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Molecular Screening of $^{bla-}$TEM-68 Gene for TEM-68 ESBLs Resistant *K. pneumoniae* and *C. freundii* from Hospital Waste in Adamawa state Specialist Hospital, Yola

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ABSTRACT

Resistant strains of *K. pneumoniae* and *C. freundii* due to under use and over use of antibiotics and biocides has contributed to economic burden due to nosocomial infections. This study focuses on screening hospital wastes for antibacterial and biocide resistant *K. pneumoniae* and *C. freundii* in Adamawa state specialist hospital, Yola. One hundred and twenty (120) hospital wastes samples were analysed. Samples were cultured on appropriate media and incubated at 37 °C for 24h. Biochemically identified isolates were molecularly confirmed and sequenced. *K. pneumoniae* being 93% identical to strain KPNIH48 and *C. freundii* being 99% identical to complex sp CFNH4 were confirmed. Resistant *K. pneumonia* and *C. freundii* were subjected to phenotypic ESBLs test, then DDS test was carried out to confirm ESBLs production. Phenotypically ESBLs positive *K. pneumoniae* were positive for DDST but negative for *C. freundii*. The DDST positive isolates were then screened for $^{bla-}$TEM-68 gene. All the DDST positive *K. pneumoniae* harboured the $^{bla-}$TEM-68 gene but none in *C. freundii.* The isolates carrying $^{bla-}$TEM-68 gene were cured using 10 % SDS and the results showed that the ESBLs genes were plasmid and/or chromosomally encoded. The presence of *K. pneumoniae* carrying $^{bla-}$TEM-68 gene indicates a possibility for increased nosocomial infections in Adamawa state and Nigeria at large and so relevant preventive recommendations were made.

Keywords- $^{bla-}$TEM-68 gene, Enterobacteriaceae, *K. pneumoniae, C. freundii*

I. INTRODUCTION

Hospital wastes are heterogeneous mixtures of general refuse, laboratory and pharmaceutical chemicals, plastic or metal containers and pathological wastes [1]. Both pathological waste and laboratory waste combine to form infectious wastes and may contain antibacterial agents. Hospital waste containing high concentration of antibiotics and disinfectants are found to harbor antibacterial resistant strains of microorganisms [2]. However, to date new studies seem to have focused on the emergence of biocide resistant bacteria in hospital environment other than waste water. Health care waste if not properly managed can pose an even greater threat than the original diseases themselves [3].

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Resistant strains of bacteria cannot be excluded from the group of organisms found in hospital wastes, this is because antibacterial agents have been used for the treatment of in-patients with many bacterial infections such as meningitis, endocarditis, skin infections, urinary tract infections and many more diseases [4] [5]. Unfortunately, the wide use of antibiotics has led to the spread of antibiotics resistant genes.

The resistance of bacteria to commonly used antibacterials has resulted in major global health care problems in the 21st century [6]. Such resistances to antibacterials have also been observed in enterobacteriaceae and have been implicated as the cause of a range of clinical infections and are a major cause of community, as well as nosocomial infections such as urinary tract, blood stream, surgical site, intra-abdominal infection, and pneumonia [7]. Throughout the world the incidence of antibacterial and biocide resistance has been increasing and in cross resistance and multi-resistance pattern has been observed [8].

Epidemiological studies of the incidence of antibiotics resistance and its spread among microorganisms globally have been reported by World Health Organisation with contineuous updates. Treatment failure to the last resort of medicine for gonorrhoea for instance (third generation cephalosporin antibiotics) has been confirmed in at least 10 countries (Australia, Austria, Canada, France, Japan, Norway, Slovenia, South Africa, Sweden and the United Kingdom of Great Britain and Northern Ireland) [9].

The widespread emergence of multidrug-resistant (MDR) bacterial pathogen is an important public health challenge worldwide [10]. Infections with MDR organisms are associated with increased mortality, longer hospital stays and inflated healthcare costs [11][12]. Recent data also indicate a trend towards increased antibiotic resistance among cases of community-onset infections [13][14]. For many

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bacterial pathogens, particularly Gram-negative organisms, high rates of antibacterial resistance present limited therapeutic options for treating serious infections.

The development of antibiotic agent is one of the most significant achievements in the medical history. Antibiotics in medical and veterinary sciences denote a substance, either a chemical agent or a product from living organism, which kills or inhibit bacteria. Since the introduction of sulfa and penicillin during 1930's and 1940's, bacteria have display a remarkable ability to develop different types of resistance mechanisms.

