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Isolation and molecular identification of Klebsiella pneumonia by polymerase chain reaction PCR in Human and Cattle

A Thesis Submitted to the Council of Veterinary Medicine College / Al-Qadisiyah University in Partial Fulfillment of the Requirements for the degree bachelor's degree in veterinary Science

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Abstract

Klebsiella pneumonia was the most important nosocomial infections pathogen. It was causing several morbidity and mortality in a sick animals and human. Its identification and detection were performed by usage of conventional cultural characters, biochemical tests and Polymerase chain reaction technique (PCR). One hundred clinical samples, were divided into (50) samples of cattle suffering from pneumonia and (50) samples of human with respiratory infection, were collected from different regions of Al.Diwaniya city in period between 1/10/2017-1/2/2018 of the K.disease between cattle and human.the positive results 34 (68%) cattle samples were positive for *K. pneumonia* identification, while 38 (76%) sputum samples of human with respiratory cases gave K. *pneumoniae* isolates. The results show only (72) isolates were identification by PCR technique *K.pneumoniae* in Al.Diwanniya.

The results of this study in revealed that the pcr technique had a high specifity in detection of Klebsiella pneumonia especially the comparison percentage

Between human and cattle by using 16SrRNA gene partial sequence, compared to cultural, biochemical testing in addition that this technique conform that source of infection in cattle and human.

Introduction

In the past decade, geographic differences have been recognized in the spectrum of disease caused by Klebsiella pneumoniae. These differences include a preponderance of severe invasive disease in Taiwan and other parts of Asia [1–8].

A characteristic syndrome has emerged in which liver abscess is accompanied by K. pneumoniae bacteremia and sometimes by endophthalmitis or meningitis [7].

This is typically a community-acquired infection that occurs in patients with diabetes mellitus. Reports of this syndrome from North America, Europe, and Australia are uncommon [2]. Additionally, K. pneumoniae has long been recognized as a possible cause of community-acquired pneumonia. Over the past 2 decades, K. pneumoniae has been an exceedingly rare cause of community-acquired pneumonia in North America, Europe, and Australia [2, 9, 10].

Yet, it remains an important cause of severe community-acquired pneumonia in Asia and Africa. In these regions, patients also have classic risk factor of alcoholism [11 [2].

In this study, we performed molecular detection of K.pneuonia by PCR polymearase chain reaction to calculate the prevalence of K. pneumonia in cattle as well as in human .diwaniyah province.

Aim of the study

- To investigate the spreading of *K pneumonia* among Human.
- To investigate the spreading of *K pneumonia* among cattle & its zoonotic importance.
- To evaluate some epidemiological features of *K. pneumonia*.
- Molecular characterization of *K.pneumonia* by PCR by using specific primer for detection of *k .pneumonia*

Chapter two Literature review

2.Literature review

2-1. The causative agent

Klebsiella pneumoniae is a Gram-negative, nonmotile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped bacterium. It appears as a mucoid lactose fermenter on MacConkey agar.

Although found in the normal flora of the mouth, skin, and intestinesit can cause destructive changes to human and animal lungs if aspirated (inhaled), specifically to the alveoli (in the lungs) resulting in bloody sputum. In the clinical setting, it is the most significant member of the *Klebsiella* genus of theEnterobacteriaceae. *K. oxytoca* and *K. rhinoscleromatis* have also been demonstrated in human clinical specimens. In recent years, *Klebsiella* species have become important pathogens in nosocomial infections. [1]

It naturally occurs in the soil, and about 30% of strains can fix nitrogen in anaerobic conditions. As a free-living diazotroph, its nitrogen-fixation system has been much-studied, and is of agricultural interest, as *K. pneumoniae* has been demonstrated to increase crop yields in agricultural conditions. [3]

Members of the *Klebsiella* genus typically express two types of antigens on their cell surfaces. The first, O antigen, is a component of the lipopolysaccharide(LPS), of which 9 varieties exist. The second is K antigen, a capsular polysaccharide with more than 80 varieties. Both contribute to pathogenicity and form the basis for serogrouping.[4]

2-2 Classification

Klebsiella has been known as human pathogen since it was first isolated in the late nineteenth century by Edwin klebs [1], and it's a genus belongs to the family *Enterobacteriaceae* which is Gram-negative straight rods bacteria, arranged singly, in pairs or short chains, and slightly shorter than other *Enterobacteriaceae* [2].

