Antimicrobial Resistance Patterns and Molecular Characterization of *Klebsiella pneumoniae* in Clinical Isolates at Mbarara Regional Referral Hospital

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Abstract

Background: *Klebsiella pneumoniae* is one of the most frequent opportunistic pathogens causing a range of infections and being resistant for beta-lactamases (ESBL) and Carbapenemases. **Aim:** The aim of the present study was to determine the antimicrobial resistance patterns and molecular characterization establishing the phenotypes and genotypes associated with drug resistance, an antibiogram of genotypically positive isolates for resistance of *Klebsiella pneumoniae* in clinical isolates at MRRH. **Materials and Methods:** A laboratory-based descriptive cross-sectional study that was conducted from September 2018 to May 2019 at MRRH. *Klebsiella pneumoniae* was identified by cultural and biochemical methods. Antibiotic sensitivity test was performed by modified Kirby-Bauer disc diffusion technique. ESBL production in *Klebsiella pneumoniae* was tested by double-disc synergy test, Carbapenemase production by MHT, Boronic Acid or EDTA test using Meropenem phenotypically and both resistance confirmed genotypically by Multiplex PCR. **Results:** Out of 1055 clinical isolates, 298 (28.2%) were found positive for *Klebsiella.*spp, 175 isolates were subcultured among which 22 (12.57%) were *K. pneumoniae* based on API 20E. Overall Sensitivity patterns of these *Klebsiella pneumoniae* isolates to Ceftriaxone, (Amoxicillin/Clavulanate), Gentamicin, Cefepime, Ciprofloxacin, Cefoxitin, Nitrofurantoin, Cefuroxime, piperacillin/tazobactam, Meropenem, Ceftazidime and cefotaxime were 72.7%, 63.7%, 54.5%, 45.5%, 31.8%, 31.8%, 27.3%, 27.3%, 22.7%, 22.7%, 18.2%, 9.1%, 9.1% respectively. ESBL producing *K. pneumoniae* was found at 68.18% (15/22) phenotypically. Genotypically; the ESBL genes were bla_{CTX-M} (100%),

bla_{SHV} (80%) and bla_{TEM} (100; 47%); 8/15 (73.3%) had CTX-M, SHV, TEM, 4/15 (26.67%) CTX-M, TEM, 3/15 (20.00%) CTX-M and SHV. Carbapenemase producing *K. pneumoniae* was found at 31.82% (7/22) phenotypically; 1/7 (14.28%) by MHT, 4/7 (57.14%) Boronic acid test and 2/7 (28.58%) EDTA test. Genotypically; 3/4 [(75%) 42.86%] had OXA-48, 1/4 [(25%) 14.28%] OXA-48 and KPC gene, 1/2 [(50%) 14.28%] KPC and VIM, 1/2 [(50%) 14.28%] KPC and KPC gene [(100%) 14.28%]. **Conclusion/Recommendations:** DDS to be used for ESBL production, MHT, Boronic Acid test and EDTA tests using Meropenem/or Imipenem for Carbapenemase-production routinely.

Keywords

Antimicrobial Resistance Patterns, ESBLs, Carbapenemase Resistance, *Klebsiella pneumoniae*

1. Introduction

Background

Klebsiella pneumoniae has traditionally been considered an opportunistic pathogen and is a common cause of nosocomial infections ranking second to *E. coli* but recently has surpassed *Escherichia coli* as the most common cause of liver abscess [1] since its discovery in 1883 by Carl Friedländer, a German pathologist [2] later given a generic name of *Klebsiella* after the German bacteriologist Edwin Klebs [3]. Its Gram-negative bacillus of the Enterobacteriaceae family, non-motile, encapsulated, lactose-fermenting, facultatively anaerobic, rod-shaped bacterium measuring 2 μm by 0.5 μm and appears as a mucoid lactose fermenter on MacConkey agar [4].

The presence of a capsule around its cell known as K antigen is a reason for its pathogenicity, which protects the bacteria from phagocytosis during the course of infection [5]. There are 77 different capsular (K) antigens denoted from K_1 through up to K_{77}, and certain serotypes are associated with certain infection sites [6]. Its infections are mostly seen in immunocompromised individuals thus grouped as opportunistic pathogen, seen mostly affecting middle-aged and older men with debilitating diseases [2]. This patient population is believed to have impaired respiratory host defenses, including persons with diabetes, alcoholism, malignancy, liver disease, chronic obstructive pulmonary diseases, glucocorticoid therapy and renal failure [7].

A new antibiotic-resistant strain of *K. pneumonia* [8] is responsible for serious disseminated infections, such as pyogenic liver abscesses, osteomyelitis, and endophthalmitis, in a generally younger and healthier population [9]. It has a range of clinical diseases which include pneumonia, thrombophlebitis, urinary tract infection, cholecystitis, diarrhea, upper respiratory tract infection, wound infection, meningitis, bacteremia and septicemia [10] with a mortality rate of 50% which can be nearly 100% for people with alcoholism and bacteremia, even

with antimicrobial therapy [11].

Resistance is attributed to plasmids as a source of the resistant genes [12] and can be categorized based on of prevalence of antimicrobial resistance genes as extended-spectrum β-lactamase (ESBLs) [13], plasmid-mediated quinolone resistance (PMQR) genes [14] especially involving Qnr proteins and the aminoglycoside acetyltransferase variant determinant (AAC(6')-Ib-cr), which has emerged and is now described worldwide [15] and 16S rRNA methylase (16S-RMTase) genes [16].

The ability to produce ESBLs, *Klebsiella pneumoniae* has become resistant to beta-lactam antibiotics [17] including fluoroquinolones, aminoglycosides, trimethoprim, and sulphamethoxazole [16] thus related infections have caused high rates of morbidity and mortality, particularly among persons with prolonged hospitalization and those critically ill and exposed to invasive devices (ventilators or central venous catheters).

Carbapenems over years have been the last line drugs for the *Klebsiella pneumoniae* related infections but carbapenem-resistant *Klebsiella pneumoniae* (CRKP) have emerged [18] arising from; hyperproduction of ampC beta-lactamase with an outer membrane porin mutation, CTX-M extended-spectrum beta-lactamase with a porin mutation or drug efflux, and carbapenemase enzyme production, where *bla*kpc gene that encodes for it is carried on a mobile piece of genetic material (a transposon; the specific transposon involved is called Tn4401), which increases the risk for dissemination [19]. Strains that harbor *bla*kpc have minimum inhibitory concentrations within the susceptible range for carbapenems thus patients with unrecognized CRKP colonization have become reservoirs for transmission during nosocomial outbreaks [17].

The mode of transmission is through person-to-person contact (contaminated hands of healthcare personnel, or other people via patient to patient); Patients with an invasive device in their bodies (neonatal ward devices, respiratory support equipment in ICUs, cannulars and urinary catheters) put patients at increased risk [20]. Prevention follows specific infection-control precautions, which include strict adherence to hand hygiene (preferably using an alcohol-based hand rub (60-90%) or soap and water if hands are visibly soiled [21]. Alcohol based hand rubs, wearing gowns and gloves when entering rooms where patients with *Klebsiella*-related illnesses are housed, are effective against these Gram-negative bacilli [22]. Healthcare facilities also must follow strict cleaning procedures to prevent the spread of *Klebsiella pneumoniae* [23].

Treatment for *K. pneumoniae* infections is difficult because of the emerging resistance to traditional antibiotics which used to be effective against them thus tests to determine which antibiotics will treat this infection is a requirement because the recommended treatment changes as the organism develop resistances [12]. The choice of a specific antimicrobial agent or agents depends on local susceptibility patterns and on the part of the body infected. For patients with severe infections, a best approach is the use of an initial short course (48 - 72 h) of

combination therapy, followed by a switch to a specific monotherapy once the susceptibility pattern is known for the specific patient [24].

2. Methodology

2.1. Study Design

This was a laboratory-based descriptive cross-sectional study that was conducted from September 2018 to April 2019 in which antimicrobial susceptibility testing and molecular characterization were performed for identified *K. pneumoniae* from clinical isolates.

2.2. Study Site and Setting

The study was conducted in the Microbiology Laboratory, Department of Microbiology, Mbarara University of Science and Technology (MUST) whose clinical Microbiology Laboratory serves for Mbarara Regional Referral Hospital (MRRH) for Antimicrobial susceptibility testing and phenotyping for resistance.

Molecular characterization for positive phenotypic isolates for antibiotic resistance was carried out at Inqaba Biotechnical Industries (Pty) Ltd, 525 Justice Mahomed Street, Muckleneuk, Pretoria 0002 South Africa which offers sequencing and primer synthesis services.

