Republic of Iraq

Ministry of Higher Education and Scientific Research

University of Diyala

College of Veterinary Medicine

Study on Multiple Drug Resistance of *Klebsiella pneumoniae* Isolated from Human and Domesticated Animals

A Thesis

Submitted to the Council of the College of Veterinary Medicine University of Diyala in Partial Fulfillment of the Requirements of the Degree of M.Sc. in Microbiology

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1442 A.H.

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Dedication

To the one who has the enlightened thought, who proudly bore his name, my dear father.

To the source of tenderness, the secret of existence , my big love mom.

To the one who has patience with me and continuously inspired me to fulfill my dreams for my loving and supportive husband.

To the most beautiful thing in my life my children.

To those who looked forward to my success, my brothers .

To all my relatives and loved ones.

Hadeel M. Oraibi

Acknowledgements

Thanks and gratitude first and foremost to ((ALLAH)) Who honored me by completing this studies.

I am heartily thankful to my supervisor Prof. Dr. Abdulrazak Shafiq Hasan for Suggesting the study, his guidance throughout my study, without his efforts this work cannot be achieved.

I would like to express my gratitude to my supervisor Prof. Dr. Amer Khazaal Al-Azzawi for all his guidance.

Thanks and appreciation to the dean of Veterinary Medicine college Prof. Dr. Talib Jawad Kazim, for all his support for post graduate students

I would like to express my thank to my role model Prof. Dr. Nazar Jabbar, Al-Khafaji , for all his guidance and for his support for my

Finally, I would like to thank everyone who helped me, and apologies to those I forgot to mention.

Summary

Antimicrobial resistance is a global threat , with deaths associated with Antimicrobial resistance(AMR) infections are expected to exceed 10 million cases per year by the year 2050. The overuse and misuse of antibiotics is the primary driver of this resistance, with up to 50% of antibiotics prescribed in the hospital setting being either unnecessary or inappropriate. *K.pneumoniae* is a Gram-negative, non-motile, encapsulated, lactose- fermenter, facultative anaerobic, rod-shaped in the family *Enterobacteriaceae*. *Klebsiella* organisms are often resistant to multiple antibiotics. Plasmids are implicated as the primary source of the resistance genes. *Klebsiella* species which producing extended-spectrum beta-lactamases are resistant to virtually all beta-lactam antibiotics, except carbapenems. Aminoglycosides, Fluoroquinolones, Tetracycline, Chloramphenicol, and Trimethoprim/Sulfamethoxazole represent other frequent resistance targets. The Carbapenems resistant *K. pneumoniae* is emerging as an important challenge in health-care settings.

This is a cross sectional study conducted for the period from October 2019 to April 2020. The study included 293 human and animal samples. Human samples were obtained from patients suffering from different infections; 112 urine, 22 sputum, 8 burns, 11 wounds 22 stool and 7 blood samples. The age range of patients was 1-65 years; 77 were males and 105 were females. Human specimens were collected from Baquba Teaching Hospital, Al-Batool Teaching Hospital for Maternity and Children and from other health care centers. Human privacy was respected as verbal consents of patients were obtained. Whereas, animal samples were; 55 stool, 23 milk and 33 urine samples from different animal species (cow, sheep, goat and chicken).

Animal specimens were collected from several fields of animal husbandry poultry farms in the cities of Bani Saad, Baquba and from Veterinary clinic in Buhriz district. These samples were submitted for standard bacteriological culture using ordinary and selective media. Plates were incubated at 37^0C for overnight. Morphological inspection, microscopically examination, biochemical tests, and VITEK2 system were used to confirm bacterial identification. Statistical analysis was done on data accumulated throughout the study using SPSS, T-test & Chi square. P values less than 0.05 were considered significant.

