بسم الله الرحمن الرحيم

Sudan University of Science and Technology

College of Graduate Studies

Multiplex Polymerase Chain Reaction Detection of *Klebsiella pneumoniae* Virulence Strains Isolated from Selected Drinking Water Sources in Khartoum State

الكشف عن تفاعل متعدد البلمره المتسلسل لسلالات كليبسيلا الالتهاب الرئوي الممرضه المعزوله من مصادر مياه الشرب المختاره في ولاية الخرطوم

A Dissertation Submitted in Partial Fulfillment of The Requirements of M.Sc in Medical Laboratory Science (Microbiology)

Submitted by

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الآيـة

بسم الله الرحمن الرحيم

(فَتَعَالَى اللَّهُ الْمَلِكُ الْحَقُّ وَلَا تَعْجَلْ بِالْقُرْآنِ مِنْ قَبْلِ أَنْ يُقْضَى إِلَيْكَ وَحْيُهُ وَقُلْ رَبِّ زِدْنِي عِلْمًا)

صدق الله العظيم

سورة طه- الاية 114

DEDICATION

I dedicate this work to my father, bless upon him, my mother who has been my constant source of support and love, my sisters, brothers, and my friends whom helped me in my life and gave me the force to continue.

Acknowledgments

All Thanks and gratefulness to my lord, ALLAH for helping me to complete this work.

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Abstract

Good health depend on a clean, drinkable water supply. This mean that water must be free of pathogens, *K.pneumoniae* is found in the environment and as a harmless commensal, but is also a frequent nosocomial pathogen and the agent of specific human infections.

This study is cross sectional study aimed to determine the frequency of virulence genes in the *K.pneumoniae* isolated from drinking water in Khartoum State (Omdurman, Khartoum, and Bahri) conducted in May to December 2019.

A total of 100 isolates were obtained from Central Public Health Laboratory in Khartoum. These isolates were isolated from drinking water in Khartoum State and subcultures in Eosin Methylene Blue (EMB) media, the isolates were then identified by routine biochemical tests, 25 (25%) 0f isolates were identified as *K.pneumoniae* . Multiplex Polymerase Chain Reaction (MPCR) was performed to detect the virulence genes (213, ent , Kfu , MRK, mag , K2and RMP). Most of the isolated bacteria were from tanke 12(48%), cooler 8(32%), and tap water 5 (20%). In addition to, the majority of them were from houses 10 (40%), dormitory 5 (20%), Cafeteria 6 (24%), pharmacy 2 (8%) and company 2 (8%). Most of them from Omdurman province 11 (44%), Khartoum 7 (28%) and Bahri 7 (28%).

The multiplex PCR assay confirmed the presence of all virulence genes of *K.pneumoniae* (213, ent, Kfu ,MRK , mag , k2 and RMP).

The study concluded that a high prevalence of virulence strains of *K.pneumonae* in drinking water in Khartoum State. The water can also be a source of transmission of disease and drug resistant isolates. detection of virulence genes by Multiplex PCR was quite satisfactory.

المستخلص

تعتمد الصحة الجيدة على إمدادات مياه نظيفة وصالحة للشرب. هذا يعني أن الماء يجب أن يكون خاليًا من مسببات الأمراض ، توجد *K.pneumoniae* في البيئة وكمعايش غير ضار ، ولكنها أيضًا عامل ممرض متكرر في المستشفيات وعامل عدوى بشرية محددة.

هذه الدراسة عبارة عن دراسة مقطعية تهدف إلى تحديد تواتر الجينات الفوعة في بكتيريا *K.pneumoniae* المعزولة من مياه الشرب بولاية الخرطوم (أم درمان ، الخرطوم ، وبحري) والتي أجريت في مايو إلى ديسمبر 2019.

تم الحصول على 100 عزلة من المختبر المركزي للصحة العامة بالخرطوم. عزلت هذه العزلات من مياه الشرب في ولاية الخرطوم و التي تم اعادة عزلها في Eosin Methylene Blue ، ثم تم التعرف على العزلات باختبارات بيوكيميائية روتينية ، وتم تحديد 25 (25٪) عزلة على أنها *K.pneumoniae* . تم إجراء تفاعل متعدد البوليميراز المتسلسل (MPCR ، 213 mag، K2، MRK، Kfu، ent) للكشف عن جينات الفوعة). معظم البكتريا المعزولة كانت من الخزان 12 (48٪) ،ثم المبرد 8 (32٪) ، ومياه الصنبور 5 RMPو (20٪). بالإضافة إلى أن غالبيتهم كانت من المنازل 10 (40٪) ،ثم الداخليات 5 (20٪) ، الكافتيريات 6 (24٪) ، الصيدليات 2 (8٪) والشركات 2 (8٪). معظمهم من محافظة أم درمان 11 (44٪) ، ثم الخرطوم 7 (28٪) وبحري 7 (28٪).

أكد اختبار PCR متعدد الإرسال وجود جميع جينات الفوعة لـ (*K.pneumoniae* و mrk و 213mag و K2(. و ent و rmp

خلصت الدراسة إلى أن انتشار سلالات الفوعة من *K.pneumoniae* في مياه الشرب بولاية الخرطوم أن الماء يمكن أن يكون أيضًا مصدرًا لانتقال الأمراض والعزلات المقاومة للأدوية وكان اكتشاف جينات الفوعة بواسطة Multiplex PCR مرضيًا للغاية.

