Emergence of Extended Spectrum β - Lactamases (ESBL) In Clinical Isolates of *Klebsiella pneumoniae* In Rada'a Thamar Cities, Yemen

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Abstract

The spread of Gram- negative bacteria with plasmid-born Extendend- Spectrum β - Lactamases (ESBLs) had become a worldwide problem, that enzymes confer resistance to penicillin, cephalosporin the first, second and third generations and aztreonam via hydrolysis of the antibiotic, that sample collected from urine, sputum and stool sample. Nine isolates from ninety- five of Klebsiella pneumoniae was production ESBL (9.5%). Tested for susceptibility using Disk diffusion method. All isolates produced Extended Spectrum beta-actamase.

Based on the inhibition zone diameter, the result shows that P. aeruginosa shows highest sensitivity toward cefepime (44%) > gentamicin (22%) = amikacin (22%) > ceftazidime (11%) > cefixime (0%) = ampicillin (0). These results were in agreement with previously reported findings in international journals.

**Keywords:** Klebsiella pneumoniae, Spectrum β – Lactamases, Rada'a, Thamar
1.1. Background

*K Klebsiella pneumoniae* is normally found in the intestinal tract of humans and animals. The number of *Klebsiella* bacteria can attain $10^7$ per gram of dry feces (Brisse et al., 2006). It may be isolated in association with several pathological processes in humans, e.g., community-acquired pneumonia or nosocomial urinary tract infection. In animals, *K. pneumoniae* may be isolated from metritis in mares, bovine mastitis, or generalized infections in captive monkeys. Environmental strains generally utilize more carbon sources than clinical strains (Grimont and Grimont, 2005). In humans, the interactions of *Klebsiella* with humans range from asymptomatic carriage to opportunistic infections (mainly in hospitalized patients), and also include community-acquired infections. Most *Klebsiella* infections now occur in hospitalized patients and many are caused by antibiotic-resistant strains, including strains that produce extended-spectrum β-lactamases (ESBLs) that are a serious cause of concern (Brisse et al., 2006).

*K. pneumoniae* is the most common nosocomial pathogen of the three, and appears to have the greatest ability to receive and disseminate multidrug resistance plasmids (Denis et al., 2010). Extended-spectrum β-lactamase-producing clinical isolates among members of the *Enterobacteriaceae* family, especially *Klebsiella pneumoniae* and *Escherichia coli*, represent one of the most important world problems of β-lactam antimicrobial resistance, commonly used in the treatment of many bacterial nosocomial community infections (Wollheim et al., 2011). β-lactam drugshave been the choice for treating *Klebsiella* infections because of the presence of resistance mechanisms such as aminoglycoside-modifying enzymes, macrolide esterases, and efflux pump systems that render many other drug classes ineffective. However, the use of β-lactams has become difficult in recent years as various classes of β-lactamases have been identified and found in clinical *Klebsiella* isolates (Broberg et al., 2014).

1.2. Aims:

The aims of this study are:
- To review previous publications regarding Emergence of Extended Spectrum β-Lactamases (ESBL) of *Klebsiella pneumoniae*.
- Collecting a clinical isolates from infected patients.
- Obtaining a pure culture of *K. pneumoniae*.
- Detection of β-lactamase from the isolates.
- Determination of the isolates susceptibility to selected antibiotics versus producing enzymes.

Chapter II. Literature Review

2.1. Description

*Klebsiellae* are Gram negative straight rods, 0.3–1.0 × 0.6–6.0 μm, arranged singly, in pairs or short chains; often surrounded by a capsule. This genus is nonmotile (except *K. mobilis*), facultative anaerobe, having both a respiratory and a fermentative type of metabolism (Grimont and Grimont, 2005). The genus *Klebsiella* tend to be somewhat shorter and thicker than the other enterobacteria with parallel or bulging sides and rounded or slightly pointed ends. In the body, diplobacilli, very like pneumococci, are commonly seen. They are nonmotile. When the capsule is pronounced, it can be demonstrated even by Gram stain. Capsular material is produced in greater amount in media containing a relative excess of carbohydrate. Most strains of *Klebsiella* are fimbriate but some of the respiratory strains form an exception (Murray et al., 2006).

2.2. Historical view

The first organism of the genus *Klebsielladescr* was a capsulated bacillus isolated from patients with rhinoscleromabv byon Frisch in 1882. Then Friedländer in 1882 described a bacterium from lungs of a patient who...
had died of pneumonias. The organism was subsequently named “Hyalococcus pneumoniae” by Schroeter, 1889, even when the designation “Klebsiella” was coined by Trevisan in 1885 to honor the German bacteriologist Edwin Klebs. The species Klebsiella pneumoniae was previously named Friedländer bacillus (Grimont and Grimont, 2005). Recently, the genus Klebsiella has undergone extensive revisions. K. pneumoniae, K. ozaenae, and K. rhinoscleromatis are now accepted as subspecies within the species K. pneumoniae (Murray et al., 2006).