2.3. Sample Size Calculation

The sample size was computed using the formula:

$$n = Z^2 p \left(1 - p\right) \big/ d^2 \quad [25]$$

where:

n = sample size

z = statistic for level of confidence

p = estimated prevalence

d = precision

Where about 13% estimated prevalence (p) for *Klebsiella spp* in a study done in Southwestern Uganda was used [26], precision (d = 0.05) and 95% level of confidence (z = 1.96), a 10% contingency was added to the calculated sample size giving an estimated sample size of 192.

2.4. Study Population

The study population comprised of 175 clinical isolates with *Klebsiella* like characteristics on growth which were collected from September 2018 to April 2019 from clinical specimens at Microbiology Laboratory at MUST/MRRH. These isolates were from urine (58), sputum (32), pus swabs (26), HVS (23), blood (9), ear swabs (6), pus, pleural fluid (3 each), aspirates, CSF, pus aspirates, tracheal aspirates and tracheal swabs (2 each), ascetic fluid, chest pus, peritoneal fluid, posto auricle abscess, stool, throat swab and tracheal tube (1 each) all clinical specimens reaching the Microbiology laboratory at MRRH.

2.5. Selection Criteria

2.5.1. Inclusion Criteria

The study included all *Klebsiella* like isolates from clinical samples (blood culture, urine, stool, peritoneal fluid, pus swab, high vaginal swab, cerebrospinal fluid, pleural fluid/aspirate, sputum, urethral and nasal swabs) collected from in patients and out patients attending Mbarara Regional Referral Hospital between January and May 2019.

2.5.2. Exclusion Criteria

The study excluded all duplicated isolates from the same patient unless they were isolated from different specimens with a distinguishable susceptibility pattern.

2.6. Sample Collection and Handling

Colonies suggestive of *Klebsiella spp* on growth (*i.e.* Colonies typically appearing large, Mucoid and red with red pigment usually diffusing into the surrounding agar, indicating fermentation of lactose and acid production) were picked and emulsified into 1 ml of Brain Heart Infusion (BHI) with 20% Glycerol later archived at −20˚C in a triple series.

2.7. Cultures

Isolation of *Klebsiella pneumoniae,* biochemical testing and the antimicrobial susceptibility testing was performed at the Clinical Microbiology Laboratory, Faculty of Medicine, Mbarara University of Science and Technology (MUST).

One hundred and seventy five *Klebsiella* spp isolates from different clinical specimens (blood, sputum, urine, pus aspirates and endotracheal secretions) were collected over five months period at MRRH. Specimens reaching the laboratory were cultured on blood agar (Global Scientific Supplies LTD, plot 1495, Roche Close) and MacConkey agar (Mast Group Ltd., Mast House, Derby Road, Bootle, Liverpool, Merseyside, U.K) except for urine samples which were plated on Cysteine Lactose Electrolytes Deficient (CLED) agar (SIGMA-ALDRICH, Co., 3050 Spruce Street, St. Louis, MO 63103 USA 314-771-5765) (Figure 1). All isolated *Klebsiella* spp strains were identified in the clinical samples by conventional microbiological methods (colonial morphology, Gram staining, and biochemical tests).

2.8. Biochemical Characterization

All clinical isolates exhibiting mucoid colonies were processed for biochemical testing. Biochemical test employed were urease production, citrate utilization and fermentation of sugars. Sugar fermentation tests performed were sucrose, glucose, mannitol, lactose, Adonitol, dulcitol, Melibiose and Esculin (using API 20E) which are attributable to the majority of *K. pneumoniae* sub species pneu moniae strain, being positive to Adonitol, Melibiose, Esculin, urease and citrate. Indole test and H2S production on TSI agar, oxidase, catalase and nitrate plus

motility were also carried out [5] following standard procedures (Appendix II omitted as it is not indicated in the manuscript). The isolates were confirmed for *K.pneum.pneumoniae* (22 Isolates) on API 20E (BioMerieux SA France 69280 Marcy, mini API) with profiles: 521(3)5773, percentage identification (%id) 97.7 for isolates 5235773, id% 97.7 (Figure 2).
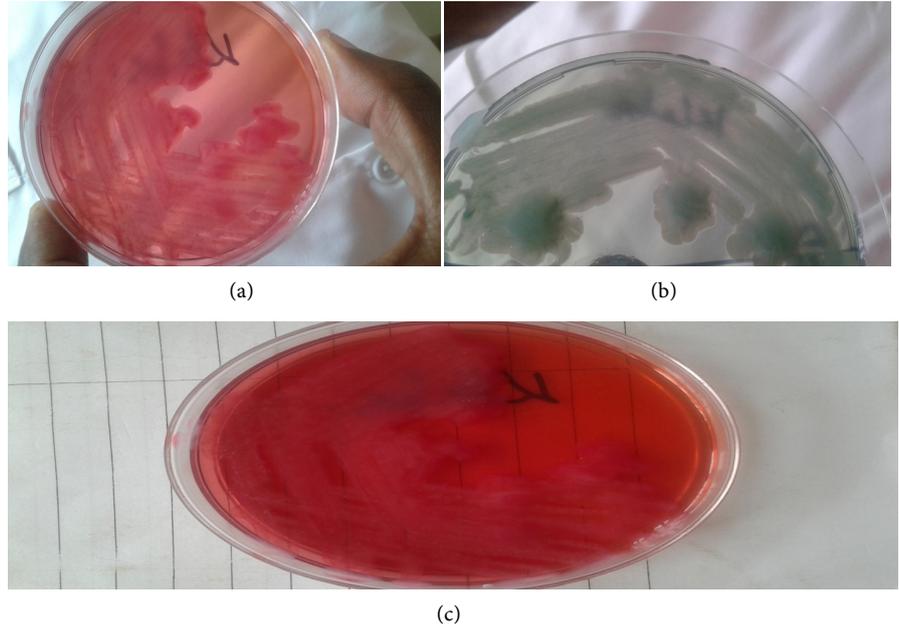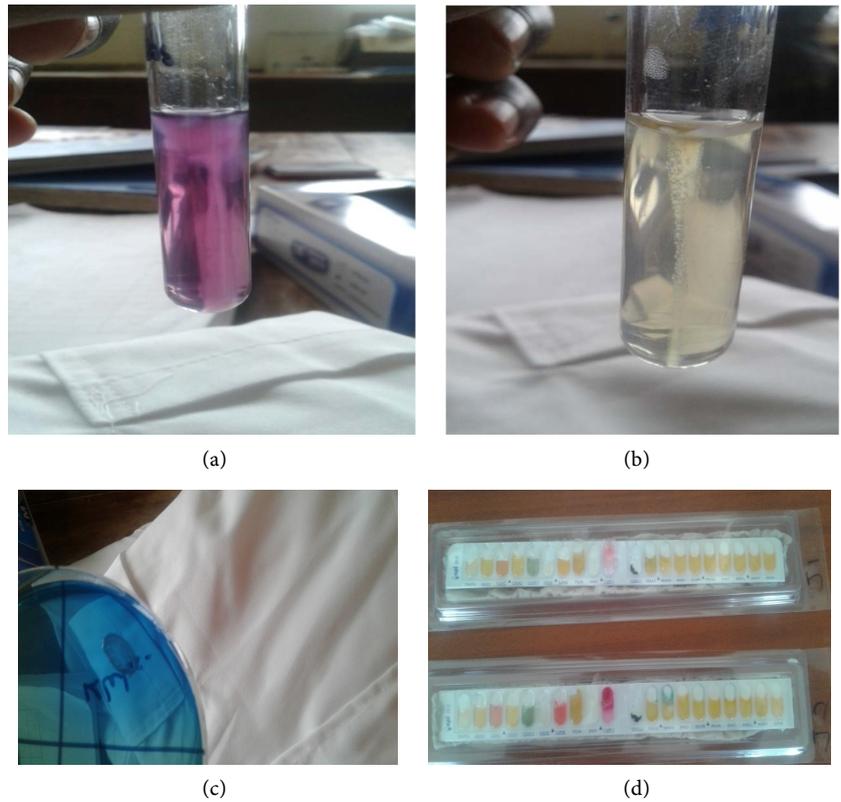

(a)

(b)

(c)

Figure 1. Mucoid colonies as seen on (a) MCA, (b) CLED, (c) BA.