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List of Abbreviations

APi20E	System For Identification Of Enterobactericae
BGBB	Birilent Green Bile Broth
BP	Base Pair
CAP	Community Acquired Pneumonia
CC23K1	Clonal Complex –Capsular Serotype
CC82K1	Clonal Complex –Capsular Serotype
CLSI	Clinical Lab Standerds Institute
CPS	Capsular Polysaccharide
DEC	Diarrheagenic Escherichia Coli
DNA	Deoxyribonuclic Acid
EMP	Eosin Methylene Blue
Ent	Enterobactin
HAIs	Hospital Acquired Infections
K2	Serotyping Capsular Antigen
Kfu	Iron Uptake system gene of Klebsiella
KIA	Keliger Iron Agar
Mag	Mucoviscosity Associated Gene
MLST	Mulilocus Sequence Typing
MPCR	Muliplex Polymerase Chain Reaction
MR/KH	Mannose Resistant –Klebsiella Like Hemagglutination
MRHA	Mannose Resistant Hemagglutinins
Mrk	Mannose Resistant Klebsiella
MSHA	Mannose Sensitive Hemagglutinins
NS	Normal Saline
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electophoresis

PLA	Pyogenic Liver Abscess
RFLP	Restriction Fragment Polymorphism
RMP	Regular Of Mucoid Phenotype
TE	Tris EDTA
U.V	Ultra-Violet
UTI	Urinary Tract Infection

Chapter I

Introduction

1.1 Introduction

Water is essential to life, but many people do not have access to clean and safe drinking water and many dies of waterborne bacterial infections (Cabral, 2010). Moreover, a negative impact of climate change on freshwater resources is expected. Recent advances in molecular detection technologies for bacterial pathogens in drinking water bear the promise in improving the safety of drinking water supplies by precise detection and identification of the pathogens (Brettar and Höfle, 2008). More importantly, the array of molecular approaches allows understanding details of infection routes of waterborne diseases, the effects of changes in drinking water treatment, and management of freshwater resources(Brettar and Höfle, 2008).

*K.pneumoniae*is an important nosocomial pathogen. Most community acquired *K.pneumoniae* infections cause pneumonia or urinary tract infections (Siu*et al*.,2012).During the past two decades, however, a distinct invasive syndrome that causes liver abscesses has been increasingly reported in Asia, and this syndrome is emerging as a global disease(Cabral, 2010).Diabetes mellitus and two specific capsular types in the bacterium predispose a patient to the development of liver abscesses and the following metastatic complications: bacteraemia, meningitis, endophthalmitis, and necrotizing fasciitis (Munoz *et al*.,2013). For patients with this invasive syndrome appropriate antimicrobial treatment, percutaneous drainage ofliver abscesses increases their chances of survival, Rapid detection of the hyper virulent strain that causes this syndrome allows earlier diagnosis and treatment,

thus minimizing the occurrence of squeal and improving clinical outcomes(Siu *et al*.,2012).

1.2Rationale

Water is essential to life, but many people do not have access to clean and safe drinking water and many die of waterborne bacterial infections (Cabral, 2010).The occurrences some pathogenic bacteria in drinking water may increase the risks of water-related diseases and health problems in local residents (Suther *et al*., 2009).The purpose of the present study was to determine the occurrence and distribution of the *Klebsiella* species in drinking water. Having done this, investigated whether environmental isolates are capable of producing putative *Klebsiella* virulence factors, such as pili, serum resistance properties, siderophores, or particular capsular types. For comparison, a group of previously described *K. pneumoniae* human clinical isolates was used (Podschun*et al*., 2001).

Change of climate increase contamination of water and increase risk of infection in water source , there was numerous reports of out breaks that follow this contamination (Hunter, 2003).

1.3Objectives

1.3.1 General objective

To detect *k.pneumoniae* virulence strains isolated from drinking water in Khartoum state, Sudan.

1.3.2 Specific Objective

1. To Isolate and identify of *K.pneumoniae* from selected water contaminated source in Khartoum state using culture technique.

2. To detect the presence and frequency of virulence factors; KFU, MRK, MAG, K2 and RMP strains in drinking water in Khartoum state by using multiplex PCR.

3. To detect association between virulence genes and sources of contaminated water.

Chapter II

Literature Review

2.1 Background

Waterborne diseases are cause by pathogenic microorganisms that are transmitted in drinking contaminated or dirty water. The water borne disease can be spread via ground water, which is contaminated with trench latrines causing of many types of disease, including cholera and other serious illnesses such as typhoid and dysentery (Moreira and Bondelind,2016).Water pollution with pathogenic microorganisms is one of the serious threats to human health, particularly in developing countries (Ashbolt,2015).An understanding of the microbial ecology of distribution systems is necessary to design innovative and effective control strategies that will ensure safe and high-quality drinking water (Ashbolt,2015).

2.2 Etiology

K.pneumoniae is the major cause of liver abscess, Gram-negative bacillary meningitis, brain and lung abscess, thoracic empyema, prostatic abscess, deep neck infection and complicated skin and soft tissue infections in Taiwan , and comprised 4.8% of the common causative pathogens of communityacquired pneumonia (CAP) in a multi-center survey(Lin *etal*., 2010). A number of studies have suggested that bacteremia is a risk factor for death in patients with CAP. Although previous studies investigated the characteristics and treatment strategy for bacteremic pneumococcoal pneumonia, few studies of bacteremic CAP due to *K.pneumoniae* have been reported and most of these were conducted before 2000(Lin *et al*., 2010). In one global study, pneumonia accounted for 29% and 62% of all cases of community-acquired *K.*

pneumonia bacteremia in Taiwan and South Africa, respectively; however, only four cases of community-acquired bacteremic *K. Pneumonia* (Brisse*et al*., 2009).

It's an important nosocomial pathogen, most frequently causing pneumonia, and urinary tract, wound or blood infections (Brisse*et al*., 2009). Nosocomial isolates are often associated with extended spectrum B-lactamases, including, recently, carbapenemases such as KPC and OXA-48 making treatment options limited. It is encapsulated, with 77 capsular types being recognized in an international scheme. The capsule is an important virulence factor, and some capsular types, particularly K1 and K2, but also K54 and K57 (Fang *et al*., 2007), are associated with a community-acquired invasive pyogenic liver abscess syndrome. In particular, the virulent clone of sequence type23, referred to as CC23(K1), or the K1 cluster, identified by MLST and PFGE, is implicated (Turton*et al*., 2010). Other putative virulence factors have been described, including the plasmid-borne rmpA (regulator of mucoid phenotype A) (Fang *et al*., 2007).