2.3. Taxonomy

Based on 16s ribosomal small subunit phylogenetic analyses and other DNA-based studies, Klebsiella belong to Gammaproteobacteria; a class comprise some of the most gram-negative overt pathogens.

Classification: (Bergey’s Manual of Systematic Bacteriology 2nd ed. 2009)

Domain: Bacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Enterobacteriales
Family: Enterobacteriaceae
Genus: Klebsiella
Species: K. pneumoniae

2.4. Habitat

Klebsiella spp. are often found in a variety of environmental sources such as soil, vegetation, and water, contributing to biochemical and geochemical processes, and has been identified as a major component of the microflora in several types of stressed nonclinical environments (Brisse et al., 2006). They also colonize the mucosal surfaces of humans, horses, and swine. Klebsiella can frequently be isolated from the root surfaces of various plants. K. pneumoniae, K. oxytoca, or K. planticola are capable of fixing nitrogen and are classified as associative nitrogen fixers (Grimont and Grimont, 2005).

2.5. Physiology of Klebsiella pneumoniae

The outermost layer of Klebsiella bacteria consists of a large polysaccharide capsule, a characteristic that distinguishes members of this genus from most other bacteria in the family (Escherichia coli strains with a heat-stable K antigen may form similar capsules). The cell wall is structured similarly to other Enterobacteriaceae. Above the cytoplasmic membrane is the peptidoglycan layer and the outer membrane containing lipopolysaccharide (LPS). In addition, Klebsiella strains possess fimbriae of I and III types (Grimont and Grimont, 2005, Madigan et al., 2015).

2.6. Culture

Best results with biochemical tests are obtained when Klebsiella cultures are incubated at 30–35°C. Klebsiella strains (except K. granulomatis) grow readily on all kinds of media commonly used to isolate Enterobacteriaceae such as nutrient agar, tryptic casein soy agar, bromuresolpurple lactose agar, MacConkey agar, eosin-methylene blue (EBM) agar, and bromothymol blue (BTB) agar. K. pneumoniae and K. oxytoca colonies are lactose positive, more or less dome-shaped, 3–4 mm in diameter after overnight incubation at 37°C or 30°C, with a mucoid aspect and sometimes stickiness depending on the strain and the composition of the medium (Grimont and Grimont, 2005).

2.7. Pathogenicity of Klebsiella pneumoniae

The genus Klebsiella can be associated with different sorts of infections. Klebsiella pneumoniae is a cause of community-acquired bacterial pneumonia (Friedländer’s pneumonia), occurring particularly in chronic alcoholics. The fatality rate is high if untreated (Grimont and Grimont, 2005). K.
pneumoniae subsp. rhinoscleromatis is the causative agent of rhinoscleroma, a chronic infection that can involve the nasal cavity (most often) or the upper airways (pharynx, larynx, and trachea). Typically, a granulomatous destructive and disfiguring process results in airway obstruction. Ultimately, extensive fibrosis and scarring occur. K. pneumoniae subsp. ozaenae has been implicated in ozena; a chronic atrophic rhinitis giving off a very bad smell. However, the pathogenic role of K. pneumoniae in this syndrome is less clear (Grimont and Grimont, 2005).

Klebsiella pneumoniae has been associated with brain abscess in diabetics and produces a gas-forming appearance on X-ray imaging (Donald, 2006). The urinary tract is the most common site of infection, and K. pneumoniae accounts for 6–17% of all nosocomial urinary tract infections. K. pneumoniae is also a frequent cause of bacteremia. In premature infants, K. pneumoniae is often involved in neonatal sepsis. Some strains will kill mice in a dose of 0.2 ml of a 24 h broth culture diluted 10^6 times; others fail to kill even with 0.2 ml of the undiluted culture (Murray et al., 2006).

Molecular pathogenesis

Klebsiella species are surrounded by a hydrophilic polysaccharide capsule, which is the first virulence factor described in klebsiellae. Experimental studies using mice (skin model) showed that strains with K1, K2, K4, and K5 capsular antigens were more virulent than strains with K6 and K above 6. The loss of K antigen resulted in the reduced virulence. This reduced virulence may be explained by a higher degree of phagocytosis as measured by chemiluminescence response of human polymorphonuclear leukocytes (PMNLs) and by enhanced killing by either human PMNLs or human serum both. However, other studies (mouse peritonitis model) found strains with antigens K1 and K2 more virulent than strains with other K antigens. The K antigen plays a crucial role in protecting the bacterium from opsonophagocytosis in the absence of specific antibodies. The antiphagocytic function consists of inhibiting the activation or uptake of complement components, especially C3b. In addition, the K antigen inhibits the differentiation and functional capacity of macrophages in vitro. The degree of virulence conferred by a particular K antigen might be connected to the mannosereceptor of the capsular polysaccharide. The most important role of the O antigen is to protect K. pneumoniae from complement-mediated killing. Almost all strains of Klebsiella produce enterobactin whereas only a few produce aerobactin; two types of high-affinity iron-chelating systems called siderophores. These systems solubilize and import the required iron from different iron-associated molecule in human body (Grimont and Grimont, 2005).