A wide repertoire among the virulence and resistance factors is present about *K. pneumoniae* genome allowing for the expression of capsule, siderophores, adhesins, and antimicrobial determinants [3]. Infections caused by *K. pneumoniae* can result in serious and life threating infections including pneumonia, urinary tract infections, intravascular line infections, soft tissue infections, intraabdominal infections and bacteremia [4].

Serotyping is one of the typing techniques used to identify microorganisms of same species that can vary in the antigenic determinants manifested on the cell surface .Capsule is a major virulence [5].

K. pneumoniae on a MacConkey agar plate
Scientific classification
Domain: Bacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Enterobacteriales
Family: Enterobacteriaceae
Genus: Klebsiella
Species: K. pneumoniae
Binomial name

Although found in the normal flora of the mouth, skin, and intestines,[1] it can cause destructive changes to human and animal lungs if aspirated (inhaled), specifically to the alveoli (in the lungs) resulting in bloody sputum. In the clinical setting, it is the most significant member of the Klebsiella genus of the Enterobacteriaceae. K. oxytoca and K. rhinoscleromatis have also been demonstrated in human clinical specimens. In recent years, Klebsiella species have become important pathogens in nosocomial infections[15]

2-3 Clinical significance

As a general rule, *Klebsiella* infections are seen mostly in people with a weakened immune system. Most often, illness affects middle-aged and older men with debilitating diseases. This patient population is believed to have impaired respiratory host defenses, including persons with diabetes, alcoholism, malignancy, liver disease, chronic obstructive pulmonary diseases, glucocorticoidtherapy, renal failure, and certain occupational exposures (such as papermill workers). Many of these infections are obtained when a person is in the hospital for some other reason (a nosocomial infection). Feces are the most significant source of patient infection, followed by contact with contaminated instruments [11]

in pneumonia, typically in the form of bronchopneumonia and also bronchitis. These patients have an increased tendency to develop lung abscess, cavitation, empyema, and pleural adhesions. It has a death rate around 50%, even with antimicrobial therapy. [5]

In addition to pneumonia, *Klebsiella* can also cause infections in the urinary tract, lower biliary tract, and surgical wound sites. The range of clinical diseases includes pneumonia, thrombophlebitis,

urinary, cholecystitis, diarrhea, upper respiratory tract infection, wound infection, osteomyelitis, meningitis, and bacteremia and septicemia. For patients with an invasive device in their bodies, contamination of the device becomes a risk; for example, neonatal ward devices, respiratory support equipment, and urinary catheters put patients at increased risk. Also, the use of antibiotics can be a factor that increases the risk of nosocomial infection with *Klebsiella* bacteria. Sepsis and septic shock can follow entry of the bacteria into the blood.

2.4. Culture and metabolic characteristics Klebsiella

species are easily cultured on media suitable for Enterobacteriaceae bacteria, including: nutrient agar, tryptic casein soy agar, bromocresol purple lactose agar, Drigalski agar, MacConkey agar, eosin-methylene blue (EMB) agar and bromothymol blue (BTB) agar [61]. No additional growth factors are required by K. pneumoniae, which is capable of both fermentative and respiratory metabolism [54]. The facultative anaerobe can have a variable mucoid appearance, which may vary between different strains and be influenced by the composition of the medium used [54,61]. Useful tests in determining enterobacterial taxonomy include carbon source utilisation Most strains can hydrolyse urea, reduce nitrates without the production of H2S gas, as well as utilise glucose and citrate as carbon sources [54]. In the case of fermentation of glucose, a gas and an acid are produced [25]. Glucose fermentation also results in the formation of acetoin and 2,3-butanediol [6]. review

2-5 Virulence factors and the role in pathogenesis of K. pneumoniae

The significant impact of K pneumoniae in the clinical setting as a healthcare-associated pathogen has prompted investigation into the factors

implicated in its pathogenesis [7]. The factors aiding in basic pathogenesis of K. pneumoniae are the fimbrial and non-fimbrial adhesins, a capsule, siderophores (particularly enterobactin), urease, lipopolysaccharide (LPS), serum resistance as well as biofilm formation [7,8,9].