2.3 General characteristic

Klebsiella species are usually identified and differentiated according to their biochemical reactions. The genus is defined as containing Gram-negative, nonmotile, usually encapsulated rod-shaped bacteria of the family Enterobacteriaceae, which produce lysine decarboxylase but not ornithine decarboxylase and are generally positive in the Voges-Proskauertest(Barati,*etal*.,2016).Typical *K.pneumoniae* is an opportunistic pathogen, which mostly affects those with weakened immune systems and tends to cause nosocomial infections. A subset of hyper-virulent *K.pneumoniae* serotypes with elevated production of capsule polysaccharide can affect previously healthy persons and cause

life-threatening community-acquired infections, such as pyogenic liver abscess, meningitis, necrotizing fasciitis, endophthalmitis and severe pneumonia(Li *et al*.,2014).

2.3.1Biotyping

Based on an extended panel of biochemical and culture tests is certainly the most practicable method of typing for smaller laboratories that are epidemiologically not optimally equipped. Bio-typing can be carried out by using macro tube tests alone or by combining a commercially available miniaturized system such as the API 20E system with additional macro tube tests. However, because of the large number of reactions to be tested and the often-long cultivation times up to 90 days for demonstration of gelatinase biotyping of *Klebsiella* spp. is not very suitable as an epidemiological tool (Trivedi*etal*., 2015).

2.3.2 Serotyping

Serotyping is currently the most widely used technique for typing *Klebsiella* spp. It is based mainly on a division according to the capsule antigens. Klebsiellae usually have well developed polysaccharide capsules, which give their colonies their characteristic mucoid appearance (Trivedi *et al*.,2015). Pathogens of the genus *Klebsiella* have been classified into distinct capsular (K) types for nearly a century. K typing of *Klebsiella* species still has important applications in epidemiology and clinical microbiology, but the serological method has strong practical limitations (Brisse*et al*., 2013).

2.4 Genomes Structure

Genus Klebsiella, are still in their infancy. Preliminary descriptions have been presented on plasmid profile. Ribotypes, multilocus enzyme analyses, and applications of pulsed-field gel electrophoresis. The

procedures vary from laboratory to laboratory and lack standardization, making it difficult to compare them (Heddelland Mitchel, 2010).

2.5Bacteriocin Typing

Bacteriocins are antibacterial proteins produced by bacteria. They also differ from traditional antibiotics in having a relatively narrow spectrum of action and being lethal only for bacteria which are closely related to the producing strains. The aim of this study was to investigate the antibacterial spectrum of the action of bacteriocins from environmental and clinical *K.pneumoniae* isolates (Al-Charrakh*et al*.,2011)

2.6 Virulence Factors

2.6.1 Capsular Antigen

The capsule is considered to be a major virulence factor for *Klebsiella*, and there are important differences in virulence between the serotypes. It is widely held that serotypes K1–K6 are more associated with severe respiratory infection and septicaemia in humans than the higher numbered serotypes (Turton *et al*., 2008). Capsule is a major virulence factor of *K.pneumoniae,* and capsular types are related to the severity of infection. The prevalence of capsular types in each *K. pneumoniae*-related disease could be crucial for disease control and prevention. However, determination of capsular types often is difficult due to the limitations of traditional serotyping. The results of serotyping also are inconsistent, except in patients with community-acquired PLA (Pan *et al*.,2013).

2.6.2 Pilli (Fimbriae)

As a critical first step in the infectious process, microorganisms must come as close possible to host mucosal surfaces and maintain this proximity by attaching to the host cell (adherence). The adhesive

properties in the Entero bacteriaceae are generally mediated by different types of pili (Schroll*et al*., 2010). Pili (otherwise known as fimbriae) are nonflagellar, filamentous projections on the bacterial surface. Thes estructures are upto10 mm long and have a diameter of 1 to 11 nm; they consist of polymeric globular protein subunits (pilin) with a molecular mass of 15 to 26 kDa. Pili are demonstrated mainly on the basis of their ability to agglutinate erythrocytes of different animal species. Depending on whether the reaction is inhibited by D-mannose, these adhesins are designated as mannose-sensitive or mannose-resistant hemagglutinins (MSHA and MRHA), respectively. Of the different types of pili described in enterobacteria, there are two predominant types inKlebsiella spp. Type1(common)pili. Type1pili are the best investigated of the bacterial adhesins. They are MSHA which agglutinate guinea pig erythrocytes(Huang*et al*., 2009).

2.7Virulence genes

2.7.1K2 virulence gene

A single multiplex PCR assay targeting virulence factors and the gene specific for the K1 and K2 capsular serotypes of *K.pneumoniae* was developed and tested, which is responsible for community acquired severe human infections. The assay is useful for the surveillance of emerging highly virulent strains (Compain*etal*.,2014).

2.7.2mrk virulence gene

Pathogenesis of nosocomial *K.pneumoniae* infections has been associated with its capacity to form biofilms, particularly on medical devices. *K. pneumoniae* biofilm development is primarily mediated by the mannose-resistant *Klebsiella*-like (MR/K) hemagglutinins or “Mrk proteins”. The Mrk proteins are encoded by an operon which comprises

the genes *mrk ABCDF*. The Mrk proteins form type 3 fimbriae, cell surface structures that can extend into long filaments (up to 2 μm in length) that attach to surfaces. Type 3 fimbriae are synthesized by the chaperone-usher pathway of protein translocation (Wilksch*etal*.,2011)

2.7.3rmp virulence gene

The *rmpA* gene was first reported to be located on the 180 kb plasmid in the K2 strain 52145, and to positively control the mucoid phenotype (Hsu*et al*.,2011). The *rmpA2* gene, sharing 80% identity with the 3′ DNA sequences of *rmpA*, was later identified on the large plasmid in a K2 strain Chedid and shown to enhance CPS .Sequence analysis of RmpA2 showed that the C-terminal region shared considerable similarity with the putative helix–turn–helix motif of transcription regulators NtrC and FixJ. The N- and C-terminal regions also exhibited some homology to RcsA, a transcription activator for colonic acid synthesis in *E. coil* (Hsu *et al*.,2011). *K. pneumoniae* CPS requires the chromosomal *cps* gene cluster encoding proteins mainly for translocation and surface assembly of polysaccharides. The *rmpA* and *rmpA2* genes in the K2 strain CG43 have been reported to activate *cps* transcription and increase the virulence of this K2 strain in mice (Hsu *et al*.,2011).

