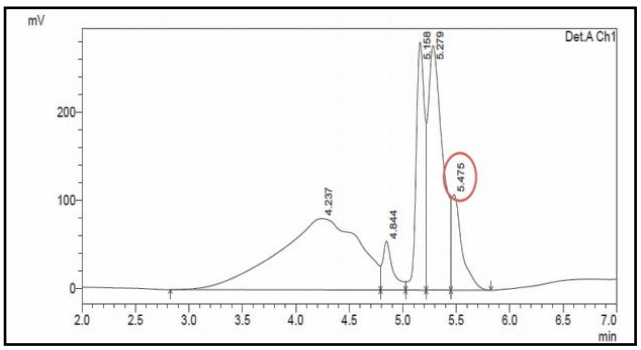


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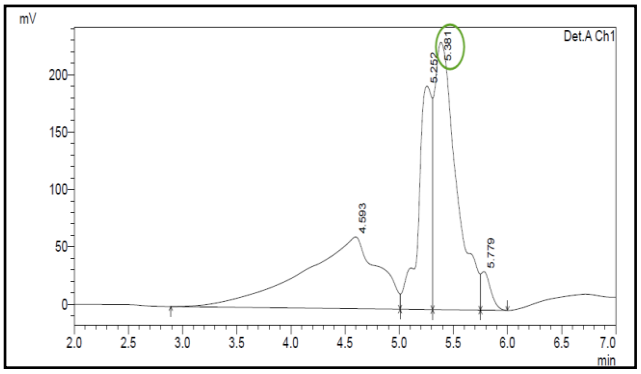


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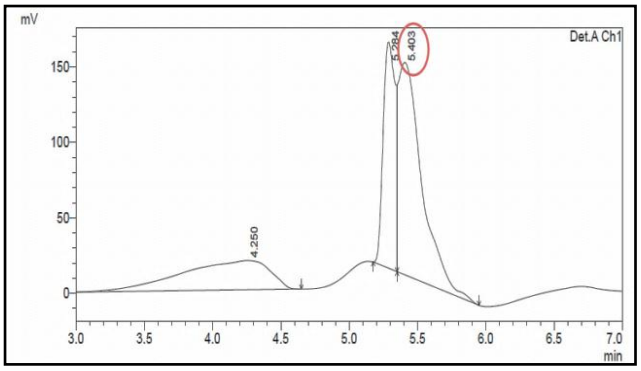


F

Figure 5. **Factors affecting the concentration of histamine by HPLC analysis in *K . pneumonia* A: Effect of(pH 6.5),B: Effect of(pH 6.), C: Effect salts Nacl(1 mg), D: Effect salts Nacl (0.25 mg),E: Effect temperature( 40 °C)and F: Effect temperature35°C.**

## DISCUSSION

This results showed that the isolates can change the color of Niven´s medium, because presence of Purple Bromocresol and PH change due to the activity of histidine decarboxylase enzyme that convert acidic histidine to alkaline histamine[16] which exploit the change of pH due to histamine formation and consequently the change in the color of the medium[17]. Results were also agreed with Bjornsdottir, K. and *et al*[18], which used primer to determine the histamine *hdc* gene and found its size was 709bp. It was also found that all the bacteria that gave positive results in the detection of the bacteria producing histamine were given as a result of gene *hdc* amplification that responsible for production of histamine.

Bacteria species belonging to the *Enterobacteriaceae* family play a role in the reproducibility of biogenic amines, may be this is part of the amines fed[19.20]. The present study is agreed with the results of[21] showed that bacterial histamine producing can be classified into two groups : bacteria have producing a huge quantities of histamine (> 100mg / 100 ml) as *Klebsiella pneumonia* and *Morganella morganii*and others species that produce less amounts of histamine (< 25 mg / 100 ml) as *E. coli* and *Citrobacter freundii.* The present study agreed with many research[22] the less concentration of some microorganisms like *E.coli* and *Citrobacter freundii* that histidine conversion into histamine depends on their enzyme action and the ability to grow in environment. Affecting factors that assist in producing histamine were studied like pH, NaCl and temperature. Acording Figure 4 a and b. In vitro data showed a wide range of beta-lactams, aminoglycosides, Flouroquinolcin and other antibiotics which are useful for treatment of *Klebsiella* infections[23,24,25]. Both Gram positive and Gram negative bacteria have cell walls which is composed of heavily cross-linked peptidoglycan layers which are stimulated by cell-wall transpeptidases also known as penicillin binding protein(PBP). β-lactam antibiotics disturb peptide bond formation by acting as competitive inhibitors to these PBPs. These result in formation of irreversible covalent bonded penicilloyl-enzyme complexes with weak cross-linked peptidoglycans, thus ease bacteria lyses and death[26]. The antibiotic treatments for *K. pneumoniae* infections are βeta-lactams as carbapenems and cephalosporins, aminoglycosides such as quinolones and gentamycin. The treatments are ineffective toward some isolates of *K. pneumoniae* that have resistance mechanisms[27]. *K. pneumoniae* contain two resistance mechanisms: enzymes production and formation of biofilm. Resistance had been shown against beta-lactams, sulfa methoxazoles, carbapenems, fluoroquinolones, trimethoprim, and aminoglycosides[28]. Research have shown that antibiotic abuse develop to selective increasing β-lactam resistance in bacteria of the genus *Enterobacteriaceae*[29]. Plasmid encoded resistance to broad spectrum cephalosporins is becoming a widespread phenomenon in clinical medicine. These antibiotics are destroyed by an array of different extended spectrum β-lactamases (ESBLs). It had developed by mutation of TEM/SHV type β-lactamases. Plasmids coding these enzymes has been encountered in many species of the enterobacteriaceae but are often harbored by *K. pneumoniae*[30].

The results[31] has been confirm that stress of the oxidation−reduction potential the reason for production of amines to enhanced the conditions to the effort to produce histamine and effectiveness of the enzyme HDC discouraging the presence of oxygen, also amines formed is strongly affected by temperature between 20-37°Care typical for bacterial growth of most those containing the enzyme remover carboxylaes and found the low temperature leads to growth reduce.

The present results have agreed with many studies[32,33] of that factors affect the growth of bacteria producing histamine and the effectiveness of the carboxyl groups enzyme and on the accumulation of histamine such as temperature incubation and pH of the medium factors. The increasment of NaCl concentration at least from histamine accumulation this agreed with[31]. A number of researchers found[34,35,36] that the degree of mild temperatures can done for the bacterial growth of histamine production in less than 3–4 hrs, studies also showed that histamine cannot break degree cooking temperature. It was found that increasing the proportion of histamine at reduced pH also was found that reduced histamine concentration if the medium treated with 5% NaCl concentration. Histamine producing bacteria is able to grow in a range of temperature[37].

## CONCLUSIONS

The *K. pneumonia* have a new virulence factor it can be cause or enhanced pathogenicity. The presence of new virulence agents in the bacterial normal flora is their ability to produce histamine, making it one of the causes of the irritable bowel disease. *K .pneumonia*have high sensitive against many antibiotics Trimethoprim, while the overall resistance pattern high resistance Ampicilin, Ambicillin/Sulbactam.

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