On the other hand, enhancing factors aiding invasion include other siderophores (aerobactin and yersiniabactin), The prerequisite to an infection is often the mucosal pathogen's ability to adhere [7,39]. Klebsiella pneumoniae expresses numerous fimbrial and non-fimbrial adhesins capable of recognising varied receptors, which in turn can facilitate the adherence to several target cells [7]. Fimbrial adhesins include mannose-sensitive type 1 fimbriae, type 3 fimbriae and plasmidencoded fimbriae designated as KPF-28, while a non-fimbrial adhesin includes the CF29K factor [7,38].

The above mentioned type 1 and type 3 fimbriae are frequently detected in K. pneumoniae isolates, particularly mediating urinary tract infections (UTIs) and biofilm formation, respectively [8,40,41]. The expression of the various fimbriae can be both beneficial in that it may facilitate attachment or disadvantage the bacterium due to the heightened host immune response that may be triggered, thus outlying the opportunistic nature of K. pneumoniae [7]. Surface saccharides that have been associated with K. pneumoniae virulence in a human host include an LPS and capsule [42]. Capsules can play an important role outside the human host by offering some protection against desiccation in the environment or in the host by resisting complement-mediated lysis or phagocytosis and possibly having a neutralising effect against antibodies through the release of excessive capsular material [42,43].

At least 78 antigenically varied capsular types have been identified in K. pneumoniae [1,44] Resistance to phagocytosis was found to be higher in K1 and

K2 capsular serotypes [1,47]. Particular types may also play a more significant role in virulence, such as the K2 capsule, which has frequently been isolated from clinical isolates implicated in urinary tract infections, pneumonia and bacteraemia [7,46,]. The LPS is, on the other hand, a component situated in the outer membrane of bacteria and part of it forms the O-antigen of which there are only 12 differing antigens [50]. Finally, the growth of K. pneumoniae in vivo necessitates essential elemental iron for which it competes with the host by producing high-affinity extracellular ferric chelators (iron-binding molecules) [7,48]. A hypervirulent strain of K. pneumoniae was found to possess greater quantities of biologically active siderophores [1,50].

Chapter three The materials and method

3. Materials and methods:

3-1 Materials

3-1-1 Equipments and Instruments

Table (3-1) Equipments and instruments used in this study

No.	Equipment & instrument	Company
1	Autoclave	Memmert/Germany
2	High Speed Cooled centrifuge	Eppendorf /Germany
3	Oven	Memmert/Germany

4	Sensitive Balance	Sartorius/Germany
5	Water Bath	Memmert/Germany
6	Vortex	CYAN/ Belgium
7	Micropipettes 5-50, 0.5-10, 100-1000µl	Epprndorf/ Germany
8	Nanodrop	Thermo/ USA
9	Refrigerator	Concord /Lebanon
10	Eppendorf tubes	Bioneer/ korea
11	Disposable container 50 ml	Sterile EO. / China
12	Exispin vortex centrifuge	Bioneer/ korea
13	MyGene PCR thermocycler	Bioneer/ Korea
14	Rack tube	Biobasic/USA
15	Cooled Box	china
16	medical Gloves	Fisher/USA
17	Camera	Samsung /China

3-1-2. Diagnostic Kits:

No.	Kits	Company	Country

	AccuPower ™ PCR Premix	**Bioneer**	**Korea**
	Taq DNA polymerase		
	dNTPs (dATP, dCTP, dGTP, dTTP)		
	Tris-HCl pH 9.0, KCl, $MgCl_2$		
	Loading dye		

Table (3-2): shown the main diagnostic kits that used throughout this study with their companies and countries of origin:

3-1-3. Chemical Materials

Table (3-3) Chemical materials used in this study

.	emical Material	nufacturer	igin
	solute Ethanol	co	Jordan
	arose	Basic	Canada
	idium Bromide	Basic	Canada
	e Nuclease Water	lab	USA
	E Buffer	Basic	nada