2.7.4mag virulence gene

magA was restricted to serotype K1. All K1 or K2 isolated were neither serotype K1 nor serotype K2 (non-K1/K2) carried *rmpA (Yehet al.,2007).* Serotype K1 or K2 isolates demonstrated significantly more phagocytic resistance and virulence than did *rmpA*-positive and -negative groups of non-K1/K2 isolates. In the non-K1/K2 group, the virulence profiles of *rmpA*-positive strains from Taiwan and Singapore were different by phagocytosis assay and in the mouse model, indicating that factors other than *rmpA* contributed to virulence *(Yehet al.,2007).*

2.8 Epidemiology

Klebsiella spp. are ubiquitous in nature .Klebsiella probably have two common habitats, one being the environment, where they are found in surface water, sewage, and soil and on plants,and the other being the mucosal surfaces of mammals such as humans, horses, or swine, which they colonize(Munoz-price*etal*.,2013). In this respect, the genus *Klebsiella*is like *Enterobacter* and *Citrobacter* but unlike *Shigella* spp. or *E. coli*, which are common in humans but not in the environment. In humans, *K.pneumoniae* is present as a saprophyte in the nasopharynx and in the intestinal tract. Carrier rates differ considerably from study to study. The detection rate in stool samples ranges from 5 to 38%, while rates in the nasopharynx range from 1 to 6. Because gram-negative bacteria do not find good growth conditions on the human skin, Klebsiella spp. are rarely found there and are regarded simply as transient members of the flora (Munoz-price,2013).

2.9 Laboratory Diagnosis

2.9.1 Gram Stain

K.pneumoniae, a member of the *Enterobacteriaceae* family of gram-negative bacilli, is part of the normal microbiomeof many humans. Among 242 healthy volunteer participants in the National Institutes of Health Human Microbiome Project, K.pneumoniae was identified in 3.8% and 9.5% of stool and nasal samples, respectively. Colonization was also detected atcutaneous, oral, and vaginal sites. There appears to be substantial geographic variation in the proportion of persons in whom the normal intestinal microbiota includes *K.pneumoniae*. For example, in a Korean study, *K.pneumonia* was detected in stool samples obtained from 21.1% of 1,174 healthy persons at least 16 years of age (Calfee, 2017).

2.9.2 Culture

K.pneumoniae on agar media, it has a mucoid phenotype that is conferred ferments lactose. *K.pneumoniae* is part of the *Enterobacteriaceae* family, which is comprised of other familiar pathogens such as *Escherichia coli*, *Yersinia* species, *Salmonella* species, and *Shigella* spp. (Martin and Bachman, 2018). *K.pneumoniae,* a leading cause of hospital-acquired infections (HAIs) in the United States has classically been considered an opportunistic pathogen, since it typically causes infections in hospitalized or otherwise immunocompromised individuals. As the virulence of these bacteria and the demographic features of the patients they infect begin to shift, understanding how *K. pneumoniae* is transmitted and the factors responsible for pathogenicity is important in treating infected patients(Martinand Bachman, 2018).

2.9.3 Biochemical test

The biochemical characteristics in bacteria are an important tool in their identification. (Borkarand Ajayasree,2018).*K.pneumoniae* infection is also reported in animals and birds leading to severe enteritis, septicaemia and death. The strain of *K.pneumonia*is also reported to infect maize plant in Yunnan province, China10 and recently in India on pomegranate crop causing wilt.The known biochemical characteristics of *K.pneumoniae* sub-spp. And *Klebsiell a*species was compared with plant pathogenic *K.pneumoniae* strain Borkar for its biochemical characteristics. *K.pneumoniae* strain Borkar was positive for ONPG, lysine utilization, urease, VP, MR, PYR, esculin hydrolysis, nitrate reduction, citrate utilization and utilization of sucrose, sorbitol, trehalose, malatiose, salicin, mannose, glucose, malonate, arabinose, rhamnose and manitol as carbohydrate sources, while it was negative for ornithine utilization, Indole, H2S, β-glucoronidase, α-galactocidase, β-xylosidase and did not

utilize cellobiose, maltose, raffinose, lactose and adonitol (Borkarand Ajayasree,2018).

2.9.4 Molecular methods

A number of nucleic acid-basedmethod, have been established for the detection and characterization of *Klebsiella*strains, the most commonly method is based on the use of Polymerase Chain Reaction (PCR) to amplify a specific genes target in *Klebsiella* strains (O ` Sullivan *et al*., 2008).

2.9.5 Previous studies

In Malaysia Baratiand his research group in 2016 isolated and characterized the aquatic-borne *K.pneumoniae* from tropical estuaries in Malaysia the results indicated that matang mangrove stawery could harbor potentially pathogenic *K.pneumonae* with risk to public health (Barati*et al*.,2016).

In 2019Liand his group study identified a rapid, specific, and sensitive method for the ureR_1 gene in *K.pneumoniae* by loop-mediated isothermal amplification method, the study showed that the LAMP target to ure_1 was the fast, specific, sensitive and suitable method foe detection of *K.pneumonae* (Li *et al*., 2019).

In China Cheng and his group studied the characterization of *K. pneumoniae* associated with cattle infections in southwest China using multi-locus sequence typing (MLST), antibiotic resistance and virulence-associated gene profile analysis, the extended spectrum beta-lactamases genes were found in 93% of strains, of which, TEM was the most prevalence 93.4%(Cheng *et al*.,2018).

In 2014 Cao and his group demonistration molecular characterization of clinical multidrug-resistant *K. pneumoniae* isolates, they founded that the most frequents distributed among 10% and 29% strains (Cao, *et al.*, 2014).