The World Health Organization (WHO) has estimate that 3.4 million people, mostly children, die every year from related diseases (mainly diarrhea). The majority of them live in 49 development countries which according to the United Nations report, are experiencing a mounting number of cases of cholera and dysentery [15]. The situation is further complicated by emergence of bacteria with the ability to produce ESBLs [16]. In some cases, the bacterial strains are resistant to all available antibacterials agent [17].

In developmed nations steps have been taken to address this menace, however, not much has been done [18][19]. In developing countries, the main contributors to human and animal diseases are the resistant strains of bacteria [20]. Diseases caused by multi-resistant bacteria are more difficult to treat and also require drugs that are not readily available, expensive and are more toxic [21].

Antibacterial resistance is not only a medical problem but also an economic problem [22][23]. Most of the developing countries like Nigeria have inadequate Health care system due to limited resources and financial capacity. The increase in cases of antibiotic resistance is further aggravated by other socio-economic factors connected with misuse of antibiotics, knowledge, expectation, poor prescriber–patient interactions, economic incentives, and characteristics of health systems, the regulatory environment and availability of resource [24]. Some common practices that also contribute to antibiotic resistances in the developing countries include self-medication, non-compliance, misinformation, misconception and false belief, pressure due to retreatment expectation [25][26]. However, the most important underlying factor in developing countries is poverty [27].

Poverty can lead to poor sanitation, malnutrition due starvation which can degenerate to weak immunity, poor access to drugs and poor health care, all of which may propagate antibacterials resistance. Poverty can be directly linked to corrupt tendencies and corruption is a major factor in propagating antibacterial resistance and cannot be ignored. In many developing countries, health care providers are influenced by financial gains and operate on bases of profit. This may lead to unnecessary prescription [24].

Most Nigerian hospitals have inadequate waste management systems due to the gaps in the knowledge of waste management among health care workers; however, this has been further worsened by the poor financial support for our health care system. Poor waste management in our hospitals has further increase the spread and transmission of biocide and antibacterial resistant enterobacteriaceae such as *K. pneumoniae* and *C. freundii* in our hospitals and can as well be traced in our communities. The ultimate result of the spread of biocide and antibacterial resistant strains results in increased mortality, morbidity and prolonged hospital stay which can be translated into higher medical cost in a developing country like Nigeria where large number of its populace are living below poverty line of less than one Dollar per day.

K. pneumoniae is a member of the family enterobacteriaceae. It is a nonfastidious, Gram-negative bacillus, which is usually encapsulated. Species of the genus Klebsiella are the bacterial pathogens most often found associated with infections in healthcare settings and infections may be endogenous or acquired through direct contact with an infected host. The mechanisms of antibiotics resistance in *K. pneumoniae* include drug inactivation or alteration, modification of drug binding sites, reduced intracellular drug accumulation, porin loss, efflux pumps and biofilm formation [28]. In recent years, many *K. pneumoniae* strains have acquired a massive variety of β-lactamase enzymes, which can destroy the chemical structure of β-lactam antibiotics such as penicillins, cephalosporins, and carbapenems. Because carbapenems are conventionally used to treat persistent infections caused by Gram-negative bacteria, the increasing prevalence of carbapenem-resistant *K. pneumoniae* (CRKP), with resistance encoded by bla_{KPC}, presents a significant challenge for physicians [29][30]. In addition, the emergence of the *K. pneumoniae* super enzyme, known as NDM-1 and encoded by bla_{NDM-1}, has increased the proportion of carbapenem-resistant *K. pneumoniae* isolates and may pose a threat to other antibiotics such as β-lactams, aminoglycosides, and fluoroquinolones [28]. Even if several intensive infection control practices are used, outbreaks of carbapenemase-mediated multidrug resistant (MDR) strains are only reduced and cannot be completely eradicated. An effective treatment is therefore needed to overcome these pathogens.