Epidemiology

K. pneumoniae is well established in the hospital environment and became a (still) leading cause of nosocomial infections. Not only is it found in the gastrointestinal tracts of patients, at frequencies as high as 80%, but high carriage rates have also been recorded for patient nasopharynges and hands. This considerable efficiency of colonization, enhanced by acquired resistance to antibiotics (see later text), enables K. pneumoniae to persist and spread rapidly in health care settings (Tzouvelekis et al., 2012). The principal reservoir of K. pneumoniae in the hospital is the gastrointestinal tract of patients. The principal vectors are the hands of personnel (Grimont and Grimont, 2005).

2.8. Virulence factors

K. pneumoniae contains several well studied virulence factors that include capsular polysaccharide, lipopolysaccharide, type 1 and type 3 fimbriae, and siderophores depicted in Figure 1 (adapted from Podschun et al., 2003).
**Capsular Polysaccharide**

Most clinical isolates of *K. pneumoniae* are encapsulated with a thick complex of acidic polysaccharide structure comprised of repeating 4-6 sugar subunits. Capsular polysaccharides (CPS) have been classified into 77 serological types, termed K-antigens. The presence of the capsule is critical for the virulence of *K. pneumoniae*. Deletion of the Orf6 gene within the CPS operon resulted in an avirulent unencapsulated *K. pneumoniae* mutant. Similarly, *wabG* mutants lack cell-attached CPS and were shown to be avirulent in animal models of both UTI and pneumonia. Deletion of *rmpA2*, a transacting activator of CPS synthesis, resulted in decreased CPS production and reduced virulence *in vivo* (Yeh et al., 2006).

**Lipopolysaccharide (LPS)**

cell walls have only a thin layer of peptidoglycan, but outside this layer is another, outer bilayer membrane composed of two different layers or leaflets. The inner leaflet of the outer membrane is composed of phospholipids and proteins, but the outer leaflet is made of lipopolysaccharide (LPS). Integral proteins called porins form channels through both leaflets of the outer membrane, allowing glucose and other monosaccharides to move across the membrane. The outer membrane is protective, allowing Gram-negative bacteria to better survive in harsh environments. LPS is a union of lipid with sugar. The lipid portion of LPS is known as lipid A. The erroneous idea that lipid A is inside Gram-negative cells led to the use of the term endotoxin for this chemical. A dead cell releases lipid A when the outer membrane disintegrates, and lipid A may trigger fever, vasodilation, inflammation, shock, and blood clotting in humans. Because killing large numbers of Gram-negative bacteria with antimicrobial drugs releases large amounts of lipid A, which might threaten the patient more than the live bacteria, any internal infection by Gram-negative bacteria is cause for concern. The Gram-negative outer membrane can also be an impediment to the treatment of disease. For example, the outer membrane may prevent the movement of penicillin to the underlying peptidoglycan, thus rendering the drug ineffectual against many Gram-negative pathogens. Between the cytoplasmic membrane and the outer membrane of Gram-negative bacteria is a periplasmic space. The periplasmic space contains the peptidoglycan and periplasm, the name given to the gel between the membranes of these Gram-negative cells. Periplasm contains water, nutrients, and substances secreted by the cell, such as digestive enzymes and proteins involved in specific transport. The enzymes function to catabolize large nutrient molecules into smaller molecules that can be absorbed or transported into the cell. Because the cell walls of Gram-positive and Gram-negative bacteria differ, the Gram stain is an important diagnostic tool. After the Gram staining procedure, Gram-negative cells appear Pink (Jawetz et al., 2013).

**Fimbriae**

*Klebsiella*fimbriae are non-flagellar, filamentous projections on the bacterial cell surface. These fimbriae are thought to play an important role during the early stages of bacterial adhesion to host cells. Fimbriae expressed by *Klebsiella* have been divided into two major categories based on whether their adhesive interaction can be inhibited by D-mannose. Type 1 fimbriae are mannose sensitive and bind to mannose containing trisaccharides on host epithelial cell glycoproteins. Interestingly, after mannose-dependent binding to

(Fig. 1) *K. pneumoniae* Virulence factors
epithelial cells, invasive, *K. pneumoniae* bacteria can turn off expression of type 1 fimbriae in order to avoid mannose-dependent binding by host phagocytic macrophages. Type 3 fimbriae are resistant to D-mannose inhibition and have been shown to promote binding to endothelial cells, respiratory epithelium, and kidney epithelial cells. The gene cluster required for expression of type 3 fimbriae in *K. pneumoniae* has been cloned and shown to contain six genes termed MrkA-F. The importance of type 3 fimbriae during infection is not well understood. However, a mutant of a *K. pneumoniae* virulent strain deficient in type 3 fimbriae production (gift from Dr. Steven Clegg, U. Iowa) was found to be no different in its pathogenicity compared to the parental strain (unpublished observation)(Lawlor et al., 2007).