(a)

(b)

(c)
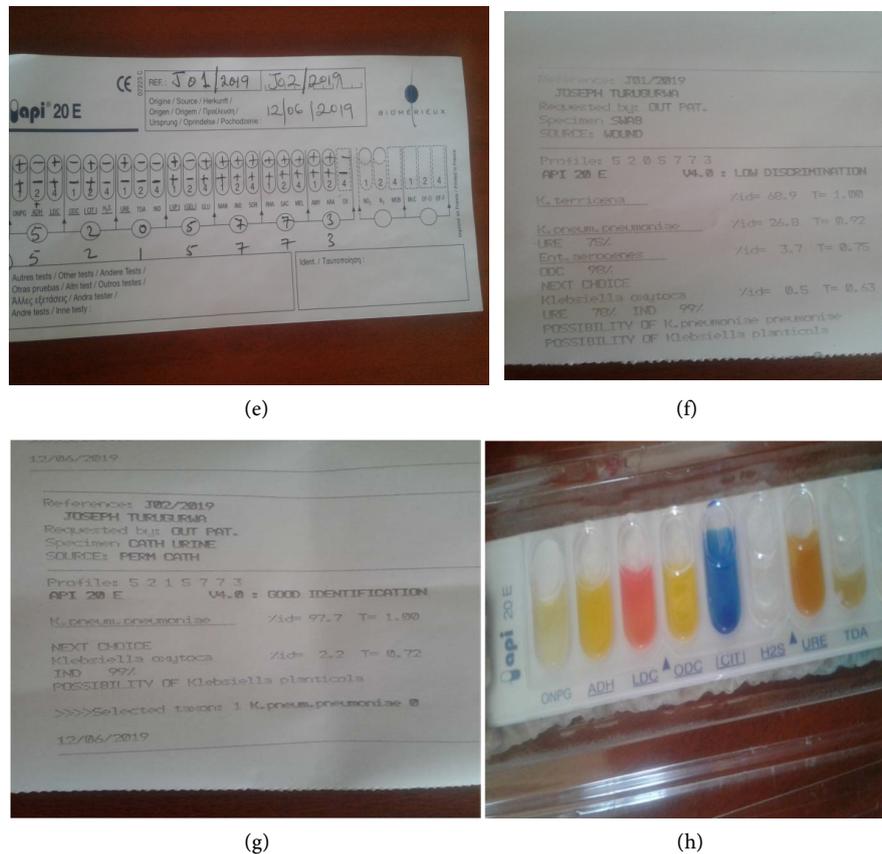

(d)

(e)

(f)

(g)

(h)

Figure 2. Biochemical tests for confirmation of *K. pneumoniae sub species pneumoniae* strain; (a) MIO (Motility, Indole, Ornithine), (b) SMI (Sulphite, Motility, Indole), (c) Citrate Utilization on Simmon's Citrate Agar (SCA), (d) API 20E following 24 hr incubation at 37˚C, addition of reagents VP$_{1,2}$, James reagent and TDA, (e) API 20E leaflet for reading tests, ((f) and (g)) results from mini API machine and (h) API 20E strip after adding KOH onto CIT well.

2.9. Antibiotic Sensitivity

Isolates were tested for the pattern of antimicrobial susceptibilities by an agar disc diffusion method using paper discs (Carramore international limited, Oxoid Ltd, Wade Road, Basingstoke, Hants, RG24 8PW, UK) on Mueller-Hinton (MH) agar including susceptibility to the following antibiotics: amoxicillin/clavulanate (20/10 μg), cefuroxime (30 μg), ceftriaxone (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), meropenem (30 μg), gentamicin (10 μg), ciprofloxacin (5 μg), nitrofurantoin (300 μg), cefepime (30 μg), piperacillin/tazobactam (100/10 μg) and cefoxitin (30 μg). Organisms were grown in BHI broth, the turbidity of the suspension adjusted to the 0.5 McFarland standard. After overnight incubation at 37˚C, inhibition zone diameters were read (**Figure 3**). The results of a disc diffusion test are interpreted by comparing the measured zone diameter with the interpretive criteria recommended by CLSI guidelines [27].

2.10. Quality Control and Data Quality Assurance

Standard operating procedures (SOPs) were strictly followed verifying that me-

dia meet expiration date and quality control parameters per CLSI 2017. Visual inspections of cracks in media or plastic petridishes, unequal fill, hemolysis, evidence of freezing, bubbles, and contaminations were done. Quality control was performed to check the quality of medium. Each new lot was quality-controlled before use by testing the *E. coli* ATCC 25922 standard control strain. During ESBLs detection, ESBL-positive *Klebsiella pneumoniae* ATCC 700603 and ESBL-negative *E. coli* ATCC 25922 control strains were used in this study. For carbapenemase detection, *E. coli* ATCC 25922 strain was used as a negative control. Culture results were recorded carefully before data entry, and the data were double-checked by a different person before analysis.

2.11. Detection of ESBLs

2.11.1. Double Disc Synergy Test

The organism to be tested was spread onto a Mueller-Hinton agar plate. The antibiotic discs used were Ceftriaxone (30 µg), Cefotaxime (30 µg), Ceftazidime (30 µg), and Amoxicillin/Clavulanic acid (20/10 µg). The three antibiotics were placed at distances of 30 mm (edge to edge) from the Amoxicillin/Clavulanic acid disc that was placed in the middle of the plate (Figure 4).

Figure 3. DST on MHA after inoculation with antibiotic disks and incubation for 24 hrs at 37°C.

Figure 4. Double Disc Synergy (DDST).

After 24-h incubation, if an enhanced zone of inhibition between either of the Cephalosporin antibiotics and the Amoxicillin/Clavulanic acid disc occurred, the test was considered positive. This indicated synergistic activity with Clavulanic acid and the presence of an ESBL [27].

2.11.2. Phenotypic Confirmatory Disc Diffusion Test (Combined Disc Method)

A Ceftazidime (30 μg) disc was used alone and in combination with Clavulanic acid (30 μg/10μg) for phenotypic confirmation of the presence of ESBLs. A ≥ 5 mm increase in zone diameter for either of the Cephalosporin discs and their respective Cephalosporin/Clavulanate disc was interpreted as ESBL producer (The antibiotics used were; ceftazidime (30 μg) and ceftazidime-clavulanic acid (20 + 10 μg), cefotaxime (30 μg) and cefotaxime-clavulanic acid (20 + 10 μg)) (Figure 5).

2.11.3. Detection of ESBLs Encoding Genes

Polymerase chain reaction (PCR) was carried out at Inqaba Biotechnical Industries (Pty) Ltd, Pretoria 0002, South Africa. The genes TEM, SHV and CTX-M, were amplified using primers (Table 2) [obtained from Inqaba Biotech 525 Justice Mahomed St, Muckleneuk, Pretoria, 0002, South Africa] and conditions as described in the Southwestern Uganda study [26]. Where archived isolates were thawed and subcultured, then incubated at 37˚C for overnight.

DNA Extraction

For PCR, bacterial genomic DNA was extracted directly from colonies on subcultures grown on Trypticase soy agar with 5% sheep blood by boiling a dense suspension of an approximate 0.1McFarland standard in sterile distilled water. As the DNA template in the PCR assays, 2–3 μL of the boiled cell suspension was used. PCR amplification of bla_{CTX-M}, bla_{SHV}, and bla_{TEM} genes in *K. pneumoniae* clinical isolates was performed by using a consensus primer pair specific to each type of β-lactamase as described [26]. A multiplex PCR was developed and used for simultaneous detection of bla_{CTX-M} (551 bp) and bla_{TEM} (972 bp) genes, other PCRs were performed for bla_{SHV} (560). PCRs were carried out by

Figure 5. Combined disc test for ESBLs using ceftazidime (30 μg) and ceftazidime-clavulanic acid (20 + 10 μg).

using the Hot Start DNA polymerase master mix (QIAGEN, Germantown, MD, USA) with 30 - 35 cycles at an annealing temperature of 52˚C for bla_{CTX-M} and $bla_{TEM,}$ and 50˚C for $bla_{SHV.}$ PCR products were analyzed by using the agarose gel electrophoresis. The specificity of PCR amplicons on representative isolates was confirmed by DNA sequencing.

DNA Sequencing

For DNA sequencing, PCR products were purified by using the PCR Purification kit (QIAGEN) according to the manufacturer's instructions. The purified DNA amplicons were sequenced by using an ABI Prism BigDye Terminator (version 1.1) cycle sequencing ready reaction kit on the ABI 3500×l DNA Analyzers, POP7™ (ThermoScientific) at Inqaba Biotechnical Industries (Pty) Ltd. The CTX-M, TEM, and SHV gene sequences were compared with sequences in GenBank by using the NCBI basic local alignment search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.12. Detection of Carbapenemase Production

The Modified Hodge test (MHT), Boronic acid synergy test and the EDTA for the detection of carbapenemase production were performed as described [27].

