Cape Peninsula
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# MOLECULAR CHARACTERISATION OF THE MULTI-ANTIBIOTIC RESISTANT BACTERIA, *KLEBSIELLA PNEUMONIAE* ISOLATED FROM NOSOCOMIAL INFECTIONS

**by**

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**DECLARATION**

I, **Marney van Ginkel**, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

**22 November 2017**

**Signed** **Date**

## ABSTRACT

**Background:** It is well established that *Klebsiella pneumoniae* (*K. pneumoniae*) is an opportunistic pathogenic organism that has been frequently identified as the cause of nosocomial and community acquired infections. Furthermore, studies have shown that over the last few decades strains of the genus *Klebsiella* have systematically developed resistance to numerous antibiotics.

**Aims and Methods:** The primary aim of this study was to investigate the prevalence of *K. pneumoniae* in nosocomial and community isolates in the Western Cape province of South Africa. Various identification techniques such as the polymerase chain reaction (PCR) using the API 20 E, the VITEK®2 system, primers specific for the 16S-23S rDNA ITS region and the Matrix-assisted laser desorption/ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) were compared for the identification of this pathogen. The VITEK 2 system was used to detect antibiotic resistant profiles of the *K. pneumoniae* isolates and to identify the extended spectrum beta-lactamase (ESBL) phenotypic among these isolates. The PCR was used to detect Beta-lactam genes viz. CTX-M ($bla_{CTX-M}$), TEM ($bla_{TEM}$) and SHV ($bla_{SHV}$) respectively in both the genome and plasmid DNA of *K. pneumoniae* using gene specific primers.

**Results:** In total 57 agar plate bacterial cultures or glycerol stock bacterial cultures were obtained during 2011. Of the 57 isolates, the API 20 E test identified 47 (82.5%) of the isolates (*n* = 57) as *K. pneumoniae* while 10 isolates (17.5%) were identified as *Raoultella* species. The VITEK 2 method and PCR identified all 57 isolates as *K. pneumoniae* (100%). Of the isolates, 82.5% (47/57) were positively identified as *Klebsiella* species, 14% (8/57) were identified as *Klebsiella variicola* and 3.5% (2/57) were shown as no reliable identification (NRI) when using the MALDI-TOF MS. Examination of the 57 isolates using primers specific for the CTX-M ($bla_{CTX-M}$), TEM ($bla_{TEM}$) and SHV ($bla_{SHV}$) respectively showed the following: PCR amplicons for the TEM gene were produced successfully for 46 (81%) of the 57 isolates included in this project, while 11 (19%) of the samples did not yield any TEM amplicons; PCR amplicons for the $bla_{SHV}$ gene were obtained successfully for 56 (98%) of the 57 DNA samples, while 1 sample (2%) did not yield any SHV amplicons; and PCR amplicons for the $bla_{CTX-M}$ gene were produced successfully by 89% (*n* = 51) of the DNA samples included in this project, while 11% (*n* = 6) did not yield any CTX-M amplicon. Extended-spectrum beta-lactamase phenotypes had been

confirmed in 84% (*n* = 48) *K. pneumoniae* isolates while nine isolates were found to be non-ESBL. Resistance rates for these 48 isolates were high and showed resistance patterns of: Amoxicillin/Ampicillin, Amoxycillin/Clavulanate, Ceftriaxone/Cefotaxime, Cefuroxime/Cefprozil and Ceftazidime (100%, n = 48); Piperacillin/Tazobactam and Cefoxitin (98%, 47/48); Cefepime (96%, 46/48); Aztreonam (94%; 45/48); Tobramycin (81%, 39/48); Gentamycin and Ciprofloxacin (77%, 37/48); Trimethoprim/Sulfamethoxazole (67%, 32/48); and Tigecycline (25% 12/48).

**Conclusion:** For the analysis by all four methods employed, a total agreement of 68.4% was obtained, indicating the positive identification of *K. pneumoniae* in 39 of the 57 samples analysed. An average agreement of 28.1% was then obtained for the comparison of results generated for three of the methods utilised, while a 3.5% average agreement was obtained for at least two methods. Furthermore, all four methods agreed that 82.5% of the isolates were *Klebsiella* species while three methods agreed that 17.5% of the isolates were *Klebsiella* species. Based on the results obtained in the current study, PCR and VITEK 2 were the methods of choice for the identification of *K. pneumoniae*. The current study also showed, that ESBL-*K. pneumoniae* strains are present in the Western Cape province, South Africa; with high resistance profiles to numerous antibiotics including the Cephalosporins.

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## GLOSSARY

| Terms/Acronyms/Abbreviations | Definition/Explanation |
|---|---|
| MAR | Multi Antibiotic Resistant |
| bla | beta-lactamase |
| NCBI | National Centre for Biotechnology Information |
| ESBL | Extended Spectrum Beta-lactamase |
| PBP | Penicillin-binding protein |
| MDR | Multi-drug resistant |
| MIC | Minimum inhibitory concentration |
| SHV | Sulphydryl variable |
| TEM | Temoniera |
| CTX-M | Cefotaxime |
| PCR | Polymerase chain reaction |
| ITS | Internal transcribed spacer |
| WHO | World Health Organization |

**KEYWORDS**

*Klebsiella pneumoniae*, nosocomial infection, multi-antibiotic or multi-drug resistant, extended-spectrum beta-lactamase, plasmid, polymerase chain reaction, Cephalosporin

# CHAPTER ONE

# 1 LITERATURE REVIEW

## 1.1 Introduction

Microbes are abundant on this planet and found in staggering amounts (Schaechter et al., 2003). Members of the domain or super kingdom bacteria are diverse and inhabit all the environments and climates known to man including extreme pH (Beveridge, 1999), boiling and sub-zero temperatures, extremely high pressure, high salinity and even in solid rock deep in the crust of the earth (Spellberg et al., 2008). Microbes are considered the basis of the biosphere as they perform critical functions necessary to support life on earth; such as degrading biological waste and cycling critical elements including carbon, nitrogen, hydrogen, sulphur and oxygen into the biosphere. This biogeochemical cycling in effect increases the availability of plant nutrients by regulating fertility of the soil and enhancing plant growth, which is crucial to human and animal life (Schaechter et al., 2003).

Relman et al. (2009) stated that the healthy human body comprise of about $10^{14}$ cells of which 90 – 99% belong to the microbiota. Normal flora in humans usually develops in an orderly sequence from birth to the stable population of bacteria which encompass the normal adult host flora. The nature of the local environment (pH, temperature, redox potential and oxygen, water and nutrient levels) is the main factor determining the composition of the normal flora. Peristalsis, saliva, lysozyme secretion and immunoglobulin secretion are other factors which play a role in controlling the normal microbial flora. The significance of normal flora lies in the fact that they perform a number of useful functions such as digestion of essential nutrients, maturation of intestinal physiology, stimulation of the immune system, systemic effects on blood lipids and the inhibition of harmful bacteria (McFarland, 2000). A diverse microbial flora populates various body regions (skin, nails, eyes, oropharynx, genitalia, and gastrointestinal tract) and usually each region is associated with specific genera (**Table 1.1**).

**Table 1.1 Body regions with their associated normal flora (adapted from Davis, 1996)**

| **Body Region** | **Skin and Nail flora** | **Nose flora** | **Oral and Upper Respiratory tract flora** | **Gastrointestinal tract flora** | **Urogenital tract flora** | **Conjunctiva flora** |
|---|---|---|---|---|---|---|
| Bacterial Organisms | Gram-positive cocci:<br>*Staphylococcus epidermidis*<br>*Staphylococcus aureus*<br>*Micrococcus luteus*<br>Diphtheroid:<br>*Corynebacterium spp.*<br>*Propionibacterium acnes*<br>*P. granulosum*<br>*P. avidum*<br>Gram-negative bacilli<br>*Enterobacter*<br>*Klebsiella spp.*<br>*Escherichia coli*<br>*Proteus spp.* | *S. aureus* | *Haemophilus spp.*<br>*Neisseria meningitidis*<br>*Bordetella pertussis*<br>*C. diphtheriae*<br>*Streptococcus spp.*<br>α-haemolytic streptococci<br>beta-haemolytic streptococci | > 400 species<br>*Helicobacter pylori*<br>*Bacteroides*<br>*Bifidobacterium*<br>*Eubacteria*<br>*Pepto-Streptococcus*<br>*Clostridium difficile*<br>Enterobacteriaceae – *Klebsiella pneumoniae* | Sparse mixed flora at various stages of life:<br>*Lactobacillus spp.*<br>Diphtheroid<br>*S. epidermidis*<br>enterococci<br>*E. coli*<br>*Proteus spp.*<br>*Neisseria* (non-pathogenic) | Conjunctiva flora are sparse and are found rarely in the healthy eye.<br>*Haemophilus*<br>*Staphylococcus spp.*<br>Corynebacterium<br>*Neisseria*<br>*Moraxella*<br>*Streptococcus spp.*<br>*Haemophilus parainfluenza* |

*Klebsiella pneumoniae* (*K. pneumoniae*) is a bacterial organism constituting part of the normal human flora, inhabiting particularly the human gastrointestinal tract (**Table 1.1**) and is classified as an opportunistic pathogen (Bester, 1988; Bridson, 1993). This implies that this microorganism usually does not cause infection in a host with a healthy immune system, but it uses the opportunity to spread and cause infection in a debilitating immune system. *Klebsiella* spp. has also frequently been identified as the causative agents in nosocomial [the World Health Organization (WHO) defines nosocomial infection as follows: "An infection acquired in hospital by a patient who was admitted for a reason other than that infection. An infection occurring in a patient in a hospital or other health care facility in whom the infection was not present or incubating at the time of admission. This includes infections acquired in the hospital but appearing after discharge, and also occupational infections among staff of the facility." (WHO, 2002)] and community acquired infections (an infection acquired outside a hospital setting).

Globally, over the last few decades, *K. pneumoniae* in particular started to emerge as a pathogen associated primarily with nosocomial infections (Ko et al., 2002). In South Africa, the problem is exacerbated as this organism targets especially hospitalised patients with compromised immune systems. For example, Hlope & McKerrow (2014) reported on an outbreak of *K. pneumoniae* in a paediatric intensive care unit in Grey's Hospital (Pietermaritzburg) between July 2003 and December 2010. Moreover, the WHO stated in their report of 30 April 2014 that antimicrobial resistance (when bacteria mutate in such a way that renders antibiotics ineffective on the bacteria that needs to be eradicated) is no longer a prediction for the future, but a real and serious threat that is happening now. The WHO report (WHO, 2014) also mentioned the high resistance rate of *K. pneumoniae* in particular against the third generation carbapenems and cephalosporins. Furthermore, the resistance to the carbapenems, the last resort treatment for life threatening *K. pneumoniae* infection, has now spread worldwide, including to South Africa (WHO, 2014). Buys et al. (2016) then reported on a multi-drug resistant (when an organism is resistant to three or more classes of antibiotics normally used to treat that specific bacterial strain) *K. pneumoniae* outbreak in the Red Cross War Memorial Children's Hospital (Cape Town) from 2006 to 2011, where a total of 109 children died as a result of the infection.

## 1.2 Introduction to the bacteria *K. pneumoniae*

### 1.2.1 Classification

According to the National Centre for Biotechnology Information (NCBI) (2009) taxonomy database (*K. pneumoniae* genome, 2009) the taxonomic outline for *K. pneumoniae* is as follows:

Super Kingdom: Bacteria;
Phylum: Proteobacteria;
Class: Gamma Proteobacteria;
Order: Enterobacteriales;
Family: Enterobacteriaceae;
Genus: *Klebsiella*;
Species: *pneumoniae*; and
Abbreviation: *K. pneumoniae*

### 1.2.2 Description and differentiation

*Klebsiella pneumoniae* is named after Edwin Klebs, a 19th century German microbiologist (Umeh & Berkowitz, 2009). The bacterium is a Gram-negative, rod shaped (bacilli) organism which measures 2 µm in length and 0.5 µm in width (The Genome Institute at Washington, 2012) (**Figure 1.1)**. It is a facultative anaerobic, lactose fermenting, non-motile and capsulated bacterium (The Genome Institute at Washington, 2012; Umeh & Berkowitz, 2009). *Klebsiella* species are usually also identified and differentiated according to their biochemical reactions and are generally positive in the Voges-Proskauer test producing lysine decarboxylase but not ornithine decarboxylase. The bacteria *Raoultella terrigena* (*R. terrigena*) was previously classified as *Klebsiella terrigena* and many clinical laboratories confuse these two organisms as they are genotypically and phenotypically similar (Shaikh & Morgan, 2011). **Table 1.2** shows the differentiating biochemical properties of *Klebsiella* species versus *R. terrigena.* Shaikh & Morgan (2011) reported that *Raoultella terrigena* was first isolated from soil in 1981 and for many years no human isolates were reported until 1991, when *R. terrigena* was isolated from the stool of healthy humans. *Raoultella terrigena* were also isolated from the respiratory tract of humans and in 2007, the first human infection caused by this organism was reported (Shaikh & Morgan, 2011).


**Figure 1.1** Scanning electron microscopic (SEM) image of a *K. pneumoniae* bacterium (Photo credit Janice Haney Carr) (Carr, 2017).

*Klebsiella pneumoniae* and *K. variicola* are also very closely related species and it is often difficult to distinguish between the two species by commonly used methods. However, Berry et al. (2015) reported that *K. pneumoniae* ferment the carbohydrate adonitol, whereas more than 95% of *K. variicola* do not ferment this pentose alcohol.

### 1.2.3 Ecology

*Klebsiella pneumoniae* is an organism that exists ubiquitously in the environment and has been isolated from a variety of habitats such as surface water, lakes, salt water, fresh water, brackish water, sewage, soil, vegetation, grain, land mammals, sea mammals and importantly, humans (Kumar et al., 2011; Jang et al., 2010). In humans it is a commensal on the mucosal surfaces where it forms part of the normal flora of the mouth, skin and intestines (Bester, 1988; Bridson, 1993).

**Table 1.2 Biochemical reactions of *Klebsiella* species and *Raoultella terrigena* (adopted from Podschun & Ullmann, 1998)**

| Characteristic | *Klebsiella pneumoniae* | | | *K. oxytoca* | *K. planticola* | *K. ornithinolytica* | *Raoultella terrigena* |
|---|---|---|---|---|---|---|---|
| | **Subsp.** *pneumoniae* | **Subsp.** *ozaena* | **Subsp.** *rhinoscleromatis* | | | | |
| Indole | - | - | - | + | - | + | - |
| Ornithine decarboxylase | - | - | - | - | - | + | - |
| Pectate degradation | + | v* | - | + | + | + | + |
| Gas from lactose at 44.5°C | + | - | - | - | - | - | - |
| Growth at 10°C | - | - | - | + | + | + | + |
| Acid from: | | | | | | | |
| D-Melezitose | - | - | - | v | - | - | + |
| L-Sorbose | v | | | + | + | | + |
| Utilization of: | | | | | | | |
| *m*-Hydroxybenzoate | - | - | - | + | - | - | + |
| Hydroxy-L-proline | v | - | - | v | + | | v |
| Malonate | + | - | + | + | + | + | + |
| Methyl red test | - | + | + | - | v | + | + |
| Voges-Proskauer reaction | + | - | - | + | + | + | + |
| Adonitol fermentation | + | | | | | | |

***v denotes variable**

### 1.2.4 Genome structure

The genome of *K. pneumoniae* consists of one circular chromosome and multiple circular plasmids. A complete genome sequence of *K. pneumoniae* MGH 78578 (ATCC 700721) was determined from a multi-drug resistant strain isolated from human sputum at the Genome Sequencing Centre at Washington University in St. Louis, United States of America (The Genome Institute at Washington, 2012). This strain consists of one circular chromosome and five circular plasmids. Another complete genome of a multidrug-resistant strain named *K. pneumoniae* subsp. *pneumoniae* HS11286, isolated from human sputum, was sequenced by Liu et al. (2012). This strain consists of one circular chromosome and six circular plasmids.

### 1.2.5 Pathogenesis of bacteria

Pathogenesis of bacteria refers to a) the mechanism of infection and b) the mechanism by which disease develops (pathogenicity). It is not in the scope of this review to discuss these mechanisms in depth, but a number of common mechanisms used by bacterial pathogens to invade their host and cause disease will be discussed.

#### 1.2.5.1 Mechanisms of infection

When a primary pathogen is isolated from a patient it is considered the probable agent of disease, e.g., when *Salmonella spp.* is isolated from a patient with diarrheal disease. An opportunistic pathogen is isolated from a patient suffering from a compromised host defence mechanism, such as a predisposed urinary tract infection with *E. coli* by catheterising. Some bacteria are non-pathogenic because they never or very rarely cause disease. Their categorisation as non-pathogenic may change as a result of chemotherapy, immunotherapy, radiation therapy and antibiotic therapy leading to the emergence of resistance mechanisms. For example, *Serratia marcescens,* a common soil bacterium, that now causes pneumoniae, urinary tract infections and bacteraemia in compromised hosts - which was previously not known to cause disease (Peterson, 1996). That said, host susceptibility to bacterial infections depends on the physiologic and immunologic conditions of the host and the virulence of the bacteria.

*Klebsiella pneumoniae* is a saprophyte in the human intestinal tract. Although it forms part of the normal flora of the human body it causes disease when it invades

areas such as in the human lungs, urinary tract and blood. The increasing prevalence of antibiotic resistant strains only serves to increase this species' clinical importance (Lawlor et al., 2005; Spanu et al., 2006; Liu et al., 2012). *Klebsiella pneumoniae* can cause destructive changes in the human lungs if aspirated especially, in the lung alveoli, resulting in bloody sputum. Furthermore, *K. pneumoniae* infection is a cause of necrosis with a high risk of death (Umeh & Berkowitz, 2009).

The first response against invading bacteria is the cell-mediated immune response of the host resistance, such as polymorph nuclear neutrophils and macrophage clearance. Although the normal flora has the ability to inhibit pathogens, any disruption of this local environment can cause the normal flora to invade the host body. In addition, failure of the normal flora to suppress transient pathogens can lead to an invasion of the human body by these pathogens and cause infection. An infection can be defined as the process by which the host is invaded by bacteria, which multiply in close association with the host's tissue (Peterson, 1996) and begins when the balance between bacterial pathogenicity and host resistance is disrupted.

Bacteria can invade the host through a variety of routes. The skin and the mucous membranes are some of the most important barriers of the body to the microbial world. When the skin and mucosa is breached an individual becomes susceptible to infection with a variety of bacteria (**Table 1.1**) (Peterson, 1996). Such a breach can be due to burns or surgical wounds or any other type of wound. Patients with cystic fibrosis, where there is an inability to clear mucus from the respiratory tract due to poor ciliary function, are highly susceptible to infections with a mucoid strain of *Pseudomonas aeruginosa* resulting in serious respiratory distress (Peterson, 1996). Tracheal intubation and catheterisation of blood vessels and the urethra cause a breach of the skin and mucous barriers, which could lead to infection (Peterson, 1996). Bacteria colonising these devises, can migrate from the outside of the tube to infect deeper tissue or the blood stream (Peterson, 1996). Patients with defective immune responses are susceptible to even the least virulent bacteria and are prone to recurrent infections (Peterson, 1996). In patients infected with the human immunodeficiency virus (HIV), the $CD4^+$ helper lymphocytes are progressively disseminated by this virus leading to a compromised and weakened immune system (Peterson, 1996). Age also influences the specific defence systems: the immune

systems in infants have not yet fully developed and they cannot mount a protective immune response against pathogens, rendering them particularly vulnerable against group B streptococci (Peterson, 1996). Aging also weakens the defence systems and the elderly can no longer effectively combat bacterial invasion. Genetic defects of the compliment or cellular defence systems in some individuals also cause an inability of polymorph nuclear neutrophils to kill bacteria (Peterson, 1996). Finally, granulocytopenia (decrease in the number of granulocytes) may develop in some patients with a predisposing disease such as cancer, or in patients undergoing immunosuppressive chemotherapy for cancer or organ transplant (Peterson, 1996).

To cause infections, many pathogens must first adhere to the host surfaces, such as the skin, mucous membranes and deeper tissues. To establish an infection at any of these sites, the bacteria must adhere to the epithelium and multiply before the mucous and extruded epithelium are swept away by mechanical forces such as saliva secretion, sneezing, coughing, blood flow, mucous flow and peristalsis. To accomplish this, bacteria have attachment mechanisms or factors that bind to molecules on host tissue cells. These attachment mechanisms or adhesins can be made of polypeptides (protein) or polysaccharides (carbohydrates). Fimbriae (also pili) are protein adhesins of which the tip frequently serves to bind the host receptor. Many organisms are also surrounded by a thick polysaccharide network, called the capsule, which encloses the entire cell and protects the bacterium from phagocytosis.

#### 1.2.5.2 Mechanisms of bacterial pathogenicity

Disease is the tissue damage caused by the invading bacteria (Bester, 1988). Pathogenicity of a bacterium reflects its ability to cause disease (Bester, 1988). Virulence refers to the measure of the degree of pathogenicity or the severity of the disease (Bester, 1988). Virulence factors are produced by bacteria that evoke disease (Peterson, 1996). Pathogenic bacteria produce species specific virulence factors that allow them to multiply in their host without being killed or expelled by the host defence mechanisms.

Virulence factors can be divided into several functional types that enable pathogenic bacteria to achieve the following; attachment to the host cells, evasion of the host's immune response, inhibition of the host's immune response, entry into and exit out of the host's cells if the pathogen is intracellular and to sequester nutrients from the host

(Peterson, 1996). Some bacteria produce and excrete large amounts of high molecular weight polysaccharides or exopolysaccharides forming the capsule mucous coating which surrounds the bacterium (Wilson et al., 2002).

Capsules are one of the major virulence factors that cause bacteria to evade clearance from the site of infection. It functions mainly to protect the bacteria against the host inflammatory response that is compliment activation and phagocyte mediated killing, as well as antibiotics (Wilson et al., 2002). Some capsules have an immunomodulatory effect. The capsule prevents opsonising antibodies to be recognised by the phagocytes (macrophages and neutrophils) of the host defence system. As there is no recognition of the pathogen by the phagocytic system, more inflammatory cytokines are produced by macrophages and neutrophils in an attempt to clear the bacteria. This process leads to enhanced inflammatory response where the host immune response causes more host tissue damage than the bacterial factors due to the ever-increasing number of neutrophils and macrophages at the site of infection (Wilson et al., 2002). *Streptococcus pneumoniae*, *K. pneumoniae Neisseria meningitides,* and *P. aeruginosa* are the most notorious bacterial species to produce capsules (Wilson et al., 2002; Kumar et al., 2011). At least 90 different capsules are known of which only a subset of 23 types are known to cause more than 90% of invasive diseases worldwide (Wilson et al., 2002).

### 1.2.6 Pathophysiology

As previously mentioned *K. pneumoniae* forms part of the normal flora but, it can also be classified as an opportunistic pathogen (Bester, 1988; Bridson, 1993), meaning that it usually does not cause infection in a host with a healthy immune system. However, in a debilitating immune system, it spreads and cause infection. This organism infects humans of all ages, especially infants, hospitalised patients, the elderly, and immunocompromised patients with an underlying condition such as chronic pulmonary obstruction, diabetes mellitus and chronic alcohol abuse (Highsmith & Jarvis, 1985). In a hospital environment, the main reservoir for transmission of *Klebsiella* are the hands of personnel and the gastrointestinal tract of patients (Podschun & Ullmann, 1998). In *K. pneumoniae*, the capsular polysaccharide contains the major virulence factors associated with pathogenesis (Kumar et al., 2011). Furthermore, it expresses two types of antigens (a lipopolysaccharide or O antigen and a capsular polysaccharide or K antigen) on its

cell surface which contributes to its pathogenicity. Additionally, multiple antibiotic resistance genes carried on the chromosome inhibits the effect of antibiotics to clear the organism from the host tissue (Amako et al., 1988). The increasing prevalence of antibiotic resistant strains only serves to increase this species' clinical importance (Lawlor et al., 2005; Spanu et al., 2006; Liu et al., 2012).

The Cephalosporin group of antibiotics are now in their fifth generation (extended spectrum antibiotics) with the third generation (broad spectrum effective against Gram-positive and Gram-negative bacteria) of antibiotics (Ceftazidime, Cefoperazone, Ceftriaxone, Ceftizoxime, Ceftaroline, Ceftiofur, Ceftibuten, Ceftamere, Cefteram, Cefpodoxime, Cefpimizole, Cefodizime, Cefmenoxime, Cefixime, Cefetamet, Cefditoren, Cefdinir, Cefdaloxime, Cefcapene) under pressure against resistance. With each new generation there are fewer antibiotics developed.

Many nosocomial infections around the world have been linked to *K. pneumoniae* and a worldwide study indicated that *K. pneumoniae* was often also linked to community acquired infections (Ko et al., 2002). Infections with *K. pneumoniae* cause a severe, rapid-onset illness associated with necrosis with mortality rates of up to 60% (Lawlor et al., 2005; Umeh & Berkowitz, 2009). In the case of lung infections, it often causes destruction of the lungs with abscess formation, inflammation, haemorrhage and necrosis. Symptoms are flu-like, and includes fever, chills, shortness of breath, severe breathing difficulties, pain in the chest, and a cough with abundant mucous described as "currant jelly" due to the presence of thick mucus and blood. The production of extended spectrum beta-lactamase (ESBL) is also a cause of growing concern as there is a lack of new antibiotics effective against these organisms.

Infections can spread to virtually every part of the body where it can cause infections such as urinary tract infection, septicaemia, respiratory tract, soft tissue infection, wound infections, septic arthritis, rhinoscleroma (chronic granulomatous disease or chronic inflammatory process involving the nasopharynx caused by *K. pneumoniae subsp. rhinoscleromatis*), ozaenae (chronic atrophic rhinitis, characterised by necrosis of nasal mucosa and mucopurulent nasal discharge caused by *K. pneumoniae subsp. ozaniae*), pyogenic liver abscess, osteomyelitis, meningitis, brain abscess, bacteraemia, thrombophlebitis (Highsmith & Jarvis, 1985; Peña et al., 1998)

and Friedlander's pneumonia [*Klebsiella* was first identified as a cause of pneumoniae in 1882 by the pathologist named Karl Friedlander (Lawlor et al., 2005), hence the name Friedlander's pneumoniae] associated with chronic alcoholics and neonatal septicaemia (Peña et al., 1998).

### 1.2.7 Epidemiology

In an international prospective study of *K. pneumoniae* bacteremia, Paterson et al. (2004) reported that out of seven participating countries, the highest proportion of nosocomial linked *K. pneumoniae* bacteremia was found in South Africa (**Table 1.3**). South Africa was ranked third for the proportion of nosocomial *K. pneumoniae* bacteremia due to ESBL-producing *K. pneumoniae* with a proportion of 37% (**Table 1.4**). Brink et al. (2008) reported an even higher proportion of 50% of ESBL-producing *K. pneumoniae* strains isolated from hospitals in major centres in South Africa namely, Bloemfontein, Cape Town, East London, Durban, Johannesburg, Port Elizabeth and Pretoria.

**Table 1.3 Proportion of nosocomial *K. pneumoniae* bacteraemia in seven participating countries (ranked from highest to lowest) (adopted from Paterson et al., 2004)**

| **Participating countries** | **% Infection** |
|---|---|
| South Africa | 31.1 |
| Taiwan | 18.9 |
| Australia | 17.2 |
| Argentina | 13.9 |
| United States | 10.2 |
| Belgium | 4.9 |
| Turkey | 3.7 |

**Table 1.4 Proportion of nosocomial *K. pneumoniae* bacteremia due to ESBL-producing *K. pneumoniae* (ranked from highest to lowest) (adopted from Paterson et al., 2004)**

| **Participating countries** | **% ESBL-producing *K. pneumoniae*** |
|---|---|
| Turkey | 78 |
| Argentina | 59 |
| South Africa | 37 |
| United States | 36 |
| Belgium | 25 |
| Australia | 12 |
| Taiwan | 7 |

## 1.3 Antibiotics

### 1.3.1 The mechanism of activity of antibiotics

Antibiotics works with five major mechanisms of activity (Shaikh et al., 2015):

(a) Interference with cell wall synthesis:

Penicillin and Cephalosporins impedes enzymes that are responsible for the formation of peptidoglycan layer.