A total of 41 confirmed isolates of *K.pneumoniae* were identified, 37 from human samples and 4 from animal samples. The antibiotic susceptibility test for *K. pneumoniae* isolates was performed against 14 antibiotics using disk diffusion method. All of the 37 human isolates (100%) were resistant to Rifampicin, Ampiclox and Methicillin. 16 (43.2%), were resistant to Ceftriaxone, 29 (78.4%) were resistant to Carbenicillin, 23 (62.2%) were resistant to Piperacillin, 15(40.5%), resistance to streptomycin, and 16 (43.2%) were resistance to Trimethoprim-Sulfamethozal. The rates of sensitivity to Imipenem Ciprofloxacin, Azithromycin, Levofloxacin, Nalidixic acid and Polymyxin B were (100%), 33 (89.2%), 31 (83.8%), 30 (81.1%), 21 (56.8%) and 25 (67.6%) respectively.

On the other hand, antibiotic susceptibility test for the 4 isolates of *K. pneumoniae* from animal were all(100%) resistant to Rifampicin, Ampiclox and Methicillin. Whereas, all isolates (100%) were sensitive to Ciprofloxacin, Imipenem, Azithromycin, levofloxacin and Polymyxin B. 3(75%) of the isolates were resistant to Carbenicillin and 2 (50%) were resistant to Trimethoprim-Sulfamethozal. Additionally, 1 (25%) of the isolates were resistant to Streptomycin, Nalidixi acid, Ceftriaxone and Piperacillin. The rate of multi-drug

resistance phenotypes in *K. pneumoniae* human isolates was (70.2%), while it was (75%) for animal isolates.

Polymerase chain reaction (PCR) technique was used in the detection of resistance genes in 30 isolates of *K. pneumoniae* (26 of human and 4 of animal) by using primers for three resistance genes (*bla- CTX-M, bla –OXA ,and StrA*). The findings showed that 15 human isolates had the *bla- CTX-M* genes, 2 isolates had *bla –OXA* genes, and 18 isolates had *StrA* gene. Whereas, only one isolate (25%) from animal samples had both *bla- CTX-M and StrA* genes, and all the isolates were devoid of the *bla- OXA* genes.

Automated DNA sequencer, for the resistance genes (*StrA , bla-OXA , bla-CTX-M*) of two isolates (one from human and the another from animal) were sequenced and compared with reference gene in Genbank. The DNA sequence of all the five resistance genes were 95-100% identical to DNA sequences of resistance gene from different species of *Enterobacteriaceae* bacteria and other bacterial species that were documented in the Genbank. Those five resistant genes diagnosed in the current study were recorded in the Genbank data bases.

The study concluded that multiple drug resistant(MDR) *K. pneumoniae* are prevalent among human population in Diyala community particularly in children and elderly hospitalized patients. Domesticated animals may play a role as an additional source of the MDR bacterium in the community.

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BP	**Base pair**
Bla- gene	**β-Lactamase gene**

CDC	**Centers for Disease Control**
CFU	**Colony forming unit**
CL	**Cell lysis**
CLSI	**Clinical and Laboratory Standard Institute**
CPS	**Capsular Polysaccharide**
CTX -M	**Cefotaximase, B-lactamase active on cefotaxime**
DNA	**Deoxyribose Nucleic Acid**
dNTP	**Deoxy nucleoside tri-phosphate**
EDTA	**Ethylene Diamine Tetraacetic Acid**
ESBL	**Extended spectrum beta lactamase**
KPC	***Klebsiella pneumoniae* Carbapenemase**
LPS	**Lipopolysaccharide**
MBL	**Metallo Beta-lactamase**
MDR	**Multi drug resistance**
MIC	**Minimum inhibitory concentrations**
MR/K-HA	**Mannose-resistant, *Klebsiella*-like hemagglutination**
OMPs	**Outer-membrane proteins**
OXA	**Oxacillinase b-lactamase active on oxacillin**
PBS	**Phosphate Buffered Saline**
PCR	**Polymerase Chain Reaction**
PCV	**Packed Cell Volume**

SHV	**β -Lactamase (Sulfhydryl Reagent Variable)**
TDR	**totally drug-resistant**
TEM	**β-lactamase named after the patient (Temoneira)**
UK	**United Kingdom**
UTI	**Urinary Tract Infection**
UV	**Ultra Violet**
WHO	**World health organization**
XDR	**extensively drug resistant**