Citrobacter species are a common cause of nosocomial infections associated with patients that are undergoing prolonged hospital treatments. *C. freundii* has recently been reported to express resistance to broad-spectrum antibiotincs including piperacillin, piperacillin tazobactam, vancomycin and cephalosporins. Isolation of ceftriaxone-resistant *C. freundii* (CRCF) has been associated with the overprescribed broad-spectrum antibiotics. The emerging new CRCF strains could suggest induction or depression of resistance genes as well as elimination of competing organisms. CRCF has

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been mostly isolated from patients with significant comorbidities including AIDS, peripheral vascular disease, and cerebrovascular disease. The usage of fluoroquinolone has also been reported to have no affect against the isolation of CRCF [31].

C. freundii is also known to contain in its chromosome a gene coding for cephalosporinase. This enzyme hydrolyses −CO−NH− bond in the lactam ring of cephalosporins and cephamycins thus rendering the bacteria resistant to this type of antibiotics. However, when exposed to new third generation cephems and cerbapenems, clinically isolated *C. freundii* showed sensitivity to those substances. A small outbreak of *C. freundii* resistant to third generation cephems has been observed in the surgical ward of Nagoya University Hospital in patients that underwent surgical procedures. The *C. freundii* was isolated from patient's bile, wound gauze, feces, pus, and ascites. It was suggested that these new strains of *C. freundii* contained a plasmid encoding AmpC celphalosporinase but upon failure to transfer the cephems resistance from *C. freundii* to *E. coli* it was concluded that the enzyme must be encoded in the chromosome of *C. freundii.* Since *C. freundii* is associated with nosocomial infections caution to this new strains are recommended [32]. Isolates of *C. freundii* resistant to cefotaxime and cefepime were found to be sensitive to entrapenem. The reason why entrapenem worked against *C. freundii* is because it is active against AmpC producing enterobacteriaceae, a group which *C. freundii* belong [33].

Studies have also confirmed the mechanisms of resistance to fluoroquinolones in two (2) *C. freundii* strains. Both strains were isolated from the same patient. This study allowed partial characterisation of the *acrA* and *acrB* genes of the strains. Expression of genes in both strains was analysed using DNA microarrays for *Escherichia coli*. Nucleotide similarity between the partially sequenced *acrA* and *acrB* genes of *C. freundii* and *E. coli* was 80.7% and 85%, respectively. The *acrA* and *acrB* genes of *C. freundii* are similar to those in *E. coli* and their overexpression may play an important role in modulating the final minimum inhibitory concentration of fluoroquinolones [34].

Resistance to Beta–lactam Antibiotics is a big challenge. The beta lactam group of antibiotics is the largest and most commonly used group of antibiotics globally. The beta-lactam-antibiotics comprises the family of penicillins, cephalosporin, monobactams, cabarpenems, and cephamycins, which are semi synthetic compounds originating from the fungi and bacteria [35]. All drugs in this group possess a beta-lactam ring as the functional part of the antibiotic**.**

Extended spectrum beta-lactamases are often acquired plasmid-mediated beta- lactamases that hydrolyse broader spectrum beta – lactams containing an oxyimino- group (e.g. ceftazidine, ceftriaxone, cefotaxime, aztreonam) as well as penicillin [36][37]. They can be inhibited by clavulanic acid and tazobactam. ESBLs have been found in the enterobacteriaceae family, mostly *E. coli and K. pneumoniae,* non – Enterobacteriaceae ESBLs-Producers are relatively rare with *Pseudomonas aeruginosa* being the most important organism [38]. ESBLs have been reported in *Acinetobacter* spp, *Burkholderia capacia* and *Alcaligenes feacalis* [36][38].

Epidemiology of *ESBLs* of the *CTX-M* type in Human Medicine cannot be overemphasized. The first Nosocomial out-break of CTX-M-J was recorded in an intensive care unit in a hospital in Paris, France [39]. Ten (10) years after documentation of what must be describe as an almost explosive global dissemination of CT-X-MS in hospital increase with high speed [40][41]. Although the majority of ESBLS- producing isolates have been derived from human's staying at hospitals [36][42][43]. Isolates producing CTX-M type enzymes can now easily be acquired in the community [44][45][46][47]. The CTX-MS have been extremely successful and are able to pass into almost any kind of settings and materials, including hospital waste [48]. The prevalence of classical ESBLS enzymes like TEM and or/ SHV has decreased but there is need for more research [49].

The aim and objectives of the present study is to screen hospital waste for $^{bla-}$TEM-68 gene in ESBL resistant *K. pneumoniae* and *C. freundii* in Yola, Adamawa State, Nigeria.