(b) Inhibition of protein synthesis:

Aminoglycosides binds to the 30S ribosomal subunit and inhibit initiation of protein synthesis.

(c) Interference with nucleic acid synthesis:

Quinolones interferes with type II topoisomerase, DNA gyrase and type IV topoisomerase which cause a double-strand to break thus inhibiting DNA synthesis during the replication cycle.

(d) Inhibition of a metabolic pathway:

Sulfonamides and trimethoprim inhibits DNA and RNA synthesis by blocking folate synthesis needed for biosynthesis of nucleotides – the building blocks of DNA and RNA.

(e) Disorganising the cell membrane:

The hypothesis is that polymyxins have an effect on cell membrane permeability. It increases membrane permeability causing leakage of the bacterial content and cell death.

The first antibiotic was discovered in 1928 by Alexander Fleming in the mould *Penicillium notatum* and was named after this mould hence the name penicillin, which was found to be active against staphylococcal strains. This penicillin is also known as Penicillin G or benzylpenicillin - a beta-lactam antibiotic. The mortality rate of individuals infected with *Staphylocccus aureus* during that time was about 80% and for the first-time clinicians could combat fatal bacterial infections. The clinical success of Penicillin G prompted the search for, and development of, additional derivatives.

Due to advances in engineering and biochemistry, the development and production of many different and novel antimicrobial agents commenced by high throughput screening strategies (HTS) (a process by which millions of chemical, pharmacological and genetic tests are conducted by the use of specialised equipment and data processing and control software to rapidly identify genetic elements, antibodies or active compounds which modulate a specific biomolecular pathway) or by altering the structure of known antimicrobial agents. The products of these tests include narrow- and extended-spectrum cephalosporins, monobactams and carbapenems that are beta-lactam antibiotics in use today, to name a few. A four membered beta-lactam ring is the common structural feature of these classes of antibiotics, which is responsible for its antibacterial activity (Drawz & Bonomo, 2010), hence the name beta-lactam antibiotic. These antibiotics are used for the treatment of infection caused by Gram-negative organisms such as *K. pneumoniae*. Broad-spectrum beta-lactam antibiotics - the Cephalosporins - were discovered with an expanded spectrum of action against Gram-positive and Gram-negative bacteria.

Today antibiotic therapy continues to be the mainstay for the treatment of bacterial infections. Environmental organisms produce antimicrobial substances as demonstrated by the mould, so it is not surprising to note that antimicrobial resistance mechanisms also should have been around for a number of years as demonstrated by the following: Beta-lactamase activity was reported in *Escherichia coli* even before penicillin became commercially available (Bradford, 2001); an ampicillin (a derivative of penicillin) resistant bacteria, estimated of being 2000 years old, was discovered in glacial samples from the Canadian Arctic Archipelago (Dancer et al., 1997); in addition, Temoniera (TEM)-type beta-lactamases were detected among clones of a metagenomic library from the cold-seep sediments of the Edison

seamount estimated as being older than 5000 years (Song et al., 2005). However, since Alexander Fleming's breakthrough discovery and the scientific advances made in the development of new antimicrobial agents to combat bacterial infections, bacteria are constantly acquiring mechanisms to neutralise the effects of antibiotics due to mutations.

These mechanisms led to the emergence of beta-lactamase-mediated antibiotic resistance, which became a significant clinical threat to the use of beta-lactam antibiotics (Drawz & Bonomo, 2010). It is these antimicrobial resistance mechanisms that cause problems in human health, because with each new antibiotic that is produced and manufactured, new antimicrobial resistance mechanisms appeared, hindering the successful treatment of certain bacterial infections.

### 1.3.2 Antibiotic resistance mechanisms

Shaikh et al. (2015) described antibiotic resistance as: "The reduction in effectiveness of a drug such as an antimicrobial or an antineoplastic in curing a disease or condition". Correspondingly, there are mainly three resistance mechanisms that can be harboured by a bacterial strain (Shaikh et al., 2015):

(a) Antibiotic inactivation:

(i) By hydrolysis - Many antibiotics have a beta-lactam ring consisting of chemical bonds such as amides and esters. These bonds can be broken by hydrolysing enzymes rendering the antibiotic inactive. These enzymes are excreted in the form of ESBL's and are effective against all Penicillins, third generation Cephalosporins (e.g. Ceftazidime, Cefotaxime, and Ceftriaxone) and Aztreonam, excluding Cephamycins (Cefoxitin) and the carbapenems (Shaikh et al., 2015).

(ii) By redox process - This is the process where pathogenic bacteria exploit oxidation or reduction of an antibiotic by reducing a critical ketone group to an alcohol at position 16.

(b) Antibiotic inactivation by group transfer:

Transferases are the most diverse family of resistant enzymes. These enzymes add adenylyl, phosphoryl or acetyl groups to the periphery of the antibiotic molecule, in effect modifying the antibiotic preventing it from bind to the target.

(c) Antibiotic resistance via target modification:

The antibiotic target site is modified, effectively preventing the antibiotic from binding to the target.

Acquisition of genetic material is largely responsible for the development and escalation of antibiotic resistant strains of bacteria (Bennet, 2008). Horizontal gene transfer is the principal mechanism by which genetic material is transferred from one cell to another with different mechanisms such as transformation, transduction and conjugation, employed (**Figure 1.2**). Conjugation is perhaps the dominant form by which genetic material is transferred from one plasmid to another or from plasmid to bacterial chromosome or from bacterial chromosome to plasmid. Plasmids are transferable, extra chromosomal, genetic material that does not form part of the bacterial genome. Plasmids do not depend on the bacterial chromosomal deoxyribonucleic acid (DNA) for replication and can carry a variety of genes, including antibiotic resistance genes. Resistance plasmids encode for antibiotic resistance against most classes of antibiotics currently in clinical use, including the commonly used Cephalosporins, Fluoroquinolones, Aminoglycosides (Bennet, 2008) and Carbapenems (Souli et al., 2008).

The transfer of genetic material within the bacterial chromosome is done by transposons. Transposons can carry a variety of resistance genes and transfer these resistance genes into the bacterial genome or plasmid by incorporating itself into the bacterial chromosome or plasmid. A well-known example is CTX-M-15. This extended spectrum beta-lactamase were initially found in *E. coli* but are now found in other members of the Enterobacteriaceae (Mshana et al., 2009). This gene is often associated with the mobile genetic element IS26, located on the highly mobile IncFII plasmid (Mshana et al., 2009). It has an extended spectrum of activity against the majority of beta-lactams including the Cephalosporins (Mshana et al., 2009).

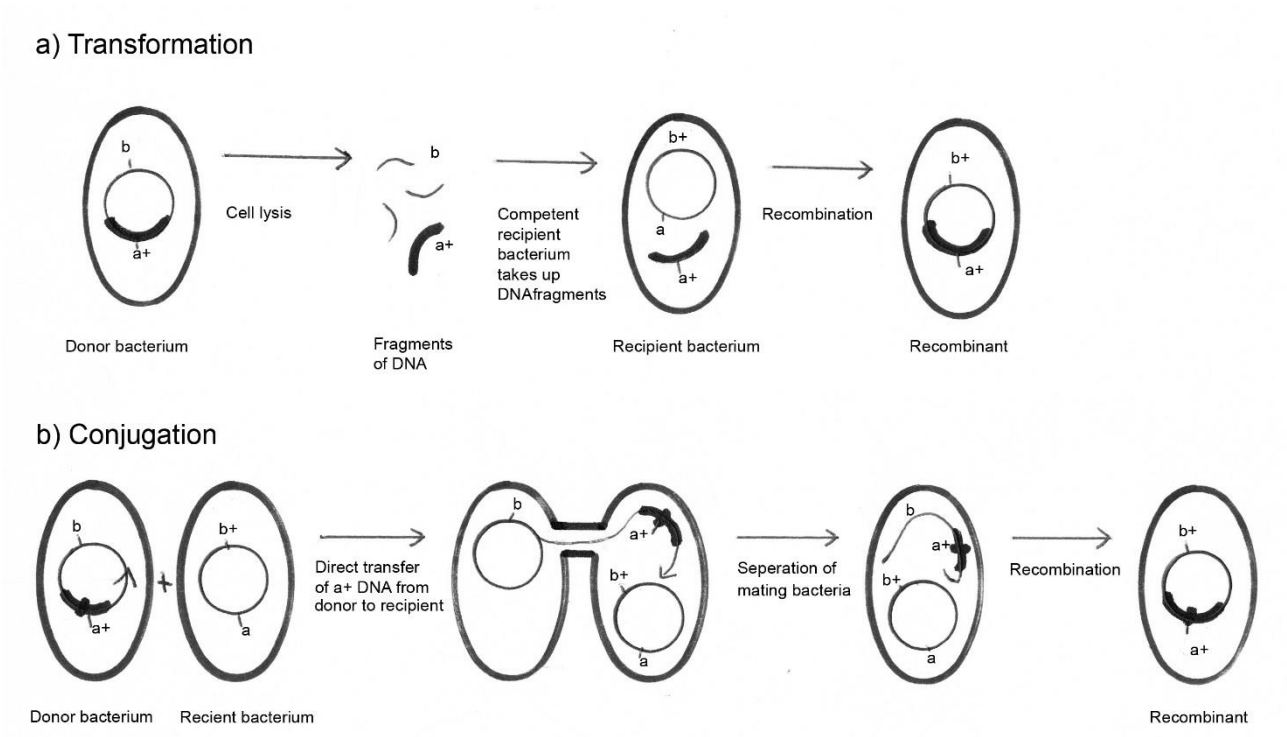


**Figure 1.2** Transfer of genetic material in bacterial cells. [Adapted from Holmes & Jobling, (1996)]

### 1.3.3 Cephalosporins

Cephalosporins are a family of antibiotics first isolated from the fungus *Cephalosporium acremonium* during 1948 by Giuseppe Brotzu. Cephalosporins contain a beta-lactam structure similar to penicillin and therefore also inhibit the transpeptidase reaction during peptidoglycan synthesis. This soon became the antibiotic of choice to treat mild to severe bacterial infections (Adesoji et al., 2016). However, resistance towards the Cephalosporins quickly emerged creating problems for treating infections caused by Gram-negative bacteria.

In response to these challenges, beta-lactam antibiotics that are able to evade bacterial enzymatic inactivation by beta-lactamases were developed or discovered, or beta-lactamases were inhibited so that the beta-lactam can reach the penicillin binding protein (PBP), the target of the beta-lactam antibiotic (Drawz & Bonomo, 2010). When bacterial cells grow and divide their cell walls are continuously being broken down and rebuild, in effect remodelling the peptidoglycan in the cell wall. In Gram-negative bacteria, the peptidoglycan molecules are cross-linked to each other by the bacterial enzyme transpeptidase in a manner resembling chain mail. When penicillin reaches the transpeptidase enzyme it binds with it to form a highly stable

penicilloyl-enzyme intermediate. This bond is irreversible and inhibits the beta-lactamase activity. Because of the interaction between penicillin and transpeptidase, this enzyme is also known as the PBP.

### 1.3.4 Beta-lactamases and Extended-spectrum beta-lactamases

Beta-lactamases are enzymes that are synthesised by Gram-negative bacteria. According to the Ambler-classification (Bonnet, 2004), beta-lactamases are classified into 4 groups: A, B, C and D according to their amino acid sequences. Classes A and C are the most frequent found beta-lactamases among bacteria. These antibiotics all have a four-atom ring known as a beta-lactam ring in their molecular structure. This ring reacts with the penicillin target. In short, the beta-lactamase breaks the beta-lactam ring open thus deactivating the antibacterial properties of the beta-lactam molecule. However, during 1985 a new group of enzymes that could hydrolyse and inactivate the oxyimino-Cephalosporins or expanded spectrum beta-lactams was detected for the first time in Germany (Turner, 2005) and was designated ESBL.

Cantón et al. (2012) stated that ESBL's are characterised by their ability to hydrolyse third and fourth generation Cephalosporins (oxyimino-cephalosporins) and monobactams, but not Cefoxitin. According to Shaikh et al. (2015), there is no consensus as to the precise definition of an ESBL. But ESBL's can more or less be defined as enzymes that are produced by members of the Enterobacteriaceae, capable of destroying broad-spectrum Cephalosporins with an oxyimino side chain (the side chain is attached to the beta-lactam nucleus and can be easily modified to have profound effects on the properties of the beta-lactam molecule such as resistance to penicillinase, gastric acid tolerance or the ability to penetrate the outer membrane of Gram-negative organisms). They are also in general susceptible to beta-lactamase inhibitors such as Clavulanate, Sulbactam and Tazobactam (Cantón et al., 2012) The amino acid configuration around the active site of the beta-lactamases are altered by mutations, thus extending their resistance to a broad spectrum of beta-lactam antibiotics. "Bad" bacteria now became "ugly" due to the over-use of group A, C and D beta-lactam antibiotics that lead to the production of ESBLs that are responsible for a multi-drug resistant (MDR) phenotype in *K. pneumoniae*. Amongst the Gram-negative MDR pathogens certain microbes are of particular interest, e.g. *Escherichia coli*, *K. pneumoniae* and

*Acinetobacter baumannii*. The reason for this is linked to the beta-lactamases (Shaikh et al., 2015).

### 1.3.5 Antibiotic treatment for *K. pneumoniae*

A study conducted by Krapivina et al. (2007) showed that Carbapenems and Cefoperazone/Sulbactam are the most active antibiotics against Gram-negative bacteria including *K. pneumoniae*. Endimiani and Paterson (2007) also revealed that the Carbapenems are the drug of choice against bacteria that produce ESBL. Wide spread use of antimicrobial substances, especially of multiple antibiotics and extended-spectrum antibiotics [this is a broad class of antibiotics that contain a beta-lactam nucleus in its molecular structure; it includes penicillin (penams), Cephalosporins (Cephems), Monobactams and Carbapenems] are considered the primary cause for the development of MDR *Klebsiella* in hospitals. *K. pneumoniae* isolates must be resistant to three or more classes of the following antibiotics in order to be classified as multi-antibiotic resistant (MAR)/ MDR: (Brink et al., 2008):

1. Anti-pseudomonal cephalosporins (Ceftazidime and Cefepime)
2. Carbapenems (Ertapenem, Imipenem and Meropenem)
3. Beta-lactam/[beta]-lactamase inhibitor combination (Piperacillin/ Tazobactam)
4. Fluoroquinolones (Ciprofloxacin or Levofloxacin)
5. Aminoglycosides (Amikacin, Gentamicin and Tobramycin)
6. Aztreonam
7. Polymyxin E
8. Tigecycline

The antibiotic frequently used for the treatment of ESBL-producing *K. pneumoniae* infections are two types of Carbapenems, namely Imipenem and Meropenem (Lee et al., 2006). However, it is predicted that the selective pressure resulting from the increasing use of Carbapenems will lead to the development of Carbapenem-resistant *K. pneumoniae* (Lee et al., 2006). The in vivo development of an Ertapenem (Carbapenem) resistant MAR strain of ESBL-producing *K. pneumoniae* was first reported in a patient with pneumonia in a hospital in Cape Town (Brink et al., 2007). The minimum inhibitory concentrations (MIC) of Imipenem and Meropenem were four- and eight-fold increased, respectively, in comparison to control strains (Brink et al., 2007). Two similar case reports were also described in Cape Town hospitals (Brink et al., 2007). However, as predicted by Lee et al. (2006), the WHO

reported in 2014 a high resistance rate of *K. pneumoniae* against the third generation Cephalosporins and even the Carbapenems according to data received from 194-member states divided into 6 regions (WHO, 2014) (**Table 1.5**).

**Table 1.5 Prevalence of *K. pneumoniae* causing infections in hospitals and in the community and the resistance patterns based on data obtained from 194-member states divided into 6 regions (adapted from WHO, 2014)**

| **Antibiotic – 3rd generations** | **Sites of Typical infections** | **Number of WHO regions with national reports of 50% or more resistance** | **Number of Member states providing data** |
|---|---|---|---|
| Cephalosporins | Bloodstream, pneumoniae (lungs), urinary tract | 6 | 87 |
| Carbapenems | | 2 | 71 |

To relieve the selection pressure resulting from over use of Carbapenems, Lee et al. (2006) proposed an alternative to Carbapenems in the form of Cephamycins i.e. Cefmetazole, Cefotetan and Flomoxef. Flomoxef also yields better in vivo activity against ESBL-producing *K. pneumoniae* than other Cephamycins, due to its unique structure of having a difluoromethyl-thio-acetamide group at carbon position seven. Moreover, according to Souli et al. (2008), Colistin (introduced in the 1950's) and Tigecycline (a semi-synthetic glycylcycline introduced in 2005) are the only options available against MDR Enterobacteriaceae. Jones (2001) then advised that the prevalent resistance patterns should be considered in selecting a treatment for a nosocomial infection.

Faure (2015) reported that the antibiotic of choice during 2010 in South Africa was Trimethoprim (Cotrimoxazole) with a consumption of 15000 to 20000 standard units per population of a 1000, with the broad-spectrum penicillin following close behind with 5000 to 10000 standard units per population of a 1000, followed by the macrolides, Quinolones and Tetracycline with less than 5000 units each. The Trimethoprim consumption in South Africa far exceeds other countries such as Brazil, Canada, China, India, Russia, United Kingdom and United States, which have a consumption of less than 5000 standard units per population of a 1000. Faure (2015) also reported on the resistance pattern for *K. pneumoniae* in South Africa namely, 32% resistance against the third generation Cephalosporins, 30% resistance against the Fluoroquinolones and 2% for the Carbapenems.

### 1.3.6 Antibiotic resistance genes found in *K. pneumoniae*

The “war” against bacteria has restricted the spread of many pathogenic bacteria and has correspondingly lessened the burden of human disease. However, for a period of 30 years up to 2009, 37 new pathogens were identified as disease threats (Relman et al., 2009) and pathogens continue to cause infectious diseases with a high morbidity and mortality throughout the world with an estimate of 26% deaths by infectious disease recorded in 2001 (Relman et al., 2009). Moreover, globally resistance mechanisms are a major problem limiting the successful treatment of infectious bacterial diseases by healthcare professionals. The production of ESBL’s is considered the most significant resistance-mechanism hindering the effective use of Enterobacteriaceae infections with available antimicrobial treatments. Numerous reports in the field of biomedical literature provide evidence that *K. pneumoniae* isolates harbour several genes associated with ESBL production of which the most important are TEM, SHV and CTX-M (Mendonca et al., 2009; Shaikh et al., 2015; Cantón et al., 2012; Dropa et al., 2010)

#### 1.3.6.1 SHV and TEM

Extended spectrum beta-lactamases are commonly of the sulphydryl variable (SHV) and TEM-type, which are generally plasmid mediated, and to date more than 50 SHV-type beta-lactamases and 130 TEM-type have been described. The TEM and SHV ESBL enzymes had their origin from point mutations from the plasmid-mediated enzymes TEM-1, TEM-2, and SHV-1. These 3 enzymes are now considered to be of great importance in hydrolysing extended-spectrum Cephalosporins (Cantón et al., 2012). SHV-1 plasmid mediated beta-lactamase was discovered in *K. pneumoniae* and *E. coli* in 1972. During 1985 in Germany a SHV was discovered in a strain of *K. pneumoniae* with the ability to hydrolyse this expanded-spectrum beta-lactam antibiotic and was named SHV-2 (Turner, 2005). Oxyimino-cephalosporins were the antibiotic of choice for the treatment of severe Gram-negative bacterial infections, but resistance rapidly emerge. During the 1980’s and 1990’s, TEM- and SHV-ESBL’s were predominant in the ESBL landscape and were associated with nosocomial outbreaks involving *K. pneumoniae*. During this time, CTX-M was less prevalent.

The acronym “TEM” was derived from the patient’s name, Temoniera, from which the strain was first isolated during 1965 in Greece. Temoniera-type beta-lactamases has rapidly spread to other members of the Enterobacteriaceae and even different

species (Turner, 2005). Oxyimino-cephalosporins were again the antibiotic of choice for the treatment of severe Gram-negative bacterial infections but, resistance was rapidly acquired. Lal et al. (2007) reported that the majority of *K. pneumoniae* isolates carry both TEM and SHV as oppose to either TEM or SHV alone.

#### 1.3.6.2 CTX

The Cefotaximase (CTX-M)-type beta-lactamases are most active against Cefotaxime (Khalaf, et al., 2009) hence the name Cefotaximase. The CTX-M family of enzymes are non-homogeneous and complex (Cantón et al., 2012). The different CTX-M variants were classified into five clusters according to the alignment of the amino acid sequences (CTX-M group 1, 2, 8, 9 and 25) (Shaikh et al., 2015). Each cluster is named after the first described member (Rossolini et al., 2008). Rossolini et al. (2008) mention a sixth cluster namely CTX-M-45, but stated that the subgroups remain to be identified, but would most likely be members of the genus *Kluyvera*. The chromosomal genes resident in members of the genus *Kluyvera* is considered the source of CTX-M determinants (Rossolini et al., 2008). From here CTX-M diverged by point mutation probably due to the selective pressure by antibiotics after the genes were mobilised and incorporated into mobile genetic elements. The CTX-M-ESBL's were first discovered during 1989 in Europe, but only came under the spotlight in the first decade of the 2000's when extraordinary dispersion was observed (Cantón et al., 2012). They are now considered as the most abundant and wide spread beta-lactamase type enzymes (Bonnet, 2004) that are now found in hospital settings as well as in the community (Cantón et al., 2012). In clinical bacterial strains, the genes encoding for CTX-M enzymes are primarily located on plasmids. CTX-M and TEM-1 can co-exist with TEM-1 on the same plasmid and may associate with TEM-2 and SHV-type enzymes (Bonnet, 2004). These plasmids vary in size from 7 kb up to 160 kb and can carry genes for resistance to a multiple of other antibiotics such as Aminoglycosides, Chloramphenicol, sulphonamide, Trimethoprim and Tetracycline. In-vitro these plasmids move around by conjugation transferring genetic material from one bacterium to the next (Bonnet, 2004).

## 1.4 Techniques used for the identification of bacteria

Bacteria are a diverse group of microscopic, rigid-walled, unicellular organisms which multiply asexually by means of binary fission. There are exceptions to the above broad definition as this heterogeneous group of living cells can vary in size, shape, structure and function. The accurate and definitive microorganism identification, including bacterial identification and pathogen detection, is essential for correct disease diagnosis, treatment of infection and trace-back of disease outbreaks associated with microbial infections.

The main objective of a medical microbiology laboratory is to culture bacterial cells successfully in order to: a) identify them and b) test their antibiotic sensitivity. This is a time-consuming procedure that can take several days before the organism is successfully isolated. Traditional methods of bacterial identification rely on phenotypic identification of the causative organism using various methods. These methods include identification by Gram-staining, morphology, culture and biochemical methods.

The shape and cell wall structure and to some extent the size of the cell, is valuable in the initial identification of the pathogenic bacterial organism. Although bacteria are microscopic they do vary greatly in size. Bacterial cells are measured in micrometre (µm) units which is $10^{-6}$ meter (m) or $10^{3}$ nanometre (nm). The smallest prokaryotic cell can be as small as 0.2 µm, but the size of bacteria is usually given as a mean due to the variation in size within the same species depending on the age, phase in the growth cycle and presence of antibiotics (Bester, 1988). Most disease-producing bacteria range in size from 0.2 to 1.4 µm in diameter and 0.4 to 13 µm in length. Typical cocci such as *Staphylococcus spp*. are 1 µm in diameter while typical bacilli such as *Klebsiella spp.* are 2 to 5 µm in length (Bester, 1988). Identification of species usually starts by performing a Gram-stain that is used to visualise the shape of the bacterium such as a cocci or bacilli, using a light microscope (Bester, 1988). The mechanism of this stain is based on the permeability differences of the cell wall due to cell wall structure and composition. The Gram-stain distinguishes between the differences in cell wall structure by producing a colour difference visible as either purple for Gram-positive and red for Gram-negative (Bester, 1988).

Biochemical tests are also widely utilised in identifying an unknown bacterial species. Bacterial species vary in their metabolic make-up producing different metabolic reactions. This forms the basis of many biochemical tests. These tests are used to test for the production of certain end products such as Indole, acetyl methyl carbinol, Niacin or urease production; test for the breakdown of certain substrates such as nitrate reduction; and/or determine whether an organism is able to utilise a particular organic substrate such as sodium citrate (Bester, 1988). Metabolic reactions are catalysed by enzymes and the biochemical tests are thus used to determine the presence or absence of a particular enzyme (Bester, 1988).

The standard methods utilised for bacterial identification have two major drawbacks. First, they can be used only for organisms that can be cultivated in vitro and secondly, some strains exhibit unique biochemical characteristics that do not fit into patterns that have been labelled as a characteristic for any known genus and species. Phenotypic identification systems such as the API systems are however the most common methods used in the rapid presumptive identification of microbial species. It is important to note that the genome of a given species is extremely complex and the biochemical properties analysed in these identification systems may not accurately reflect the organism's metabolism and chemistry. Phenotypic properties can also be unstable at times as their expression is dependent upon changes in the environmental conditions such as growth substrate, temperature and pH levels (Janda & Abbott, 2002). Furthermore, the configuration of the biochemistry panels in these identification systems rarely changes after commercial production although formulation of tests does occur (Janda & Abbott, 2002). For example, the 20 biochemical tests on the API 20 E, patented on February 3rd, 1976 (Janin, 1976), are still the same biochemical test on the API 20 E strip used today (BioMérieux, 2010). Despite the above-mentioned drawbacks of phenotypic methods, it serves the clinical laboratories well in the identification of medically important bacterial strains and remains the method of choice for preliminary identification.

### 1.4.1 Molecular based techniques

In the past decade molecular techniques have proven beneficial in overcoming some limitations of traditional phenotypic procedures for the detection and characterisation of bacterial phenotypes. Using a DNA based assay, one can easily detect bacterial strains directly from clinical samples or from small amounts of cultured bacterial cells,

thus improving the sensitivity and decreasing the time required for bacterial identification. The polymerase chain reaction (PCR) has been particularly useful in this regard and relies on primer sequences designed to facilitate bacterial identification at any level of specificity: strain, species or genus. Many of the problems encountered with phenotypic tests can be overcome with specific PCR primers and will be very useful in the diagnosis of culture-negative infections. However, PCR also has several disadvantages: it is costly, it is labour intensive and technically complex and human error occurs more frequently than with phenotypic methods.

#### 1.4.1.1 Polymerase chain reaction amplification

Molecular-based detection methods, such as PCR amplifies specific target DNA of the organism investigated. Ribosomal DNA (rDNA), more specifically the 16S (small subunit) rDNA genes were, used for studies at subgeneric level, but such studies were deemed not appropriate by Boyer et al. (2001) as the 16S rDNA genes were found to be highly conserved (Liu et al., 2008). Probes were then designed to target the 16S-23S internal transcribed spacer (ITS) (Boyer et al., 2001). This 16S-23S internal transcribed spacer is more genetically variable and species specific than 16S or 23S (Liu et al., 2008). The primers used in this project was designed by Liu et al. (2008) to target the 16S-23S ITS of *K. pneumoniae* and were employed in specificity testing. The great advantage of PCR is its specificity and DNA can be targeted with specific primers, out-performing culture-based methods. The PCR is also an excellent technique for the rapid detection of pathogens, including those difficult to culture (Valones et al., 2009). Limitations to this technique are its inability to distinguish between dead and viable cells, which could lead to false positive results.