2.12.1. Modified Hodge Test (MHT)

A Preparation of a 0.5 McFarland standard suspension (using direct colony suspension) of *E. coli* ATCC® a25922 (the indicator organism) in saline, and was diluted to a ration 1:10 in saline, inoculated an MHA plate and a meropenem (10 µl) disk on the plate, picked fresh test colonies, inoculated in a straight line out from the edge of the disk to the plate rim. Interpretation of both negative and positive tests was done according to CLSI 2017. [*K. pneumoniae* ATCC® BAA-1705™ used] suggestive of *KPC* or OXA-48.

2.12.2. The Boronic Acid Synergy Test

Was done by adjusting the inoculum to a 0.5 McFarland turbidity standard and then streaked on a plate by swabbing. The disks of 10 µg meropenem and 400 µg of phenyl Boronic Acid (PBA) (Carramore international limited, Oxoid Ltd, Wade Road, Basingstoke, and Hants, RG24 8PW, UK) were then placed on the inoculated plate 15 mm apart center to center, and incubated for 24 hours. The plate was then examined for the presence of an enhanced growth inhibition zone between the carbapenem disk and Boronic acid disk. The test with an enhanced growth inhibition zone was considered positive for the Carbapenem-Resistant Enterobacteriaceae, detection of KPC enzyme production, as described elsewhere [28].

2.12.3. The Ethylenediammine Tetra Acetic Acid (EDTA) Test

The Ethylenediamine tetra acetic acid (EDTA) test was used as performed earlier [29]; an overnight liquid culture of the test isolate was adjusted to a turbidity of 0.5 McFarland standard and spread on the surface of a Mueller Hinton Agar plate. Two 10 µg imipenem discs were placed on the agar 15 mm apart (center to

center). 10 μl of 0.5 MEDTA was added to one of the imipenem disc to get the desired concentration of 750 μg. After incubation at 37˚C overnight, increase of inhibition zone diameter of more ≥ 5 mm in the disc potentiated with the EDTA was interpreted as positive for metallo-β-lactamase production as described elsewhere (Figure 6) [30].

2.12.4. Detection of Carbapenemase Encoding Genes

Polymerase chain reaction (PCR) was carried out at Inqaba Biotechnical Industries (Pty) Ltd, Pretoria 0002, South Africa. The genes *blaKPC, bla*OXA-48 and *bla*VIM were amplified using primers (Table 5) [obtained from Inqaba Biotech 525 Justice Mahomed St, Muckleneuk, Pretoria, 0002, South Africa] and conditions as described in the [31]; where the presence of carbapenemase encoding genes was determined using primers targeting*bla*KPC, blaOXA-48 and blaVIM.

DNA Extraction

For PCR, bacterial genomic DNA was extracted directly from colonies on from fresh subcultures grown on Trypticase soy agar with 5% sheep blood (SIGMA-ALDRICH, Co., 3050 Spruce Street, St. Louis, MO 63103 USA 314-771-5765) by boiling a dense suspension of an approximate of 0.1 McFarland standard in sterile distilled water. As the DNA template in the PCR assays, 2–3 μL of the boiled cell suspension was used. PCR amplification of $bla_{OXA\text{-}48}$, bla_{VIM}, and bla_{KPC} genes in *K. pneumoniae* clinical isolates was performed by using a consensus primer pair specific to each type of Carbapenemase as described [31]. A multiplex PCR was developed and used for simultaneous detection of $bla_{OXA\text{-}48}$ and bla_{KPC} genes, other PCRs were performed for bla_{VIM}. PCRs were carried out by using the HotStart DNA polymerase master mix (QIAGEN) with 30–35 cycles at an annealing temperature of 52˚C for $bla_{OXA\text{-}48}$ and bla_{kpc}, and 50˚C for $bla_{VIM.}$ PCR products were analyzed by agarose gel electrophoresis. The specificity of PCR amplicons on representative isolates was confirmed by DNA sequencing.

DNA Sequencing

For DNA sequencing, PCR products were purified by using the PCR Purification kit (QIAGEN) according to the manufacturer's instructions. The purified DNA amplicons were sequenced by using an ABI Prism BigDye Terminator (version 1.1) cycle sequencing ready reaction kit on the ABI 3500×l DNA Analyzers POP7^{TM} (ThermoScientific) at Inqaba Biotechnical Industries (Pty) Ltd. The CTX-M,
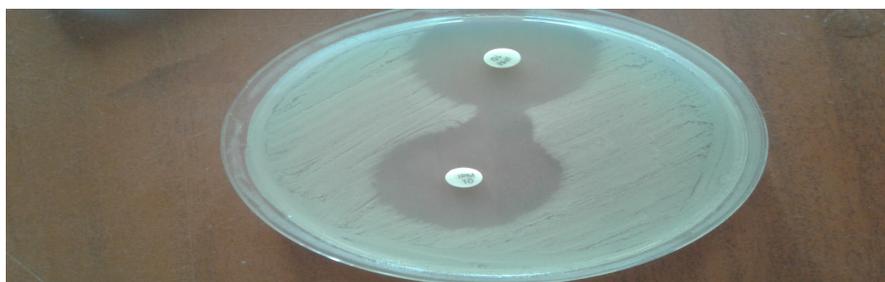

Figure 6. EDTA for metallo-β-lactamase testing.

TEM, and SHV gene sequences were compared with sequences in GenBank by using the NCBI basic local alignment search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.13. Phenotypic Screening for Hypervirulence (HV)

For HV-phenotype determination, a standard bacteriologic loop (Greiner Bio-One Hungary Kft) was used to stretch a mucoviscous string vertically from a colony. The formation of a viscous string of >5 mm was confirmatory for the HV-positive phenotype (**Figure 7**) [1].

Detection of Hypervirulence Encoding Genes

No isolate passed string test thus no need to subject to conventional PCR for *rmpA*, *rmpA*2, *magA* and *K*2 genes as described in previous studies [32].

2.14. Data Analysis

Data was coded and entered into the Microsoft Excel. Then, data was exported to STATA (version 12) for analysis. Descriptive statistics were done and results were presented in tables. A test of association was done using binary logistic regression. All the statistical tests were set for significance at P value 0.05. Odds ratios (OR) will be determined between wards and clinics with the following outcomes; 1) resistance to one antibiotic, 2) resistance to three different classes of antimicrobial agents: multi-drug resistance (MDR), phenotypic characteristics ESBLs, Carbapenem resistance and Hypervirulence.

2.15. Ethical Consideration

Ethical clearance was obtained from the Department of Microbiology, Mbarara University of Science and Technology (MUST), Faculty of Medicine Review Committee (FRC), Institutional Ethical Review Committee (IERC) of MUST.

Figure 7. String test for the phenotypic testing of hypervirulence.