Chapter One
Introduction

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Introduction

1.1. Preface:

Two main ways through which modern medicine saves lives are antibiotic treatment of serious infections and the success of surgical and medical procedures under the control of antibiotics (Nathan and Cars 2014). Antibiotics are the most important type of antimicrobial substances that either inhibit or kill the growth of bacteria. Antibiotics (e.g. penicillin) are those formed naturally by certain microorganisms battling others, whereas non-antibiotic antibacterials (e.g. sulfonamides and antiseptics) are fully synthetic, both are included in antimicrobial chemotherapy and both have the ability of killing or preventing the growth of bacteria (Leekha *et al*., 2011; Gould, 2016). However, ability of several bacterial pathogens to create resistance to antibiotics is the legacy of the golden era of antibiotic discovery, the 1930s -1960s (Nathan and Cars, 2014).

Antimicrobial resistance (AMR) is the capability of a microbe to resist the effects of antimicrobials which previously could successfully treat the microbe. The statement antibiotic resistance (AR) is a subset of AMR as it refers just to those bacteria that become antibiotic resistant. Resistant bacteria are harder to treat; requiring various or large doses of antibiotics. These Strategies may be toxic , expensive, or both. Bacteria resistant to multiple antimicrobials are called(MDR). Those considered extensively drug resistant (XDR) or totally drug-resistant (TDR) are sometimes called "superbugs" (WHO, 2015; CDC, 2017).

Antimicrobial resistance is global widespread problem, with AMR-related deaths expected to reach 10 million annually by the year 2050. The

overuse and misuse of antimicrobials is the primary cause of this phenomenon, with up to 50 per cent of antibiotic drugs prescribed in the hospitals settings being either unnecessary or inappropriate (de Kraker *et al.*, 2016 ;Tagliabue and Rappuoli, 2018). Worldwide disaster of antimicrobial resistance, potentially, has a devastating effect on global economy, human beings and the livestock. It has been predicted that over the next years ,300 million people will die from drug resistance (Editorial, 2014). This will knock catastrophically on the economy, reducing global gross domestic product (GDP) by 2 to 3.5% more than it should otherwise has been in 2050 (CDC, 2019).

At least some clinical isolates of many pathogenic bacterial species; *Mycobacterium tuberculosis, Neisseria gonorrhoeae, Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and species of *Enterobacter*, *Salmonella,* and *Shigella* are resistant to most antibiotics. The problem seems out of control (Nathan and Cars ,2014).

The *K. pneumoniae* is a Gram negative, non-motile, with large capsule, fermenting to lactose, rod shaped, also the bacterium is facultative anaerobic of the *Enterobacteriaceae* family. On MacConkey agar it appears as a mucoid fermenting lactose colony. Although is present as part of the normal residential flora of the intestines, skin and mouth, it can cause harmful changes to human and animal lungs if inspired, specifically to the air vesicles resulting in bloody jelly like sputum. *Klebsiella species*; in recent years, have become prominent pathogens in nosocomial infections (Li *et al*., 2014; Bing *et al*., 2019). In addition to community acquired or nosocomial pneumonia which are typically in the form of bronchopneumonia, *K. pneumoniae* can also cause urinary tract infections, lower ducts of bile infections, and surgical wound infections. Many clinical diseases, including upper respiratory tract;

infection of urinary tract (UTI), cholecystitis, pneumonia, thrombophlebitis; diarrhea, wound infection; meningitis, osteomyelitis can lead to (bacteremia ; sepsis and septic shock) that may result from bacteria entering the blood stream (Jung *et al*., 2012). Also *Klebsiella pneumoniae* can cause diarrhea especially in children (Siham *et al*., 2016) .