In indiaKumar and Kumari. Studied *Klebsiella* in drinking water,the percentage of isolated *K.pneumonae* was 15%(Kumar and Kumari.,2013).

In 2016Mekonnen. Assessed bacteriological and Parasitological Quality and Safety of Public Municipal Drinking Water Sources in Addis Ababa, Ethiopia, the result of this study revealed that were 10% of all samples were positive bacteriological parameters(Mekonnen, 2016).

Chapter III

Material and Method

3.1Study design

This was descriptive cross sectional and laboratory-based study.

3.2 Study area

The study was conducted at Central Public Health Laboratory at Khartoum state (Sudan).

3.3 Study duration

The study was carried out during the period from May to December 2019.

3.4 Sampling

Non- probability sampling - Quota water samples were selected randomly from Central Public Health Laboratory at Khartoum state.

3.4.1 Sample Size

Hunderd samples from drinking water have been collected from Central Public Health Laboratory, Khartoum, Sudan.

3.5 Data Collection

General data were collected and samplling based on constrected questionnaire that included date, place, type of water.

3.6 Sample processing

Hunderd isolated from drinking water were culture in EMB media and then subculture in MaCconky agar and stored at 4 C within three days..

3.7 Laboratory works

The Gram stain was used to detect the Gram –negative bacilli, then cultured in EMB media, biochemical test was done to identify

the*K.pnuemonae* bacterium, the susceptibility test was used to detect the sensitivity of antibiotic, The extractionof DNA, PCR and gel electrophoresis was used to detect the virulence factors in strains.

3.8.1 Collection of samples

Water samples were obtained from places suspected of having fecal contamination, some important factors were considered (Cheesbrough *et al*.,2011). The inside and outside the tap was cleaned and disinfected carefully. The tap was opened and the water was flowed for 2-3 minutes. The tap was turned off and the spout was sterilized by alcohol -Water sample was taken with the sample in sterile container "bottles"

3.8.2Culturesof water samples

The cultures was used for the detection of coliform bacteria in water using the presence-absence coliform test (Vaz*et al*.,2011).The bottle content (50ml) of sterile selective culture broth containing lactose and an indicator "Laurytryptose (lactose) broth" with the Durham tube was added to water sample. After incubation (48 hours at 37°C) the bottle in which lactose fermentation with acid and gas production has been cultured in two bottles of Birilent Green Bile Broth (BGBB) media with the Durham tube. Two bottles were incubated in (BGBB) media for 48 hours in 37°C for one bottle to detect the coliform and the other one in 44°C to detect the heat- tolerant coliform. If a bottle in 37°Cshowed turbidity with acid andgas in the Durham tube in less than 48 hours this will be indicated for the presence of coliform bacteria.Then the bacteria were identified by sub cultcuring on EMB and Indole test (Vaz and Moreira.,2011).

3.8.3 Identification of K. pnuemoniae

3.8.3.1 Gram stain method

A primary stain “Crystal violete” was applied to the dry – heat – fixed smear of microorganism for 1 minute. Then the stain was washed with distilled water and cover with Lugol’s iodine for 1 minute. Stain was washed with distilled water.and decolorized by acetone, alcohol and washed with distilled water. Then the stain was covered with safarnin for 2 minutes. The slide was placed in a rack to dry. The specimen was examined at (X100) (oil-immersion lens) (Cheesbrough *et al*., 2011).

3.8.3.2. Bacterial isolates

One loopful of the sample was placed in the top of the EMB agar plate and was streaked down, the plate was then incubated overnight in 37°C in an incubator. Apenkish-redish color was the indicator for a positive reaction of the presence of *K.pnuemonae* in water specimen (Cheesbrough *et al*., 2011).

3.8.3.3 Biochemical tests

Four biochemical tests were used for the identification of bacterial isolates, Indole test – Urease test – Citrate test – KIA test

3.8.3.3.1 Indole test method

Several colonies of the microorganism were rubbed into the tube of tryptophan peptone water media with sterile loop, the tube was incubated overnight in 37°C in an incubator, after 18-24 hours Kovacs reagent was added, a bright pink – red color ring was developed in positive reaction (Niemi *et al.*,2009).

3.8.3.3.2 Urease test method

Colonies of the microorganism to be inoculated in the urea media by sterile loop, the tube was incubated overnight in 37°C , after incubation the media was tested for change in color if pink the test will be positive (Maroncle,*etal*.,2006).

3.8.3.3.3 Citrate test method

Colonies of the microorganism were picked up by a straight wire and inoculated in sloped Simmon's citrate agar and incubated overnight at 37°C in an incubator, after incubation the media was tested for change in color if blue the test is positive (Cheng *et al*.,2012).

3.8.3.3.4 KIA test method

Colonies of the microorganism were picked up by a straight wire and inoculated in sloped (KIA) media, then incubated overnight at 37°C in an incubator after incubation if there was achange throughout the medium, butt and slant are yellow, gas bubbles in the butt and no blackening in the butt this may be *K.pnuemoniae* (Hassan and Hammed,2017).

3.8.4 Molecular identification

3.8.4.1 DNA extraction

The DNA was extracted byboiling centrifugation method as described by (Fazhan *et al*., 2016).

3.8.4.2 Boiling centrifugation method

Several colonies of the isolated organism (*K.pneumonae*) were subcultured in Nutrient Agar media, after overnight incubation at 37°C, 1-3 colonies were washed with 1ml sterile Normal Saline (NS) in sterile 1.5 ml Eppendorf tube(Queipo-Ortuno.,2008). The tube was vortexed at 10.000r/min for 1-2 minutes. The supernatant was discarded.The pellet

was resuspendedin 200/μL, of distilled water. Then the tube was boiled at 95°C for 15 minutes. The tube was centrifuged at 13.000r/min for 3 minutes. The supernatant (that content the DNA) was removed to a new sterile Eppendorf tube. The tube was stored at - 20°C till used (Queipo-Ortuno *et al*.,2008).