Rapid yet effective surveillance methods are vital in determining the measures needed to control antibiotic resistance. Additionally, identification of potential new sources for different antibiotic resistance genes is critical. These goals require tools that can be used for profiling of antibiotic resistance genes from various types of samples. New and rapid laboratory methods are necessary to facilitate this important effort. Several antibiotic resistance genes are prevalent in a variety of sample types including sputum, urine, blood samples to name but a few and molecular techniques such as PCR have overcome the limitations of traditional culture-based methods.

### 1.4.2 Automated Techniques

Over the last three decades automated systems have been developed for the identification of Gram-negative rods. However, these systems are culture-based techniques and the success of the identification rely on pure and viable cultures.

#### 1.4.2.1 MALDI-TOF

Matrix-assisted laser desorption/ ionisation time of flight mass spectrometry (MALDI-TOF MS) is a promising rapid detection method that is extensively used in clinical microbiology laboratories for the identification of microorganisms (Loff et al., 2014). The initial cost of the instrument is high, but the technique is relatively inexpensive for the routine biotyping of bacteria. Furthermore, it is a powerful tool for the identification of clinical isolates due to short turnaround times, low reagent cost, and low sample volume required (Pavlovic et al., 2013). The MALDI-TOF MS technique is used to identify and analyse proteins by generating a spectral profile of the abundant bacterial proteins from whole bacterial extracts (Loff et al., 2014). The ability of this method to detect a large spectrum of proteins enables it to differentiate between species that are very closely related (Loff et al., 2014). However, when this technique is used for biotyping, the isolates needs to be cultured on general media and only culturable organisms present are considered. As mentioned previously, for the benefit of a clinical laboratory the ideal solution would be to develop approaches that do not require culture-based steps.

#### 1.4.2.2 VITEK

The VITEK 2 system, is an automated system that is widely used in clinical microbiology today. It differs considerably from its predecessors in its ability to provide definitive identification results for Gram-negative rods including the Enterobacteriaceae, within three hours (Funke et al., 1998). It utilises a new fluorescence-based technology that is much more sensitive in detecting metabolic changes and is continuously monitoring the reactions resulting in the faster identification of the test organism (Sanders et al., 2001). Moreover, the software identifies the bacterial strain then interprets the antibiotic resistance patterns. It is thus a reliable and efficient tool for the accurate detection of possible ESBL producing strains (Robin et al., 2008).

The Advanced Expert System (AES) incorporated into the VITEK 2 system has several functions: it analyses results generated by the VITEK 2 system for biologic validity and then provides comments on the results; it looks for inconsistencies between the identification of the organism and the antimicrobial susceptibility of the isolate; it ascertains the antimicrobial phenotype of the isolate based on results of susceptibility tests; it uses the antimicrobial susceptibility data generated to suggest the phenotype of the tested isolate and thereby determines susceptibility or resistance to antibiotics tested (Sanders, et al., 2001). The VITEK 2 is thus considered a powerful tool for the rapid and definitive identification of Gram-negative rods and its phenotypes such as ESBL *K. pneumoniae*. However, VITEK 2 is a growth-based application that requires live and pure bacterial cultures for its methodology. Turnaround times for the identification of bacteria are thus lengthened.

### 1.5 Hypothesis and objective of this study

From the literature it is clear that *K. pneumoniae* is not merely a harmless member of the human body's normal flora. Given the opportunity it can develop into a deadly opportunistic pathogen. Frequently it has been identified as the culprit to cause nosocomial and community acquired infections. This organism infects humans of all ages, especially the elderly, infants and immunocompromised patients with an underlying condition, such as chronic pulmonary obstruction, diabetes mellitus and chronic alcohol abuse. Over the last few decades strains of the genus *Klebsiella* have systematically developed resistance to numerous antibiotics and by the 1970's *Klebsiella* strains were resistant to the aminoglycosides (the antibiotics which could previously control them). By 1982 ESBL producing strains resistant to the extended-spectrum cephalosporins, in other words, the third generation Cephalosporins, emerged. Major outbreaks of *K. pneumoniae* were already reported in major centres in South Africa including the Western Cape – these outbreaks caused severe illness and even death especially under neonatal ICU infants. A study conducted by Brink et al. (2008) at institutions in major centres in South-Africa (Bloemfontein, Cape Town, East London, Durban, Johannesburg, Port Elizabeth and Pretoria) revealed that 50% of *K. pneumoniae* strains isolated, were producers of ESBLs. The emergence of, and extensive spread of antibiotic resistant strains and ESBL producing strains, are a major cause of concern as it restricts and complicates the choice and use of antibacterial drugs in the treatment of serious infections and poses

challenging infection control issues. Infections with ESBL-producing *K. pneumoniae* also significantly increases the mortality rates of infected humans (Umeh & Berkowitz, 2009).

The objective of this study is to determine the prevalence of ESBLs in nosocomial isolates of MAR *K. pneumoniae*. The aim is to establish the presence of known and novel beta-lactamases in MAR *K. pneumoniae* isolated from hospital patients in the Western Cape. The aim was achieved as follows:

1. PCR using primers specific for the 16S-23S rDNA ITS region, the MALDI-TOF MS and the VITEK 2 analyser were compared for the identification of the pathogen *K. pneumoniae*.
2. The VITEK 2 system was used to detect the antibiotic resistant profiles of *K. pneumoniae* isolates and to identify the ESBL phenotype.
3. The PCR was used to detect Beta-lactam genes viz. CTX-M ($bla_{CTX-M}$), TEM ($bla_{TEM}$) and SHV ($bla_{SHV}$) respectively in both the genome and plasmid DNA of *K. pneumoniae* using gene specific primers.

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# CHAPTER TWO

# 2 MATERIALS and METHODS

## 2.1 Comparison of the MALDI-TOF Mass Spectrometry versus PCR, API 20 E and VITEK 2 for the identification of clinical *K. pneumoniae* strains

### 2.1.1 Sample Collection and isolation of *Klebsiella* spp.

Ethical clearance was obtained from the Ethics Committee of the Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology to conduct this study. Furthermore, written consent was granted by a local Central Pathology laboratory (Reference Laboratory) to utilise *K. pneumoniae* presumptive positive bacterial cultures cultivated by the facility during 2011. In total 57 agar plate cultures and glycerol stock cultures were obtained during 2011 as follows: one University facility provided 10 glycerol stock samples and a second Central Pathology laboratory provided 47 MacConkey agar plate (Biolab, South Africa) cultures (29 from nosocomial infections and 18 from community acquired infections). The site of origin of the latter 47 samples was as follows: 29 urine samples, two bronchial aspirates samples, nine sputum samples, four tracheal aspirates and three samples were obtained from urine in a catheter. The exact sites of origin of the ten samples from the first Pathology facility are unknown. However, these samples were known to be associated with nosocomial infections.

Cultures from the Reference laboratory were received on either blood agar plates or MacConkey agar plates. These cultures were assigned with an alpha-numerical number. This alpha-numerical number consisted of the letter “P” (P-samples) to identify the culture plates received as those coming from the Pathology lab, followed by the numerical value to distinguish the different culture plates from each other. Fresh pure cultures were prepared by streak plating one single colony from each of these plates onto fresh MacConkey agar plates followed by incubation at 37°C for 12 to 18 hours. A similar procedure was performed on the original stock cultures obtained from the University facility. Again, an alpha-numerical number were assigned to each sample. In this case the letter “J” (J-samples) was used to distinguish these samples from the P-samples, followed by a numerical value to distinguish each J-sample. A fellow master’s student passed away during this time,

and the letter “J” was used in memory of him. In this case nutrient agar (Biolab, South Africa) was used instead of MacConkey agar. To preserve these cultures for future analysis glycerol stock cultures were prepared: the MacConkey agar and nutrient agar cultures were streak plated onto Mueller-Hinton Agar (Biolab, South Africa) to produce fresh single colonies. The inoculated Mueller-Hinton plates were incubated at 37°C for 12 to 18 hours. One well isolated single colony from each culture was used to prepare the glycerol stock cultures.

#### 2.1.1.1 Glycerol stock cultures

One well isolated single colony from Mueller-Hinton agar plates was inoculated into 5 ml Luria Bertani Broth (Biolab, South Africa). The Luria Broth culture was incubated overnight at 37°C. A 20% (v/v) glycerol stock culture was then prepared by mixing 20 µl sterile glycerol with 80 µl Luria Bertani Broth culture. Five glycerol stock cultures were made for each sample to ensure ample stock. These glycerol stock cultures were stored at minus 80°C until needed and for future reference. Each glycerol culture was checked every three months for viability by plating onto Nutrient agar and incubated overnight at 37°C.

### 2.1.2 API 20 E analysis

Glycerol stock cultures (*n* = 57) were streaked onto Nutrient agar and incubated at 37°C for 12-18 hours. A single bacterial colony was re-streaked unto MacConkey agar (Biolab, South Africa) to ensure selective cultures of *Klebsiella spp*. were obtained and incubated at 37°C for 12-18 hours. One single colony from the MacConkey agar was inoculated into 5 ml sterile saline (0.85%) and carefully emulsified to obtain a homogenous suspension. An aliquot of the bacterial suspension was then transferred into the mini reagent compartments of an API 20 E strip (bioMérieux, South Africa) and was prepared according to manufacturer’s instructions. The strips were incubated at 37°C for 18 to 24 hours. After the incubation period all spontaneous reactions were recorded on the reaction sheet provided with the API 20 E kit. Tryptophan deaminase (TDA), Voges-Proskauer (VP) and Indole tests were revealed by the addition of the relevant API reagents (bioMérieux, South Africa). The strips were analysed according to the guidelines outlined in the reading table in the package insert provided with the API 20 E kit (bioMérieux, 2010). Identification was obtained by referring to the Analytical Profile Index or using the API Web identification software, version 1.2.1.

### 2.1.3 VITEK 2 Compact

Glycerol stock cultures (*n* = 57) were streaked unto Nutrient agar and incubated at 37°C for 18 hours. A single colony was re-streaked onto MacConkey agar (incubated at 37°C for 12-18 hours) to ensure a selective growth of *Klebsiella spp.* After incubation, a single colony was re-streaked onto blood agar plates (obtained from the Reference laboratory) as per the instruction from the Reference lab (Cape Town) performing the VITEK 2 testing. Blood agar cultures were incubated at 37°C for 12 hours for identification using the VITEK® 2 GN ID card on the VITEK 2 Compact with VITEK 2 Systems version 05.04 (bioMérieux, South Africa). Briefly, colonies were inoculated into a sterile test tube containing 3 ml sterile saline solution (0.45 - 0.5% NaCl). A homogenous organism ID suspension with a density equivalent to the recommended McFarland standard of 0.5 – 0.63 (target 0.6) for Gram-negative bacilli was prepared. VITEK 2 reaction panels for the ID, together with the ID solutions were then loaded into the VITEK 2 Compact instrument according to manufacturer's operator instructions. Growth based technology and advanced colorimetric readings, with colorimetric reading cards, was utilised to determine the identification of the test microorganisms. The colorimetric cards were incubated at 35.5°C; a colorimetric measurement is then made every 15 minutes for a maximum incubation period of 10 hours after which data was interpreted automatically. Test data of the unknown organism was then compared against the VITEK 2 database to determine a quantitative value for proximity to each of the database taxa.

### 2.1.4 MALDI-TOF MS

The 57 isolates were streaked onto Nutrient agar and incubated at 37°C for 24 hours prior to analysis. Analyses were performed at the Proteomic Unit of the University of the Western Cape using the UltrafleXtreme™ MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) as outlined in Loff et al. (2014). Briefly, utilising a sterile Eppendorf 20 µl tip, the respective 57 freshly grown cultures were directly transferred from the agar plates in triplicate as very thin layers/smears onto MTP 384 polished-steel target strips (Bruker Daltonics, Bremen, Germany) and were allowed to air dry. Each spot was further over-laid with 1.0 µl of freshly prepared CHCA matrix solution (10mg/ml alpha-cyano-4-hydroxycinnamic acid, 50% acetonitrile, 2.5% trifluoroacetic acid). Thereafter, the spots were processed in the UltrafleXtreme™ MALDI-TOF mass spectrometer (Bruker Daltronics, Bremen,

Germany) using the Bacterial Test Standard (BTS) (*E. coli* DH5α protein extract; Bruker Daltonics ref 255343) as a positive control. The analyses were performed via the MALDI Biotyper RealTime Classification (RTC) Module, which visualises AutoXecute operation of flexControl with colour coded classifications. Laser shots were employed using the Smartbeam-II Technology (1 kHz repo rate, 7_MBT spot parameter, 337 nm) to eject sample material from each spot at a rate of 100 per raster area (limited to b2000 µm) in a random walk mode. Positive ions generated from the MALDI-TOF were extracted with an accelerating voltage of 20 kV, and thereafter 40 kB spectra were collected at a 0.5 GS/s rate on a MCP detector with a 29x detector gain (3 kV). The RTC Module automated the matching of deionised, peak processed, smoothed and normalised mass spectra against the reference spectra in the Bruker Taxonomy/Listeria database (4110 cellular organisms, Bruker Daltonics, Bremen, Germany) (Loff et al., 2014).

### 2.1.5 DNA extraction

The boiling method as outlined in Ndlovu et al. (2015) was used for DNA extraction. Briefly, one colony was selected and inoculated into 5 ml Luria-Bertani broth (Merck, South Africa) with a sterile pipette tip. This inoculum was incubated at 37°C overnight. Two ml of fresh cell suspension was transferred into a sterile Eppendorf tube and centrifuged at 14000 x *g* for 15 min. One hundred microliter of Tris-EDTA buffer (TE-buffer) (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was added to the cell pellet. The cell suspension was then heated for 15 min at 95°C and immediately placed on ice for 10 min. The resulting suspension was centrifuged at 14000 x *g* for 10 min. The supernatant was transferred to a sterile Eppendorf tube and stored at -20°C for future use.

### 2.1.6 Polymerase chain reaction identification of *K. pneumoniae*

Once DNA had been extracted from all the isolates, the PCR was used to amplify the 16S-23S rDNA ITS region (Liu et al., 2008) within *K. pneumoniae*. Each PCR, with a total volume of 50 µl, contained 0.1 mM dNTP's (Thermo Scientific, South Africa), 1X PCR buffer, 1 mM $MgCl_2$, 1 µM KPF and KPR primer (**Table 2.1**) respectively, 0.5 units GoTaq® Flexi DNA Polymerase (Promega, South Africa) and 5 µl template DNA. The PCR amplification was performed in a MyCycler™ Thermal Cycler (Bio-Rad, USA) utilising the cycling conditions as indicated in **Table 2.1**. *Klebsiella*

*pneumoniae* ATCC 10031 was used as positive control and nuclease free water was used as a negative control in all the PCR reactions.

**Table 2.1 Primer sequences and PCR cycling parameters for the detection of *K. pneumoniae* (adapted from Liu et al., 2008)**

| Primer sequence (5'-3') | Primer name | Target gene | Product size | PCR Cycling conditions |
|---|---|---|---|---|
| F:ATTTGAAGAGGTTGCAAACGAT<br>R:TTCACTCTGAAGTTTTCTTGTG TT | KPF<br>KPR | 16S-23S rDNA ITS | 130 bp | 35 cycles consisting of:<br>94ºC for 30 sec,<br>55ºC for 30 sec,<br>72ºC for 30 sec. |

The amplification of the PCR products (130 bp) was confirmed by agarose gel electrophoresis on 0.8% (w/v) agarose gels, stained with 0.5 µg/mL ethidium bromide in Tris-acetate-EDTA (TAE) buffer. The size of each PCR product was confirmed by comparing it to a Thermo Scientific Generuler 1 kb Plus DNA Ladder (Thermo Scientific, Massachusetts, United States). The PCR products ($n$ = 57) were then sent to the Central Analytical Facility (CAF) (DNA Sequencing Facility) at Stellenbosch University for purification and DNA sequencing. Sequence identification was done using the National Centre for Biotechnology Information (NCBI) and the Local Basic Alignment and Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to find the closest match of local similarity between isolates and the international database in GenBank.

### 2.1.7 Statistical analysis

The statistical software package Statistica Ver 11.0 (Stat Soft Inc, Tulsa, USA) was used to compare data obtained in the current study. The data generated from the PCR, MALDI-TOF MS, API 20 E and VITEK 2 assays were assigned values to represent the presence or absence of *Klebsiella* in tested samples. A positive identification was assigned the value 1 (present) and a negative reaction was assigned the value 0 (absent). In order to determine the co-detection and non-co-detection, the Pearson Chi-square was then used to compare the observed frequencies of *Klebsiella* using each assay. In all hypothesis tests, a significant level of 5% was used as standard.

## 2.2 Correlation between genes that confer antibiotic resistance and the antibiotic profiles of *K. pneumoniae* isolates

Extended-spectrum beta-lactamases (ESBLs) are plasmid-encoded enzymes that are produced by members of the Enterobacteriaceae family and destroy Cephalosporins with an oxyimino side chain (Cefotaxime, Ceftriaxone, and Ceftazidime, as well as the oxyimino-monobactam Aztreonam) and related oxyimino beta-lactams by lysing the beta-lactam ring and then proceeding to deactivate the antibacterial properties of the beta-lactam molecule. The plasmids that encode the ESBLs also carry resistance genes for other antibiotics including Aminoglycosides, Chloramphenicol, Sulphonamides, Trimethoprim and Tetracycline. Thus, Gram-negative bacilli containing these plasmids are classified as multidrug-resistant.

### 2.2.1 Amplification of SHV and TEM Extended spectrum beta-lactamases

Extended spectrum beta-lactamases are commonly of the sulphydryl variable (SHV) and Temoniera (TEM)-type, which are generally plasmid mediated. Lal *et al*. (2007) reported that the majority of *K. pneumoniae* isolates carry both TEM and SHV. The SHV-1 beta-lactamase is most commonly found in *K. pneumoniae* and is responsible for plasmid-mediated ampicillin resistance in this species.

Amplification of the SHV- and TEM-type ESBL genes in all the isolates (*n* = 57) was conducted using PCR. The TEM and SHV oligonucleotide primer sets used in this study are listed in **Table 2.2.**

**Table 2.2 Primer sets for detection of ESBL genes (adopted from Lal et al., 2007)**

| Target Gene | Primer sequences | Fragment sizes |
|---|---|---|
| $bla_{TEM}$ | F 5'-CTTCCTGTTTTTGCTCACCCA-3' | 717 bp |
| | R 5'-TACGATACGGGAGGGCTTAC-3' | |
| $bla_{SHV}$ | F 5'-TCAGCGAAAAACACCTTG-3' | 471 bp |
| | R 5'-TCCCGCAGATAAATCACC-3' | |

The PCR reaction mixtures were prepared as outlined in **Table 2.3**. Cycling conditions for TEM were two minutes at 94°C for an initial denaturation followed by 30 cycles of one minute at 94°C for denaturation, 30 s at 52°C for annealing and 45 s at 72°C for elongation and one cycle of a final extension of five minutes at 72°C.
Cycling conditions for SHV were two minutes at 94°C for an initial denaturation followed by 30 cycles of one minute at 94°C for denaturation, one minute at 58°C for annealing and one minute at 72°C for elongation and one cycle of a final extension of seven minutes at 72°C.

**Table 2.3 Reaction mixture for TEM and SHV PCRs (adapted from Lal et al., 2007)**

| **Reaction mixture** | **Total volume: 50 µl** |
|---|---|
| DNA | 5 µl |
| GoTaq buffer 5x | 10 µl |
| $MgCl_2$ (25 mM) | 5 µl |
| Deoxynucleoside- triphosphates mix (dNTPs) (10 mM) | 1 µl |
| Forward Primer (10µM) | 5 µl |
| Reverse Primer (10µM) | 5 µl |
| $H_2O$ (nuclease free) | 18.75 µl |
| *Taq* DNA polymerase (5U/ µl) | 0.25 µl |

Ten microliters of the amplified products were then visualised on a 1% (w/v) agarose gel in 1X TAE buffer stained with 0.5 µg/mL ethidium bromide. The PCR bands corresponding to the correct respective band size were excised from the gel and were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, South Africa). The purified products were sent for DNA sequencing at the Stellenbosch University, Central Analytical Facility (DNA Sequencing Facility). The obtained sequences were analysed using the online Basic Local Alignment Search Tool (BLAST), available at http://blast.ncbi.nlm.nih.gov/Blast.cgi, to find the closest match of local similarity of the isolates to the online international database in GenBank.

### 2.2.2 Amplification of the Cefotaximases

The Cefotaximase (CTX-M)-type beta-lactamases are most active against Cefotaxime (Khalaf et al., 2009). Cefotaximase beta-lactamases are not closely related to TEM or SHV ESBLs, however their amino acid sequences are highly identical to the chromosomal beta-lactamases of *Kluyvera spp.* (Khalaf et al., 2009). There are currently 40 CTX-M gene sequences in the NCBI GenBank database.

Based on the amino acid sequence similarities of CTX-M-beta-lactamases, it can be divided into five groups (**Table 2.4**) (Pitout et al*.,* 2004).

**Table 2.4 Groups of CTX-M-beta-lactamases (Shaikh et al., 2015)**

| | |
|---|---|
| **Group I** | CTX-M-1, -3, -10, -11, -12, -15, -22, -23, -28, -29, -30 |
| **Group II** | CTX-M-2, -4, -5, -6, -7, -20, Toho 1 |
| **Group III** | CTX-M-8 |
| **Group IV** | CTX-M-9, -13, -14, -16 to -19, -21, -27, Toho 2 |
| **Group V** | CTX-M-25, -26 |

Amplification of CTX-M-type ESBL genes in all the isolates (*n* = 57) was conducted using the PCR technique. The CTX-M oligonucleotide primer sets used in this study are listed in **Table 2.5.**

**Table 2.5 Primer sets for detection of CTX-M type ESBL genes (adopted from Edelstein et al., 2003)**

| **Target Gene** | **Primer sequences** | **Fragment sizes** |
|---|---|---|
| $bla_{CTX-M}$ | F 5'-TTTGCGATGTGCAGTACCAGTAA-3' | 544 bp |
| | R 5'-CGATATCGTTGGTGGTGCCATA-3' | |

The PCR reaction mixtures were prepared as outlined in **Table 2.6**. Cycling conditions for CTX-M were two minutes at 94°C for initial denaturation followed by 35 cycles of 20 s at 95°C for denaturation, 30 s at 51°C for annealing, and 30 s at 72°C for elongation and one cycle of a final extension of three minutes at 72°C.

Ten microliters of the amplified products were then visualised on a 1% (w/v) agarose gel in 1X TAE buffer stained with 0.5 µg/mL ethidium bromide. The PCR bands corresponding to the correct respective bands were excised from the gel and were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). The purified products were sent for sequencing at the Stellenbosch University, CAF (DNA Sequencing Facility). The obtained sequences were analysed using the online BLAST.

**Table 2.6 Reaction mixture for CTX-M PCR (adopted from Edelstein et al., 2003 and optimised)**

| **Reaction mixture** | **Total volume: 50 µl** |
|---|---|
| DNA | 5 µl |
| GoTaq buffer 5x | 10 µl |
| $MgCl_2$ (25 mM) | 5 µl |
| Deoxynucleoside- triphosphates mix (dNTPs) (10 mM) | 1 µl |
| Forward Primer (10µM) | 0.5 µl |
| Reverse Primer (10µM) | 0.5 µl |
| $H_2O$ (nuclease free) | 27.75 µl |
| *Taq* DNA polymerase (5U/ µl) | 0.25 µl |

### 2.2.3 Detection of ESBL genotype

An ESBL-producing isolate may be resistant to ceftazidime, but susceptible to Cefotaxime. Accordingly, ESBL-producing isolates may not be detected if susceptibility testing is limited to single third-generation Cephalosporins. Gupta et al. (2003) recommended routine screening for ESBL activity in *K. pneumoniae* isolates by determining susceptibility to several Cephalosporins. The VITEK 2 ESBL test (bioMérieux) is based on the simultaneous assessment of the antibacterial activity of Cefepime, Cefotaxime and Ceftazidime, measured either alone or in the presence of Clavulanate. This test has wells containing 1.0 mg/L of Cefepime, or 0.5 mg/L of Cefotaxime or Ceftazidime, either alone or associated with 10 or 4 mg/L of Clavulanate, respectively. After inoculation, the cards are introduced into the VITEK 2 machine, and for each antibiotic tested, turbidity is measured at regular intervals. The proportional reduction of growth in wells containing a Cephalosporin combined with Clavulanate is then compared with that achieved by the Cephalosporin alone and is interpreted as ESBL-positive or – negative through a computerised expert system known as the Advanced Expert System (AES) (Drieux *et al.,* 2008).

### 2.2.4 Determination of antibiotic sensitivity using the VITEK 2 system

*Klebsiella pneumoniae* isolates must be resistant to three or more classes of the antibiotics listed below (Brink et al., 2008) in order for the isolate to be classified as MAR/MDR:

1. Anti-pseudomonal Cephalosporins (Ceftazidime and Cefepime)
2. Carbapenems (Ertapenem, Imipenem and Meropenem)
3. Beta-lactam/[beta]-lactamase inhibitor combination (Piperacillin/ Tazobactam)
4. Fluoroquinolones (Ciprofloxacin or Levofloxacin)

5. Aminoglycosides (Amikacin, Gentamicin and Tobramycin)
6. Aztreonam
7. Polymyxin E
8. Tigecycline

The antibiotic profiles of the isolated colonies (*n* = 57) identified as *K. pneumoniae* were determined using the VITEK 2 Gram-negative cards AST-N134 or AST-N256 (due to unavailability of the AST-N134 card at a certain time the AST-N256 card was used as replacement; the AST-N256 card lacks Aztreonam and the disc diffusion method had to be used). The respective cards were loaded onto the VITEK 2 Compact with VITEK 2 Systems version 05.04 (bioMérieux, South Africa). Glycerol stock cultures of the isolates (*n* = 57) were streaked unto Nutrient agar and incubated at 37°C for 18 hours. A single colony was re-streaked onto MacConkey agar to ensure a selective growth of *Klebsiella spp.* A single colony was obtained and re-streaked onto blood agar plates as per the instruction from the reference lab performing the VITEK 2 testing. Blood agar cultures were incubated at 37°C for 12 hours. Briefly, single colonies were removed from the blood agar plates and inoculated into a sterile test tube containing 3 ml sterile saline solution (0.45 - 0.5% NaCl). A homogenous organism suspension with a density equivalent to the recommended McFarland standard of 0.5 – 0.63 (target 0.6) for Gram-negative bacilli was prepared. VITEK 2 reaction panels (Smart Cards) for the antimicrobial susceptibility test (AST), together with the AST suspension tube were then loaded into the VITEK 2 Compact instrument according to manufacturer's operator instructions. The AST suspensions were prepared by pipetting 145 µl from the ID tube into another tube containing the appropriate saline solution. Growth based technology and advanced colorimetric reading, with colorimetric reading cards, was utilised to determine the AST of the test microorganisms. The colorimetric cards are incubated and interpreted automatically. Antibiotic susceptibility results are reported in terms of Minimum Inhibitory Concentrations (MIC's) (**Table 2.7**) (Cockerill *et al*., 2012). The computerised AES (Advanced Expert System) that is responsible for analysing the data correlates identification with sensitivity and determines the presence of resistance mechanisms.

### 2.2.5 Quality control.

For quality control purposes for ESBL testing *K. pneumoniae* ATCC 700603 was used as a positive control for cephalosporin resistance (Lal et al., 2007), $bla_{KPC}$-carrying *K. pneumoniae* strain 490 for carbapenem resistance (Schechner *et al.*, 2009), while *Escherichia coli* ATCC 25922 was utilised as a negative control (Lal *et al.*, 2007).