The *Klebsiella* organisms are frequently resistant to several antibiotics. Evidence suggests plasmid as the central source of resistance genes. *Klebsiella* species that have the capability to producing extended-spectrum beta-lactamases (ESBL) were resistant to almost total beta-lactam antibiotics, except carbapenems. Other common resistance targets include, fluoroquinolones, Aminoglycoside, Tetracycline, Trimethoprim-Sulfamethoxazole and Chloramphenicol(Nathisuwan *et al*., 2001; Hudson *et al*., 2014). Carbapenems-resistant *Klebsiella pneumoniae* (CRKP) is one of carbapenems-resistant *Enterobacteriaceae* (CREs), which are emerging as an important oppose in healthcare settings. A progressive rise in CRKP has been observed globally over the last 10 years and is possibly best known for an outbreak within the healthcare system; In the United States, the most popular carbapenems resistant is *Enterobacteriaceae* (Schwaber *et al* 2008; Limbago *et al*., 2011). Carbapenems-resistant *K.pneumoniae* is resistant to about all available antimicrobials, it's infections are responsible for high morbidity and mortality, especially in risky people and may need synergistic combination of antibiotics (Azza *et al*., 2010; Yu *et al*., 2019).

Mechanisms which cause carbapenems resistance include hyper-production of (*AmpC*beta-lactamase) with mutation in external porin membrane, *CTX-M* extended spectrum beta-lactamase (ESBL) in drug efflux or a mutation in porin, and the most important mechanism is carbapenemase production(*bla – KPC)*. The gene encoding to the enzyme *bla-kpc* is carried on a mobile piece of genetic material. Anastasia *et al*.

(2018), showed that antimicrobial resistance and virulence genotypes & phenotypes of *K. pneumoniae* isolated from the urine (30%); respiratory system (57%); wounds (5%); blood (3%); cerebrospinal fluid (4%) and rectal swab (1%) revealed that the majority (98%) were (MDR) strains carrying *bla-SHV* (91%); *bla-CTX-M* (74%); *bla-TEM* (51%); *bla-OXA* (38%) and *bla-NDM* (1%) beta-lactamase genes; class 1 integrons (38%); and the porin protein gene (*ompK36*) were (96%) . Genomic analysis to identify the sequencing of *K. pneumoniae* Producing (ESBL) were detected between swine and human source in and across abattoirs (Founou *et al*., 2018).

The hyper-virulent *Klebsiella pneumoniae* (hvKp) has raised as a more virulent worldwide pathogen as compared to classical *K. pneumoniae* that is capable to induce community-acquired(CA) infections in normal healthy persons . HvKp is carried in the gastrointestinal tract(GIT); which participates in its spread in the healthcare settings and society. The hvKp has spread around the world and caused variable infections, beside pyogenic liver suppuration; it has the potential to extend to distant sites, most frequently lung, eyes, central nervous system, illnesses of soft tissue and bacteremia. The hyper virulence genetic determinants are usually located on chromosomal mobile genetic elements and also in large virulence plasmids. These different virulence determinants contain up to four siderophore systems for iron acquisition; K1 and K2 capsule types; Increased liberation of capsules; the coli-bactin toxin; biofilm formation plus the hyper-mucoviscosity a descriptive phenotypic of hvKp. Worrying that these (MDR)hypervirulent strains have appeared as additional struggle in the fight against these dangerous pathogens (Choby *et al.*, 2020).

Even an outbreak of these Carbapenems-Resistant and hyper-virulent *K. pneumoniae* was recorded (Zhao *et al*., 2019).The wide variety of the gene for antimicrobial resistance and the higher rates of resistance to

antibiotics of *K. pneumoniae* e.g. carbapenems and colistin is indicative of an extremely mutable strain and highlights the urgency of infection control steps, continuous monitoring of antimicrobial resistance and the prudent use of antibiotics to avoid further selection of resistant isolates and the emergence of pan-resistant clones (Berglund *et al*., 2019).

1.2. Aims of the study:

This study was designed and conducted in Diyala province, for Isolation, characterization and purification of *K. pneumoniae* isolates from different types of clinical specimens of human and domesticated animals to achieve the following goals:-

1. Exploration of the antibiotic susceptibility of *K. pneumoniae* isolates to different antibiotic or antibacterial types agents.
2. Detection of certain genes associated with antimicrobial resistance of *K. pneumonia* via polymerase chain reaction technique.