**Siderophores**

Iron is an essential element for bacterial growth and is procured from the host environment via the bacterial secretion of high affinity, low molecular weight iron chelators called siderophores. Enteric siderophores belong to three major groups, enterobactin, aerobactin, and yersiniabactin. A previous report indicated that enterobactin production did not correlate with virulence cloned from a virulent strain conferred virulence. However, more recently, Lawlor et. al. showed that *K. pneumoniae* yersiniabactin production is increased during pulmonary infection while during *in vitro* iron-limiting growth conditions, enterobactin is produced at higher levels when compared to yersiniabactin. The discrepancies in these results can be attributed to the complexity of these siderophores systems *in vivo* and also the lack of definitive experiments addressing the importance of these iron acquisition systems. (Lawlor et al., 2007)

**Other Virulence Factors**

In addition to the above mentioned four major classifications of virulence factors, recent studies have identified other putative genes associated with *K. pneumoniae* pathogenicity. Outer membrane protein A (OmpA) in *K. pneumoniae* binds to human and mouse macrophages and dendritic cells via TLR2, resulting in cytokine secretion. OmpA also binds to bronchial epithelial cells, inducing chemokine production and neutrophil recruitment. In addition, several studies have identified novel, previously uncharacterized genes present in virulent strains of *K. pneumoniae* using signature-tagged mutagenesis, *in vivo* expression technology, and PCR-based subtractive hybridization. (Lau et al., 2007).

**2.9. β-lactamases:**

**Beta-lactamases** are enzymes produced by some bacteria that provide resistance to β-lactam antibiotics like penicillins, cephamycins, and carbapenems (ertapenem), although carbapenems are relatively resistant to beta-lactamase. Beta-lactamase provides antibiotic resistance by breaking the antibiotics' structure. These antibiotics all have a common element in their molecular structure: a four-atom ring known as a β-lactam. Through hydrolysis, the lactamase enzyme breaks the β-lactam ring open, deactivating the molecule's antibacterial properties. Beta-lactam antibiotics are typically used to treat a broad spectrum of Gram-positive and Gram-negative bacteria. Beta-lactamases produced by Gram-negative organisms are usually secreted, especially when antibiotics are present in the environment (Bush et al., 2010).

2.9.1. B-lactam drugs and *Klebsiella pneumoniae* Mechanisms of resistance

Although *K. pneumoniae* is not inherently resistant to antibiotics, except penicillins (Murray et al., 2006) since it produces moderate amounts of chromosomal penicillinases, *K. pneumoniae* is a notorious “collector” of multidrug resistance plasmids. During the 1970s to 1980s, these were commonly plasmids encoding resistance to aminoglycosides. Later, however, *K. pneumoniae* became the index species for plasmids encoding extended-spectrum β-lactamases (ESBLs) along with a variety of genes conferring resistance to
drugs other than β-lactams (Tzouvelekis et al., 2012).

2.9.2. B-lactam drugs
This group includes penicillin and its derivatives, cephalosporins, monobactams, and carbapenems have a bactericidal activity. Both penicillins and cephalosporins are the common antibacterial drugs used among this group.

Penicillins
The penicillins are a group of natural and semisynthetic antibiotics containing the chemical nucleus 6-aminopenicillanic acid, which consists of a β-lactam ring fused to a thiazolidine ring (Fig. 2). The naturally occurring compounds are produced by a number of Penicillium spp. The penicillins differ from one another in the substitution at position “R” (see figure below), where changes in the side chain may modify the pharmacokinetic and antibacterial properties of the drug (Yao et al., 2007).

(C. N. CH-CH-S CH₃
O
OH
R-C-N-CH-CH-S-CH₃
C-N-CH-COOH
O)
(Fig. 2). β-lactams penicillin core structure, where ”R” is the variable group.

The initial step in penicillin action is binding of the drug to cell receptors. These receptors are Penicillin-binding Proteins (PBPs), at least some of which are enzymes involved in transpeptidation reactions. From 3 to 6 (or more) PBPs per cell can be present. After penicillin molecules have attached to the receptors, peptidoglycan synthesis is inhibited as final transpeptidation is blocked. A final bactericidal event is the removal or inactivation of an inhibitor of autolytic enzymes in the cell wall. This activates the autolytic enzymes and results in cell lysis (Brooks et al., 2013).

Cephalosporins
Cephalosporins are derivatives of the fermentation products of Cephalosporium acremonium (also designated Acremonium chrysogenum). They contain a 7-aminoccephalosporanic acid nucleus, which consists of a β-lactam ring fused to a dihydrothiazinering (Fig. 3). Various substitutions at positions 3 and 7 alter their antibacterial activities and pharmacokinetic properties. The addition of a methoxy group at position 7 of the β-lactam ring results in a new group of compounds called cephamycins, which are highly resistant to a variety of β-lactamases (Yao et al., 2007).