3.8.4.3Detection of DNA concentration

Concentration of extracted DNA was read using gel electrophoresis to show the presence and quality of DNA in the sample, when compared with a DNA marker of known concentration.

3.8.5Multiplex Polymerase Chain Reaction (PCR)

Multiplex PCR was used for the detection of target genes.

3.8.5.1 Primers

PCR amplification was performed using published primer pairs (213, ent,KFu, mrK, mag, K2, rmp).

3.8.5.2Preparation of Primers

For 100pmol/μL, each primer was dissolved in D. Was follows;

213 – F in two hundred and twenty μL D.W, 213 – R in two hundred and fiftyμLD.W, ent – F in two hundred and twenty μLD.W, ent, R in two hundred and twenty μLD.W, kfu-F in two hundred and fifty μLD.W, kfu-R in two hundred and forty μLD.W, mrk – F in two hundred and fifty μLD.W, mrk-R in two hundred and twenty μL D.W. And for 10 pmol/μL, 10μL of each primer was dissolved in 90μL of D.W.

3.8.5.3Prparation of 10X TE buffer

Amount of one hundred and eight grams of Tris base were added to fifty five grams of boric acid and 40ml of 0.5 EDTA, and then dissolved into 1liter of distilled water (pH8.0). 4-Preparation of IX TE buffer 10 ml of

10X was added to 90mL distilled water and heated until completely dissolved.

3.8.5.4Preparation of ethidium bromide

(10mg/ml) Five milligrams of ethidium bromide powder were dissolved in 50ml DW and kept in brown bottle(Lee *et al.*,2012).

3.8.5.5Preparation of agarose gel (2%)

Amount of 2.0g of agarose powder were dissolved in 100mL TE buffer. Then the mixture was heated in microwave for 90seconds and cooled to 60°C in room temperature. Then 3μL of (10mg/ml) ethidium bromide were added. Mixed well and poured into a casting tray with a comb. Any bubbles were removed and the gel was allowed to set at room temperature. After solidification of the gel, the comb was gently removed(Lee *et al*.,2012).

3.8.5.6Master Mix

Master mix (iNtRoN Biotechnology, Korea(was a remixed ready used solution containing all reagents required for PCR (except water, template DNA and primers) and additional compound needed for direct loading onto an agarose gel(Hindson *et al*.,2013).

3.8.5.7Preparation of reaction Mixture

The following reagents were used for each reaction in the following volumes (total reaction volume was 25μL) in 0.2 ml eppendroff tube.

1- Seventeen μL distilled water
2- Five μL master mix (iNtRoN Biotechnology, Korea).
3- One half forward primer (Macrogen Company, Korea).
4- One halfμL reverses primer (Macrogen Company, Korea).
5- twoμL DNA (Template DNA)

3.8.5.8Protocol used for amplification of the virulance genes

The amplification was done by using Techne Tc.312Thermal cycle (UK). The PCR mixture was subjected to initial denaturation step at 94°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds and the final extension at 72°C for 5 minutes(Skyberg *et al*.,2006).

3.8.5.9Visualization of PCR products

The gel casting tray was flooded by 10X TBE buffer near the gel cover surface, then 5μL of amplified PCR products of each sample was put into each well. Then to the first well of casting tray 3 μL of DNA ladder (100bp) was injected for each run. The gel electrophoresis apparatus was connected to the power supply (primer, 125V, 500 MA, UK). The electrophoresis were done at 100V/cm for 30min, after that, the gel was removed by gel holder and visualized by U.V. Transilluminater (Uvite-UK), the gel results were photographed using the Polaroid film(Skyberg*etal*.,2006).

Table(3-1) Demonstrate names of the primers ,DNA sequences (5'to3') and ampilicons size (bp)

Primer name	DNA sequence (5'to3')	Amplicons size (bp)
mrk_ for mrk_rev	AAGCTATCGCTGTACTTCCGGCA GGCGTTGGCGCTCAGATAGG	340
ent_for	GTCAACTGGGCCTTTGAGCCGTC	400

ent_rev	TATGGGCGTAAACGCCGGTGAT	
rmp_for rmp_rev	CATAAGAGTATTGGTTGACAG CTTGCATGAGCCATCTTTCA	461
K2_for K2_rev	CAACCATGGTGGTCGATTAG TGGTAGCCATATCCCTTTGG	531
Kfu_for Kfu_rev	GGCCTTTGTCCAGAGCTACG GGGTCTGGCGCAGTATGC	638
mag_for mag_rev	GGTGCTCTTTACTCATCATTGC GCAATGGCCTTTGCGTTAG	1283

(Saad *et al.*, 2013).

3.8.6Statistical data analysis

For analysis the statistical package of social science (SPSS) version 11.5 (SPSS Inc., IL, USA) software was used. Statistical tests were carried out and Frequency was calculated

Chapter IV

Results

4.Result

A total of 100 water samples were collected from different sources tank, cooler, and tap water. Out of them 75were coliform and 25 were *K.pneumoniae*.

As presented in table (Table 4-1); according to the source *K.pneumoniae* isolates were distributed as follow: tank 12 (48%), cooler 8 (32%), and tap water 5 (20%).

K.pneumoniae was isolated from different places: houses 10 (40%) ; Cafeteria 6 (24%), Dormitory 5 (20%), Pharmacy 2 (8%) and company 2(8%) as in (Table 4-2).

Distribution of collected samples was: Omdurman 11 (44%), Khartoum 7(28%) and Bahri7 (28%)as a province as showed in (Table 4-3).

Among *K.pneumoniae* isolates virulence genes' frequencies were K2 7(28%) (in cooler 3(9.3%),tap water 3(15%) and tank 1(2%)), 213 6(24%) (tape water 3(15%),cooler 3(9.3%)), mrk 4(16%) (in tape water 4(20%)), rmp 3(12%) (in tape water 2(10%) and cooler 1(3.1%)), ent 2 (8%) (in tape water 1(5%) and cooler 1(3.1%)) , mag 2(8%) (in tape water2(10%)) as presented in (Table 4.4).