**Table 2.7 List of antibiotics that were tested against *Klebsiella pneumoniae***

| **Antibiotic class** | **Antibiotic agent** | **[Disc]** | **MIC'S in µg/ml** | | |
|---|---|---|---|---|---|
| | | | **R** | **I** | **S** |
| Penicillins | Ampicillin | | ≤8 | 16 | ≥32 |
| Beta-lactamase Inhibitors | piperacillin/tazobactam | | ≤16/4 | 32/4-64/4 | ≥128/4 |
| | Amoxicillin/clavulanic | | ≤8/4 | 16/8 | ≥32/16 |
| Cephalosporins | Ceftriaxone | | ≤1 | 2 | ≥4 |
| | Cefuroxime - parenteral | | ≤8 | 16 | ≥32 |
| | Cefuroxime - oral | | ≤4 | 8-16 | ≥32 |
| | Cefepime | | ≤8 | 16 | ≥32 |
| | Cefoxitin | | ≤8 | 16 | ≥32 |
| | Cefotaxime | | ≤1 | 2 | ≥4 |
| | Ceftazidime | | ≤4 | 8 | ≥16 |
| Carbapenems | Imipenem | | ≤1 | 2 | ≥4 |
| | Meropenem | | ≤1 | 2 | ≥4 |
| | Ertapenem | | ≤0.5 | 1 | ≥2 |
| Aminoglycosides | Gentamycin | | ≤4 | 8 | ≥16 |
| | Tobramycin | | ≤4 | 8 | ≥16 |
| | Amikacin | | ≤16 | 32 | ≥64 |
| Fluoroquinolone | Ciprofloxacin | | ≤1 | 2 | ≥4 |
| Folate pathway Inhibitors | Trimethoprim/ Sulfamethoxazole | | ≤2/38 | - | ≥4\76 |
| Other | Tigecycline | | ≤ | | ≥ |
| | Colistin | | ≤ | | ≥ |
| Monobactam | Aztreonam | | ≤4 | 8 | ≥16 |
| | Aztreonam - Disc | 30 µg | ≤21 | 18-21 | ≥17 |

R - Resistant; I - intermediate; S - sensitive

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## CHAPTER THREE

# 3. RESULTS and DISCUSSION

## 3.1 Comparison of MALDI-TOF Mass Spectrometry, PCR, API 20 E and VITEK 2 for the identification of clinical *K. pneumoniae* strains

### 3.1.1 Sample Description

As indicated in the materials and methods chapter, a total of 57 samples (e.g. urine samples, bronchial aspirates, nosocomial infection samples, etc.) were obtained from two local pathology laboratories during 2011. Pure cultures of presumptive positive *K. pneumoniae* strains isolated from the respective samples (*n* = 57) were subjected to MALDI-TOF mass spectrometry, API 20 E, VITEK 2 and species-specific PCR analysis for the identification of *K. pneumoniae*.

### 3.1.2 API 20 E analysis

A suspension of the respective bacterial cultures (*n* = 57) was introduced into 20 miniaturised reaction chambers on a single strip, each containing a different specific dehydrated biochemical reagent. Incubation of the strip was required for bacterial metabolism to start, which produces biochemical reactions with colour changes that are spontaneous or revealed by the addition of reagents. The presence or absence of each reaction was subsequently transformed to a multi-digit numerical profile, which serves to identify the microorganism by means of a numerical profile register. A reaction sheet was used to facilitate the transformation of the test result data to the numerical profile. The resultant numerical profile was then entered into the API database and compared to the profile register in APIWEB (BioMérieux, 2014) that allows for the identification of the bacterial organisms. APIWEB generates a report for each profile expressed as percentage identification. The oxidase test is the 21$^{st}$ test to be performed and forms an integral part of the final profile as all Enterobacteriaceae are oxidase negative.

Of the 57 samples, the API 20 E test identified 47 (82.5%) of the isolates (n = 57) as *K. pneumoniae* [*K. pneumoniae pneumoniae* (44/47), *K. pneumoniae ozaenae* (2/47) and *K. pneumoniae* (1/47)]. The 47 isolates were classified into 10 numerical profile

groups according to the positive reactions: 5215673 (P48) ortho nitrophenyl-betaD-galactopyranosidase (ONPG), lysine decarboxylase (LDC), citrate utilization (CIT), urease (URE), Voges Proskauer (VP), glucose fermentation-oxidation (GLU), mannitol fermentation-oxidation (MAN), inositol fermentation-oxidation (INO), sorbitol fermentation-oxidation (SOR), rhamnose fermentation-oxidation (RHA), saccharose fermentation-oxidation (SAC), melibiose fermentation-oxidation (MEL), amygdaline fermentation-oxidation (AMY), arabinose fermentation-oxidation (ARA); 5215573 (P7): ONPG, LDC, CIT, URE, TDA, VP, GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY, ARA; 5235773 (P9, P11, P12, P13, P1, P18, P20, P21): ONPG, LDC, CIT, URE, TDA, IND, VP, GEL, GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY, ARA; 5215773 (P2, P3, P4, P25, P26, P27, P29, P30, P31, P32, P35, P36, P37, P38, P39, P43, P46, P51, J1, J3, J5, J6): ONPG, LDC, CIT, URE, VP, GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY, ARA; 7215773 (P23, P33) ONPG, ADH, LDC, CIT, URE, VP, GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY, ARA; 5214773 (P40, P42, P50, P55, J7, J10, J11, J12) ONPG, LDC, CIT, URE, GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY, ARA; 5014773 (J9) ONPG, LDC, CIT, URE, GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY, ARA; 5204731 (P28) ONPG, LDC, CIT, GLU, MAN, INO, SOR, RHA, SAC, AMY, ARA; 7204773 (P47) ONPG, ADH, LDC, CIT, GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY, ARA; 7235773 (P10, P14) ONPG, ADH, LDC, CIT, URE, TDA, VP, GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY, ARA. The results for the API 20 E identification of the isolates (*n* = 57) are tabulated in **Table 3.1** and **Appendix 1.**

**Table 3.1 MALDI-TOF MS, VITEK, PCR and API identification**

| Site | Sample number | MALDI-TOF MS | Score | VITEK | Species Specific PCR | Seq. ID % | GenBank Accession numbers | API 20E | ID % |
|---|---|---|---|---|---|---|---|---|---|
| Source one: Nosocomial | | | | | | | | | |
| Unknown | J1 | *K. pneumoniae* | 1.744 | *K. pneumoniae* | *K. pneumoniae* | 99 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.6 |
| | J3 | *K. pneumoniae* | 2.100 | *K. pneumoniae* | *K. pneumoniae* | 99 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.6 |
| | J5 | *K. pneumoniae* | 1.798 | *K. pneumoniae* | *K. pneumoniae* | 98 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.6 |
| | J6 | *K. pneumoniae* | 2.118 | *K. pneumoniae* | *K. pneumoniae* | 95 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.6 |
| | J7 | *K. pneumoniae* | 2.004 | *K. pneumoniae* | *K. pneumoniae* | 90 | CPC024191.1 | *K. pneumoniae pneumoniae* | 95.0 |
| | J8 | *K. pneumoniae* | 1.892 | *K. pneumoniae* | *K. pneumoniae* | 98 | CPC024191.1 | *R. terrigena* | 68.9 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | J9 | *K. pneumoniae* | 1.935 | *K. pneumoniae* | *K. pneumoniae* | 98 | CPC024191.1 | *K. pneumoniae pneumoniae* | 94.5 |
| | J10 | *K. pneumoniae* | 2.016 | *K. pneumoniae* | *K. pneumoniae* | 96 | CPC024191.1 | *K. pneumoniae pneumoniae* | 95.0 |
| | J11 | *K. pneumoniae* | 1.848 | *K. pneumoniae* | *K. pneumoniae* | 99 | CPC024191.1 | *K. pneumoniae pneumoniae* | 90.6 |
| | J12 | *K. pneumoniae* | 1.935 | *K. pneumoniae* | *K. pneumoniae* | 100 | CPC024191.1 | *K. pneumoniae pneumoniae* | 95.0 |
| Source two: Nosocomial | | | | | | | | | |
| Bronchial | P2 | *K. pneumoniae* | 2.015 | *K. pneumoniae* | *K. pneumoniae* | 100 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.5 |
| | P6 | *K. pneumoniae* | 2.095 | *K. pneumoniae* | *K. pneumoniae* | 100 | CPC024191.1 | *R. terrigena* | 62.3 |
| Tracheal | P3 | *K. pneumoniae* | 1.971 | *K. pneumoniae* | *K. pneumoniae* | 99 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.5 |
| | P28 | *K. pneumoniae* | 2.080 | *K.* | *K.* | 100 | CPC024191. | *K. pneumoniae* | 54.7 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *pneumoniae* | *pneumoniae* | | 1 | *ozaenae* | |
| | P34 | *K. variicola* | 1.806 | *K. pneumoniae* | *K. pneumoniae* | 100 | CPC024191.1 | *R. terrigena* | 44.2 |
| | P37 | *K. pneumoniae* | 1.823 | *K. pneumoniae* | *K. pneumoniae* | 100 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.6 |
| Urine | P4 | *K. pneumoniae* | 2.067 | *K. pneumoniae* | *K. pneumoniae* | 100 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.5 |
| | P18 | *K. pneumoniae* | 1.804 | *K. pneumoniae* | *K. pneumoniae* | 99 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.6 |
| | P20 | *K. variicola* | 2.065 | *K. pneumoniae* | *K. pneumoniae* | 99 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.6 |
| | P23 | *K. pneumoniae* | 1.759 | *K. pneumoniae* | *K. pneumoniae* | 100 | CPC024191.1 | *K. pneumoniae* | 97.4 |
| | P26 | *K. pneumoniae* | 1.882 | *K. pneumoniae* | *K. pneumoniae* | 97 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.4 |
| | P29 | *K. variicola* | 2.808 | *K.* | *K.* | 100 | CPC024191. | *K.* | 97.4 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *pneumoniae* | *pneumoniae* | | 1 | *pneumoniae pneumoniae* | |
| | P31 | *K. pneumoniae* | 1.963 | *K. pneumoniae* | *K. pneumoniae* | 98 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.6 |
| | P32 | *K. pneumoniae* | 1.943 | *K. pneumoniae* | *K. pneumoniae* | 98 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.6 |
| | P33 | *K. pneumoniae* | 1.831 | *K. pneumoniae* | *K. pneumoniae* | 99 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.5 |
| | P43 | *K. pneumoniae* | 2.236 | *K. pneumoniae* | *K. pneumoniae* | 99 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.6 |
| | P46 | NRI | 1.552 | *K. pneumoniae* | *K. pneumoniae* | 96 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.6 |
| | P47 | *K. pneumoniae* | 1.761 | *K. pneumoniae* | *K. pneumoniae* | 100 | CPC024191.1 | *K. pneumoniae ozaenea* | 57.2 |
| | P48 | *K. pneumoniae* | 1.992 | *K. pneumoniae* | *K. pneumoniae* | 99 | CPC024191.1 | *K. pneumoniae pneumoniae* | 98.7 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | P50 | *K. pneumoniae* | 1.905 | *K. pneumoniae* | *K. pneumoniae* | 99 | CPC024191.1 | *K. pneumoniae pneumoniae* | 95.0 |
| | P51 | *K. pneumoniae* | 1.968 | *K. pneumoniae* | *K. pneumoniae* | 98 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.6 |
| Catheter urine | P25 | *K. variicola* | 1.771 | *K. pneumoniae* | *K. pneumoniae* | 97 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.4 |
| | P27 | *K. pneumoniae* | 1.872 | *K. pneumoniae* | *K. pneumoniae* | 98 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.4 |
| | P30 | *K. pneumoniae* | 1.845 | *K. pneumoniae* | *K. pneumoniae* | 92 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.4 |
| Sputums | P5 | *K. pneumoniae* | 2.052 | *K. pneumoniae* | *K. pneumoniae* | 100 | CPC024191.1 | *R. terrigena* | 62.3 |
| | P19 | *K. pneumoniae* | 1.721 | *K. pneumoniae* | *K. pneumoniae* | 99 | CPC024191.1 | *R. terrigena* | 68.9 |
| | P21 | *K. pneumoniae* | 1.849 | *K. pneumoniae* | *K. pneumoniae* | 97 | AP014950.1 | *K. pneumoniae pneumoniae* | 97.6 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | P39 | *K. pneumoniae* | 2.118 | *K. pneumoniae* | *K. pneumoniae* | 99 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.6 |
| | P40 | *K. pneumoniae* | 1.920 | *K. pneumoniae* | *K. pneumoniae* | 100 | CPC024191.1 | *K. pneumoniae pneumoniae* | 95.0 |
| | P53 | *K. pneumoniae* | 2.039 | *K. pneumoniae* | *K. pneumoniae* | 99 | CPC024191.1 | *R. terrigena* | 68.9 |
| | P54 | *K. pneumoniae* | 1.938 | *K. pneumoniae* | *K. pneumoniae* | 99 | CPC024191.1 | *R. terrigena* | 62.3 |
| | P55 | *K. variicola* | 1.948 | *K. pneumoniae* | *K. pneumoniae* | 100 | CPC024191.1 | *K. pneumoniae pneumoniae* | 95.0 |
| | P56 | *K. variicola* | 1.952 | *K. pneumoniae* | *K. pneumoniae* | 100 | CPC024191.1 | *R. terrigena* | 68.9 |
| Source two: Community | | | | | | | | | |
| Urine | P7 | *K. variicola* | 1.798 | *K. pneumoniae* | *K. pneumoniae* | 99 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.6 |
| | P8 | *K. pneumoniae* | 2.058 | *K. pneumoniae* | *K. pneumoniae* | 99 | CPC024191.1 | *R. terrigena* | 62.3 |
| | P9 | *K. pneumoniae* | 1.995 | *K.* | *K.* | 99 | CPC024191. | *K.* | 97.6 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *pneumoniae* | *pneumoniae* | | 1 | *pneumoniae pneumoniae* | |
| | P10 | *K. pneumoniae* | 2.005 | *K. pneumoniae* | *K. pneumoniae* | 99 | CPC024191.1 | *K. pneumoniae pneumoniae* | No % available |
| | P11 | *K. pneumoniae* | 2.081 | *K. pneumoniae* | *K. pneumoniae* | 99 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.6 |
| | P12 | *K. pneumoniae* | 1.920 | *K. pneumoniae* | *K. pneumoniae* | 97 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.6 |
| | P13 | *K. pneumoniae* | 1.776 | *K. pneumoniae* | *K. pneumoniae* | 100 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.6 |
| | P14 | *K. pneumoniae* | 1.962 | *K. pneumoniae* | *K. pneumoniae* | 100 | CPC024191.1 | *K. pneumoniae pneumoniae* | No % available |
| | P15 | *NRI* | 1.674 | *K. pneumoniae* | *K. pneumoniae* | 99 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.6 |
| | P35 | *K. pneumoniae* | 2.001 | *K. pneumoniae* | *K. pneumoniae* | 100 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.6 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | P36 | *K. variicola* | 1.722 | *K. pneumoniae* | *K. pneumoniae* | 99 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.6 |
| | P38 | *K. pneumoniae* | 2.147 | *K. pneumoniae* | *K. pneumoniae* | 99 | CPC024191.1 | *K. pneumoniae pneumoniae* | 97.4 |
| | P41 | *K. pneumoniae* | 1.974 | *K. pneumoniae* | *K. pneumoniae* | 98 | CPC024191.1 | *R. terrigena* | 82.8 |
| | P42 | *K. pneumoniae* | 2.027 | *K. pneumoniae* | *K. pneumoniae* | 95 | CPC024191.1 | *K. pneumoniae pneumoniae* | 95.0 |

NRI - No Reliable Identification

An example is showed in **Figure 3.1** of a result sheet of an API 20E test strip with a positive profile for *K. pneumoniae* with a 5215773 numerical profile.

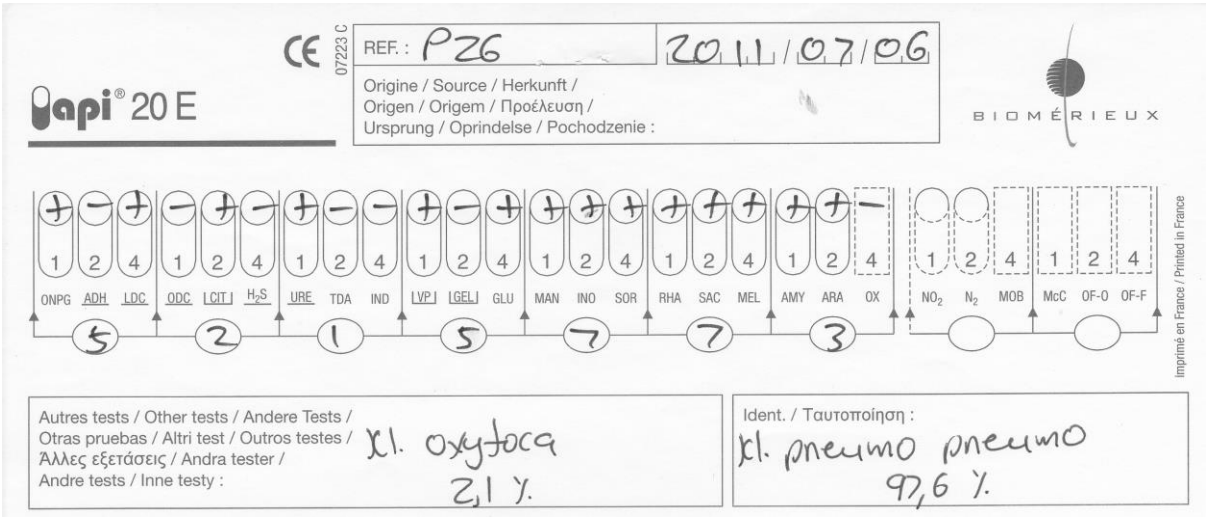


**Figure 3.1** Example of an API 20 E profile positive for *K. pneumoniae* with an identification profile of 5215773

In the current study, 10 isolates (17.5%) were identified as *Raoultella* species using the API20E. There were four profiles for *R. terrigena* with the following positive biochemical reactions: numerical profile 7205773 (P5, P6, P54) was positive for ONPG, ADH, LCD, CIT, VP, GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY, ARA; numerical profile 5205773 (P8, P19, P53, P56, J8) had the following positive biochemical reactions: ONPG, LCD, CIT, VP, GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY, ARA; profile 5205370 (P34) had the following positive biochemical reactions: ONPG, LDC, CIT, VP, GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY, ARA and profile 5204773 (P41) had the following positive biochemical reactions: ONPG, LDC, CIT, GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY, ARA (**Appendix 1**).

The accuracy of the API 20 E strips has been tested numerous times over the years. Holmes et al. (1978) conducted one of the first analyses utilising the API 20 E system for the identification of 206 strains belonging to taxa within the Enterobacteriaceae family. Results indicated that 88% (*n* = 206) of strains could be positively identified. Similarly, O'Hara (1993) reported that 77% (*n* = 252) of strains within the Enterobacteriaceae family were correctly identified using API 20 E analysis.

O'Hara (2005) reported on a similar study where 93% (*n* = 129) were accurately identified as isolates belonging to the Enterobacteriaceae family up to species level. However, according to Shaikh & Morgan (2011), the *R. terrigena* can often be incorrectly identified as *K. pneumoniae* as the API 20 E fails to correctly differentiate between these two genera. As indicated in **Table 3.1**, in the current study *Raoultella* species were identified using the API 20 E system. Isolates P28 and P47 were however, identified as *K. pneumoniae*, but with a very low percentage identification. In fact, the percentage identification for these isolates ranged from 54.7% to 57.2%, which is similar to the identification percentages obtained for the *Raoultella* species which ranged from ~44% to ~82% (**Table 3.1**). All isolates that obtained a percentage identification of >90% (*n* = 43) were identified as *K. pneumoniae pneumonia* except for P10 and P14 for which the online API database could not assign a percentage. All isolates other than those identified as *Raoultella* species that obtained a percentage identification of <90% were either identified as *R. terrigena* (P34 = 44.2%, P5 = 62.3%, P8 = 62.3%, P54 = 62.3%, P19 = 68.9%, P53 = 68.9%, P56 = 68.9% and P41 = 82.8%) or *K. pneumoniae ozaenae* (P28 = 54.7% and P47 = 57.2%) (**Table 3.1**). Chander et al. (2011) also reported that current methods such as the API system often fail to differentiate between species of *Klebsiella*.

Furthermore, certain species of *Klebsiella* are urease positive (e.g. *K. pneumoniae*). In this study all strains identified as *K. pneumoniae* subsp. *pneumoniae* (44 of 57 *K. pneumoniae* positive samples; **Table 3.1**), tested positive for urease. One sample could only be identified as *K. pneumoniae* (P23) and was urease positive with an identification percentage of 97.6%. The two isolates P28 and P47 were identified as *K. pneumoniae* subsp. *ozaenae* and were urease negative. All *R. terrigena* strains (*n* = 10) tested negative for urease with percentage identifications of <83%.

Drancourt (2001) reported that a phylogenetic tree based on the sequence comparison confirmed that the genus *Klebsiella* is heterogeneous and composed of species which form three clusters. Within the tree, the clusters also included members of other genera, including *Enterobacter aerogenes*, *Erwinia* clusters I and II and *Tatumella*. Cluster 1 includes *K. pneumoniae* subsp. *pneumoniae*, *K. pneumoniae* subsp. *rhinoscleromatis* and *K. pneumoniae* subsp. *ozaenae*; cluster

2 includes organisms that grow at 10°C and ferment L-sorbose namely *K. ornithinolytica*, *K. planticola*, *K. trevisanii* and *K. terrigena*; cluster 3 includes only *Klebsiella oxytoca*. Based on DNA-DNA hybridisation data, Drancourt (2001) proposed to rename cluster 2 to the genus name *Raoultella* with amended definitions for the *Klebsiella* species.

*Raoultella terrigena* (previously known as *K. terrigena*) was first isolated from soil in 1981 and for many years no human isolates were reported until 1991 when *R. terrigena* was isolated from the stool of healthy humans (Shaikh & Morgan, 2011). In 1992, clinical isolates of *K. pneumoniae* mostly from the respiratory tract was subjected to further tests and 0.4% was identified as *R. terrigena*, but whether these isolates caused infection remained to be clarified (Shaikh & Morgan, 2011). Two cases of actual clinical infections by *R. terrigena* were then reported in 2007 when *R. terrigena* was isolated from sputum, blood cultures and drain fluid (Shaikh & Morgan, 2011). Shaikh & Morgan (2011) proposed that *K. pneumoniae* could be differentiated from *R. terrigena* by the ability of *R. terrigena* to ferment dulcitol, melezitose and adonitol at 30°C, and its ability to assimilate histamine. Shaikh & Morgan (2011) futher proposed that the identification of *R. terrigena* can be confirmed by its ability to grow at 10°C and the inability to produce gas from lactose at 44.5°C. Another alternative method could be routine histamine assimilation testing to detect *Raoultella* species among *Klebsiella* isolates, followed by the use of conventional tests (i.e. indole and ornithine decarboxylase tests) on the *Raoultella* species to differentiate between the organisms up to the species level.

Other tests to differentiate between *R. terrigena* and *K. pneumoniae* are thus required but these tests are not commonly employed in the routine microbiology laboratory (Shaikh & Morgan, 2011). Since*, R. terrigena* is only occasionally isolated from human clinical specimens and rarely isolated from infection, and differentiation between *R. terrigena* and *K. pneumoniae* is difficult, it can thus be argued that the urease negative strains identified as *Raoultella* were incorrectly identified due to limitations presented by the API system. It is thus essential to employ further identification analyses to fully elucidate and confirm the identity of the isolates.

### 3.1.3 VITEK 2 analysis

Colorimetric readings of the Gram-negative (GN) card in VITEK 2 analysis is based on established biochemical methods as well as newly developed substrates that measure carbon source utilisation, enzymatic activity and resistance to growth in the presence of certain inhibitory substances (Pincus, 2006). Different wavelengths in the visible spectrum are used in a transmittance optical system to allow for the interpretation of test reactions. The GN card is incubated and read every 15 minutes for the duration of the incubation period to measure turbidity and/or colour products of the substrate metabolism. The reaction of each test is then determined by calculations on raw data and is compared to thresholds. Test data of the unknown organisms are subsequently compared to the VITEK 2 database to determine a quantitative value for proximity to each of the database taxa. If the value or identification pattern is not recognised, a list of possible organisms is provided, or the strain is determined to be outside the scope of the database. The VITEK 2 also utilises the Advanced Expert System (AES) during the biological validation phase for examining the antimicrobial susceptibility data to analyse for inconsistencies between the species identification of the organism and the minimum inhibitory concentrations (MIC's) (Sanders et al., 2001). If the AES only detects a single error, it will recommend a biological correction in the identification of the strain or it will recommend a numerical change in the MIC to render it more consistent with the identification profile. These single errors are considered biological due to the AES's assumption that the VITEK 2 generated a data error; data is atypical to the strain; a false negative result occurred; or an incorrect result was entered by the laboratory worker. When several possibilities for corrections exist to remove the inconsistency or there are multiple biological errors, the result is considered inconsistent.

In the current study a total of 57 Enterobacteriaceae strains were analysed and identified by a reference laboratory as *K. pneumoniae* (100%) (**Table 3.1**) using the VITEK 2 method. The identification percentage for all isolates was > 92% and the isolates previously identified as *R. terrigena* using the API 20 E analysis were identified as *K. pneumoniae* with high identification percentages obtained. Moreover, isolates P28 and P47 were confirmed to be *K. pneumoniae* with 100% identification using the VITEK 2 system. Similarly, Funke et al. (1998) reported that of the 48 *K. pneumoniae* strains included in their study, 92% (44/48) were correctly identified

using the VITEK system. In a study conducted by Bourbeau & Heiter (1998) a total of 304 isolates were tested with the VITEK system and 287 (94.4%) were correctly identified to species level. Within their study 27 isolates of *K. pneumoniae* were included of which 26 (96%) were corrrectly identified. Joyanes et al. (2001) analysed 198 clinical isolates of non-fermenting Gram-negative rods [*Pseudomonas aeruginosa* (*n* = 146), *Acinetobacter baumannii* (*n* = 25) and *Stenotrophomonas maltophilia* (*n* = 27)]. All the *S. maltophilia* (100%) isolates were correctely identified, while 91.6% *P. aeruginosa* strains and 76% *Acinetobacter baumannii* strains were correctly identified. Furthermore, Ling et al. (2001) demonstrated analysed 281 strains, with 95% (267/281) correctly identified up to species level using the VITEK 2 system. These studies corroborate the data obtained in the current study and validate the integrity of the VITEK 2 microbial identification system.

### 3.1.4 MALDI-TOF MS

Matrix-assisted laser desorption ionization of flight mass spectrometry (MALDI-TOF MS) is a chemotaxonomic method that detects different biomolecules such as sugars, nucleic acids, proteins and peptides that are desorbed from bacteria (Pavlovic et al., 2013). The analyte is first crystallised in a matrix compound and then bombarded with a laser beam. The energy from the laser beam vaporises the matrix and transfers ions to the analyte (ionization) causing desorption of the analyte. The vaporised analytes obtain a defined kinetic energy by an electric field. After the analyte molecules are accelerated they are allowed to leave the electric field to drift freely in a field-free flight tube towards a detector. Their flight speeds depend on their mass and are separated according to their mass-to-charge value (m/z) in a mass analyser, then quantified by the detector (Pavlovic et al., 2013). Highly abundant proteins, mostly from bacterial ribosomes, generate fingerprints (a characteristic mass spectral peak pattern) for each isolate based on their mass-to-charge ratio (Pavlovic et al., 2013; Loff et al., 2014). The acquired spectral peak patterns of the test bacterium is compared to a library database with known peak positions, peak intensity, and peak frequencies that allows for the identification of the bacterial species. A manufacturer-defined algorithm produces a log score to express identification levels: a ≥2 score indicates species identification; 1.7 to 1.9 indicates genus identification; and <1.7 indicates no identification (Zhang & Sandrin, 2016).