(Fig. 3). β-lactams cephalosporins core structure, where ”R” are the variable groups.

The mechanism of action of cephalosporins is analogous to that of penicillins: binding to specific PBPs that serve as drug receptors on bacteria, inhibiting cell wall synthesis by blocking the transpeptidation of peptidoglycan, and activating autolytic enzymes in the cell wall that can produce lesions resulting in bacterial death (Brooks et al., 2013).

Other β-Lactam Antibiotics
Monobactams
Monobactams are monocyclic (no fused secondary ring) β-lactam molecules (Fig. 4) and are resistant to β-lactamases. They are active against gram-negative rods primarily through binding to PBP3 but not against gram-positive bacteria or anaerobes because of poor binding to PBPs (Bush, 2010), (Brooks et al., 2013). Certain monobactams and nocardicins, are active in vitro against Gram-negative bacteria. In contrast to penicillins and cephalosporins, which are commonly produced by fungi and...
actinomycetes, naturally occurring monobactams are produced by bacteria (Bush, 2010).

Monobactams are hydrolyzed poorly by many serine β-lactamases and all metallo-β-lactamases, but can be hydrolyzed by ESBLs and serine carbapenemases (Bush, 2010).

**Carbapenems**

These drugs are structurally related to the β-lactam antibiotics. Dozens of carbapenems have been isolated from fermentation products of various streptomyces (Bush, 2010). Imipenem, the first drug of this type, has good activity against many gram-negative rods, gram-positive organisms, and anaerobes. It is resistant to β-lactamases. It is useful for treatment of complicated infections not involving hospital pathogens (Brooks et al., 2013).

**Sulbactam**

Sulbactam is a semisynthetic 6-desaminopenicillin sulfonewith weak antibacterial activity. It functions as an effective inhibitor of certain plasmid-mediated and chromosomally mediated β-lactamases of *S. aureus*, many *Enterobacteriaceae* and *H. influenzae*, *Neisseria* spp., *Legionella* spp., the *B. fragilis* group, *Prevotella* spp., *Porphyromonas* spp., and *Mycobacterium* spp. Like clavulanic acid, sulbactam does not inhibit the β-lactamases of *Enterobacter*, *Citrobacter*, *Providence*, indole-positive *Proteus*, *Pseudomonas* spp., or *S. maltophilia* (Bush, 2010).
Tazobactam

Tazobactam (formerly YTR 830) is a penicillanic acid sulfonederivative structurally related to sulbactam. Like clavulanic acid and sulbactam, tazobactam acts as a suicidal β-lactamase inhibitor and binds to bacterial PBP 1 or PBP 2. Despite having very poor intrinsic antibacterial activity by itself, it is comparable to clavulanate and sulbactamin lowering the MICs up to 20-fold for many organisms when combined with various β-lactams against β-lactamase producing organisms. Tazobactam actively inhibits the β-lactamases of staphylococci, H. influenzae, N. gonorrhoeae, E. coli, the B. fragilis group, Prevotella spp., and Porphyromonas spp. It also has activity against the class I β-lactamases of Acinetobacter, Citrobacter, Proteus, Providencia, and Morganella spp., but it remains inactive against those of Enterobacter spp., Pseudomonas spp., S. maltophilia, and some Klebsiella spp. (Bush, 2010).

Chapter III. Materials and methods

3.1. Materials:

3.1.1. Culture media:

- Nutrient Agar (Peptic digest of animal tissue 5.000 gL⁻¹, Beef extract 3.000 gL⁻¹, Potassium nitrate 1.000 gL⁻¹, Agar 12.000 gL⁻¹, HIMEDIA).
- Blood Agar (Peptic digest of animal tissue 5.000 gL⁻¹, Beef extract 3.000 gL⁻¹, Potassium nitrate 1.000 gL⁻¹, Agar 12.000 gL⁻¹, HIMEDIA).
- MacConkey Agar (Peptone 17.0 gL⁻¹, Proteose Peptone 3.0 gL⁻¹, Bile Salts 1.5 gL⁻¹, Sodium Chloride 5.0 gL⁻¹, Crystal Violet 0.001 gL⁻¹, Neutral Red 0.03 gL⁻¹, Agar 13.5 gL⁻¹, Microxpress).
- Motility Indol Urea (MIU) Medium Base (Casein enzymic hydrolysate 10.000 gL⁻¹, Dextrose 1.000 gL⁻¹, Sodium chloride 5.000 gL⁻¹, Phenol red 0.010 gL⁻¹, Agar 2.000 gL⁻¹, HIMEDIA).

3.1.2. Media, Reagents and Disks:

All media (for *k. pneumoniae*), anti-biotic (disks, and powder), Petri dishes, glassware, and reagents were privately purchased.