The presence of virulence genes from *K.pneumoniae* isolates according to place was as follow: 213 2(8%), k2 3(12%), rmp 1 (4%) and kfu 1(4%) from house samples, 213 1 (4%) and k2 1(4%) from pharmacy, 213 1(4%), k2 1(4%) from company, k2 3(12%), 213 1(4%), rmp 1(4%) from dormitory, and ent 1(4%), mrk 1(4%), rmp1(4%), K2 2(8.3%) in cafeteria as in (Table 4.6).

The presence of virulence genes from *K.pneumoniae* isolates according to province was: 213 3(6.8%), Mrk 3(6.3%), K2 2(4.5%), Mag, Rmp and Kfu 1(3.5%) from Omdurman, K2 3(10.7%), 213 1(3.5%), Ent 1(3.5%), Mag 1(3.5%) and Rmp 1(3.5%) from Khartoum, and 213, k2 2(7.1%), ent, mrk and rmp 1(3.5%) from Bahri as in Table (4.6).

Table(4-1): Frequency of *K.pneumoniae* according to source of water

Source	Frequency	Percent
Cooler	8	32%
Tap water	5	20%
Tanke	12	48%
Total	25	100%

Table(4.2):Frequency of *K.pneumoniae* according to place

Place	**Frequency**	**Percent**
House	10	40%
Cafeteria	6	24%
Dormitory	5	20%
Pharmacy	2	8%
Company	2	8%
Total	25	100%

Table (4.3):*K.pneumoniae* according to ward and the province

Province	**Frequency**	**Percent**
Omdurman	11	44%
Bahri	7	28%
Khartoum	7	28%
Total	25	100%

Table(4.4): The presence of virulence genes from *K.pneumoniae* isolates according to source of water

Genes	Water Sources			Total
	Tap water(5)	Cooler(8)	Tank(12)	
K2 +	3 (15%)	3 (9.3%)	1 (2%)	7 (28%)
213 +	3(15%)	3 (9.3%)	0 (0%)	6 (24%)
Mrk +	4 (20%)	0 (0%)	0 (0%)	4 (16%)
Ent +	1 (5%)	1 (3.1%)	0 (0%)	2 (8%)
Mag +	2 (10%)	0 (0%)	0 (0%)	2 (8%)
Rmp +	2 (10%)	1 (3.1%)	0 (0%)	3 (12%)
Kfu +	1 (5%)	0 (0%)	0 (0%)	1 (4%)
Total	16 (64%)	8 (32%)	1 (4%)	25(100%)

Key: + Positive

Table (4.5):The presence of virulence genes from *K.pneumoniae* isolates according to place

Source	Gene						
	213 +	Ent +	Mrk +	Mag +	K2 +	Rmp +	Kfu +
House (10)	2 (8%)	0(0.0%)	0(0.0%)	1(4%)	3(12%)	1(4%)	1(4%)
Pharmacy ((2)	1(4%)	0(0.0%)	0(0.0%)	0(0.0%)	1(4%)	0(0.0%)	0(0.0%)
Company (2)	1(4%)	0(0.0%)	0(0.0%)	0(0.0%)	1(4%)	0(0.0%)	0(0.0%)
Dormitoy (5)	1(4%)	0(0.0%)	0(0.0.%)	0(0.0%)	3(12%)	1(4%)	0(0.0%)
Cafeteria (6)	0(0.0%)	1(4%)	1(4%)	0(0.0%)	2(8.3%)	1(4%)	0(0.0%)

Key: + Positive

Table (4.6):The presence of virulence genes from *K.pneumoniae* isolates according to province

Source	Genes							Total
	213 +	Ent +	Mrk +	Mag +	K2 +	Rmp +	Kfu +	
Omdurman (11)	3 (6.8%)	0 (0.0%)	3 (6.8%)	1 (3.5%)	2 (4.5%)	1 (3.5%)	1 (3.5%)	11 (44%)
Khartoum (7)	1 (3.5%)	1 (3.5%)	0 (0.0%)	1 (3.5%)	3 (10.7%)	1 (3.5%)	0 (0.0%)	7 (28%)
Bahri (7)	2 (7.1%)	1 (3.5%)	1 (3.5%)	0 (0.0%)	2 (7.1%)	1 (3.5%)	0 (0.0%)	7 (28%)
Total	6 (24%)	2 (8%)	4 (16%)	2 (8%)	7 (28%)	3 (12%)	1 (4%)	25 (100%)