The possibility of identification at species level is more likely with higher peak levels (Loff et al., 2014).

**Figure 3.2** is representative of a positive *K. pneumoniae* subsp. *pneumoniae* identification where the high m/z peaks are notable. Fifty-seven cultures were spotted in triplicate and analysed by a linear MALDI-TOF MS method. Of these samples, 82.5% (47/57) were positively identified as *Klebsiella* species (**Table 3.1**), of which 38% had a score of > 2, confirming the identification of *K. pneumoniae*. In addition, 62% of the 47 isolates identified as *Klebsiella* species had a score in the range of 1.7 – 1.9, confirming the genus identification. Fourteen percent (8/57) of the isolates were identified as *K. variicola* of which 25% (2/8) had a score of > 2 and 75% (6/8) had a score in the range of 1.7 – 1.9. In addition, no reliable identification (NRI) was obtained for 3.5% (2/57) of the isolates with scores of < 1.7 recorded (**Table 3.1**). It should however be noted that both samples (viz. P15 and P46) reported as NRI using the MALDI-TOF MS method, were identified as *K. pneumoniae* using the API 20 E and VITEK 2 identifications methods. The discordance in results could possibly be attributed to contamination of the culture. Similar to the VITEK 2 results, *R. terrigena* was not identified using MALDI-TOF MS analysis.

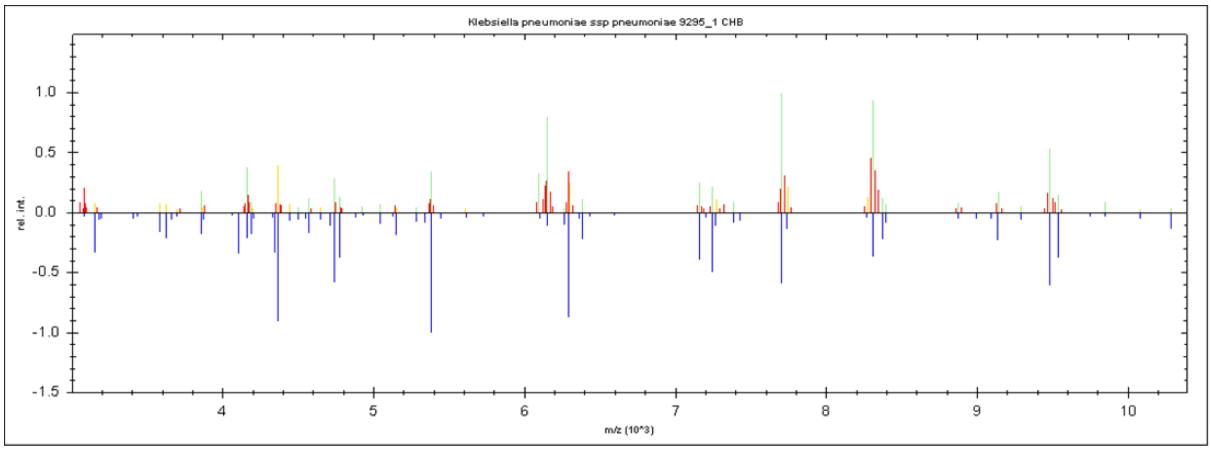


**Figure 3.2** Mass spectrum (MS) profile of a presumptive *K. pneumoniae* isolate positively identified as a *K. pneumoniae* subsp. *pneumoniae*.

However, as indicated 14% of the 57 isolates were identified as *K. variicola*. *Klebsiella variicola* is a newly described species (Rosenblueth et al., 2004) and seems to be genetically related to *K. pneumoniae* strains. It has also been isolated from plants and clinical settings (Rosenblueth et al., 2004). Rosenblueth et al. (2004) distinguished *K. variicola* from other *Klebsiella* spp. by establishing that while many *Klebsiella* spp. ferment adonitol (pentose alcohol formed by the reduction of ribose), the new species they proposed to name *K. variicola* does not ferment adonitol.

The main target for MALDI-TOF MS is the ribosomal proteins. Similar composition in these ribosomal proteins can lead to difficulty in distinguishing between closely related species (Patel, 2012) such as *K. pneumoniae* and *K. variicola*. Loff et al. (2014) reported that MALDI-TOF MS was able to identify 95% presumptive *E. coli* isolates to species level. Seng et al. (2009) reported in their study that MALDI-TOF MS was able to identify 95.4% up to genus and species level; 2.8% with no identification and 1.7% were incorrectly identified. It is argued by Seng et al. (2009) that the lack of identification and incorrect identification is due to improper database entries. Patel (2012) validated this statement and indicated that while the database is in need of updating, in general MALDI-TOF MS can be utilised for the rapid and accurate identification of bacteria and fungi isolated from clinical samples.

### 3.1.5 Species-specific PCR

Species specific primers were used in combination with PCR to screen for the 16S-23S rDNA ITS sequence present in *K. pneumoniae* (Liu et al., 2008). Analysis was performed on all samples (*n* = 57). Based on the species-specific analysis, *K. pneumoniae* was detected in 100% of the samples analysed (**Table 3.1**) yielding a PCR product band size of 130 bp (**Figure 3.3**). Furthermore, all products were sequenced and had >90% identity to *K. pneumoniae* [Example of GenBank accession numbers: CP024191.1 and AP014950.1 (**Table 3.1**)] on a nucleotide level (**Table 3.1**). The specificity of these primers was validated by Liu et al. (2008) by testing it on 31 known *K. pneumoniae* strains and 79 non-*K. pneumoniae* strains. Their results showed that only one band of 130 bp were produced for all (100%) of the known *K. pneumoniae* strains. None (0%) of the non-*K. pneumoniae* strains

yielded positive results. The high specificity of this primer pair for the detection of *K. pneumoniae* was demonstrated by this result.

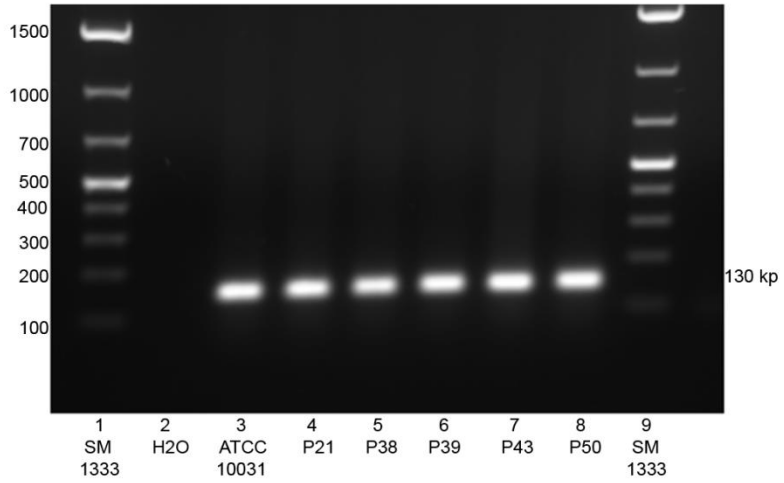


**Figure 3.3**. Agarose electrophoresis photograph of the PCR amplification of representative samples using species specific primers: lanes 1 and 9 - GeneRuler 1 kb Plus DNA Ladder; Lane 2 - negative control; lane 3 - positive control ATCC 10031; lanes 4 to 8 - representative clinical samples corresponding to 130 bp.

### 3.1.6 Statistical analysis

A summary of the comparison of PCR, MALDI-TOF MS, API 20 E and VITEK 2 for the identification of *K. pneumoniae* in clinical samples is indicated in **Table 3.2**.

**Table 3.2 Comparison of the MALDI-TOF MS with API, VITEK 2 and PCR for the identification of *Klebsiella pneumoniae***

| **Organism Identification** | **API 20 E** | **VITEK 2** | **MALDI-TOF** | **PCR** |
|---|---|---|---|---|
| *K. pneumoniae* | 82.5% (47/57) | 100% (57/ 57) | 82.5% (47/57) | 100% (57/ 57) |
| *Klebsiella* sp. | - | - | 14% (8/57) | - |
| Other | 17.5% (10/57) | - | 3.5% (2/57) | - |

VITEK 2 and species-specific PCR and DNA sequencing identified *K. pneumoniae* in 100% (*n* = 57) of the samples analysed. In comparison, both MALDI-TOF and API 20 E identified 82.5% of the isolates (albeit often not the same isolates) as *K. pneumoniae*, with *K. variicola* and *R. terrigena* also identified by these two

analysis methods, respectively. Moreover, 14% (8/57) of the isolates could only be identified to genus level using MALDI-TOF MS analysis. Both *K. variicola* and *R. terrigena* are genotypically similar to *K. pneumoniae* (Drancourt, 2001; Rosenblueth et al., 2004) and it could be hypothesised that the discrepancy in results obtained could be due to a lack of detailed and updated information in the databases of these two identification methods. For example, the API 20 E system is not currently routinely utilised in clinical laboratory settings and has to a large extent been replaced with genus or species-specific PCR and DNA sequencing.

As indicated in the materials and methods section, the Pearson Chi-square test was then used to determine the total agreement and total disagreement and compare the frequency identification of *K. pneumoniae* generated from the PCR, MALDI-TOF MS, API 20 E and VITEK 2 assays (**Table 3.3 and Appendix 2**). By definition the Pearson chi-squared test is a statistical test that is applied to sets of categorical data arising from random sampling to evaluate if the likelihood of any of the differences observed between the data sets rise by chance. It is also known as the test of goodness of fit and independence (Pearson, 1900 and Chernhoff & Lehmann, 1953).

Comparison of the results obtained by MALDI-TOF MS to VITEK 2 and PCR yielded an 82.5% agreement for the identification of *K. pneumoniae* (**Table 3.3 and Appendix 2**). A total disagreement of 17.5% (10/57) was then recorded as MALDI-TOF MS identified eight of the samples as *Klebsiella* spp., while a “non-reliable identification” was recorded for two of the samples.

**Table 3.3 Total agreement and total disagreement of two methods vs. each other for identification of *K. pneumoniae***

| Method comparison | Total Agreement | | Total Disagreement | |
|---|---|---|---|---|
| | +/+ | -/- | +/- | -/+ |
| MALDI-TOF MS vs VITEK2 | 47/57 (82.5%) | - | - | 10/57 (17.5%) |
| MALDI-TOF MS vs PCR | 47/57 (82.5%) | - | - | 10/57 (17.5%) |
| MALDI-TOF MS vs API 20 E | 39/57 (68%) | 2/57 (4%) | 8/57 (14%) | 8/57 (14%) |
| VITEK 2 vs PCR | 57/57 (100%) | - | - | - |
| VITEK 2 vs API 20 E | 47/57 (82.5%) | - | 10/57 (17.5%) | - |
| PCR vs API 20 E | 47/57 (82.5%) | - | 10/57 (17.5%) | - |

+/+ indicates that both tests showed a positive result
-/- indicates that both tests showed a negative result
+/- indicates that the first test was positive while the latter test was negative
-/+ indicates that the first test was negative while the latter test was positive

The greatest discrepancy in total agreement was recorded for MALDI-TOF MS versus the API 20 E system (**Table 3.3 and Appendix 2**). Based on the results obtained, a 68% (39/57) total agreement was recorded between the two methods indicating the positive identification of *K. pneumoniae.* Thus, a total disagreement between the two methods of 32% (18/57) was recorded. To clarify, samples (14%) identified by the API 20 E system as *Raoultella* sp. were identified by MALDI-TOF MS as *K. pneumoniae*. Similarly, where MALDI-TOF MS identified 6/57 (14%) samples as *Klebsiella* sp. and 2/57 and "non-reliable identification", the API 20 E system identified these samples as *K. pneumoniae.* For all the samples (16/57) where discrepancies were recorded between the MALDI-TOF MS and API 20 E system, either PCR or VITEK 2 corroborated the positive identification of *K. pneumoniae*.

Comparison of the VITEK 2 versus PCR then yielded a total agreement of 100% (57/57) as *K. pneumoniae* was identified in all the samples (**Table 3.3 and Appendix 2**). This was further confirmed when all PCR products were sequenced and had

>90% identity to *K. pneumoniae* on a nucleotide level. Comparison of the VITEK 2 to the API 20 E as well as the VITEK 2 to the PCR analysis yielded a total agreement for 47/57 (82.5%) of the samples, respectively, confirming the identification of *K. pneumoniae* in the clinical samples. A total disagreement between the API 20E and the VITEK 2 and PCR technique was then recorded 10 (17.5%) samples. Upon further investigation, it was found that the API 20 E system did not identify these samples as *K. pneumonia* but rather as *R. terrigena*, which clarifies the disagreement in results recorded.

The average agreements, as to whether the isolates were indeed *K. pneumoniae*, as identified by all the methods employed, three of the methods and two of the methods are indicated in **Table 3.4**. For the analysis by all four methods employed, a total agreement of 68.4% was obtained, indicating the positive identification of *K. pneumoniae* in 39 of the 57 samples analysed. An average agreement of 28.1% was then obtained for the comparison of results generated for three of the methods utilised, while a 3.5% average agreement was obtained for at least two methods (**Table 3.4**). Furthermore, all four methods agreed that 82.5% of the isolates were *Klebsiella* species (**Table 3.4**) while three methods agreed that 17.5% of the isolates were *Klebsiella* species.

**Table 3.4 Average agreements on the identification of *K. pneumoniae* and *Klebsiella* sp. between all the methods, 3 of the methods and 2 of the methods**

| Agreement Category | 4 Agree | 3 Agree | 2 Agree |
|---|---|---|---|
| Average Agreement: *K. pneumoniae* | 68.4% | 28.1% | 3.5% |
| Average Agreement: *Klebsiella* sp. | 82.5% | 17.5% | - |

Currently, most phenotypic or genotypic systems used for the identification of bacteria have limitations, as the results obtained from any single test method are not 100% accurate (Janda & Abbott, 2002). The genome of a given species is also extremely complex which implies that the biochemical characterisation monitored in most phenotypic methods may vary and may produce conflicting results. Moreover,

phenotypic properties of an organism can be unstable as its expression is dependent upon changes in environmental conditions such as growth substrate, temperature and pH levels (Janda & Abbott, 2002). Furthermore, the configuration of the biochemistry panels rarely changes after commercial production (Janda & Abbott, 2002). For example, the 20 biochemical tests on the API 20 E that was patented on February 3rd, 1976 (Janin, 1976) and are still the same biochemical tests on the API 20 E strip used today (BioMérieux, API 20E Identification system for Enterobacteriaceae and other non-fastidious Gram-negative rods, 2010).

It is also well-documented that in the late 19th and early 20th centuries, when the first biochemical investigation of bacterial species occurred, a limited number of phenotypic and biochemistry tests were available which implies that the characterisation of the proposed species was inaccurate and vague (Janda & Abbott, 2002). This led to confusion as slight differences in morphological, culture and phenotypic criteria observed by different investigators caused the discovery and rediscovery of the same bacterial species and subsequently resulted in the constant renaming of the same taxa (Janda & Abbott, 2002). For example, between 1885 and 1928 the bacterium known today as *K. pneumoniae* was listed under six different genera with seven different species (Janda & Abbott, 2002). Between 1918 and 1960, 84 different species were published of which only 6% were properly described (Janda & Abbott, 2002). Despite the above-mentioned drawbacks of phenotypic methods, it serves the clinical laboratories well in the identification of medically important bacterial strains and remains the method of choice for presumptive identification.

### 3.2 Correlation between genes that confer antibiotic resistance and the antibiotic profiles of *Klebsiella pneumoniae*

Acquisition of genetic material is largely responsible for the development and escalation of antibiotic resistant strains of bacteria (Bennet, 2008). Genetic material containing resistance genes may be transferred from one cell to another by various mechanisms. Conjugation (also horizontal or lateral gene transfer) is perhaps the most dominant form by which genetic material is transferred from one plasmid to another or from plasmid to bacterial chromosome or from bacterial chromosome to

plasmids. Plasmids are transferable, extra chromosomal, genetic material that does not form part of the bacterial genome. Furthermore, plasmids do not depend on the bacterial chromosomal deoxyribonucleic acid (DNA) for replication and can carry a variety of genes, including antibiotic resistance genes. Resistance plasmids encode for antibiotic resistance against most classes of antibiotics currently in clinical use, including the commonly used cephalosporins, fluoroquinolones and aminoglycosides (Bennet, 2008) and carbapenems (Souli et al*.,* 2008).

All the *K. pneumoniae* strains used in the current study and sourced from the local pathology laboratories were identified as extended-spectrum beta-lactamases (ESBL) producing strains. Extended-spectrum beta-lactamases are enzymes that are produced by members of the Enterobacteriaceae that mediate resistance to third generation cephalosporins (cefotaxime, ceftriaxone, and ceftazidime), as well as the oxyimino-monobactam aztreonam (Gupta, 2003).

The wide-spread use of antimicrobial substances, especially of multiple antibiotics simultaneously and extended-spectrum antibiotics [this is a broad class of antibiotics that contain a beta-lactam nucleus in its molecular structure, and includes Penicillin (penams), Cephalosporins (Cephems), Monobactams and Carbapenems] are considered the primary cause of the development of MDR in *Klebsiella* strains (Gupta et al., 2003). These strains are especially prevalent in hospitals and are frequently isolated from clinical samples.

### 3.2.1 Amplification of SHV and TEM Extended spectrum beta-lactamases by PCR

As indicated in the materials and methods chapter, the boiling method as outlined in Ndlovu et al. (2015) was used to isolate DNA from all the samples (*n* = 57). This technique makes use of alternate heating and freezing of bacterial cells to subsequently denature or break the cell walls to release the DNA. This method does not however, distinguish between genomic DNA and plasmid DNA. A mixture of two sources of DNA was thus employed in all PCR analysis.

Polymerase chain reaction assays for the amplification of SHV and TEM were performed on all the samples (*n* = 57). In addition, the VITEK 2 analysis for antibiotic profiling was also performed.

For quality control purposes for all the PCR assays a water blank was used as negative control, while the *K. pneumoniae* ATCC 10031 was used as positive control.

### 3.2.1.1 Amplification of the TEM gene by PCR and VITEK 2 analysis

The PCR amplicons for the TEM gene (**Figure 3.4**) were produced successfully for 46 (81%) of the 57 samples included in this project, while 11 (19%) of the samples did not yield any TEM amplicons (**Appendix 3**). The expected amplified product size of 717 base pairs (bp) was obtained with the primers specific for $bla_{TEM}$.

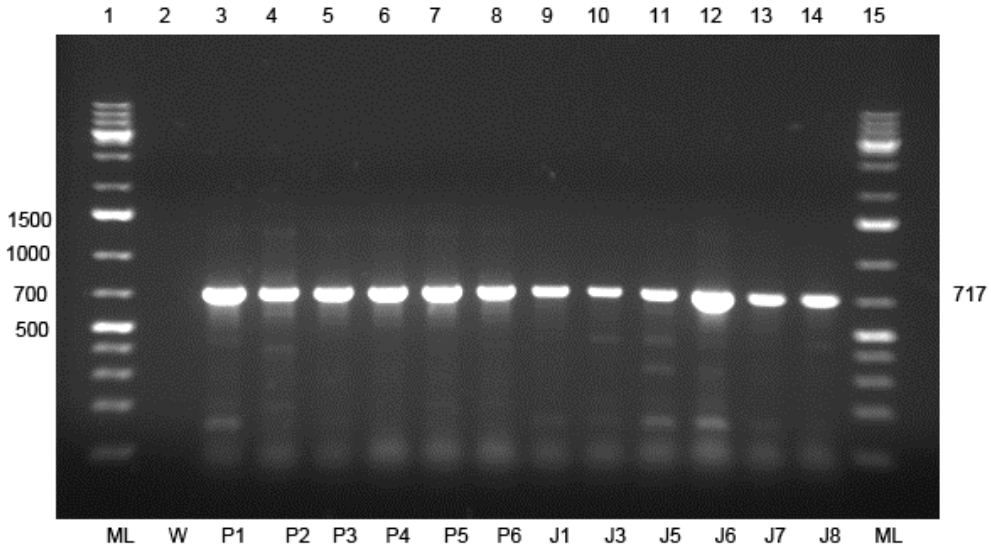


**Figure 3.4** Agarose electrophoresis photo depicting bands that correspond to the TEM gene: lanes 1 and 9 - GeneRuler 1 kb Plus DNA Ladder; Lane 2 - negative control; lane 3 - 14 representative clinical samples producing the expected 717 bp product.

When comparing the antibiotic resistance patterns for the classes of antibiotics (**Table 3.5; Appendix 4**) as provided by the VITEK 2, the antibiotic resistance profiles were as follows for samples that tested positive for TEM with PCR (*n* = 46) (**Table 3.6**): Ampicillin/Amoxicillin 100% (*n* = 46); Amoxicillin/Clavulanic acid 96% (44/46); Piperacillin/Tazobactam 93% (43/46); Aztreonam 89% (41/46); Tobramycin 87% (40/46); Ciprofloxacin 83% (38/46); Gentamycin 78% (36/46); Trimethoprim/Sulfamethoxazole 76% (35/46); Tigecycline 28% (13/46) and Colistin

9% (4/46). Correspondingly, research has shown that the presence of the TEM resistance gene often confers resistance to the broad-spectrum penicillin (Schmitt et al., 2007) and the beta-lactam group of drugs (Jain & Mondal, 2008).

**Table 3.5 Antibiotic classes tested by VITEK and the genes that cause bacterial resistance (Cockerill et al., 2012)**

| Antibiotic class | Antibiotic agent | Gene |
|---|---|---|
| Penicillins | Ampicillin/Amoxicillin (A/A) | SHV, TEM |
| Beta-lactamase Inhibitors | Piperacillin/Tazobactam (P/T) | SHV, TEM |
| | Amoxicillin/Clavulanic acid (A/C) | SHV, TEM |
| Cephalosporins | Ceftriaxone/Cefotaxime (C/C) | CTX-M |
| | Cefuroxime/Cefprozil (CFM) | CTX-M |
| | Cefepime (CEF) | CTX-M |
| | Cefoxitin (FOX) | CTX-M |
| | Ceftazidime (CFZ) | CTX-M |
| Carbapenems | Imipenem (IMI) | KPC |
| | Meropenem (MER) | KPC |
| | Ertapenem (ERT) | KPC |
| Aminoglycosides | Gentamycin (GEN) | SHV, TEM |
| | Tobramycin (TOB) | SHV, TEM |
| | Amikacin (AMK) | SHV, TEM |
| Fluoroquinolone | Ciprofloxacin (CIP) | SHV, TEM |
| Folate pathway Inhibitors | Trimethoprim/ Sulfamethoxazole (T/S) | SHV, TEM |
| Other | Tigecycline (TEG) | Unknown |
| | Colistin (COL) | Unknown |
| Monobactam | Aztreonam (AST) | SHV, TEM |

**Table 3.6 Resistance profiles for samples positively amplified by PCR for SHV and TEM**

| *Anti-biotic Agents | | SHV | | | | | | TEM | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | PRODUCT (56) | | | NO PRODUCT (1) | | | PRODUCT (46) | | | NO PRODUCT (11) | | |
| | | R | I | S | R | I | S | R | I | S | R | I | S |
| A/A | n | 56 | 0 | 0 | 1 | 0 | 0 | 46 | 0 | 0 | 11 | 0 | 0 |
| | % | 100 | | | 100 | | | 100 | | | 100 | | |
| P/T | n | 52 | 1 | 3 | 0 | 0 | 1 | 43 | 1 | 2 | 9 | 0 | 2 |
| | % | 93 | 2 | 5 | | | 100 | 93 | 2 | 4 | 82 | | 18 |
| A/C | n | 53 | 0 | 3 | 0 | 0 | 1 | 44 | 0 | 2 | 9 | 0 | 2 |
| | % | 95 | | 5 | | | 100 | 96 | | 4 | 82 | | 18 |
| C/C | n | 52 | 0 | 4 | 0 | 0 | 1 | 43 | 0 | 3 | 9 | 0 | 2 |
| | % | 93 | | 7 | | | 100 | 93 | | 7 | 82 | | 18 |
| CFM | n | 53 | 1 | 2 | 0 | 0 | 1 | 44 | 1 | 1 | 9 | 0 | 2 |
| | % | 95 | 2 | 4 | | | 100 | 96 | 2 | 2 | 82 | | 18 |
| CEF | n | 46 | 2 | 8 | 0 | 0 | 1 | 37 | 2 | 7 | 9 | 0 | 2 |
| | % | 82 | 4 | 14 | | | 100 | 80 | 4 | 15 | 82 | | 18 |
| FOX | n | 48 | 3 | 5 | 0 | 0 | 1 | 39 | 3 | 4 | 9 | 0 | 2 |
| | % | 86 | 5 | 9 | | | 100 | 85 | 7 | 9 | 82 | | 18 |
| CFZ | n | 52 | 0 | 4 | 0 | 0 | 1 | 43 | 0 | 3 | 9 | 0 | 2 |
| | % | 93 | | 7 | | | 100 | 93 | | 7 | 82 | | 18 |
| GEN | n | 38 | 2 | 13 | 0 | 0 | 1 | 36 | 1 | 9 | 2 | 1 | 8 |
| | % | 68 | 4 | 24 | | | 100 | 78 | 2 | 20 | 18 | 9 | 73 |
| TOB | n | 43 | 3 | 10 | 0 | 0 | 1 | 40 | 3 | 3 | 3 | 0 | 8 |
| | % | 77 | 5 | 18 | | | 100 | 87 | 7 | 7 | 27 | | 73 |
| AMK | n | 0 | 7 | 49 | 0 | 0 | 1 | 0 | 6 | 40 | 0 | 1 | 10 |
| | % | | 13 | 88 | | | 100 | | 13 | 87 | | 9 | 91 |
| CIP | n | 43 | 1 | 12 | 0 | 0 | 1 | 38 | 1 | 7 | 5 | 0 | 6 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | % | 77 | 2 | 21 | | | 100 | 83 | 2 | 15 | 45 | | 55 |
| T/S | n | 38 | 0 | 18 | 0 | 0 | 1 | 35 | 0 | 11 | 3 | 0 | 8 |
| | % | 68 | | 32 | | | 100 | 76 | | 24 | 27 | | 73 |
| TEG | n | 13 | 5 | 38 | 0 | 0 | 1 | 13 | 3 | 30 | 0 | 2 | 9 |
| | % | 23 | 9 | 68 | | | 100 | 28 | 7 | 65 | | 18 | 82 |
| COL | n | 4 | 0 | 52 | 0 | 0 | 1 | 4 | 0 | 42 | 0 | 0 | 11 |
| | % | 7 | | 93 | | | 100 | 9 | | 91 | | | 100 |
| AST | n | 48 | 3 | 5 | 0 | 0 | 1 | 41 | 2 | 3 | 7 | 1 | 3 |
| | % | 86 | 5 | 9 | | | 100 | 89 | 4 | 7 | 64 | 9 | 27 |

R = resistant

I = intermediate

S = sensitive

*n* = calculated for each individual antibiotic based on the totals (value in brackets) for each gene with product and with no product against the resistance profile for each individual antibiotic i.e. R, I, or S (**Table 2.7**).

% = percentage calculated for each individual gene product based on the n value for each gene resistance profile i.e. R, I or S.