3.1.3. Isolation Media:

Thom (1970) developed a medium based on the MacConkey agar in which lactose is replaced by inositol, with the addition of 100 mg of carbenicillin per ml. Bagley and Seidler (1978) devised a similar medium with only 50 mg of carbenicillin per ml. MacConkey-inositol-carbenicillin Agar (Bagley and Seidler, 1978).

MacConkey agar base (Difco) 40g
Inositol
10g
Distilled water to 1 liter

Autoclave at 121°C for 15 min, cool to 50°C in a water bath, add 0.05 g of carbenicillin dissolved in 5 ml of sterile distilled water, and mix and dispense into sterile Petri dishes. Keep at use within three days. About 95% of pink-to-red colonies were verified to be *Klebsiella* spp.

3.1.4. Materials used in the laboratory work.

- Glassware:
  - Bakers, Cover glasses, Culture tubes, Petri dishes and Slides.
  - Instruments and sets:
    - Autoclave, Balance, Bunsen burner, incubator, Laminar flow chamber, microscope and Refrigerator
- Others:
  - Antibiotic desks, Cotton and foil, Distilled water, Gloves, Gram stain kit, Inoculation needle and wire loop, Media (Nutrient agar, Macconkey agar, Mueller Hinton agar), Penicillin powder, Pipettes, Starch and Swabs.

3.1.5. Selecting of antimicrobial agents for testing

Several principles should be followed in order to select the antibacterial agents to be tested: (i) the widely used antibiotics should be included; (ii) the species to be tested, and (iii) the availability of the antibacterial agent (Turnidge et al., 2003). Parameters of choosing a testing method and antibacterial agent can be found in the Clinical and Laboratory Standards Institute (CLSI). This system has been followed in this project. The selected antimicrobial agents have been used are given in table ......

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Strength (μg)</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>30</td>
<td>AK</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>10</td>
<td>Amp</td>
</tr>
<tr>
<td>Cefazidime</td>
<td>30</td>
<td>Caz</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10</td>
<td>Ge</td>
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<tr>
<td>Cefixime</td>
<td>5</td>
<td>cfm</td>
</tr>
<tr>
<td>Cefapime</td>
<td>30</td>
<td>cpm</td>
</tr>
</tbody>
</table>

To prepare a 0.5 McFarland Standard solution (Cavalieri et al., 2005):
- Add 0.5 mL of 1.0% (wt/vol) anhydrous Barium Chloride (BaCl2) to 99.5 mL of 1.0% (vol/vol) cold pure sulfuric acid (H2SO4) solution.
- Stir to maintain a suspension.
- Thoroughly mix immediately before the next step:
- Distribute about 5 mL of the 0.5 McFarland Standard solutions into screw-top tubes and store them in a dark at room temperature.

Note: The diameter of these tubes should be the same as those used for adjusting the density of culture suspensions prior to inoculation. When these standards are thoroughly shaken the turbidity equals that of a culture containing about 1.5 x 10^8 cells.

3.2. Methods:

3.2.1. Collecting the Samples:

Clinical samples from infected patients were collected at Hospital. Isolates were sent for labeling and storage and analysis at the Microbiology Laboratory, College of Applied Science, Thamar University.

1) stool
2) urine
3) sputum

3.2.2. Isolation and Identification of *k. pneumoniae*:

Most of the samples plated directly on *MacConkey* Agar for Pyocyanin medium after a transport me lesser than 48 hours.

**Incubation and obtaining of pure cultures**

All the plates incubated aerobically for 24 hour at 37oC then the colonies that match the description of *k. pneumoniae* colonies morphologies streaked on *MacConkey* Agar for Pyocyanin plates to obtain pure cultures and further incuba on at 370C to eliminate the fluorescent group members.

**Identification**

1. **Identification of Bacteria:**

The colonies that appeared visually dissimilar were chosen and subculture to nutrient agar and incubated at 37oC for 24 hours. Identification of microorganisms did not commence until it was evident that a pure culture had been obtained.

<table>
<thead>
<tr>
<th>Colonies diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Garm stain</td>
</tr>
<tr>
<td>Morphology</td>
</tr>
<tr>
<td>Motility</td>
</tr>
<tr>
<td>Catales</td>
</tr>
<tr>
<td>Oxidaes</td>
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<tr>
<td>Indal</td>
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</table>

**Table(): Identification characters of the isolates of *k. pneumoniae***

2. **Cultural characterization:**

After growing of colonies we diagnosed it initially depending on the culturing, shaping features that include "size, shape, color, edge of colonies, high and transparency".

3. **Microscopic observation:**

Through preparing typical smear from pure colony and stain it with gram stain and testing it under microscope to notice the shape and color.