Key: + positive

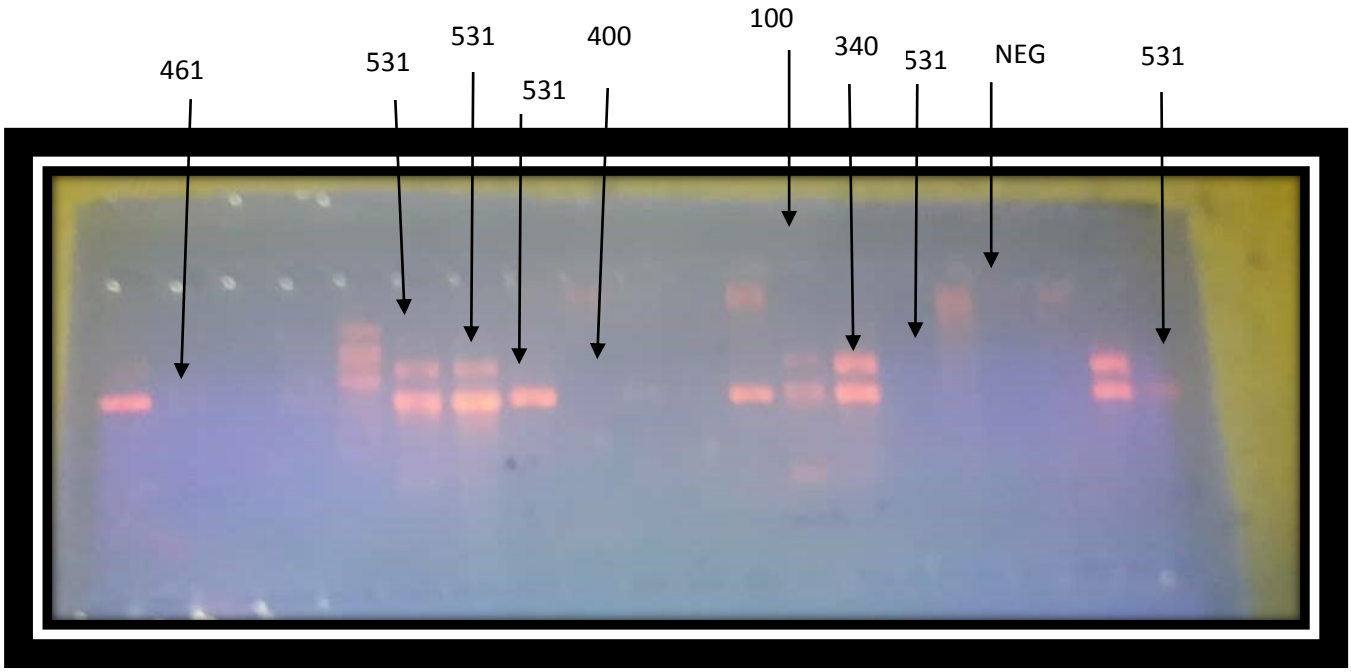

Figure 1: Multiplex PCR of *K.pnuemonae* strains using virulent specific primers

Lane 1:rmp (461bp), Lane 6 lader (100bp), Lane 3,4,8,10: K2(531 bp), Lane 5: ent gen (400 bp), Lane 7: mrk (340 bp). Lane 9: negative sample.

Chapter V

5.1 Discussion

This study was focused on the molecular detection of virulence genes of *K. pnuemoniae* in drinking water in Khartoum State, Sudan. A total of *K.Pnuemonae* isolates were collected from drinking water from different regions in Khartoum State. The present study showed a high prevalence of *K.Pnuemonae* in Khartoum State drinking water. Most of them from tank 12(48%)and cooler 8(32%), this finding is in agreement with a study in Malaysia Anis Barati *et al.* (2016),whom reported that *K. Pnuemonae* was the most common from the estuarine waters.

The prevalence of *K.Pnuemonae* are more in Omdurman province 11(44%),then Bahri province 7(28%) and Khartoum 7(28%), this may be due to that Omdurman province, is bigger than Bahri and Khartoum province and consist of many wards, and the Omdurman has an old sanitation system with multi broken pipes-line(Mayors).

In this study revealed that the frequency of virulence genes (K2, mag, Kfu,213,ent,MRk and Rmp) associated with *Klebsiella* was done by using PCR,the most prevalent gene detected during this study was K2 7(28%) followed by 213 6(42%) and Rmp 3(12%) isolated for each which in agreement with Pods and Pietsch (2001) who found that equatic inviroment was contaminated by 53% of Klebsiella that capable of expression virulence factors ,and disagreement with Luo and Yan (2014) who found that virulence genes were found in patient with bloodstream infection.

The study found that most of contamination from houses 10(40%) , followed by Cafeteria 6(24%), Dormitory 5(20%) and pharmacy 2(8%)

which is disagreement with Olds and Corsi (2018) who found that contamination come from sewage .

In this study K2 gene 7(28) is common cause of disease and this is agreement with Compain and Babosan (2014) who found that K2 was responsible for community acquired sever human infection .

5.2 Conclusions

There is a high frequency of virulence strains of *K.pneumonae* in drinking water in Khartoum State(213,Ent, Mrk, Mag, K2, Rmp and Kfu).The water can also be a source of transmission of disease and drug resistant isolates. There is a high prevalence of *Klebsiella*in drinking water in Khartoum State. Detection of virulence genes by Multiplex PCR was quite satisfactory. There is a high percentage in tank 48%and cooler 32% and low percentage in tap water 20% ;this indicate that the contamination of drinking came from the source.

5.3 Recommendations

1- The drinking water should be periodically screened for the presence of bacterial contaminates to prevent serious health risk

2- Large sample size is critical for best result.

3- Multiplex PCR technique should be used beside traditional laboratory methods as a routine technique in the diagnosis of Klebsiella strains in drinking water.

4-Advance technique such as sequencing and Restriction fragment length polymorphism (RFLP) should be used to confirm the patho type of Klebsiella strains.

5-The houses should use filters in taps' spouts to filter the water before use.

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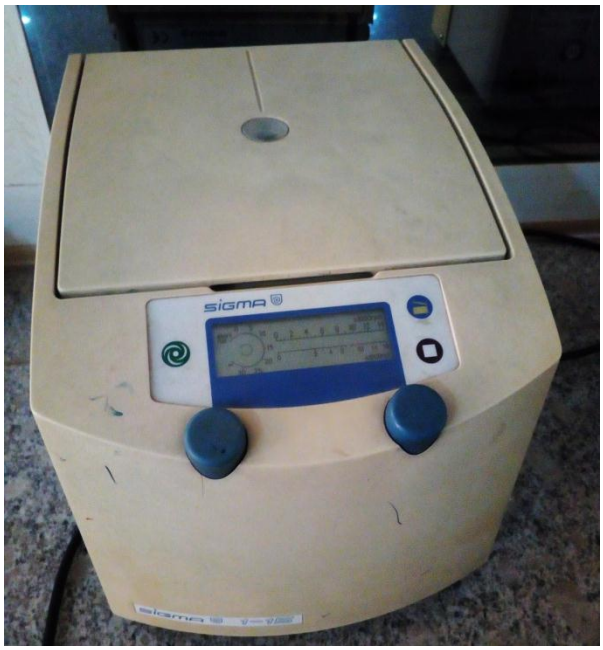
Appendix I

(A) U.V Light Machine

(B)Muliplex PCR Machine

AppendixΠ

(A)Microwave



(B)Centrifuge

Appendix III

(A) Waterpath

(B) Sensitive Balance

Appendix IV

(A) Gell Electrophoresis Machine