II. METHODOLOGY

Study Area

Adamawa State is located in the North-Eastern part of Nigeria part of Nigeria. It lies between Latitude 7° and 11°N and Longitude 11° and 14 ° E. It share boundary with Taraba State in the South-Western part, Gombe State in the North-Western, and Borno in the North. The state has international boundary with the Republic of Cameroun along the East side. The climate of Yola-Adamawa State is generally of the hot humid Tropical type, with two distinct seasons: The dry seasons lasts for a minimum of five months (November-March), and wet season spans from April to October [50].

Collection of Samples

Using necessary aseptic techniques, a total of one hundred and twenty (120) samples in all were collected and analyzed. These include cotton swabs, bandages, tissue papers, discarded catheter tubes, syringes and waste water. The samples were from Microbiology Laboratory, male ward, female ward, chemical pathology laboratory, the Intensive Care Unit (ICU) and the laundry unit of the specialist Hospital were analysed over a period of four (4) weeks.

About 30 g and/or 30 ml of the waste samples were aseptically collected in sterile opened-mouthed universal sample containers. The sample containers were quickly capped to prevent bacterial contamination and were labeled accordingly. The samples were carefully

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packed in an ice box and transported within 1h in cool condition to the Post-graduate laboratory of Microbiology Department of the Modibbo Adama University of Technology, Yola for further analysis. Sample collection, conveyance and time interval of analysis were in accordance with the internationally accepted method of APHA [51]. The two commonly used biocides in the hospital were commercially purchased after verifying the expired date and coded as biocide A and B for the biocide susceptibility test.

Isolation and Characterization of K. pneumoniae and C. freundii from Hospital Wastes

The isolation of the test isolates was carried out following standard aseptic procedure from sample collection to sample preparation and inoculation.

Preparations of Samples for Inoculation

The samples were prepared and/or processed using sterile scissors and scalpels to cut the solid non-sharp wastes which include bandages, tissue papers and cotton swabs into tiny pieces of about 3 grams. Five pieces each of the processed waste was inoculated aseptically into four (4) tubes containing MacConkey broth while the sharps were directly inoculated into a sterile tube containing 10 ml of MacConkey broth. Five (5) ml of the waste water sample was withdrawn using sterile pasture pipettes and then transferred into the test tubes containing 10ml MacConkey broth placed in a rack. The test tubes were tightly clogged with cotton wool and incubated at 37 ^{o}C to revive any of the enterobacteriaceae present in the various waste samples. All sample preparation was carried out using aseptic techniques and under aseptic condition [52].

Isolation of K. pneumoniae and C. freundii

Using aseptical techiniques, the pre-poured MacConkey agar and glycerol MacConkey agar since *C. freundii* can ferment glycerol as a single source of carbon. The plates were dried out in a hot air oven at a minimal temperature for 10-15 minute. Using spread plate techniques, 1ml sample was aseptical withdrawn from the resulting dilution of the broth suspension of the resuscitated microorganisms using sterile Pasteur pipette and were aseptically inoculated on to the dry surface of the MacConkey agar and glycerol MacConkey agar plates and in each case were spread on the dried surface of the agar by using sterile glass spreader as a standard recommended inoculation method [52]. The inoculated plates were then incubated at 37 ^{o}C for 24h.

Characterisation of K. pneumoniae and C. freundii Isolates

The characterization of the *K. pneumoniae* and *C. freundii* isolates were carried out using routine but standard laboratory methods which include macroscopic observation of colonial morphology for lactose and glycerol fermentation and utilization respectively. The appearance of larger raised mucoid colonies with undulated margin with a size of about 4mm which are nonmotile [53], while isolates which appeared distinctly lower or convex and mucoid pinkish colonies of 2-4 mm and actively motile microscopically. The distinct nasty foul-smelling odour further strongly differentiates the isolate from *K. pneumoniae* isolates. Furthermore, confirmatory biochemical tests such as Indole Methyl Red Vorges proskauer and Citrate (IMViC) test was employed to confirm the species of isolates obtained from the various hospital wastes sampled [54][55].