**Gram Stain:**

- Cover the smear with crystal violet and let stand for one minute. Briefly wash off the stain, using a wash bottle of distilled water and drain off excess water.
- Cover the smear with Gram’s iodine solution and let it stand for one minute. Then pour off the Gram’s iodine and flood the smear with 95% ethyl alcohol for 10 to 20 seconds. This step is critical. Thick smears will require more time than thin ones. Decolorization has occurred when the solvent flows colorlessly from the slide.
- Stop action of the alcohol by rinsing the slide with water from wash bottle for a few seconds. Then cover the smear with safranin for one minute. After that wash gently for a few seconds, blot dry with bibulous paper, and air-dry. The slide should be now ready to be examined under oil immersion lens.

4. **Biochemical test:**

To be done the biochemical tests on the different colonies:

**i- Catalase test:**

- Taking a clean slide.
- Patting a drop from H2O2 on the slide.
- Taking part from colony and mix it with H2O2 by a woody rod.
- Noticing appearance of bubbles or not.

**ii- Oxidase test:** (Oxidase reagent)

- **Filter Paper Method:**
  - Taking filter paper.
- Saturating the filter paper by Oxidase reagent.
- Taking apart from growing and pat it in the filter paper.
- Noticing the colour changing.

-oxidase discs:

-Moisten the impregnated discs with sterile distilled water before placing on the suspected colonies.

-Leave abaut 20-30 minutes bofor checking for any colour changes.

iii- Motility test:

A.slide method:

- Taking a clean slide.
- Patting one drop from distilled water on the slide.
- Taking apart from growing and mixing with distilled water.
- Putting a cover slide.
- Noticing the motility under microscope.

B.In MIU Media:

- Preparing (MIU) according to company instruction in tubes and sterilized.
- Incubating in 37oC for 48 hours.

-If bacteria is motile, there will be growth going out away from the stab line, and test is positive. If bacteria is not motile, there will only be growth along the stab line. A colored indicator can be used to make the results easier to see.

iv-Indol test:

- Preparing (MIU) according to company instruction in tubes and sterilized.

- Culturing bacteria by pricking method.
- Incubating in 37oC for 48 hours.
- After incubating, adding 5drops from Kovac's reagent to the media.
- Noticing appearance a red circle

3.2.3. Detection of β-lactamase production:

- The iodometric method:

The iodometric method was employed to detect β-lactamase production. This method is cited in (Miles et al., 1994) and to be carried out according to the following steps:

- From an overnight culture, a heavy suspension (approximately 109CFU/ml) is made in distilled water containing 6g/L of penicillin.

- Place 0.1 ml of the suspension into a well of microtiter plate. After incubation for 1 hour at 37oC, tow drops of the freshly prepared 1% soluble starch solu on (prepared by dissolving the starch at 100oC) to be added to each well.

- Add a drop of iodine reagent (consisting of 2.03g iodine and 5.32g potassium iodine in 100 ml distilled water).

If the blue color is lost within 10 minutes, the presence of β-lactamase is inferred.

3.2.4. Antibiotic sensitivity testing of the isolates:

All the procedures in susceptibility testing were done according to the guidelines of Clinical Laboratory Standard Institute (CLSI) published in 2013. All the clinical isolates subjected to susceptibility test against the antibiotics shown in table 1. McFarland standard solution has been used to calibrate the turbidity of bacterial suspensions used in performing Kirby-Bauer method to achieve a turbidity equal to a suspension contains 1.5×108 CFU/ml (Cavalieri et al., 2005).
Performing the Kirby-Bauer method

Antibiotic disks have been used to perform the diffusion method on Mueller-Hinton (MH) agar medium (HiMedia Co., India) using 24-hour-old colonies grown on nutrient agar. Bacterial suspension have been made in a grease-free cleaned glass tube containing 4ml distilled water and the turbidity had been adjusted to match McFarland's solution 0.5, comparison was done by comparing the two tubes at black background. Cotton swab had been used to deliver a thin layer of bacteria on the surface of MH agar. Four disks applied to each 90 cm² plate and incubated immediately in ambient air at 35°C. After 18 hours the inhibition, zones had been measured by a scale and compared with breakpoint values of CLSI guidelines.

Table 1. The American CLSI guidelines (2014) of K. pneumonia Breakboits.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Susceptible (mm)</th>
<th>Intermediate (mm)</th>
<th>Resistant (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>≥15</td>
<td>12-14</td>
<td>≤11</td>
</tr>
<tr>
<td>Amikcin</td>
<td>≥17</td>
<td>15-16</td>
<td>≤14</td>
</tr>
<tr>
<td>Cefixime</td>
<td>19≥</td>
<td>16-18</td>
<td>≤15</td>
</tr>
<tr>
<td>Cefbime</td>
<td>≥18</td>
<td>15-17</td>
<td>≤14</td>
</tr>
<tr>
<td>Ceftazidim</td>
<td>≥18</td>
<td>15-17</td>
<td>≤14</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>≥15</td>
<td>13-14</td>
<td>≤12</td>
</tr>
</tbody>
</table>

Table 2. Distribution K. pneumoniae in various clinical specimens.