3. Results

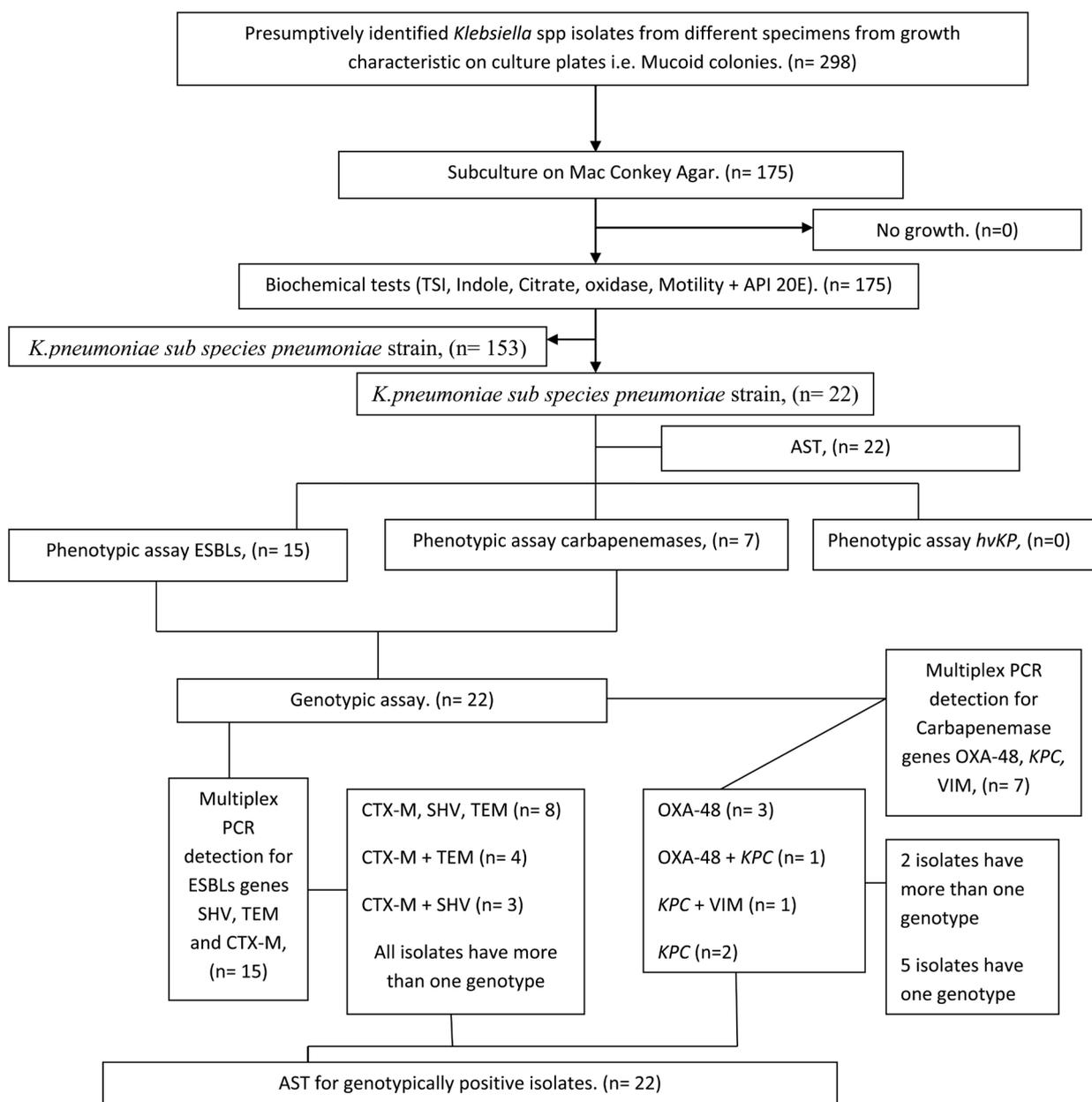### 3.1. Study Flow (Figure 8)

Figure 8. Study flow.

3.2. Prevalence of *Klebsiella* spp.

Out of 1055 samples from clinical patients between September 2018 and April 2019, *Klebsiella* spp. was reported in 298 cases through standard cultural and biochemical tests which later were archived at −20˚C. The prevalence rate of *Klebsiella* spp was about 28% and non *Klebsiella* spp was 72% (**Figure 9**).

Highest prevalence was observed in urine sample at 36.56% followed by sputum 18.29%, pus swabs 14.28%, HVS 13.14%, blood 8.56%, ear swabs 3.43%, pus

aspirate, pleural fluid 1.71%, aspirate, CSF, pus, tracheal aspirate, tracheal swabs 1.14%, and ascetic fluid, chest pus, peritoneal fluid, posto auricle abscess, stool, throat swab, tracheal tube 0.57% (Figure 10). Females were more affected (63.43%) with *Klebsiella* spp. than Males (36.57%). Persons younger than 50 years irrespective of gender were more affected with *Klebsiella* spp., 70.86%, and 29.14% greater or equal to 50years. High number of specimen came from OPD 50.85% followed by Medical ward 10.29%, pathology 6.29%, post natal 5.51%, TOTO 4.00%, MMIC 3.43%, Ear, Nose, Throat(ENT) 2.86%, ICU 1.71%, gyn, nl, pead, private, emergency 1.14%, chest clinic, female ward, ISS, must, oncology, pnw, post OPD, prison and TB ward 0.57% (Figure 11).

3.3. Subculturing and Biochemical Testing

On subculturing, out of 175 isolates picked from archived samples gave out growth exhibiting colonies similar to *Klebsiella* species and were tested biochemically. Both the standard (*Klebsiella pneumoniae* ATCC 700603) and clinical isolates of *Klebsiella ssp* (175) were subjected to biochemical tests. Standard *K. pneumoniae* cultures showed positive reactions for urease production, citrate utilization, catalase reaction and fermentation of sugars like glucose, lactose, sucrose, and mannitol, Adonitol, Melibiose and Esculin. Organisms showed negative
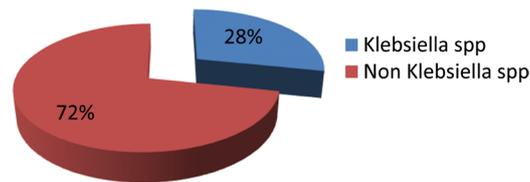
Figure 9. Prevalence rate of Klebsiella spp isolates among clinical specimens collected from September 2018 to April 2019.
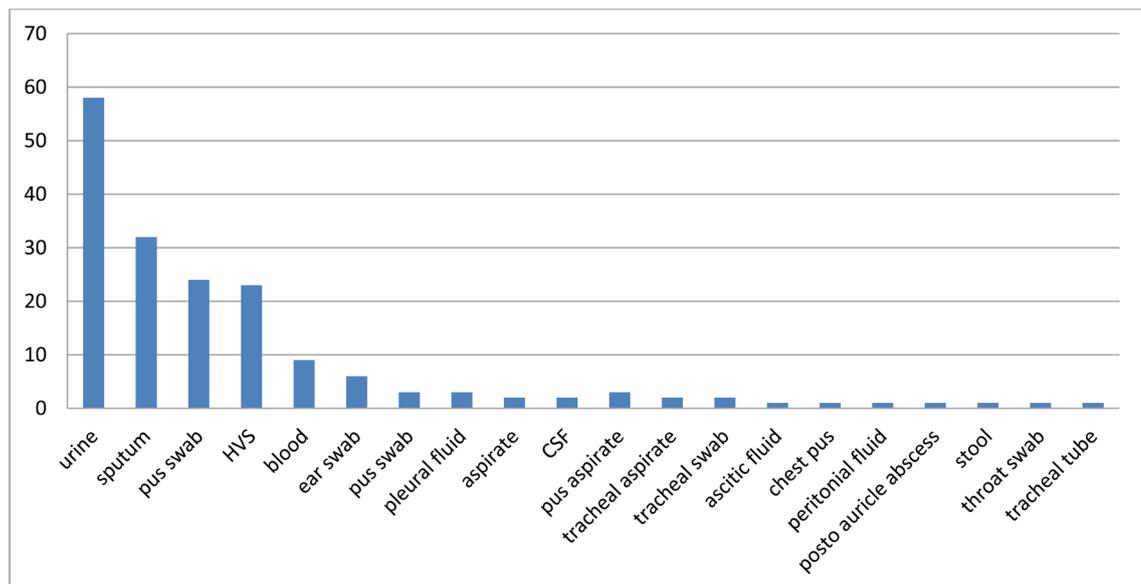
Figure 10. Prevalence of Specimens received at Microbiology Laboratory, Department of Microbiology, MUST received between September 2018 and April 2019 end.

test for indole production, motility, and oxidase. There was no H_2S production on Triple Sugar Iron agar (TSI) giving A/A for both slope and butt. Fifty eight (58) clinical isolates showed common biochemical reaction pattern for *Klebsiella pneumoniae* being positive to glucose, lactose, sucrose, and mannitol and negative to oxidase and indole. On subjection onto some rare sugars (Adonitol, Melibiose, Esculin and dulcitol) on API 20E, twenty two (22) of them exhibited reactions attributable to majority of *K. pneumoniae sub species pneumoniae* strain (Figure 12), being positive to Adonitol, Melibiose, Esculin, urease and citrate. The rest of the isolates showed a variation in reaction to the used biochemical tests.