*Abbreviations for the antibiotic names are provided within **Table 3.5**.

Of the 46 samples with the TEM gene, 43 samples also had the CTX-M gene present which confers plasmid mediated resistance to the following CTX-M associated antibiotics (**Table 3.5** and **Table 3.7**): Cefuroxime/Cefprozil 98% (42/43); Ceftriaxone/Cefotaxime 100% (*n* =43/43); Ceftazidime 98% (42/43); Cefoxitin 88% (38/43) and Cefepime 86% (37/43). The antibiotic profiles for the three samples that contained the TEM amplicon but did not contain the CTX-M gene, as expected, showed resistance to the TEM/SHV-associated antibiotics (**Table 3.5** and **Table 3.6**). The antibiotic resistant profiles for these three samples did not show any resistance towards the CTX-M associated antibiotics.

### 3.2.1.2 Amplification of the SHV gene by PCR and VITEK 2 analysis

The PCR amplicons for the $bla_{SHV}$ gene (**Figure 3.5**) were obtained successfully for 56 (98%) of the 57 DNA samples included in this project, while 1 sample (2%) did not yield any SHV amplicons (**Appendix 5**). The amplified product obtained with primers specific for $bla_{SHV}$ was 471 bp, which was the expected product size.

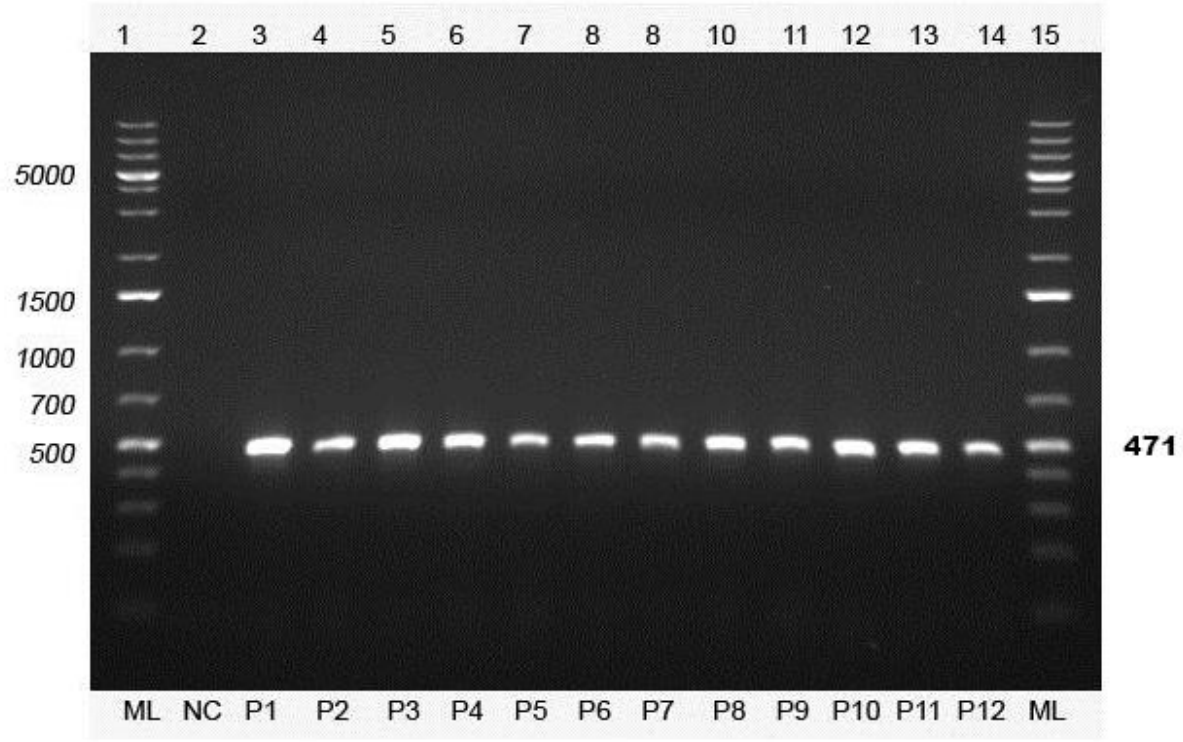


**Figure 3.5** Agarose electrophoresis photo depicting band sizes corresponding to the SHV gene: lanes 1 and 15 - GeneRuler 1 kb Plus DNA Ladder; Lane 2 - negative control; lane 3 - 14 representative clinical samples producing the expected 471 bp product.

When comparing the antibiotic resistance patterns for the classes of antibiotics (**Table 3.5; Appendix 4**) as provided by the VITEK 2, the antibiotic resistance profiles were as follows for the strains that tested positive for SHV with PCR (46 positive for both TEM and SHV and 10 positive for SHV only) (**Table 3.6**): Ampicillin/Amoxicillin 100% (n=56); Amoxicillin/Clavulanic acid 95% (53/56); Piperacillin/Tazobactam 93% (52/56); Aztreonam 86% (48/56); Tobramycin 77% (43/56); Ciprofloxacin 77% 43/56); Gentamycin 68% (38/56); Trimethoprim/Sulfamethoxazole 68% (38/56); Tigecycline 23% (13/56) and Colistin 7% (4/56). Accordingly, research has shown that the presence of the SHV resistance gene often confers resistance to broad-spectrum penicillins (Paterson et

al., 2004). The one sample that tested negative for SHV was resistant to only Ampicillin/Amoxicillin (**Table 3.6**).

Of the 56 samples with the SHV gene, 51 samples also tested positive for the CTX-M gene. The 51 samples thus also showed resistance to the following CTX-M associated antibiotics (**Table 3.5** and **Table 3.6**): Cefuroxime/Cefprozil 100% (51/51); Ceftriaxone/Cefotaxime 100% (51/51); Ceftazidime 100% (51/51); Cefoxitin 90% (46/51) and Cefepime 88% (45/51). The antibiotic profiles for the five samples that contained the SHV gene but did not contain the CTX-M gene, as expected showed resistance to the TEM/SHV associated antibiotics (**Table 3.5** and **Table 3.6**). The antibiotic resistant profiles for the CTX-M associated antibiotics for the five samples not containing the CTX-M gene were as follows: Cefuroxime/Cefprozil 40% (*n* = 2); Cefoxitin 40% (*n* = 2); Ceftazidime 20% (*n* = 1); Cefepime 20% (*n* = 1) and Ceftriaxone/Cefotaxime 20% (*n* = 1). These five samples could possibly have contained another type of ESBL such as the PER-1-type beta-lactamase which hydrolyses Cephalosporins and penicillins or GES-1-type beta-lactamase which hydrolyse extended-spectrum cephalosporins as well as penicillins (Shaikh et al., 2015).

In summary, 46 samples (80.7%) were positive for both TEM and SHV genes, while only one sample (P20) which was isolated from urine (**Table 3.1**) was negative for both the TEM or SHV genes (**Appendix 3 and 5**). In addition, ten samples were positive for SHV only (**Appendix 3 and 5**). Results obtained in the current study thus confirmed that *K. pneumoniae* isolates generally contain both TEM and SHV in contrast to only containing TEM or SHV (Jain & Mondal, 2008).

### 3.2.2 Amplification of the Cefotaximases by PCR and VITEK 2 analysis

The PCR amplicons for the $bla_{CTX\text{-}M}$ gene (**Figure 3.6**) were produced successfully by 89% (*n* = 51) of the DNA samples included in this project, while 11% (*n* = 6) did not yield any CTX-M amplicons (**Appendix 6**). The amplified product obtained with primers specific for $bla_{CTX\text{-}M}$ was 544 bp, which was the expected product size. Of the 51 samples that yielded CTX-M amplicons, 49% (25/51) were identified as CTX-M-15; 43% (22/51) as CTX-M-71; 4% (2/51) CTX-M-14b; 2% (1/51) CTX-M-1 and 2% (1/51) as CTX-M-17 (**Table 3.7**).

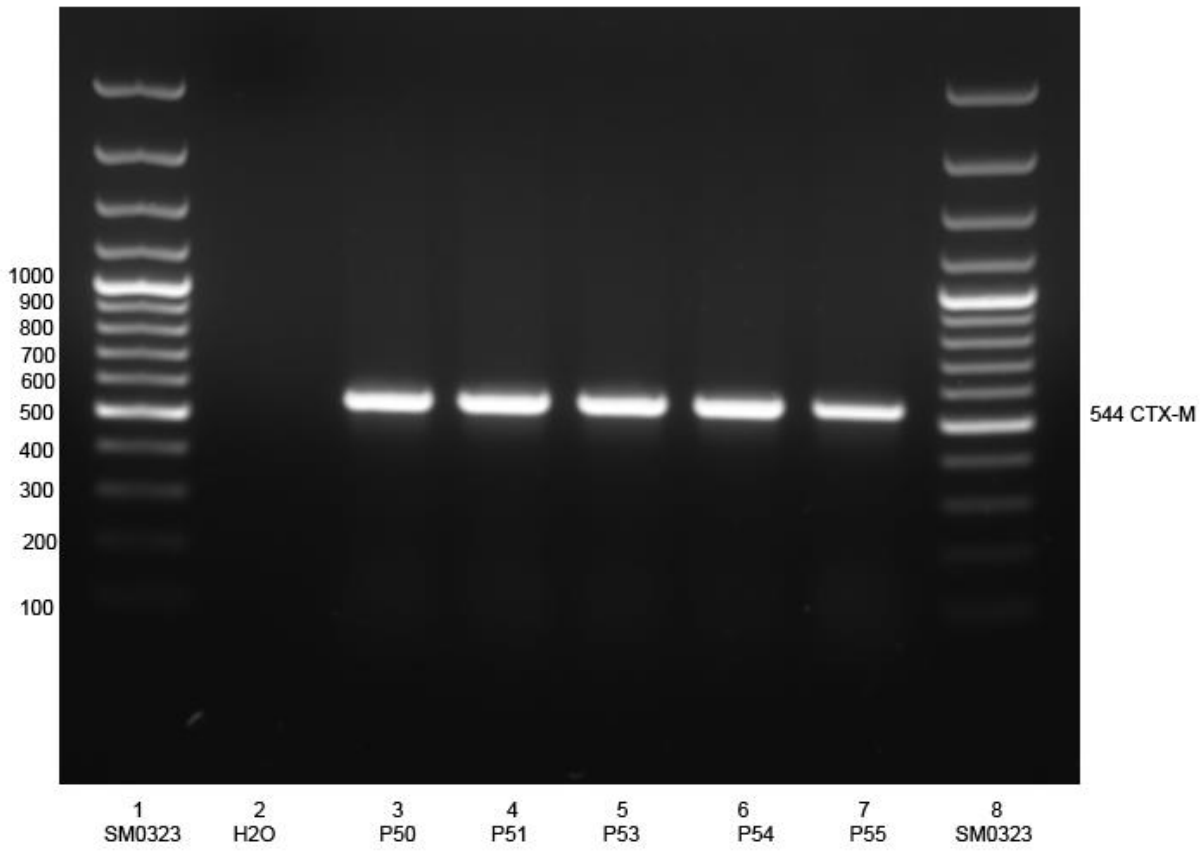


**Figure 3.6** Agarose electrophoresis photo depicting band sizes corresponding to the CTX-M gene: lanes 1 and 8 - GeneRuler 100 bp Plus DNA Ladder; Lane 2 - negative control; lane 3 - 7 representative clinical samples at producing the expected 544 bp product.

When comparing the antibiotic resistance patterns for the classes of antibiotics (**Table 3.5; Appendix 4**) as provided by the VITEK 2, to samples that tested positive for CTX-M (*n* = 51) with PCR, the profiles for those antibiotics associated with the CTX-M gene were as follows (**Table 3.7**): Cefuroxime/Cefprozil 100% (*n* = 51); Ceftazidime 100% (*n* = 51); Ceftriaxone/Cefotaxime 93% (n=51); Cefoxitin 90% (46/51); Cefepime 88% (45/51); Tigecycline 24% (12/51); and Colistin 8% (4/51). Accordingly, research has shown that the presence of the CTX-M resistance gene often confers resistance to the Cephalosporins [Bonnet (2004) and Edelstein, (2003)]. Of the 51 samples that contained the CTX-M gene, all 51 samples also contained the SHV gene and as previously explained, 43 samples also contained the TEM amplicon. The antibiotic profiles for the 51 SHV and CTX-M positive samples were thus identical to those provided previously for the 51 samples that contained the SHV amplicon. The antibiotic profile for the 43 samples that contained both the TEM and CTX-M gene was provided within Section 3.2.1.1.

In the current study, the samples with amplicons for CTX-M (*n* = 51) notably exhibited and increased resistance against the Cephalosporins [100% (*n* = 51) resistance against Ceftriaxone/Cefotaxime; 100% (*n* = 51) resistance against Cefuroxime/Cefprozil; 100% (*n* = 51) against Ceftazidime; 90% (*n* = 46) against Cefoxitin; and 88% (*n* = 45) against Cefepime] than against the aminoglycosides, Ciprofloxacin, Trimethoprim/Sulfamethoxazole, Tigecycline, Colistin and Aztreonam (**Table 3.7; Appendix 3**). No notable differences in resistance were noted when compared to the penicillins and beta-lactamase inhibitors except that all samples (100%) were resistant to the penicillin's. Hackman et al. (2014) reported the following antibiotic resistance profile for 70 samples containing the CTX-M gene: Ampicillin 100% (*n* = 70); Cefotaxime 100% (*n* = 70); Trimethoprim/Sulfamethoxazole 99% (*n* = 69); Gentamicin 89% (*n* = 62); Ciprofloxacin 71% (*n* = 50); Piperacillin/Tazobactam 61% (*n* = 43); Ceftazidime 43% (*n* = 30); Amoxicillin/Clavulanic acid 29 (*n* = 20); Cefoxitin 13% (*n* = 9); Cefepime 9% (*n* = 6); and Amikacin 0% (*n* = 0). This is similar to the profile obtained in the current study.

**Table 3.7 Resistance profiles for samples positively amplified by PCR for CTX-M**

| *Anti-biotic Agents | | CTX-M | | | | | |
|---|---|---|---|---|---|---|---|
| | | PRODUCT (51) | | | NO PRODUCT (6) | | |
| | | R | I | S | R | I | S |
| A/A | n | 51 | 0 | 0 | 6 | 0 | 0 |
| | % | 100 | | | 100 | | |
| P/T | n | 50 | 1 | 0 | 2 | 0 | 4 |
| | % | 98 | | | 33 | | 67 |
| A/C | n | 51 | 0 | 0 | 2 | 0 | 4 |
| | % | 100 | | | 33 | | 67 |
| C/C | n | 51 | 0 | 0 | 1 | 0 | 5 |
| | % | 100 | | | 17 | | 83 |
| CFM | n | 51 | 0 | 0 | 2 | 1 | 3 |
| | % | 100 | | | 33 | 17 | 50 |
| CEF | n | 45 | 2 | 4 | 1 | 0 | 5 |
| | % | 88 | 4 | 8 | 17 | | 83 |
| FOX | n | 46 | 2 | 3 | 2 | 1 | 3 |
| | % | 90 | 4 | 6 | 33 | 17 | 50 |
| CFZ | n | 51 | 0 | 0 | 1 | 0 | 5 |
| | % | 100 | | | 17 | | 83 |
| GEN | n | 36 | 0 | 15 | 2 | 2 | 2 |
| | % | 71 | | 29 | 33 | 33 | 33 |
| TOB | n | 39 | 3 | 9 | 4 | 0 | 2 |
| | % | 76 | 6 | 18 | 67 | | 33 |
| AMK | n | 0 | 4 | 47 | 0 | 3 | 3 |
| | % | | 8 | 92 | | 50 | 50 |
| CIP | n | 40 | 1 | 10 | 3 | 0 | 3 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | % | 78 | 2 | 20 | 50 | | 50 |
| T/S | n | 36 | 0 | 15 | 2 | 0 | 4 |
| | % | 71 | | 29 | 33 | | 67 |
| TEG | n | 12 | 4 | 35 | 1 | 1 | 4 |
| | % | 24 | 8 | 69 | 17 | 17 | 67 |
| COL | n | 4 | 0 | 47 | 0 | 0 | 6 |
| | % | 8 | 0 | 92 | 0 | 0 | 100 |
| AST | n | 47 | 3 | 1 | 1 | 0 | 5 |
| | % | 92 | 6 | 2 | 17 | 0 | 83 |
| IMI | n | 0 | 0 | 51 | 0 | 0 | 6 |
| | % | | | 100 | | | 100 |
| MER | n | 0 | 0 | 51 | 0 | 0 | 6 |
| | % | | | 100 | | | 100 |
| ERT | n | 0 | 1 | 50 | 0 | 0 | 6 |
| | % | | 2 | 98 | | | 100 |

R = resistant

I = intermediate

S = sensitive

*n* = calculated for each individual antibiotic based on the totals (value in brackets) for each gene with product and with no product against the resistance profile for each individual antibiotic i.e. R, I, or S (**Table 2.7**).

% = percentage calculated for each individual gene product based on the n value for each gene resistance profile i.e. R, I or S

*Abbreviations for the antibiotic names are provided within **Table 3.5**.

### 3.3 Detection of ESBL phenotype

Research has indicated that an ESBL-producing isolate may be resistant to Ceftazidime, but susceptible to Cefotaxime (Gupta et al., 2003). This implies that if susceptibility testing is limited to a single third-generation Cephalosporin, ESBL-producing isolates may not be detected. Gupta et al. (2003) recommended the

routine screening for ESBL activity in *K. pneumoniae* isolates by determining susceptibility to several Cephalosporins. The VITEK 2 ESBL test (bioMérieux) is based on the simultaneous assessment of the antibacterial activity of Cefepime, Cefotaxime and Ceftazidime, measured either alone or in the presence of clavulanate. The proportional reduction in growth in wells containing a Cephalosporin with clavulanic acid compared with those containing the Cephalosporin without clavulanic acid was considered indicative of ESBL production (Drieux et al., 2008). VITEK identified 84% (48/57) of the samples as ESBL *K. pneumoniae*.

### 3.4 Determination of antibiotic sensitivity using the VITEK 2 system

In order to obtain a general overview of resistance levels for the 57 samples, a panel of 19 antibiotics effective against Gram-negative organisms was analysed using VITEK 2.

Based on the results obtained, all (100%, *n* = 57) (**Table 3.8**) isolates were resistant to Amoxicillin/Ampicillin. Ninety three percent (53/57) were resistant to Amoxycillin/Clavulanate as well as Cefuroxime/Cefprozil (93%; 53/57). Antibiotic resistance profiles were then also obtained as follows: Ceftriaxone/Cefotaxime (91%; 52/57), Ceftazidime (91%; 52/57), Piperacillin/Tazobactam (91%; 52/97), Aztreonam (84%; 48/57), Cefoxitin (84%; 48/57), Cefepime (81%; 46/57), Tobramycin (75%; 43/57), and Gentamycin (67%; 38/57) (**Table 3.8**).

**Table 3.8 Tabulation of SHV, TEM and CTX-M genes, ESBL detection and panel of 19 antibiotics showing their respective *n* values and percentages as identified or tested**

| | SHV | TEM | CTX-M | ESBL | A/A | P/T | A/C | C/C | CFM | CEF | FOX | CFZ | IMI | MER | ERT | GEN | TOB | AMK | CIP | T/S | TEG | COL | AST |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P n | 56 | 46 | 51 | | | | | | | | | | | | | | | | | | | | |
| % | 98 | 81 | 89 | | | | | | | | | | | | | | | | | | | | |
| NP n | 1 | 11 | 6 | | | | | | | | | | | | | | | | | | | | |
| % | 2 | 19 | 11 | | | | | | | | | | | | | | | | | | | | |
| Pos n | | | | 48 | | | | | | | | | | | | | | | | | | | |
| % | | | | 84 | | | | | | | | | | | | | | | | | | | |
| Neg n | | | | 9 | | | | | | | | | | | | | | | | | | | |
| % | | | | 16 | | | | | | | | | | | | | | | | | | | |
| R n | | | | | 57 | 52 | 53 | 52 | 53 | 46 | 48 | 52 | 0 | 0 | 0 | 38 | 43 | 0 | 43 | 38 | 13 | 4 | 48 |
| % | | | | | 100 | 91 | 93 | 91 | 93 | 81 | 84 | 91 | | | | 67 | 75 | | 75 | 67 | 23 | 7 | 84 |
| I n | | | | | 0 | 1 | 0 | 0 | 1 | 2 | 3 | 0 | 0 | 0 | 1 | 2 | 3 | 7 | 1 | 0 | 5 | 0 | 3 |
| % | | | | | | 2 | | | 2 | 4 | 5 | | | | 2 | 4 | 5 | 12 | 2 | | 9 | | 5 |
| S n | | | | | 0 | 4 | 4 | 5 | 3 | 9 | 6 | 5 | 57 | 57 | 56 | 17 | 11 | 50 | 13 | 19 | 39 | 53 | 6 |
| % | | | | | | 7 | 7 | 9 | 5 | 16 | 11 | 9 | 100 | 100 | 98 | 30 | 19 | 88 | 23 | 33 | 68 | 93 | 11 |

P = Product; NP = No Product

Pos = Positive for ESBL; Neg = Negative for ESBL

R = Resistant; I= Intermediate; S = Sensitive

*n* = Sample size of 57

A/A = Ampicillin/Amoxicillin; P/T = Piperacillin/tazobactam; A/C = Amoxicillin/Clavulanic; CEF = Cefepime; FOX = Cefoxitin;

C/C = Ceftriaxone/Cefotaxime; CFZ = Ceftazidime; IMI = Imipenem; MER = Meropenem; ERT = Ertapenem; GE*N* = Gentamycin;

TOB = Tobramycin; AMK = Amikacin; CIP = Ciprofloxacin; T/S = Trimethoprim/Sulfamethoxazole; TEG = Tigecycline;

COL = Colistin; AST = Aztreonam; CFM = Cefuroxime/Cefprozil

Antibiotic profiles based on results obtained for the ESBL positive (*n* = 48) isolates, showed that all (100%) were resistant to Amoxicillin/Ampicillin, Amoxycillin/Clavulanate, Ceftriaxone/Cefotaxime, Cefuroxime/Cefprozil and Ceftazidime. Furthermore, the following antibiotic resistance profiles were also obtained for the ESBL positive isolates: Piperacillin/Tazobactam and Cefoxitin (98%, 47/48), Cefepime (96%, 46/48) Aztreonam (94%; 45/48), Tobramycin (81%, 39/48), Gentamycin and Ciprofloxacin (77%, 37/48), Trimethoprim/Sulfamethoxazole (67%, 32/48), and Tigecycline (25% 12/48).

In contrast, all isolates (100%, *n* = 57) exhibited sensitivity to Imipenem and Meropenem, 98% (56/57) were sensitive to Ertapenem, while most samples were sensitive to Colistin (88%; 49/57), Amikacin (86%; 48/57) and Tigecycline (63%; 35/57). Thirty four percent (19/57) were sensitive to Trimethoprim/Sulfamethoxazole; while 25% (14/57) were sensitive to Gentamycin and Ciprofloxacin, respectively; 18% (10/57) were sensitive to Tobramycin; 16% (9/57) were sensitive to Cefepime; 11% (6/57) were sensitive to Cefoxitin and Aztreonam; 9% (5/57) were sensitive to Piperacillin/Tazobactam, Ceftriaxone/Cefotaxime and Ceftazidime, respectively; 7% (4/57) were sensitive to Amoxicillin/Clavulanic; and 5% (3/57) were sensitive to Cefuroxime/Cefprozil (**Table 3.8**). All ESBL positive samples (100%, n = 48) exhibited sensitivity to Imipenem and Meropenem while, 98% (47/48) were sensitive to Ertapenem.

Fourteen percent (8/57) of the isolates produced an intermediate result (the sensitivity of a bacterial strain to a given antibiotic is said to be intermediate when it is inhibited in vitro by a concentration of this antibiotic that is associated with an uncertain therapeutic effect – it implies clinical efficacy in body sites where the antibiotics are physiologically concentrated such as beta-lactams in urine, while the same antibiotic may not be adequately effective against the same organism if it is located at other sites such as the meninges.) against Tigecycline. In addition, intermediate profiles were obtained as follows; 13% (7/57) against Amikacin; 5% (3/57) against Cefoxitin and Tobramycin, respectively; 4% (2/57) against Cefepime, Gentamycin and Aztreonam, respectively; and 2% (1/57) against Piperacillin/Tazobactam, Cefuroxime/Cefprozil and Ciprofloxacin and Ertapenem, respectively. Some intermediate results were also obtained for the ESBL positive isolates: Amikacin (10%, 5/48), Aztreonam and Tigecycline (6%, 3/48),

Piperacillin/Tazobactam (2% 1/48), Cefepime, Ertapenem, Gentamycin, and Ciprofloxacin (2%, 1/48).

Beta-lactamase inhibitors prevent bacterial degradation of beta-lactam antibiotics. It was originally anticipated that these inhibitors would extend the life-span of the current beta-lactams and also extend the range of bacteria the drugs are effective against (Drawz & Bonomo, 2010). Beta-lactamase production by Gram-negative bacteria is an important contributor to beta- lactam resistance in these pathogens and the most important use of beta-lactamase inhibitors is in the treatment of infections caused by these pathogens. In the current study, the Beta-lactamase inhibitors (Amoxycillin/Clavulanate and Piperacillin/Tazobactam) showed very high resistance rates of 93% and 91%, respectively (**Table 3.8**) and thus could be of little value against the treatment of this pathogen.

As can be seen from **Table 3.9**, all the isolates contained the TEM, SHV and CTX-M genes in combination or two or more of the genes as follows: TEM and SHV (*n* = 46); or TEM and CTX-M (*n* = 43); or SHV and CTX-M (*n* = 51); or TEM, SHV and CTX-M (*n* = 43). Moreover, while sample P20 did not contain an amplicon for either TEM, SHV or CTX-M, this specific *K. pneumoniae* strain was sensitive to all antibiotics with the exception of Ampicillin/Amoxicillin and tested ESBL negative. All (100%) tested sensitive against the Carbapenems and all (100%) tested resistant against Ampicillin/Amoxicillin.

**Table 3.9 Comparison of antibiotic resistance patterns of ESBL producing *K. pneumoniae* on the basis of genotypes and resistance patterns of ESBL's**

| Antibiotic | Resistance % | | | |
|---|---|---|---|---|
| | TEM+ SHV+ CTXM Positive *n*=43 | TEM+ SHV Positive *n*=46 | SHV+ CTXM Positive *n*=51 | TEM+ CTXM Positive *n*=43 |
| Ampicillin/Amoxicillin | 100 | 100 | 100 | 100 |
| Piperacillin/Tazobactam | 100 | 91 | 98 | 100 |
| Amoxicillin/Clavulanic | 100 | 96 | 100 | 100 |
| Ceftriaxone/Cefotaxime | 100 | 89 | 100 | 100 |
| Cefuroxime/Cefprozil | 100 | 96 | 100 | 100 |
| Cefepime | 88 | 83 | 88 | 86 |
| Cefoxitin | 88 | 85 | 90 | 88 |
| Ceftazidime | 100 | 93 | 100 | 100 |
| Gentamycin | 86 | 83 | 75 | 84 |
| Tobramycin | 86 | 87 | 76 | 86 |
| Amikacin | 2 | 2 | 2 | 2 |
| Ciprofloxacin | 79 | 80 | 76 | 79 |
| Trimethoprim/Sulfamethoxazole | 79 | 78 | 73 | 79 |
| Tigecycline | 28 | 28 | 24 | 30 |
| Colistin | 16 | 15 | 14 | 16 |
| Aztreonam | 95 | 89 | 92 | 95 |
| Imipenem | 0 | 0 | 0 | 0 |
| Meropenem | 0 | 0 | 0 | 0 |
| Ertapenem | 0 | 0 | 0 | 0 |

Furthermore, the ESBL isolates (*n* = 48) show more resistance against the Cephalosporins where only P41 tested sensitive against Cefepime and Cefoxitin while P42 tested intermediate against Cefepime; all other ESBL isolates tested resistant against the Cephalosporins. Forty-seven of the 48 ESBL producing isolates were found to contain at least 2 of the three (CTX-M, TEM and SHV) ESBL gene products (**Appendix 4** and **Appendix 7**).