<table>
<thead>
<tr>
<th>Source/site</th>
<th>No. of samples</th>
<th>No. of K. pneumoniae isolates</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stool</td>
<td>50</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Urine</td>
<td>30</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Spatum</td>
<td>15</td>
<td>2</td>
<td>13.3</td>
</tr>
</tbody>
</table>

4.1. Results

A total of 95 clinical samples were collected. About sample were obtained between 2-2015-7-2015.

Resistance through β-lactamase production

All the isolates were subjected to β-lactamase detection. The disappearance of the blue colour of starch and iodine was indicative of β-lactamase production. And all the isolates were β-lactamase producers (100%). Results obtained for disk diffusion method (Inhibition zone diameters) for the different antibiotics used in this experiment are shown in tables 5. Statistical calculations had been done using Microsoft Excel 2007.
Table(3)

| Table(4) Antimicrobial susceptibility pattern of klebsiella pneumonia isolates |
|-----------------|-----------------|-----------------|
| Antibiotic      | Susceptibility pattern | susceptible | Intermediade susceptibility | Resistant |
| Ampicillin      | S | I | R | % | % | % |
| Amikacin        | 2 | 3 | 4 | 22 | 33 | 44 |
| Celixime        | 0 | 0 | 9 | 0 | 0 | 100 |
| Cefbime         | 4 | 2 | 3 | 44 | 22 | 33 |
| Ceftazidim      | 1 | 0 | 8 | 11 | 0 | 89 |
| Gentamicin      | 2 | 2 | 5 | 22 | 22 | 56 |

Figure 12. Percentage of resistant isolates in the studied sample (sample size=9)
It is clear that the spectrum rank order of the antimicrobial agents against *k.pneumoniae* in terms of percentage of susceptibility was: cefepime (44%) > gentamicin (22%) > amikacin (22%) > ceftazidime (11%) > celiixm (0%) = ampicillin (0%). Significant proportion of the sample were intermediately susceptible to few antibiotic in the following order: Amikacin (33%) > cefepime (22%) = gentamicin (22%) = ampicillin (0%) = ceftazidime (0%) = celiixm (0%).

4.2 Discussion:

This is the first study in the Department of Microbiology and in the University of Themar that evaluated the emergence of extended spectrum β–lactamases (ESBL) in clinical isolates of *klebsiella pneumonia*. The phenotypic Identification of producing ESβL enzyme can do by tow stebs. The first is antimicrobial susceptibility test and the second stebs is synergy between oxyimino cephalosporine and another. Disk approximation Method used to phenotypic method, the result showed 9 isolat (9.5%) from 95 isolat producing ESβL. All the isolates were subjected to β-lactamase detection. The disappearance of the blue colour of starch and iodine was indicative of β-lactamase production. And all the isolates were β-lactamase producers (100%) this agree with (Abas ,2011). susceptibility was: cefapime (44%) > gentamicin (22%) > amikacin (22%) > ceftazidime (11%) > celiixm (0%) = ampicillin (0%). In spite of the difficulties in comparing results that show regional differences, the results obtained from this study are in agreement with those obtained for cefapime in previous study (2011) for antibiotic. However, the studied isolates in this show less susceptibility for cefepime, but higher than that reported for gentamicin (2011). In another recent study (Jasim et al., 2012) this study published in 2014, study showed high resistance to antibiotic, 100% to (ceftazidime, Ampicillin) 20% to Amikacin. The isolates of sputum 13.3% then stool 10% finally urine 6.7% this disagree (Abas ,2011). But it very agree (Rampure et al., 2013).

**Recommendation**

- The mechanism of resistance was beyond the scope of this study. It requires advance techniques, more time and availability of materials, which is limited under the current circumstances and can be an interesting project in the future.
- Specially-design study to investigate the MICs in a concentration-dependent manner is required for each antibiotic.
- Larger sample size study is required to understand the prevalence of *P. aeruginosa* resistance rate in Yemen.
- Patients, especially those with open wounds and/or with low immunity should protect themselves from being in contact with contaminated materials.
- Health care staff must realize the potential emergence of multidrug resistant *P. aeruginosa* strains.
- Hygienic procedures in the hospitals should be improved to limit the spread of such devastating microorganisms.
- Clinically significant infections with *P. aeruginosa* should not be treated with single-drug therapy, because the bacteria can rapidly develop resistance when single drugs are employed.
- The resistant strains could become a real challenge if the administration/dissension of the antibiotics are misused.

**References:**


[36]. Wollheim,C;Guerra,I.M.F;Conte,V.D;Holffman,S.P;Schreiner,F.J;Delamare,A.P.L;Barth, A.L;Echeverrigaray,S.(2011): Nosocomial and community infections due to class A extended-spectrum β-lactamase (ESBLA)-producing Escherichia coli and Klebsiella spp. in southern Brazil, Braz J Infect Dis;15(2):138-143.