3.4. Antibiotic Sensitivity Patterns of *Klebsiella pneumoniae*

Antibiotic sensitivity testing of twenty two (22) confirmed *K. pneumoniae* clinical isolates was done on Muller-Hinton agar plates. On the basis of resistance to antibiotic, strains were categorized into three groups *i.e.* susceptible(S), resistant (R) and intermediate (I) as shown in Table 1. Twelve antibiotics which included
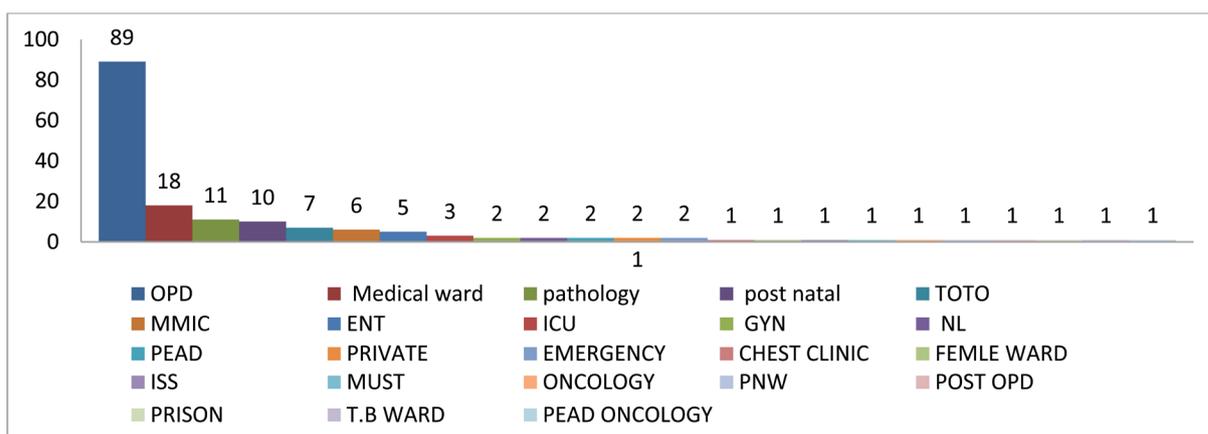

Figure 11. Occurrence of Specimens by ward received at Microbiology Laboratory, Department of Microbiology, MUST received between September 2018 and April 2019 end.
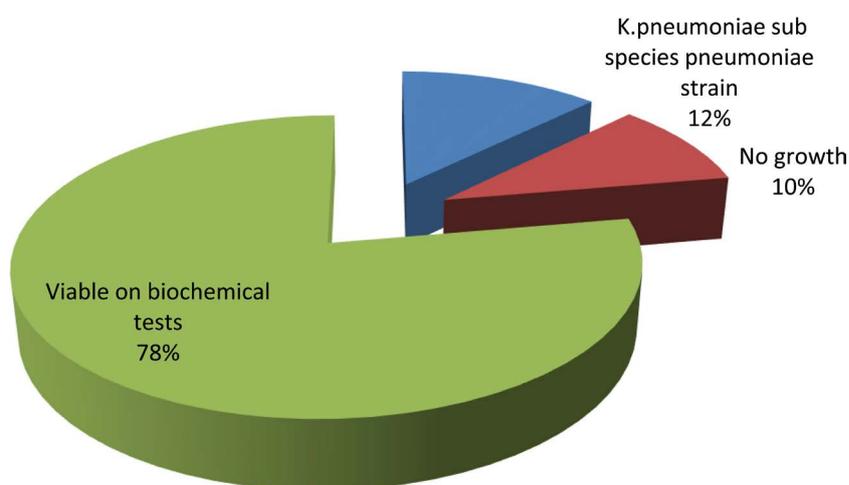

Figure 12. Prevalence of K. pneumoniae sub species pneumoniae strain from clinical isolates.

amoxicillin/clavulanate (20/10 μg), cefuroxime (30 μg), ceftriaxone (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), meropenem (30 μg), gentamicin (10 μg), ciprofloxacin (5 μg), nitrofurantoin (300 μg), cefepime (30 μg), piperacillin/tazobactam (100/10 μg) and cefoxitin (30 μg) were used.

The pattern of antimicrobial susceptibility among the 22 *K. pneumoniae* isolates showed that 72.7% was resistant to Ceftriaxone, 63.7% was resistant to (Amoxicillin/Clavulanate), 54.5% was resistant to Gentamicin, 45.5% was resistant to Cefepime, 31.8% was resistant to Ciprofloxacin and Cefoxitin, 27.3% was resistant to Nitrofurantoin and Cefuroxime, 22.7% was resistant to piperacillin/tazobactam, 18.2% was resistant to Meropenem, Ceftazidime and cefotaxime was resistant to 9.1% (Table 1, Figure 13).

MDR was observed at 72.7% but unique isolate that kept appearing in the zones of clearance which at first was thought to be a contaminant was subcultured and

Table 1. Susceptibility patterns of confirmed *K. pneumoniae* isolates ($N = 22$).

| Antibiotics	Sensitive	Intermediate	Resistant
Amoxicillin/Clavulanate (20/10μg)	3 (13.64%)	5 (22.73%)	14 (63.65%)
Cefuroxime (30 μg)	11 (50.00%)	5 (22.73%)	6 (27.27%)
Ceftriaxone (30 μg)	1 (4.45%)	5 (22.82%)	16 (72.73%)
Ceftazidime (30 μg)	14 (63.36%)	6 (27.27%)	2 (9.09%)
Meropenem (30 μg)	17 (77.28%)	1 (4.54%)	4 (18.18%)
Gentamicin (10 μg)	5 (22.73%)	5 (22.73%)	12 (54.54%)
Ciprofloxacin (5 μg)	13 (59.09%)	2 (9.09%)	7 (31.82%)
Nitrofurantoin (300 μg)	8 (36.36%)	8 (36.36%)	6 (27.28%)
Cefepime (30 μg)	9 (40.90%)	3 (13.63%)	10 (45.47%)
Piperacillin/tazobactam (100/10μg)	15 (68.18)	2 (9.09%)	5 (22.73%)
Cefoxitin (30 μg)	10 (45.47%)	5 (22.73%)	7 (31.82%)
Cefotaxime (30 μg)	14 (63.36%)	6 (27.27%)	2 (9.09%)
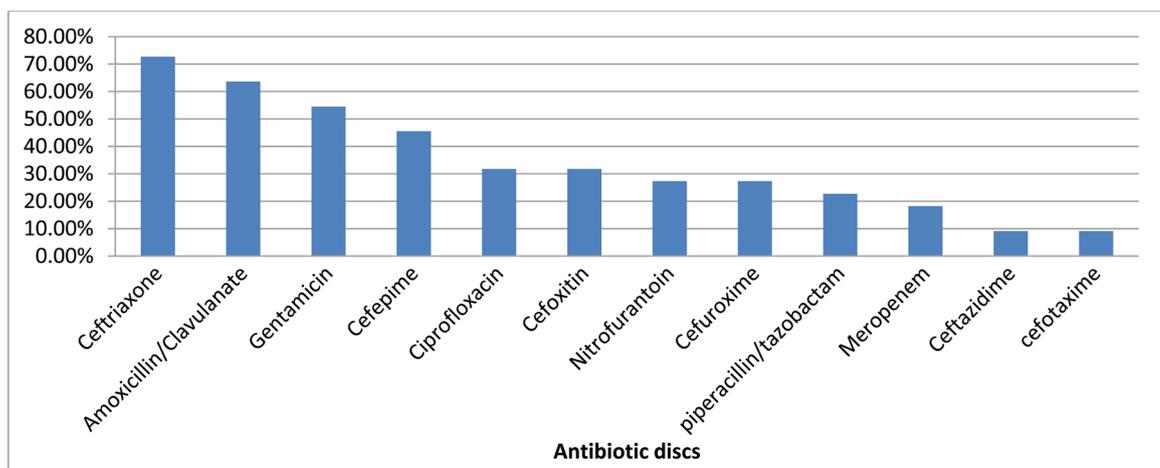
Figure 13. Percentage Resistance of confirmed *K. pneumoniae* isolates to different used antibiotics.

later revealed to be a *Klebsiella* spp on biochemical test basis was found out to be resistant to all tested 12 antibiotics.

3.5. Potential ESBL among Biochemically Positive *Klebsiella pneumoniae* Isolates

All the confirmed 22 bacterial pathogens were tested. In order to improve the sensitivity, we used ceftazidime, cefotaxime, and ceftriaxone antibiotics to screen for ESBL activity. The combined disk test indicated that 15 (68.18%) isolates presented inhibition of clavulanic acid.

E. coli (ATCC 25922) negative ESBLs and *K. pneumoniae* (ATCC 700603) positive ESBLs were used as controls.

3.5.1. PCR Amplification for ESBLs Genes

Table 2. Primer sets for amplification of ESBLs resistance genes.

Gene	Primer sequence (5' → 3')	TM (˚C)	Amplicons size (bp)
CTX-M	Forward: CGCTTTGCGATGTGCAG Reverse: ACCGCGATATCGTTGGT	52	551
SHV	Forward: ATGCGTTATATTCGCCTGTG Reverse: TGCTTTGTTATTCGGGCCAA	52	972
TEM	Forward: AAACGCTGGTGAAAGTA Reverse: AGCGATCTGTCTAT	45	560

3.5.2. Prevalence of ESBLs Genes

All ESBL *K. pneumoniae* tested on PCR assay had at least two ESBL genes showing that 8/15 (53.33%) possessed all the genes (*i.e.* CTX-M, SHV, TEM), 4/15 (26.67%) had both CTX-M and TEM while 3/15 (20.00%) had CTX-M and SHV. CTX-M (100%) *i.e.* in all isolates, TEM (80%) and SHV (73.3%) (Table 3).