3-1-4 Primers

Two PCR primers were used in this study for identification and genotyping of *E. granulosus* based on sequencing of two mitochondrial genes that amplified by using PCR technique. These primers were designed as in

Nikmanesh *et al*., (2014) and provided by (Bioneer Company, Korea) as following table: Table (3-4): The Primers that used in the study

nplicon	quence	Primer
gyrB	AA GTC ATC ATG ACC GTT CTG CA)	**1256bp**
	C AGG GTA CGG ATG TGC GAG CC)	

3-2.Methood

3-2-1. Samples collection:

One hundred samples divided in to fifty fecal of cattle were collected from cattle suffered from diarrhea and fifty samples of human suffered from diarrhea in different sites of Al- qadissyiah province. These samples were placed in cold container and transported to bacteriology Lab. in Vet Med. College for isolation and identification of *k. pneumonia*.

3-2-2 .Isolation and identification of *K.pnemonia*:

The samples were inoculated in nutrient, blood and MacConkey agar to isolate *k. pneumonia.* from other enterobacteriacea bacteria. Single pure colony were cultured on Orientation chrome agar and EMB agar to identification *k. pneumonia.*

From other lactose ferment enterobacteriacea. Biochemical tests were used to confirm *k. pneumonia* identification.

3-2-3. DNA extraction of bacteria genome:

Genomic DNA of *K. pneumonia isolates* was extracted by using (PrestoTM Mini gDNA Bacteria Kit. Geneaid. USA). 1ml of new bacterial growth (for overnight) in nutrient broth was put in 1.5ml microcentrifuge tubes. These tubes were centrifuged at 10000 rpm for 1 minute. The supernatant was removed and the bacterial cells pellets were used to extract the genomic DNA according to company instruction. Nanodrop spectrophotometer was used to check the concentration and purification of the extracted DNA and storage it in -20°C at freezer until perform PCR assay.

3-2-4. Polymerase chain reaction (PCR):

Specific primer were using in PCR assay to detect *k. pneumonia* the housekeeping gene *gyrB* ,F (GAA GTC ATC ATG ACC GTT CTG CA) and R(AGC AGG GTA CGG ATG TGC GAG CC) with product size ١٢٥٦bp and specific primer (GGAGTTAGTGCAGCCTCCAG) primers, were designed by NCBI-GenBank, [54] were provided by (Bioneer Company. Korea) used to amplify a 1256bp fragment of highly conserved regions gyrB gene of Preparation of PCR master mix according to (Bioneer. Korea). PCR premix tube contains freeze-dried pellet of (Taq DNA polymerase 1U, dNTPs 250µM, Tris-HCl (pH 9.0) 10mM, KCl 30mM, MgCl2 1.5mM, stabilizer, and tracking dye). PCR reaction was acted by added 5µl of purified genomic DNA and 1.5µl of 10pmole of forward primer and 1.5µl of 10pmole of reverse primer to PCR premix tube and complete the volume into 20µl total volume by deionizer PCR water according to kit instructions. Briefly, it mixed by Exispin vortex centrifuge (Bioneer. Korea). The reaction was performed in a thermocycler (Mygene

Bioneer. Korea) by set up the following thermocycler conditions; A total of 30 cycles of amplification was performed with template DNA denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and primer extension at 72°C for 2 finally extension at 72 °C for 10 min for *Gyr B* .and 95 °C initial denaturation temperature for 5 min ; next 30 cycles at 95 °C denaturation for 30 s , annealing 58 °C for 30 s, and extension 72 °C for 1min and finally extension for 10 min .The PCR products were examined by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide, under UV transilluminator.