All non-ESBL (n = 9) isolates, excluding P35, tested sensitive to at least one of the Cephalosporins. Eight non-ESBL isolates tested sensitive against Cefepime and one (P35) tested intermediate. Eight of the non-ESBL isolates were found to contain at least one of the three (CTX-M, TEM and SHV) ESBL gene products (**Appendix 4** and **Appendix 7**). Four of the 9 non-ESBL producing isolates were revealed to carry CTX-M (P8, P30, P32 and P35). These four samples have almost identical resistance profiles: all tested resistant against Penicillin, the Beta-lactamase inhibitors, Ciprofloxacin, Trimethoprim/Sulfamethoxazole, Colistin and the monobactam Aztreonam, and the same three Cephalosporins (Ceftriaxone/Cefotaxime, Cefuroxime/Cefprozil and Ceftazidime). P8 is also resistant to Tobramycin. The non-ESBL producing isolates tested sensitive to all above mentioned except the Penicillin which is resistant for all isolates. The resistance rates for the non-ESBL producing strains without CTX-M are much lower than for those with CTX-M (**Appendix 8**).

In all the isolates in which a gene product could be amplified (SHV, TEM or CTX-M) an ESBL gene were revealed with the NCBI BLAST blastn suite. The multidrug resistant phenotypes of these isolates can thus be explained by the presence of at least one of the CTX-M, TEM or SHV ESBL genes. It is noteworthy that all but one of the ESBL producing isolates was shown to possess the CTX-M gene product. The P28 isolate is the exception and contain only SHV-1 though similar resistance patterns as the other ESBL isolates were seen. The dominant CTX-M gene isolated by CTX-M specific PCR was CTX-M-15 (*n* = 30), followed by CTX-M-28 (*n* = 17), CTX-M-14 (*n* = 2) then CTX-M-1 (*n* = 1) and CTX-M-188 (*n* = 1) (**Appendix 6 and 7**). CTX-M-15 (P30 and P35) and CTX-M-28 (P8 and P32) were also found in the non-ESBL isolates. CTX-M-15 has been previously described as the dominant type of ESBL in gram-negative pathogens to cause outbreaks in nosocomial and community settings (Calbo et al., 2011). Bourouis et al., (2013) reported on MDR *Enterobacter*

*clocae* (also a member of the Enterobacteriaceae) where all strains (*n* = 8) in their study contained CTX-M-28 and predicted that this CTX-M-28 encoded plasmid may become an epidemiological problem. It has now been demonstrated in 17 *K. pneumoniae* isolates from nosocomial and community settings in the Western Cape Province (**Appendix 7, 9 and 10)**. However, no difference could be observed in isolates containing CTX-M-28 as oppose to those containing any of the other CTX-M-subtypes mentioned above concerning the ESBL isolates. Furthermore, P8 and P32 were identified as non-ESBL producing isolates, though CTX-M-28 was amplified with resistance profiles identical to P30 and P35 with CTX-M-15 (resistant to Penicillin, Beta-lactamase inhibitors, Ciprofloxacin, Trimethoprim/Sulfamethoxazole, Colistin Aztreonam, Ceftriaxone/Cefotaxime, Cefuroxime/Cefprozil and Ceftazidime).

Another interesting phenomena is the presence of TEM-116 (**Appendix 3**) that were found in eight of the J-samples (*n* = 10), the other two J-samples were both TEM-1 (**Appendix 3**) by TEM specific PCR. None of the TEM-116 genes were found in the P-samples. The dominant TEM gene sub-types found in all the isolates were TEM-1 (*n* = 38), followed by TEM-116 (*n* = 8) (**Appendix 3**). The dominant TEM subtype amplified in the ESBL isolates were TEM-1 (n = 34) (**Appendix 9**) while TEM-116 was amplified in five of the ESBL producing J-samples isolates (**Appendix 9**)**.** No difference in antibiotic resistance profiles could be observed in the isolates containing TEM-116 as oppose to those with TEM-1 subtypes.

The dominant SHV gene found in all the isolates were SHV-11 (*n* = 42) followed by SHV-28 (*n* = 4), SHV-61 (*n* = 3), SHV-2 (n = 1), SHV-12 (*n* = 1), SHV-27 (*n* = 1), SHV-38 (*n* = 1) and SHV-98 (*n* = 1) (**Appendix 5**). All of the SHV-subtypes demonstrated in this study have been reported before (Liakopoulos et al., 2016).

The antibiotic profiles of the ESBL producing strains remained similar irrespective of the gene sub-types the isolates harboured (**Appendix 11**).

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## CHAPTER FOUR

## 4. CONCLUSION

The MALDI-TOF MS, VITEK 2, API 20 E systems and PCR, employed in the current study for the identification of *K. pneumoniae* from clinical samples, are methods in which sample preparation is a crucial factor for accuracy and repeatability of the results. Any variations in the protocol can affect the outcome of the results. For the MALDI-TOF MS, VITEK 2 and API 20 E systems, pure cultures are needed for an accurate result as any contamination may have a profound impact on the end result. The disadvantage in the preparation of pure cultures is the prolonged handling of bacterial pathogens by laboratory staff. A further disadvantage of the API 20 E system is the time required in preparing the API strip as well as the high input required from laboratory staff for the manual reading of the strip and interpretation of the reactions. An advantage of the API system is the lack of expensive equipment other than an incubator.

The MALDI-TOF MS also utilises pure growth cultures in the culturable state as microorganisms in a viable but non-culturable state cannot be detected (Loff et al., 2014). A major disadvantage of the MALDI-TOF MS is the initial cost of the instrument but it is a powerful tool for the identification of clinical isolates due to short turnaround times, low reagent cost, and low sample volume needed (Pavlovic et al., 2013). Data is also generated within 16 hours and numerous studies have demonstrated that the MALDI-TOF MS is a rapid and reliable method for identification of bacteria (Romero-Gómez et al., 2012; Pavlovic et al., 2013 and Barnini et al., 2015). Similar to the MALDI-TOF MS technique, the major disadvantage of the VITEK 2 is the initial capital cost involved in the purchasing of the instrument. However, the relatively short turnaround times and the minimum input required from laboratory staff concerning interpretation of the result are major advantages.

Species specific PCR amplification that screen for the 16S rDNA gene is the most common molecular technique that is used today for bacterial identification. Vast amounts of sequenced data for a large consortium of living organisms, particularly bacteria, have been accumulated (Woo et al., 2008). Comparison of the16S rDNA genes of the smaller subunit of the ribosomes has shown that it is highly conserved

within the same genus or species but differs between organisms of other species which makes it a powerful tool in identifying or distinguishing between bacterial species. Disadvantages encountered with phenotypic tests can thus be overcome with specific PCR primers and will be very useful in the diagnosis of culture-negative infections. However, this method also has several disadvantages: it is costly, labour intensive and technically complex and human error occurs more frequently than with the phenotypic analysis methods.

Based on the results obtained in the current study, PCR and VITEK 2 are the methods of choice for the identification of *K. pneumoniae*. A further advantage of the VITEK 2 is that while PCR does not indicate the pathogenicity of a particular *K. pneumoniae* strain, VITEK 2 can indicate pathogenicity with antibiotic resistance profiles and extended-spectrum beta-lactamase confirmation.

Based on the current study, it is clear that MDR *K. pneumoniae* is a real and current threat in the Western Cape region of South-Africa as demonstrated with the high resistance profiles and the high prevalence of CTX-M, SHV and TEM gene products in these isolates. This is alarming as the Cephalosporins are prescribed and administered many times as a first-line therapy for mild uncomplicated urinary tract infections to severe bacteraemia or septic shock (Adesoji et al., 2016). The bacterial organisms usually involved in conferring resistance to the Cephalosporins include *Enterococcus faecium, Staphylococcus aureus, Clostridium difficile, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and other Enterobacteriaceae (Adesoji et al., 2016). It is necessary to study the trend in antibiotic resistance in general and likewise efforts made towards sustaining the relevance of the use of antibiotic to the present-day world. Also, increasing our knowledge about bacterial genetics will be conclusive for the effective treatment of bacterial infections while at the same time help to slow down the development of new resistance traits, helping in the aim to sustain the use of antibiotics as a treatment option.

In this study fifty-seven bacterial isolates were considered with the aim to assess the genetic make-up of this isolates to ascertain the reason for the resistance associated with the MAR profiling. These strains were highly resistant to Penicillins, Cephalosporins (Ceftriaxone/Cefotaxime, Cefuroxime/Cefprozil, Cefepime, Cefoxitin

and Ceftazidime) and the beta-lactamase inhibitors (Piperacillin/Tazobactam and Amoxicillin/Clavulanic). Furthermore, a high prevalence of the three most important ESBL genes (viz. TEM, SHV and CTX-M) were demonstrated by the MDR *K. pneumoniae* strains in the Western Cape. As mentioned before, CTX-M has undergone a rapid and global spread in Enterobacteriaceae. A high prevalence of CTX-M-15 and CTX-M-28 [both belonging to the cluster CTX-M-1 (Calbo et al., 2011)] were found. Bourouis et al. (2013) mentioned that all the MDR strains in their study harboured the CTX-M-28 gene. The first report of CTX-M-28 was made in 2007 according to Bourouis et al. (2013) and has now spread to other parts of the world including South-Africa. CTX-M-15 have also been reported previously (Calbo et al., 2011; Xu et al., 2007) reported that this subtype has already spread worldwide, includng Africa and is associated with major outbreaks of MDR bacterial infections. Calbo et al. (2011) also stated that CTX-M-15 and CTX-M-14 (also amplified in this sudy) are by far the most important subtypes within the CTX-M enzymes. Furthermore, CTX-M-1 was also amplified in this study which is also associated with MDR outbreaks (Xu et al., 2007).

All of the TEM (TEM-1 and TEM-116) and SHV (SHV-1, SHV-2, SHV-11, SHV-12, SHV,27, SHV-28, SHV-38, SHV-61 and SHV-98) subtypes demonstrated in this study had been previuosly reported as being associated with multi-drug resistance in *K. pneumoniae*. It is worth mentioning that TEM-116 was isolated only from the J-samples – the origin of these samples is unknown except that it is nosocomial isolates that were used in a previous study. Since there is no knowledge of the origin for the J isolates, the reason for the presence of the TEM-116 genes in these (or the absence of it from the P-samples) can thus not be fully explained; it can be assumed that these J-samples had the same clonal origin and from a different part of the population than the P-samples. All the ESBL producing strains used in this study show similar resistance patterns, but it should however, be noted that irrespective of the gene combination or the gene sub-types the ESBL producing isolates harboured, their antibiotic resistance patterns remained similar (**Appendix 11**).

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## APPENDICES

## Appendix 1:

## API 20E Biochemical profiles

| Code name | Biochemical Reactions | | | | | | | | | | | | | | | | | | | | | Numerical Profiles | Organism ID | % Identification |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ONPG | ADA | LDC | ODC | CIT | H2S | URE | TDA | IND | VP | GEL | GLU | MAN | INO | SOR | RHA | SAC | MEL | AMY | ARA | OX | | | |
| P2 | + | + | + | - | + | - | + | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 7215773 | *K. pneumoniae pneumoniae* | 97.5 |
| P3 | + | + | + | - | + | - | + | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 7215773 | *K. pneumoniae pneumoniae* | 97.5 |
| P4 | + | + | + | - | + | - | + | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 7215773 | *K. pneumoniae pneumoniae* | 97.5 |
| P5 | + | + | + | - | + | - | - | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 7205773 | *R. terrigena* | 62.3 |
| P6 | + | + | + | - | + | - | - | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 7205773 | *R. terrigena* | 62.3 |
| P7 | + | - | + | - | + | - | + | - | - | + | - | + | + | - | + | + | + | + | + | + | - | 5215573 | *K. pneumoniae pneumoniae* | 97.6 |
| P8 | + | - | + | - | + | - | - | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 5205773 | *R. terrigena* | 68.9 |
| P9 | + | - | + | - | + | - | + | + | - | + | - | + | + | + | + | + | + | + | + | + | - | 5235773 | *K. pneumoniae pneumoniae* | 97.6 |
| P10 | + | + | + | - | + | - | + | + | - | + | - | + | + | + | + | + | + | + | + | + | - | 7235773 | *K. pneumoniae pneumoniae* | no percentage |
| P11 | + | - | + | - | + | - | + | + | - | + | - | + | + | + | + | + | + | + | + | + | - | 5235773 | *K. pneumoniae pneumoniae* | 97.6 |
| P12 | + | - | + | - | + | - | + | + | - | + | - | + | + | + | + | + | + | + | + | + | - | 5235773 | *K. pneumoniae pneumoniae* | 97.6 |
| P13 | + | - | + | - | + | - | + | + | - | + | - | + | + | + | + | + | + | + | + | + | - | 5235773 | *K. pneumoniae pneumoniae* | 97.6 |
| P14 | + | + | + | - | + | - | + | + | - | + | - | + | + | + | + | + | + | + | + | + | - | 7235773 | *K. pneumoniae pneumoniae* | no percentage |
| P15 | + | - | + | - | + | - | + | + | - | + | - | + | + | + | + | + | + | + | + | + | - | 5235773 | *K. pneumoniae pneumoniae* | 97.6 |
| P18 | + | - | + | - | + | - | + | + | - | + | - | + | + | + | + | + | + | + | + | + | - | 5235773 | *K. pneumoniae pneumoniae* | 97.6 |
| P19 | + | - | + | - | + | - | - | + | - | + | - | + | + | + | + | + | + | + | + | + | - | 5225773 | *R. terrigena* | 68.9 |
| P20 | + | - | + | - | + | - | + | + | - | + | - | + | + | + | + | + | + | + | + | + | - | 5235773 | *K. pneumoniae pneumoniae* | 97.6 |
| P21 | + | - | + | - | + | - | + | + | - | + | - | + | + | + | + | + | + | + | + | + | - | 5235773 | *K. pneumoniae pneumoniae* | 97.6 |
| P23 | + | - | + | - | + | - | + | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 5215773 | *K. pneumoniae* | 97.6 |
| P25 | + | - | + | - | + | - | + | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 5215773 | *K. pneumoniae pneumoniae* | 97.6 |
| P26 | + | - | + | - | + | - | + | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 5215773 | *K. pneumoniae pneumoniae* | 97.6 |
| P27 | + | - | + | - | + | - | + | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 5215773 | *K. pneumoniae pneumoniae* | 97.6 |

| | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P28 | + | - | + | - | + | - | - | - | - | - | - | + | + | + | + | + | + | - | + | - | - | 5204731 | *K. pneumoniae ozaenae* | 54.7 |
| P29 | + | - | + | - | + | - | + | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 5215773 | *K. pneumoniae pneumoniae* | 97.6 |
| P30 | + | - | + | - | + | - | + | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 5215773 | *K. pneumoniae pneumoniae* | 97.6 |
| P31 | + | - | + | - | + | - | + | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 5215773 | *K. pneumoniae pneumoniae* | 97.6 |
| P32 | + | - | + | - | + | - | + | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 5215773 | *K. pneumoniae pneumoniae* | 97.6 |
| P33 | + | + | + | - | + | - | + | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 7215773 | *K. pneumoniae pneumoniae* | 97.5 |
| P34 | + | - | + | - | + | - | - | - | - | + | - | + | + | + | - | + | + | + | - | - | - | 5205370 | *Raoultella terrigena* | 44.2 |
| P35 | + | - | + | - | + | - | + | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 5215773 | *K. pneumoniae pneumoniae* | 97.6 |
| P36 | + | - | + | - | + | - | + | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 5215773 | *K. pneumoniae pneumoniae* | 97.6 |
| P37 | + | - | + | - | + | - | + | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 5215773 | *K. pneumoniae pneumoniae* | 97.6 |
| P38 | + | - | + | - | + | - | + | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 5215773 | *K. pneumoniae pneumoniae* | 97.6 |
| P39 | + | - | + | - | + | - | + | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 5215773 | *K. pneumoniae pneumoniae* | 97.6 |
| P40 | + | - | + | - | + | - | + | - | - | - | - | + | + | + | + | + | + | + | + | + | - | 5214773 | *K. pneumoniae pneumoniae* | 95 |
| P41 | + | - | + | - | + | - | - | - | - | - | - | + | + | + | + | + | + | + | + | + | - | OOO773 | *R. terrigena* | 82.8 |
| P42 | + | - | + | - | + | - | + | - | - | - | - | + | + | + | + | + | + | + | + | + | - | 5214773 | *K. pneumoniae pneumoniae* | 95 |
| P43 | + | - | + | - | + | - | + | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 5215773 | *K. pneumoniae pneumoniae* | 97.6 |
| P46 | + | - | + | - | + | - | + | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 5215773 | *K. pneumoniae pneumoniae* | 97.6 |
| P47 | + | + | + | - | + | - | - | - | - | - | - | + | + | + | + | + | + | + | + | + | - | 7204773 | *K. pneumoniae ozaenae* | 57.2 |
| P48 | + | - | + | - | + | - | + | - | - | + | - | + | - | + | + | + | + | + | + | + | - | 5215673 | *K. pneumoniae pneumoniae* | 98.4 |
| P50 | + | - | + | - | + | - | + | - | - | - | - | + | + | + | + | + | + | + | + | + | - | 5214773 | *K. pneumoniae pneumoniae* | 95 |
| P51 | + | - | + | - | + | - | + | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 5215773 | *K. pneumoniae pneumoniae* | 97.6 |
| P53 | + | - | + | - | + | - | - | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 5205773 | *R. terrigena* | 68.9 |
| P54 | + | + | + | - | + | - | - | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 7205773 | *R. terrigena* | 62.3 |
| P55 | + | - | + | - | + | - | + | - | - | - | - | + | + | + | + | + | + | + | + | + | - | 5214773 | *K. pneumoniae pneumoniae* | 95 |
| P56 | + | - | + | - | + | - | - | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 5205773 | *R. terrigena* | 68.9 |
| J1 | + | - | + | - | + | - | + | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 5215773 | *K. pneumoniae pneumoniae* | 97.6 |
| J3 | + | - | + | - | + | - | + | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 5215773 | *K. pneumoniae pneumoniae* | 97.6 |
| J5 | + | - | + | - | + | - | + | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 5215773 | *K. pneumoniae pneumoniae* | 97.6 |
| J6 | + | - | + | - | + | - | + | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 5215773 | *K. pneumoniae pneumoniae* | 97.6 |
| J7 | + | - | + | - | + | - | + | - | - | - | - | + | + | + | + | + | + | + | + | + | - | 5214773 | *K. pneumoniae pneumoniae* | 95 |

| | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| J8 | + | - | + | - | + | - | - | - | - | + | - | + | + | + | + | + | + | + | + | + | - | 5205773 | *R. terrigena* | 68.9 |
| J9 | + | - | + | - | - | - | + | - | - | - | - | + | + | + | + | + | + | + | + | + | - | 5014773 | *K. pneumoniae pneumoniae* | 94.5 |
| J10 | + | - | + | - | + | - | + | - | - | - | - | + | + | + | + | + | + | + | + | + | - | 5214773 | *K. pneumoniae pneumoniae* | 95 |
| J11 | + | + | + | - | + | - | + | - | - | - | - | + | + | + | + | + | + | + | + | + | - | 7214773 | *K. pneumoniae pneumoniae* | 90.6 |
| J12 | + | - | + | - | + | - | + | - | - | - | - | + | + | + | + | + | + | + | + | + | - | 5214773 | *K. pneumoniae pneumoniae* | 95 |

## Appendix 2:

## Data generated from the PCR, MALDI-TOF MS, API 20 E and VITEK 2 assays

| Code name | MALDI | VITEK | PCR | API20E | ALL | %Agreement | 4 agree | 3 agree | 2 agree |
|---|---|---|---|---|---|---|---|---|---|
| J1 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| J3 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| J5 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| J6 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| J7 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| J8 | 1 | 1 | 1 | 0 | 3 | 75% | 0 | 1 | 0 |
| J9 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| J10 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| J11 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| J12 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P2 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P3 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P4 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P5 | 1 | 1 | 1 | 0 | 3 | 75% | 0 | 1 | 0 |
| P6 | 1 | 1 | 1 | 0 | 3 | 75% | 0 | 1 | 0 |
| P7 | 0 | 1 | 1 | 1 | 3 | 75% | 0 | 1 | 0 |
| P8 | 1 | 1 | 1 | 0 | 3 | 75% | 0 | 1 | 0 |
| P9 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P10 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P11 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P12 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P13 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P14 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P15 | 0 | 1 | 1 | 1 | 3 | 75% | 0 | 1 | 0 |
| P18 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P19 | 1 | 1 | 1 | 0 | 3 | 75% | 0 | 1 | 0 |
| P20 | 0 | 1 | 1 | 1 | 3 | 75% | 0 | 1 | 0 |
| P21 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P23 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P25 | 0 | 1 | 1 | 1 | 3 | 75% | 0 | 1 | 0 |
| P26 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P27 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P28 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P29 | 0 | 1 | 1 | 1 | 3 | 75% | 0 | 1 | 0 |
| P30 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P31 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P32 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P33 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P34 | 0 | 1 | 1 | 0 | 2 | 50% | 0 | 0 | 1 |
| P35 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| P36 | 0 | 1 | 1 | 1 | 3 | 75% | 0 | 1 | 0 |
| P37 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P38 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P39 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P40 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P41 | 1 | 1 | 1 | 0 | 3 | 75% | 0 | 1 | 0 |
| P42 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P43 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P46 | 0 | 1 | 1 | 1 | 3 | 75% | 0 | 1 | 0 |
| P47 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P48 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P50 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P51 | 1 | 1 | 1 | 1 | 4 | 100% | 1 | 0 | 0 |
| P53 | 1 | 1 | 1 | 0 | 3 | 75% | 0 | 1 | 0 |
| P54 | 1 | 1 | 1 | 0 | 3 | 75% | 0 | 1 | 0 |
| P55 | 0 | 1 | 1 | 1 | 3 | 75% | 0 | 1 | 0 |
| P56 | 0 | 1 | 1 | 0 | 2 | 50% | 0 | 0 | 1 |
| | 47 | 57 | 57 | 47 | | | 39 | 16 | 2 |
| | 57 | 57 | 57 | 57 | | | 57 | 57 | 57 |
| | MALDI | VITEK | PCR | API20E | | Average agreement | 4 agree | 3 agree | 2 agree |
| | 82.5% | 100.0% | 100.0% | 82.5% | | 91.0% | 68.4% | 28.1% | 3.5% |

## Appendix 3:

## TEM subtypes isolated by PCR

| Code name | TEM - Subtype | % Identity | Lactamase type | Accession number |
|---|---|---|---|---|
| J1 | TEM-116 | 99 | ESBL | KY496572.1 |
| J3 | TEM-116 | 99 | ESBL | KY496572.1 |
| J5 | TEM-116 | 99 | ESBL | KY496572.1 |
| J6 | TEM-1 | 98 | ESBL | LT853897.1 |
| J7 | TEM-116 | 99 | ESBL | KY496572.1 |
| J8 | TEM-116 | 99 | ESBL | KY496572.1 |
| J9 | TEM-116 | 99 | ESBL | KY496572.1 |
| J10 | TEM-116 | 94 | ESBL | KY496572.1 |
| J11 | TEM-1 | 95 | ESBL | LT853897.1 |
| J12 | Product obtained: No sequencing results obtained | | | |
| P2 | TEM-1 | 99 | ESBL | LT853897.1 |
| P3 | TEM-1 | 100 | ESBL | LT853897.1 |
| P4 | TEM-1 | 100 | ESBL | LT853897.1 |
| P5 | TEM-1 | 100 | ESBL | LT853897.1 |
| P6 | TEM-1 | 99 | ESBL | LT853897.1 |
| P7 | NO PRODUCT | | | |
| P8 | TEM-1 | 97 | ESBL | LT853897.1 |
| P9 | TEM-1 | 99 | ESBL | LT853897.1 |
| P10 | NO PRODUCT | | | |
| P11 | TEM-1 | 99 | ESBL | LT853897.1 |
| P12 | TEM-1 | 96 | ESBL | LT853897.1 |
| P13 | TEM-1 | 94 | ESBL | LT853897.1 |
| P14 | TEM-1 | 94 | ESBL | LT853897.1 |
| P15 | NO PRODUCT | | | |
| P18 | TEM-1 | 98 | ESBL | LT853897.1 |
| P19 | TEM-1 | 99 | ESBL | LT853897.1 |
| P20 | NO PRODUCT | | | |
| P21 | TEM-1 | 87 | ESBL | LT853899.1 |
| P23 | TEM-1 | 92 | ESBL | LT853897.1 |
| P25 | NO PRODUCT | | | |
| P26 | TEM-1 | 92 | ESBL | LT853898.1 |
| P27 | TEM-1 | 96 | ESBL | LT853897.1 |
| P28 | NO PRODUCT | | | |
| P29 | NO PRODUCT | | | |
| P30 | TEM-1 | 92 | ESBL | LT853897.1 |
| P31 | TEM-1 | 96 | ESBL | LT853898.1 |
| P32 | TEM-1 | 94 | ESBL | LT853898.1 |
| P33 | TEM-1 | 92 | ESBL | LT853897.1 |
| P34 | TEM-1 | 95 | ESBL | LT853897.1 |
| P35 | TEM-1 | 93 | ESBL | LT853897.1 |
| P36 | TEM-1 | 90 | ESBL | LT853897.1 |
| P37 | TEM-1 | 97 | ESBL | LT853897.1 |
| P38 | TEM-1 | 94 | ESBL | LT853897.1 |

| | | | | |
|---|---|---|---|---|
| P39 | TEM-1 | 95 | ESBL | LT853897.1 |
| P40 | TEM-1 | 96 | ESBL | LT853897.1 |
| P41 | TEM-1 | 94 | ESBL | LT853897.1 |
| P42 | TEM-1 | 94 | ESBL | LT853897.1 |
| P43 | TEM-1 | 94 | ESBL | JN043384.1 |
| P46 | TEM-1 | 93 | ESBL | LT853898.1 |
| P47 | TEM-1 | 95 | ESBL | LT853897.1 |
| P48 | TEM-1 | 97 | ESBL | LT853897.1 |
| P50 | TEM-1 | 91 | ESBL | LT853897.1 |
| P51 | NO PRODUCT | | | |
| P53 | NO PRODUCT | | | |
| P54 | TEM-1 | 87 | ESBL | LT853898.1 |
| P55 | NO PRODUCT | | | |
| P56 | NO PRODUCT | | | |