3.6. Prevalence of Carbapenemase Active Isolates Based on Phenotypic Tests

Regardless of their ESBL result, all the 22 isolates were tested for carbapenemase production. Among the 22 *K. pneumoniae* isolates, 7 (31.82%) were positive for the production of carbapenemases which of these, 1/7 (14.28%) was detected by MHT method, 4/7 (57.14%) by Boronic acid test and 2/7 (28.58%) by the EDTA test (Table 4).

Prevalence of Carbapenemase Genes

Based on the PCR assay, 3/4 [(75%) 42.86%] detected carbapenemases by Boronic

Table 3. Prevalence of ESBLs Genes ($N = 15$).

Genes	Isolates	% age
CTX-M, SHV, TEM	8	53.33
CTX-M, TEM	4	26.67
CTX-M, SHV	3	20.00

Acid test all showed to be positive for OXA-48 gene for which 1/4 [(25%) 14.28%] of them had both OXA-48 and KPC gene, 1/2 [(50%) 14.28%] isolates detected by EDTA test was positive for both KPC and VIM the other 1/2 [(50%) 14.28%] had KPC and that detected by MHT method showed KPC gene [(100%) 14.28%]. The Boronic Acid test showed a strong association with detection of OXA-48 gene type (P = 0.000) and KPC (P = 0.045) carbapenemases compared to VIM type metallo-β-lactamases (P >0.05) (Table 6).

3.7. Prevalence of the Hypervirulent *K. pneumoniae*

No isolate exceeded a 3 mm stretch with a biological loop.

3.8. Antibiogram for the Genotypically Confirmed *K. pneumoniae* for ESBLs and Carbapenemases Isolates

Seven antibiotics which included Cefoperazone/Sulbactam, Ciprofloxacin, Ertapenem, Imipenem, Levofloxacin, Meropenem and Colistin were used.

The pattern of antimicrobial susceptibility among the 22 *K. pneumoniae* isolates irrespective of the confirmed resistance showed that 31.8% was resistant to Ciprofloxacin, 18.2% was resistant to Meropenem, 13.63% was resistant to Colistin, 9.09% was resistant to Ceftazidime/avibactam and Levofloxacin, 4.54%

Table 4. Phenotypic tests.

Test	Isolates	%age
MHT	1	14.28
Boronic Acid	4	57.14
EDTA	2	28.58

Table 5. Primer sets for amplification of carbapenemase resistance genes.

Gene	Primer sequence (5' → 3')	TM (˚C)	Amplicons size (bp)
bla-VIM	Forward: GATGGTGTTTGGTCGCATA Reverse: CGAATGCGCAGCACCAG	52	390
bla-KPC	Forward: CATTCAAGGGCTTTCTTGCTGC Reverse: ACGACGGCATAGTCATTTGC	52	498
bla-OXA-48	Forward: GCTTGATCGCCCTCGATT Reverse: GATTTGCTCCGTGGCCGAAA	50	238

Table 6. Prevalence of carbapenemase genes.

Genes	Isolates	% per Isolate	% per Resistance
OXA-48 (Boronic Acid)	3	75	42.86
OXA-48 and *KPC* (Boronic Acid)	1	25	14.28
KPC and VIM (EDTA)	1	50	14.28
KPC (EDTA)	1	50	14.28
KPC (MHT)	1	100	14.28

was resistant to Ertapenem and there was no resistance for Imipenem (Table 7, Figure 14).

4. Discussion

4.1. Prevalence of *Klebsiella* spp.

The increased prevalence of *Klebsiella* spp among clinical isolates has been of a greater concern worldwide as it has been one the organism showing a higher resistance to available antibiotics. In the present study, a 28% prevalence rate of *Klebsiella* was observed among clinical isolates at MRRH arriving at MUST Microbiology Laboratory which is an increased prevalence rate compared to the past studies done at the same institution with a rate of 20.26%, 12.7% [26] [33] respectively but lower in a study done by Mutiibwa and Tumusiime at 37.9% [34] at the same institution. Different rates were seen at different institution but same regions like 52% at Kabale, 22.4% at Mulago, 14.4% at Mbale and 10.2% in Tanzania [35] [36] [37] [38] may be acceptable because the prevalence of *Klebsiella* infections varies in different geographical locations [39].

4.2. Prevalence of *K. pneumoniae*

A number of studies have shown different prevalence of *K. pneumoniae* among

Table 7. Susceptibility patterns of genotypically confirmed *K. pneumoniae* isolates ($N = 22$).

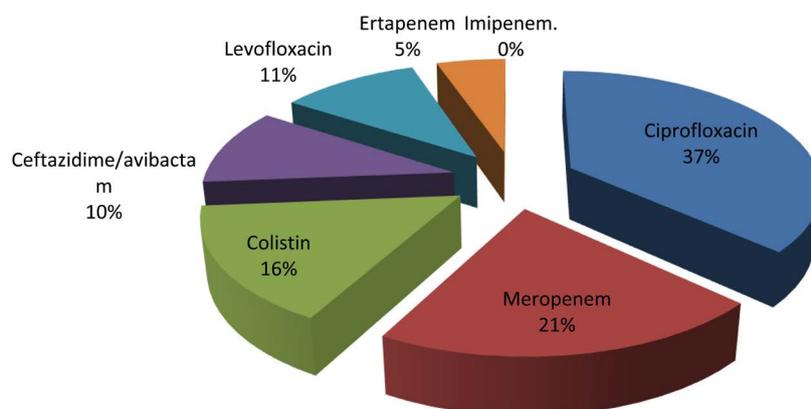| Antibiotics	Sensitive	Intermediate	Resistant
Meropenem (10 μg)	17 (77.28%)	1 (4.54%)	4 (18.18%)
Ciprofloxacin (5 μg)	13 (59.09%)	2 (9.09%)	7 (31.82%)
Ceftazidime-avibactam (30/20μg)	18 (81.82)	2 (9.09%)	2 (9.09%)
Ertapenem (10 μg)	20 (90.92%)	1 (4.54%)	1 (4.54%)
Imipenem (10 μg)	22 (100.00%)	0 (0.00%)	0 (0.00%)
Levofloxacin (5 μg)	16 (72.73%)	4 (18.18%)	2 (9.09%)
Colistin.	2 (9.09%)	17 (77.28%)	3 (13.63%)

Figure 14. Number of Resistant Isolates against antibiotic discs. (Renamed from Figure 13 to Figure 14 and percentage calculated).

isolates determined in different study places putting it at around 3% to 12%; 3% [40], 5% [41]. The prevalence rate of *Klebsiella pneumoniae* was at 12.57% in this study which is in the agreement with the previous study done at MRRH, Bushenyi and central districts of Uganda [26] [40] [42] [43] but lower to the other study done at the same institution putting it at 18.6% [44], same region 20% at Mulago [45], 15% [35] but the most alarming figures are seen in studies conducted at Mulago, MRRH at 52.2%, 28.7%, 50.0% [29] [46] [47].

4.3. Distribution of *Klebsiella* spp among Different Specimens, Age and Gender

Prevalence was higher in Females (63.43%) than in the Male (36.57%) due to mechanical, hormonal and physiological changes at adolescence, during pregnancy and pre/post menopause. Females who were 50 years of age and above were found to be more infected with *Klebsiella* spp associated infection probably due to reduced immunity and pre/post menopause whereas Males between 18 and 50 years of age had the highest incidence of infection on age group greater than 50 years due to poor health living styles like increased sexual activities and living a dangerous life which is much supported by a number studies done around [40] [46].

High number of specimens came from OPD at 50.85% and most of the *Klebsiella* were recovered from urine samples irrespective of age and gender, followed by sputum and pus swab Kateregga *et al.*, Ampaire *et al.*, also reported that urine is the principal source of *Klebsiella* [44] [46] but not supported by a number of studies which have different specimen for isolation and different ward which could be due to a number of reasons like the aim of the study or even the months of collection since due to environmental pressure, during the warmest months may lead to an increase in *Klebsiella* infection which could be due to characteristics of *Klebsiella* which might have some role on such seasonal variation. *Klebsiella* is the most heat tolerant among all enteric pathogens [48] having maximal specific growth rate at 37°C. In addition, they are believed to survive at higher humidity [49].