4-The Result:

4-1.Isolation and identification:

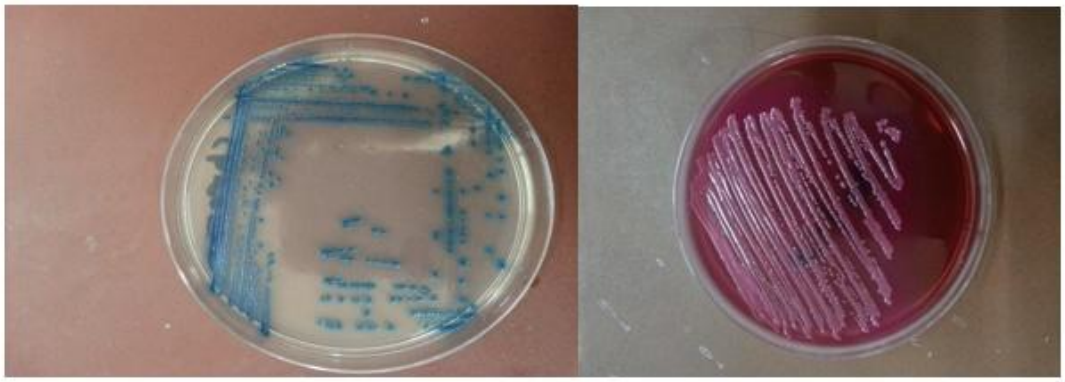
Cultural characters of *K. pneumonia* growth on macConky and Orientation media showed in table (1). Pink ,mucoid ,lactose fermented colonies were considered to be *Klebsiella* spp on MacConky agar while on orintation medium, colonies is metalic blue color, large ,rounded.(Figure 1). These isolates were positive citrate utilization and catalase while negative for H_2S production and oxidase reaction.[12].

Table (1): identification of *Klebsiella Pneumonia* by biochemical test.

Bacteria	Biochemical test														
	Cat.	Oxi.	Ind	MR.	VP.	Urs.	Cit.	H_2S	Moti.	Gel.	Nit.	Glu.	Lac.	Suc.	Man.
K.pneur	+	-	-	-	+	+	+	-	-	-	+	+	+	+	+

Y=yellow . H_2S=sulfurehydrate production, EMB=Eosin Methyline Blue.

Table (2): cultural characters of *Klebsiella Pneumonia* on selective media



Figure(1): Metallic blue ,rounded and large colonies of *Klebsiella pneumonia* on Orientation chrome agar and MacConkey agar.

Bacterial isolates	**EMB**	**Triple Sugar Iron(TSI) Slant/butt**	**MacConky agar**	**Orientation chrome agar**
Klebsiella Pneumonia	**purple**	**y/y, gas, no H_2S**	**pink coloines**	**metallic blue color**

Thirty four (68%) samples from50 nasal swap collected from sheep suffering from respiratory singes were positive for *K. pneumoniae* ,while 38 (76) of human with sputum cases gave *K. pneumoniae* isolates table (2).

Detection of K.pnemonia gyrB

17(50%) three isolates from total 34 isolates were positive to Detection of gyrB gene, these positive isolates divided to 19 isolate (50%) for human from total human 38 for detection of were performed by PCR by usage specific primer sequences with product sizes of 1256 bp. (Figure 4) . In this study we investigated the epidemiology of K. pneumoniae in a defined geographic area that

included a general hospital and surrounding canals and farms. Our findings support the suggestion that clinical K. pneumoniae have evolved mechanisms to better adapt to survival in the clinical setting. Virulence factors were more frequent in clinical isolates and had been acquired on more than one occasio

theses results of human more appear than of sheep because of prevalence k.pnemonia appear as nosocomial pathogen in hospital .while the disease was also prevalence in diseased sheep

5.1 Conclusions:

1-*K.pnemonia* was main cause respiratory signs that most of them were aged persons and the time of spreading was related to the pilgrimage season.

2- *K.pnemonia* was widely speeded in cattle which were mainly apparently diseased that mean camel play a role in the zoonotic transmission as a reservoir or intermediate host

3-The bacteria more prevalent in adult animal than young &there were geographical variation & high infection rate in the western borders

5.2: Recommendations

1. Sequencing of k.pnemonia in cattle and human
2. Phylogenetic tree of K. Pneumonia and efficient laboratory techniques for accurate detection & design a data base k.pnemonia in Iraq
3. Further molecular study and full genome sequencing & study the role of alteration the sequence of genome especially all virulence gene & the effect of mutation on the bacteria transmission from human to human in addition to investigation of drug efficiency on the bacteria

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