## Appendix 4:

## SHV, TEM and CTX-M products with corresponding VITEK antibiotic profiles for each sample

| | Gene products | | | | Penicillin | Beta-lactamase inhibitors | | Cephalosporins | | | | | Carbapenems | | | Aminoglycosides | | | Fluoroquinolone | Folate pathway inhibitors | Other | | Monobactam | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CODE NAME | SHV | TEM | CTX-M | ESBL | A/A | P/T | A/C | C/C | CFM | CEF | FOX | CFZ | IMI | MER | ERT | GEN | TOB | AMK | CIP | T/S | TEG | COL | AST | Total resistant |
| J1 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | S | S | R | 13 |
| J3 | P | P | NP | N | R | S | S | S | I | S | I | S | S | S | S | I | R | I | R | S | I | S | S | 3 |
| J5 | P | P | NP | N | R | S | S | S | R | S | R | S | S | S | S | R | R | S | R | R | R | S | S | 8 |
| J6 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | I | R | R | S | R | R | R | S | R | 14 |
| J7 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | S | S | R | 13 |
| J8 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | S | S | S | S | R | S | S | R | 10 |
| J9 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | I | R | S | S | S | R | 12 |
| J10 | P | P | NP | N | R | R | R | S | S | S | S | S | S | S | S | R | R | I | R | R | S | S | S | 7 |
| J11 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | S | R | R | S | R | 13 |
| J12 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | I | R | R | S | S | R | 13 |
| P2 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | S | S | R | 13 |
| P3 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | S | S | R | 13 |
| P4 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | R | S | R | 14 |
| P5 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | S | S | R | 13 |
| P6 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | S | S | R | 13 |
| P7 | P | NP | P | P | R | R | R | R | R | R | R | R | S | S | S | S | S | S | R | R | S | S | R | 11 |

| | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P8 | P | P | P | N | R | R | R | R | R | S | S | R | S | S | S | S | R | S | R | R | S | R | R | 11 |
| P9 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | R | S | R | 14 |
| P10 | P | NP | P | P | R | R | R | R | R | R | R | R | S | S | S | S | S | S | R | S | I | S | R | 10 |
| P11 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | S | S | R | 13 |
| P12 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | S | S | S | R | 12 |
| P13 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | S | S | R | 13 |
| P14 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | S | S | S | R | 12 |
| P15 | P | NP | P | P | R | R | R | R | R | R | R | R | S | S | S | S | S | S | R | R | S | S | S | 10 |
| P18 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | S | R | S | R | 13 |
| P19 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | S | R | S | R | 13 |
| P20 | N | NP | NP | N | R | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | 1 |
| P21 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | S | S | I | 12 |
| P23 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | R | S | R | 14 |
| P25 | P | NP | P | P | R | R | R | R | R | R | R | R | S | S | S | S | S | S | R | R | S | S | I | 10 |
| P26 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | S | S | S | R | 12 |
| P27 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | R | S | R | 14 |
| P28 | P | NP | NP | P | R | R | R | R | R | R | R | R | S | S | S | I | R | I | S | S | S | S | R | 10 |
| P29 | P | NP | NP | N | R | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | 1 |
| P30 | P | P | P | N | R | R | R | R | R | S | S | R | S | S | S | S | I | S | R | R | S | R | R | 10 |
| P31 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | I | R | R | S | S | R | 13 |
| P32 | P | P | P | N | R | R | R | R | R | S | I | R | S | S | S | S | I | S | R | R | I | R | R | 10 |
| P33 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | S | S | S | R | R | S | S | R | 11 |
| P34 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | S | R | S | S | I | 11 |
| P35 | P | P | P | N | R | R | R | R | R | I | I | R | S | S | S | S | I | S | R | R | S | R | R | 10 |
| P36 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | R | S | R | 14 |
| P37 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | S | R | S | S | R | 12 |
| P38 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | R | S | R | 14 |
| P39 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | S | R | S | S | R | 12 |
| P40 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | S | S | R | 13 |
| P41 | P | P | P | P | R | I | R | R | R | S | S | R | S | S | S | S | R | S | I | R | S | S | R | 8 |

| | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P42 | P | P | P | P | R | R | R | R | R | I | R | R | S | S | S | S | R | S | R | S | R | S | R | 11 |
| P43 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | S | S | S | R | 12 |
| P46 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | S | S | S | S | R | S | S | R | 10 |
| P47 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | S | I | S | R | 12 |
| P48 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | R | S | R | 14 |
| P50 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | I | R | S | S | S | R | 12 |
| P51 | P | NP | P | P | R | R | R | R | R | R | R | R | S | S | S | S | S | S | R | S | I | S | R | 10 |
| P53 | P | NP | P | P | R | R | R | R | R | R | R | R | S | S | S | S | S | S | S | S | S | S | R | 9 |
| P54 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | S | R | S | S | R | 12 |
| P55 | P | NP | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | S | S | S | S | R | 11 |
| P56 | P | NP | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | S | S | S | S | R | 11 |

P = Product; NP = No Product; R = Resistant; S = Sensitive; I= Intermediate; P = ESBL positive; *N* = ESBL negative

A/A = Ampicillin/Amoxicillin; P/T = Piperacillin/Tazobactam; A/C = Amoxicillin/Clavulanic; CEF = Cefepime; FOX = Cefoxitin; C/C = Ceftriaxone/Cefotaxime; CFZ = Ceftazidime; IMI = Imipenem; MER = Meropenem; ERT = Ertapenem; GEN = Gentamycin; TOB = Tobramycin; AMK = Amikacin; CIP = Ciprofloxacin; T/S = Trimethoprim/Sulfamethoxazole; TEG = Tigecycline; COL = Colistin; AST = Aztreonam; CFM = Cefuroxime/Cefprozil

## Appendix 5:

## SHV subtypes isolated by PCR

| Code name | SHV - Subtype | % Identity | Lactamase type | Accession number |
|---|---|---|---|---|
| J1 | SHV-11 | 95 | ESBL | HM060536.1 |
| J3 | SHV-11 | 96 | ESBL | HM060536.1 |
| J5 | SHV-11 | 95 | ESBL | HM060535.1 |
| J6 | SHV-11 | 99 | ESBL | HM060535.1 |
| J7 | SHV-11 | 94 | ESBL | HM060536.1 |
| J8 | SHV-11 | 97 | ESBL | GQ470428.1 |
| J9 | SHV-11 | 96 | ESBL | HM060536.1 |
| J10 | SHV-11 | 95 | ESBL | HM060536.1 |
| J11 | SHV-2 | 99 | ESBL | LT628506.1 |
| J12 | SHV-11 | 95 | ESBL | HM060536.1 |
| P2 | SHV-11 | 96 | ESBL | HM060536.1 |
| P3 | SHV-11 | 97 | ESBL | GQ470428.1 |
| P4 | SHV-11 | 99 | ESBL | HM060538.1 |
| P5 | SHV-11 | 99 | ESBL | HM060536.1 |
| P6 | SHV-28 | 99 | ESBL | HQ877609.1 |
| P7 | SHV-11 | 99 | ESBL | LT628512.1 |
| P8 | SHV-11 | 99 | ESBL | HM060535.1 |
| P9 | SHV-11 | 99 | ESBL | HM060536.1 |
| P10 | SHV-11 | 99 | ESBL | HM060536.1 |
| P11 | SHV-11 | 99 | ESBL | HM060536.1 |
| P12 | SHV-11 | 99 | ESBL | HM060536.1 |
| P13 | SHV-38 | 92 | ESBL | NG_050077.1 |
| P14 | SHV-28 | 95 | ESBL | HQ877609.1 |
| P15 | SHV-11 | 98 | ESBL | LT854835.1 |
| P18 | SHV-28 | 97 | ESBL | HQ877609.1 |
| P19 | SHV-28 | 98 | ESBL | HQ877609.1 |
| P20 | NO PRODUCT | | | |
| P21 | SHV-61 | 96 | ESBL | KY496584.1 |
| P23 | SHV-11 | 95 | ESBL | KY496584.1 |
| P25 | SHV-27 | 90 | ESBL | KY496584.1 |
| P26 | SHV-11 | 99 | ESBL | HM060536.1 |
| P27 | SHV-11 | 94 | ESBL | HM060536.1 |
| P28 | SHV-12 | 93 | ESBL | LT853894.1 |
| P29 | SHV-1 | 92 | ESBL | LT628514.1 |
| P30 | SHV-11 | 99 | ESBL | HM060535.1 |
| P31 | SHV-11 | 93 | ESBL | HM060531.1 |
| P32 | SHV-11 | 99 | ESBL | HM060535.1 |
| P33 | SHV-11 | 99 | ESBL | HM060535.1 |
| P34 | SHV-11 | 99 | ESBL | GQ470428.1 |
| P35 | SHV-11 | 99 | ESBL | HM060535.1 |
| P36 | SHV-11 | 98 | ESBL | GQ470428.1 |
| P37 | SHV-11 | 99 | ESBL | HM060536.1 |
| P38 | SHV-11 | 99 | ESBL | HM060536.1 |

| | | | | |
|---|---|---|---|---|
| P39 | SHV-11 | 99 | ESBL | LT628505.1 |
| P40 | SHV-61 | 99 | ESBL | KY496584.1 |
| P41 | SHV-11 | 99 | ESBL | DQ166779.3 |
| P42 | SHV-11 | 98 | ESBL | HM060536.1 |
| P43 | SHV-11 | 99 | ESBL | HM060536.1 |
| P46 | SHV-11 | 99 | ESBL | HM060535.1 |
| P47 | SHV-11 | 99 | ESBL | HM060536.1 |
| P48 | SHV-11 | 93 | ESBL | HM060536.1 |
| P50 | SHV-11 | 99 | ESBL | HM060536.1 |
| P51 | SHV-11 | 99 | ESBL | HM060536.1 |
| P53 | SHV-61 | 99 | ESBL | KY496584.1 |
| P54 | SHV-11 | 99 | ESBL | HM060536.1 |
| P55 | SHV-1 | 90 | ESBL | LT628510.1 |
| P56 | SHV-98 | 89 | ESBL | NG_050129.1 |

## Appendix 6:

## CTX-M subtypes isolated by PCR

| Code name | CTX-M - Subtype | % Identity | Lactamase type | Accession number |
|---|---|---|---|---|
| J1 | CTX-M-15 | **99** | Beta-lactamase | KM058752.1 |
| J3 | NO PRODUCT | | | |
| J5 | NO PRODUCT | | | |
| J6 | CTX-M-15 | 99 | Beta-lactamase | JX129220.1 |
| J7 | CTX-M-15 | 99 | Beta-lactamase | KM058752.1 |
| J8 | CTX-M-1 | 96 | Beta-lactamase | JX268711.1 |
| J9 | CTX-M-15 | 98 | Beta-lactamase | JX129220.1 |
| J10 | NO PRODUCT | | | |
| J11 | CTX-M-15 | 99 | Beta-lactamase | JX129220.1 |
| J12 | CTX-M-15 | 99 | Beta-lactamase | JX129219.1 |
| P2 | CTX-M-15 | 94 | Beta-lactamase | KY496541.1 |
| P3 | CTX-M-15 | 99 | Beta-lactamase | JX129220.1 |
| P4 | CTX-M-15 | 99 | Beta-lactamase | JX129220.1 |
| P5 | CTX-M-15 | 99 | Beta-lactamase | JX129220.1 |
| P6 | CTX-M-28 | 95 | ESBL | LT853900.1 |
| P7 | CTX-M-15 | 92 | ESBL | EU118593.1 |
| P8 | CTX-M-28 | 96 | ESBL | LT853900.1 |
| P9 | CTX-M-28 | 95 | ESBL | LT853900.1 |
| P10 | CTX-M-15 | 99 | Beta-lactamase | JX129220.1 |
| P11 | CTX-M-15 | 97 | Beta-lactamase | JX129220.1 |
| P12 | CTX-M-15 | 99 | Beta-lactamase | JX129220.1 |
| P13 | CTX-M-15 | 97 | Beta-lactamase | JX129220.1 |
| P14 | CTX-M-15 | 92 | ESBL | EU118593.1 |
| P15 | CTX-M-28 | 97 | ESBL | LT853900.1 |
| P18 | CTX-M-15 | 98 | Beta-lactamase | JX129220.1 |
| P19 | CTX-M-15 | 98 | Beta-lactamase | JX129220.1 |
| P20 | NO PRODUCT | | | |
| P21 | CTX-M-15 | 96 | ESBL | EU118593.1 |
| P23 | CTX-M-28 | 95 | ESBL | LT853900.1 |
| P25 | CTX-M-28 | 95 | ESBL | LT853900.1 |
| P26 | CTX-M-28 | 97 | ESBL | LT853900.1 |
| P27 | CTX-M-15 | 99 | Beta-lactamase | JX129220.1 |
| P28 | NO PRODUCT | | | |
| P29 | NO PRODUCT | | | |
| P30 | CTX-M-15 | 97 | ESBL | EU118593.1 |
| P31 | CTX-M-15 | 99 | Beta-lactamase | JX129220.1 |
| P32 | CTX-M-28 | 97 | ESBL | LT853900.1 |
| P33 | CTX-M-28 | 95 | ESBL | LT853900.1 |
| P34 | CTX-M-28 | 94 | ESBL | LT853900.1 |
| P35 | CTX-M-15 | 99 | Beta-lactamase | JX129220.1 |
| P36 | CTX-M-28 | 98 | ESBL | LT853900.1 |
| P37 | CTX-M-15 | 98 | ESBL | KY496541.1 |
| P38 | CTX-M-188 | 98 | ESBL | KY496555.1 |

| | | | | |
|---|---|---|---|---|
| P39 | CTX-M-28 | 94 | ESBL | LT853900.1 |
| P40 | CTX-M-28 | 99 | ESBL | LT853900.1 |
| P41 | CTX-M-28 | 98 | ESBL | LT853900.1 |
| P42 | CTX-M-15 | 96 | ESBL | EU118593.1 |
| P43 | CTX-M-15 | 97 | ESBL | EU118593.1 |
| P46 | CTX-M-28 | 93 | ESBL | LT853900.1 |
| P47 | CTX-M-15 | 98 | Beta-lactamase | JX129220.1 |
| P48 | CTX-M-28 | 94 | ESBL | LT853900.1 |
| P50 | CTX-M-28 | 96 | ESBL | LT853900.1 |
| P51 | CTX-M-15 | 99 | Beta-lactamase | JX129220.1 |
| P53 | CTX-M-15 | 100 | Beta-lactamase | JX129220.1 |
| P54 | CTX-M-15 | 99 | Beta-lactamase | JX129220.1 |
| P55 | CTX-M-14 | 97 | Beta-lactamase | KX639428.1 |
| P56 | CTX-M-14 | 99 | Beta-lactamase | KX639428.1 |

## Appendix 7:

## Summary of gene subtypes isolated for each sample

| Code name | ESBL gene subtypes (from **Appendix 6, 7 and 8)** | | |
|---|---|---|---|
| J1 | CTX-M-15 | TEM-116 | SHV-11 |
| J3 | No product | TEM-116 | SHV-11 |
| J5 | No product | TEM-116 | SHV-11 |
| J6 | CTX-M-15 | TEM-1 | SHV-11 |
| J7 | CTX-M-15 | TEM-116 | SHV-11 |
| J8 | CTX-M-1 | TEM-116 | SHV-11 |
| J9 | CTX-M-15 | TEM-116 | SHV-11 |
| J10 | No product | TEM-116 | SHV-11 |
| J11 | CTX-M-15 | TEM-1 | SHV-2 |
| J12 | CTX-M-15 | Product but no PCR sequence obtained | SHV-11 |
| P2 | CTX-M-15 | TEM-1 | SHV-11 |
| P3 | CTX-M-15 | TEM-1 | SHV-11 |
| P4 | CTX-M-15 | TEM-1 | SHV-11 |
| P5 | CTX-M-15 | TEM-1 | SHV-11 |
| P6 | CTX-M-28 | TEM-1 | SHV-28 |
| P7 | CTX-M-15 | No product | SHV-11 |
| P8 | CTX-M-28 | TEM-1 | SHV-11 |
| P9 | CTX-M-28 | TEM-1 | SHV-11 |
| P10 | CTX-M-15 | No product | SHV-11 |
| P11 | CTX-M-15 | TEM-1 | SHV-11 |
| P12 | CTX-M-15 | TEM-1 | SHV-11 |
| P13 | CTX-M-15 | TEM-1 | SHV-38 |
| P14 | CTX-M-15 | TEM-1 | SHV-28 |
| P15 | CTX-M-28 | No product | SHV-11 |
| P18 | CTX-M-15 | TEM-1 | SHV-28 |
| P19 | CTX-M-15 | TEM-1 | SHV-28 |
| P20 | No product | No product | No product |
| P21 | CTX-M-15 | TEM-1 | SHV-61 |
| P23 | CTX-M-28 | TEM-1 | SHV-11 |
| P25 | CTX-M-28 | No product | SHV-27 |
| P26 | CTX-M-28 | TEM-1 | SHV-11 |
| P27 | CTX-M-15 | TEM-1 | SHV-11 |
| P28 | No product | No product | SHV-12 |
| P29 | No product | No product | SHV-1 |
| P30 | CTX-M-15 | TEM-1 | SHV-11 |
| P31 | CTX-M-15 | TEM-1 | SHV-11 |
| P32 | CTX-M-28 | TEM-1 | SHV-11 |
| P33 | CTX-M-28 | TEM-1 | SHV-11 |
| P34 | CTX-M-28 | TEM-1 | SHV-11 |

| | | | |
|---|---|---|---|
| P35 | CTX-M-15 | TEM-1 | SHV-11 |
| P36 | CTX-M-28 | TEM-1 | SHV-11 |
| P37 | CTX-M-15 | TEM-1 | SHV-11 |
| P38 | CTX-M-188 | TEM-1 | SHV-11 |
| P39 | CTX-M-28 | TEM-1 | SHV-11 |
| P40 | CTX-M-28 | TEM-1 | SHV-61 |
| P41 | CTX-M-28 | TEM-1 | SHV-11 |
| P42 | CTX-M-15 | TEM-1 | SHV-11 |
| P43 | CTX-M-15 | TEM-1 | SHV-11 |
| P46 | CTX-M-28 | TEM-1 | SHV-11 |
| P47 | CTX-M-15 | TEM-1 | SHV-11 |
| P48 | CTX-M-28 | TEM-1 | SHV-11 |
| P50 | CTX-M-28 | TEM-1 | SHV-11 |
| P51 | CTX-M-15 | No product | SHV-11 |
| P53 | CTX-M-15 | No product | SHV-61 |
| P54 | CTX-M-15 | TEM-1 | SHV-11 |
| P55 | CTX-M-14 | No product | SHV-1 |
| P56 | CTX-M-14 | No product | SHV-98 |

## Appendix 8:

## Correlation between antibiotic profiles and PCR products for the non-ESBL isolates

| | Gene products | | | | Penicillin | Beta-lactamase inhibitors | | Cephalosporins | | | | | Carbapenems | | | Aminoglycosides | | | Fluoroquinolone | Folate pathway inhibitors | Other | | Monobactam | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CODE NAME | CTX-M | TEM | SHV | ESBL | A/A | P/T | A/C | C/C | CFM | CEF | FOX | CFZ | IMI | MER | ERT | GEN | TOB | AMK | CIP | T/S | TEG | COL | AST | Total resistant |
| J3 | NP | P | P | N | R | S | S | S | I | S | I | S | S | S | S | I | R | I | R | S | I | S | S | 3 |
| J5 | NP | P | P | N | R | S | S | S | R | S | R | S | S | S | S | R | R | S | R | R | R | S | S | 8 |
| J10 | NP | P | P | N | R | R | R | S | S | S | S | S | S | S | S | R | R | I | R | R | S | S | S | 7 |
| P8 | P | P | P | N | R | R | R | R | R | S | S | R | S | S | S | S | R | S | R | R | S | R | R | 11 |
| P20 | NP | NP | NP | N | R | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | 1 |
| P29 | NP | NP | P | N | R | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | 1 |
| P30 | P | P | P | N | R | R | R | R | R | S | S | R | S | S | S | S | I | S | R | R | S | R | R | 10 |
| P32 | P | P | P | N | R | R | R | R | R | S | I | R | S | S | S | S | I | S | R | R | I | R | R | 10 |
| P35 | P | P | P | N | R | R | R | R | R | I | I | R | S | S | S | S | I | S | R | R | S | R | R | 10 |

## Appendix 9:

## Correlation between sample sites of origin, PCR products and organism identification of the ESBL producing isolates

| Sites | Code names | PCR products | | | Identification | | | | ESBL |
|---|---|---|---|---|---|---|---|---|---|
| | | CTX-M | TEM | SHV | MALDI-TOF MS | VITEK | Species specific PCR | API 20 E | |
| Nosocomial | | | | | | | | | |
| Unknown sites | J1 | CTX-M-15 | TEM-116 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | J6 | CTX-M-15 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | J7 | CTX-M-15 | TEM-116 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | J8 | CTX-M-28 | TEM-116 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *R. terrigena* | ESBL |
| | J9 | CTX-M-15 | TEM-116 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | J11 | CTX-M-15 | TEM-1 | SHV-2 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | J12 | CTX-M-15 | TEM-116 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| Bronchial aspirates | P2 | CTX-M-15 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P6 | CTX-M-28 | TEM-1 | SHV-28 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *R. terrigena* | ESBL |
| Tracheal aspirates | P3 | CTX-M-15 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P28 | NP | NP | SHV-12 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae ozaenae* | ESBL |
| | P34 | CTX-M-28 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *R. terrigena* | ESBL |
| | P37 | CTX-M-15 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| Sputum | P5 | CTX-M-15 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *R. terrigena* | ESBL |
| | P19 | CTX-M-15 | TEM-1 | SHV-28 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *R. terrigena* | ESBL |
| | P21 | CTX-M-15 | TEM-1 | SHV-61 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P39 | CTX-M-28 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P40 | CTX-M-28 | TEM-1 | SHV-61 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P53 | CTX-M-15 | NP | SHV-61 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *R. terrigena* | ESBL |
| | P54 | CTX-M-15 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *R. terrigena* | ESBL |
| | P55 | CTX-M-14 | NP | SHV-1 | *K. variicola* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P56 | CTX-M-14 | NP | SHV-98 | *K. variicola* | *K. pneumoniae* | *K. pneumoniae* | *R. terrigena* | ESBL |
| Catheter urine | P25 | CTX-M-28 | NP | SHV-27 | *K. variicola* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P27 | CTX-M-15 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Urine | P4 | CTX-M-15 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P18 | CTX-M-15 | TEM-1 | SHV-28 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P23 | CTX-M-28 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P26 | CTX-M-28 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P31 | CTX-M-15 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P33 | CTX-M-28 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P43 | CTX-M-15 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P46 | CTX-M-28 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P47 | CTX-M-15 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae ozaenae* | ESBL |
| | P48 | CTX-M-28 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P50 | CTX-M-28 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P51 | CTX-M-15 | NP | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| Community acquired | | | | | | | | | |
| Urine | P7 | CTX-M-15 | NP | SHV-11 | *K. variicola* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P9 | CTX-M-28 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P10 | CTX-M-15 | NP | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P11 | CTX-M-15 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P12 | CTX-M-15 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P13 | CTX-M-15 | TEM-1 | SHV-38 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P14 | CTX-M-15 | TEM-1 | SHV-28 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P15 | CTX-M-28 | NP | SHV-11 | No reliable identification | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P36 | CTX-M-28 | TEM-1 | SHV-11 | *K. variicola* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P38 | CTX-M-188 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |
| | P41 | CTX-M-28 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *R. terrigena* | ESBL |
| | P42 | CTX-M-15 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | ESBL |

## Appendix 10:

## Correlation between sample sites of origin, PCR products and organism identification of the non-ESBL producing isolates

| Sites | Code names | PCR products | | | Identification | | | | non-ESBL |
|---|---|---|---|---|---|---|---|---|---|
| | | CTX-M | TEM | SHV | MALDI-TOF MS | VITEK | Species specific PCR | API 20 E | |
| Nosocomial | | | | | | | | | |
| | J3 | NP | TEM-116 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | non-ESBL |
| | J5 | NP | TEM-116 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | non-ESBL |
| | J10 | NP | TEM-116 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | non-ESBL |
| | P30 | CTX-M-15 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | non-ESBL |
| | P20 | NP | NP | NP | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | non-ESBL |
| | P29 | NP | NP | SHV-1 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | non-ESBL |
| | P32 | CTX-M-28 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | non-ESBL |
| Community acquired | | | | | | | | | |
| | P8 | CTX-M-28 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *R. terrigena* | non-ESBL |
| | P35 | CTX-M-15 | TEM-1 | SHV-11 | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae* | *K. pneumoniae pneumoniae* | non-ESBL |

## Appendix 11:

## Correlation between antibiotic profiles and PCR products for the ESBL isolates

| | Gene products | | | | Penicillin | Beta-lactamase inhibitors | | Cephalosporins | | | | | Carbapenems | | | Aminoglycosides | | | Fluoroquinolone | Folate pathway inhibitors | Other | | Monobactam | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CODE NAME | CTX-M | TEM | SHV | ESBL | A/A | P/T | A/C | C/C | CFM | CEF | FOX | CFZ | IMI | MER | ERT | GEN | TOB | AMK | CIP | T/S | TEG | COL | AST | Total resistant |
| J1 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | S | S | R | 13 |
| J6 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | I | R | R | S | R | R | R | S | R | 14 |
| J7 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | S | S | R | 13 |
| J8 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | S | S | S | S | R | S | S | R | 10 |
| J9 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | I | R | S | S | S | R | 12 |
| J11 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | S | R | R | S | R | 13 |
| J12 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | I | R | R | S | S | R | 13 |
| P2 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | S | S | R | 13 |
| P3 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | S | S | R | 13 |
| P4 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | R | S | R | 14 |
| P5 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | S | S | R | 13 |

| | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P6 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | S | S | R | 13 |
| P7 | P | NP | P | P | R | R | R | R | R | R | R | R | S | S | S | S | S | S | R | R | S | S | R | 11 |
| P9 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | R | S | R | 14 |
| P10 | P | NP | P | P | R | R | R | R | R | R | R | R | S | S | S | S | S | S | R | S | I | S | R | 10 |
| P11 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | S | S | R | 13 |
| P12 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | S | S | S | R | 12 |
| P13 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | S | S | R | 13 |
| P14 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | S | S | S | R | 12 |
| P15 | P | NP | P | P | R | R | R | R | R | R | R | R | S | S | S | S | S | S | R | R | S | S | S | 10 |
| P18 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | S | R | S | R | 13 |
| P19 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | S | R | S | R | 13 |
| P21 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | S | S | I | 12 |
| P23 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | R | S | R | 14 |
| P25 | P | NP | P | P | R | R | R | R | R | R | R | R | S | S | S | S | S | S | R | R | S | S | I | 10 |
| P26 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | S | S | S | R | 12 |
| P27 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | R | S | R | 14 |
| P28 | NP | NP | P | P | R | R | R | R | R | R | R | R | S | S | S | I | R | I | S | S | S | S | R | 10 |
| P31 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | I | R | R | S | S | R | 13 |
| P33 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | S | S | S | R | R | S | S | R | 11 |
| P34 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | S | R | S | S | I | 11 |
| P36 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | R | S | R | 14 |
| P37 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | S | R | S | S | R | 12 |
| P38 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | R | S | R | 14 |
| P39 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | S | R | S | S | R | 12 |
| P40 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | S | S | R | 13 |
| P41 | P | P | P | P | R | I | R | R | R | S | S | R | S | S | S | S | R | S | I | R | S | S | R | 8 |
| P42 | P | P | P | P | R | R | R | R | R | I | R | R | S | S | S | S | R | S | R | S | R | S | R | 11 |
| P43 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | S | S | S | R | 12 |
| P46 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | S | S | S | S | R | S | S | R | 10 |

| | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P47 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | S | I | S | R | 12 |
| P48 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | R | R | R | S | R | 14 |
| P50 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | I | R | S | S | S | R | 12 |
| P51 | P | NP | P | P | R | R | R | R | R | R | R | R | S | S | S | S | S | S | R | S | I | S | R | 10 |
| P53 | P | NP | P | P | R | R | R | R | R | R | R | R | S | S | S | S | S | S | S | S | S | S | R | 9 |
| P54 | P | P | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | S | R | S | S | R | 12 |
| P55 | P | NP | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | S | S | S | S | R | 11 |
| P56 | P | NP | P | P | R | R | R | R | R | R | R | R | S | S | S | R | R | S | S | S | S | S | R | 11 |