4.4. Antibiotics Susceptibility Pattern for Confirmed *K. pneumoniae* Isolates

All were highly resistant to ceftriaxone 72.73%, 54.5%Gentamicin, 45.5% Cefepime, 31.8% Ciprofloxacin and Cefoxitin, 27.3% Nitrofurantoin and Cefuroxime, 18.2%Ceftazidime and cefotaxime 9.1%. Gentamicin like aminoglycosides has a good record against clinically important Gram-negative bacilli [50] as it is reflected in this study since resistance is moderate. Among the *β*-lactamase inhibitor combination agents, piperacillin-tazobactam was most active, inhibiting 68.18% of the strains; 13.64% were inhibited by Amoxicillin/Clavulanate which is supported elsewhere like studies done. The isolates showed a variation to a number of studies which could be due to the misuse or overuse of the antibiotics coupled with weak infection control measures and this alerts the health care sys-

tem to work hard on the health facilities infection control.

4.5. Multi-Drug Resistance

The present study showed that the level of MDR in *K. pneumoniae* (72.7%) which was comparable with studies conducted in Sierra Leone (73.3%) [51]. However, lower than studies conducted in Brazil (84%), Gondar, Ethiopia (95.6%) and Equatorial Guinea (91.7%) [52] [53] [54]. The difference in MDR level among *K. pneumoniae* in this study might be due to most *K. pneumoniae* being isolated from Urine specimens collected from hospital inpatients. Co-existence of genes in the same isolate with at least one or two other ESBLs or CP genes in these isolates among which it was detected, probably explain why these isolates were multidrug resistant.

4.6. Prevalence of Phenotypic ESBL-Producing *K. pneumoniae*

The occurrence of ESBL among clinical isolates greatly varies worldwide and geographically, and is rapidly changing over time [55]. In the present study, ESBL phenotypes were found to be positive in 15 (68.18%) isolates demonstrating a high prevalence of ESBL production in *K. pneumoniae* isolates, at MRRH, south western Uganda.

Similar results were reported in studies done in Mulago (72.7%), Bahir-Dar, Ethiopia (69.8%) and Jimma, Ethiopia (70.4%) [46] [56]. However, our result is higher compared to other reports from Kenya (31.4%), Kasese (23%) [42] [57]. But a number of studies around the region have reported high percentages of ESBL-producing isolates, 92.3% in Uganda [58], 92% at Kabale Regional Referral Hospital [35] 76% *K. pneumoniae* [59], 84.2% [60] in Ethiopia. The high prevalence of ESBL-producing isolates described in this study may be due to the selective pressure caused by the use of 3rd generation cephalosporins [61]. Lack of antibiotic surveillance, antibiotics misuse, and weak infection control measures may also contribute to the high magnitude of ESBL.

4.7. Prevalence of Genotypic ESBL-Producing *K. pneumoniae*

In the present study, genotypic survey on 15 confirmed ESBL phenotype strains by Multiplex PCR revealed that all isolates (100%) were positive genotypically for at least two of the studied genes. The PCR results showed that among ESBL gene families, *bla*CTX-M (100%)was the most prevalent in the isolates, followed by *bla*TEM (80%) and then *bla*SHV (73.3%). These results are in agreement with other studies, such as that done at MRRH [26], which reported that, 70% of CTX-M was the highest followed by TEM (47%) and SHV (34); the study in Kenya [62] showed that CTX-M gene was found in 88.5% followed by TEM (34.6%) and in Sudan; showed that CTX-M gene was found in 86.4% of *K. pneumonia* [63]. Expression of more than one resistance gene; bla_{CTX-M}/bla_{SHV}, bla_{CTX-M}/bla_{TEM}, bla_{SHV}/bla_{TEM} and bla_{CTX-M}/bla_{SHV}/bla_{TEM} was observed in 15/15 isolates which are supported by a number of studies like that done by Acaku *et*

al., showing that one bacterial isolate can express more than one resistance gene [26].

4.8. Prevalence of Phenotypic Carbapenem-Producing *K. pneumoniae*

Carbapenem resistance has shown a varying prevalence rate all over the world has been reported in places worldwide, such as Argentina, Egypt, India, Nigeria, and the Netherlands [64]-[69] which are representative of each continent. In the present study, the prevalence of carbapenemase-producing *K. pneumoniae* was 31.82% which is supported by a number of studies done around like; 23.2% in Kenya [70], 22.4% Uganda [29], 12.8% [71], which is much lower than rates portrayed in others studies like; 10.3% [44] at MRRH, Ethiopia 12.1% [60], 1.87% [59], but lower than prevalence rate in Tanzania 35% [31] and 59.1% [67]. The high prevalence in this study might be due to the fact that no restricted rules governing antibiotics in Uganda.

A great concern is occurrence of their resistance is worrying because these enzymes-encoding genes for this kind of resistance might be imported from abroad probably, or they emerge locally and spread by gene transfer [12].

The emergence of these carbapenemase-producing *K. pneumoniae* isolates in the hospital settings may be due to multiple factors including uncontrolled antibiotic usage, inappropriate dosing regimens, widespread counterfeit and substandard antibiotics, and local hospital practices concerning isolation of patients with multiresistant pathogens which is poorly managed but can also be community-acquired as they have also been isolated from the common water sources [67].

4.9. Prevalence of Genotypic Carbapenem-Producing *K. pneumoniae*

In the present study, the genes characterized included; *bla* OXA-48 and *bla*KPC at 57.14% and *bla* VIM at 14.28 appearing in *CPKP* (31.82%) which is different from a study at Mulago; *bla*VIM (10.7%), followed by *bla*OXA-48 (9.7%), *bla*IMP (6.1%), *bla*KPC (5.1%) and *bla*NDM-1 (2.6%) appearing in (52.2%) *Klebsiella pneumoniae* [29], while at Mbarara hospital, VIM and OXA-48 CR determinants were registered appearing in 30.60% *CPKP* supporting our study [44], in Tanzania Molecular analysis of CRE at a tertiary hospital in Mwanza established by multiplex PCR revealed that the principal CR genes were IMP (21.6%), followed by VIM (12.3%), OXA-48 (4.9%), then KPC (3.5%), and NDM (3.1%) appearing in 11% of *K. pneumoniae* [31]. In Kenya, CP *K. pneumoniae*VIM-2 genes were isolated in Nairobi [72] were Seven carbapenem-resistant NDM-1-positive *Klebsiella pneumoniae* isolates were recovered from patients hospitalized between 2007 and 2009 and all isolates carried the bla_{NDM-1} carbapenemase gene while Whole Genome sequencing (WGS) was employed to identify NDM-1 like CR genes in *K. pneumoniae* isolates at Kilifi County Hospital were no isolates were found to be resistant to carbapenems but a plasmid with the genetic architecture

of a known New Delhi metallo-β-lactamase-1 (NDM)-carrying plasmid in 25 isolates [71].Variation of these genes in different studies maybe due to the fact that beta-lactamases by gram-negative organisms are usually secreted especially when antibiotics are present in the environment [73].

5. Study Limitations

The study was limited to MRRH, thus observation bias may have occurred during observational assessment

Samples were not collected from Health workers because it is a sensitive issue thus association was not done.

No history was taken about antibiotic intake, thus may not have given us the association to development of resistance among patients.

Limited funds have prevented us from using more advanced molecular typing technologies such as multi-locus sequence typing (MLST) in order to investigate the molecular epidemiology of ESBLS-KP and CRKP in this geographical region.

6. Conclusions and Recommendations

The high prevalence rate for both ESBL-producing (68.18%) and Carbapenemase-producing (31.82%) *K. pneumoniae* among clinical isolates is an important problem for both microbiologists and clinicians thus recommending as follows;

In resource-limited settings like at MRRH, double-disk synergy method can be implemented for screening and confirming ESBL production, MHT, Boronic Acid test and EDTA tests for all resistant isolates to Meropenem/or Imipenem can be implemented for screening and confirming Carbapenemase-production that might give valuable information for appropriate antibiotics selection and controlling the spread of these modes of resistance.

Routine infection preventions strategies such as compliance to hand hygiene principles, rational use of antimicrobial agents and surveillance of AMR are urgently needed to prevent and control the spread of antimicrobial-resistant pathogens in the healthcare facilities.

The increasing prevalence of CRE mainly due to carbapenemase production is alarming in clinical practice, specifically in areas where no carbapenems are sold. This study provides a clearer picture of the current CRE scenario in the hospital setup, and hence, identifying factors that induce carbapenemase production in the absence of carbapenems prescription is essential for control of CRE dissemination within the community.

Further studies are needed to identify the most common clones of both ESBLs-KP and CRKP genes at MRRH.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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