Gram Staining Techniques

The Gram staining technique was carried out as described by [53]. Smear was prepared from each of the pure isolates, accordingly and was allowed to properly air dried and fixed by passing over the flame. Fixed smears were covered with crystal violet stain for 30 seconds. The stain was rapidly washed off with clean water, and the water tapped off immediately from the slide. Each smear was covered with Lugol's iodine for 30seconds, and the iodine was washed off with clean water. The stained slides were decolorized rapidly within few seconds with acetone alcohol and washed off immediately with clean water. Each smear was covered with counter stain (safranin) for 60 seconds. The stain was then washed off with clean water. The back of each slide was wiped clean, and placed in the draining rack for the smear to air dry. When completely air dried, each of the smears were examined under the microscope using oil immersion objective, and the isolates observed were recorded as Gram-positive (purple colour) or Gram-negative (pink/red colour).

Hanging Drop Technique for Motility Test

A clean coverslip was held by its edges and the corners carefully dabbed with plasticine using a toothpick. A loop full of the suspected colonies of the isolates to be tested was placed in the center of the prepared coverslip. The clean concavity slide was then turned upside down over the drop on the coverslip so that the plasticine seals the coverslip to the slide around the concavity. The slide was then turned over so that the coverslip is on top and the drop can be observed hanging from the coverslip. The preparation was placed correctly on the microscope and the drop examined under the low power objectives with the diaphragm closed to produce a dark field. Distinct movement observed around cells that appear either like dark or slightly greenish, very small rods, is positive for motility to confirm *C. freundii* from the motility negative *K. pneumoniae* [52].

Indole Test

The test isolates were inoculated in peptone water, contained in a sterile test tube. The peptone water contains amino acids, tryptophan and incubated at 37 ^{o}C. Following the incubation few drops of Kovac's reagent was added. Kovac's reagents consist of Para dimethyl amino benzaldehyde, isoamyl alcohol and concentrated HCl. The set were observed for the formation of a red or pink coloured ring at the top of the test tube. If no pink or red ring seen this comfirms *K. pneumoniae* and this is also the same for *C. freundii.*

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Methyl Red (MR) Test

The isolate to be tested was inoculated into glucose phosphate broth contained in sterile test tube. The phosphate broth contains glucose and phosphate buffer and was inoculated at 37 °C for 48h.The mixed-acid producing organism will produce sufficient acid to overcome the phosphate buffer and remained acidic. The pH was tested by the addition of 5drops MR reagent. Development of a yellow colour was taken as negative result. *K. pneumoniae* is negative while *C. freundii* is positive for MR test.

Voges Proskauer (VP) Test

The various isolates were inoculated into phosphate broth and incubated for at least 48 hours then 0.6 ml of alpha-naphtol was added to the test broth and shaken after which 0.2 ml of 40 % KOH was added to the broth and shaken all together. The mixture and the tube were allowed to stand for 15 minutes. Appearance of red colour is taken as positive. The negative tubes were held for one hour after addition of reagent. The negative organism will produce yellow colour. *K. pneumoniae* is positive while *C. freundii* is negative.

Citrate Utilization

Bacterial colonies to be tested were picked up using a sterile straight wire and inoculated into a slope of Simmon's citrate agar and then incubated overnight at 37 °C. The ability to utilize citrate by the organisms was interpreted from the colour change of the medium from green to blue. *K. pneumoniae* and *Citrobacter freundii* are both positive for citrate utilization test.

III. MOLECULAR IDENTIFICATION OF ISOLATES

DNA Extraction

DNA was extracted using the protocol stated by [56]. Briefly, Single colonies grown on medium were transferred to 1.5 ml of liquid medium and cultures were grown on a shaker for 48 h at 28 ºC. After this period, cultures were centrifuged at 4600 g for 5 min. The resulting pellets were resuspended in 520 µl of TE buffer (10 mMTris-HCl, 1mM EDTA, pH 8.0). Fifteen microliters of 20 % SDS and 3 µl of Proteinase K (20 mg/ml) were then added. The mixture was incubated for 1 hour at 37 ºC, then 100 µl of 5 M NaCl and 80 µl of a 10 % CTAB solution in 0.7 M NaCl were added and votexed. The suspension was incubated for 10 min at 65 ºC and kept on ice for 15 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 minutes and centrifugation at 7200 g for 20 min. The aqueous phase was then transferred to a new tube and isopropanol (1: 0.6) was added and DNA precipitated at –20 ºC for 16 h. DNA was collected by centrifugation at 13000 g for 10 min, washed with 500 µl of 70% ethanol, air-dried at room temperature for approximately three hours and finally dissolved in 50 µl of TE buffer.

Polymerase Chain Reaction (PCR) to Identify the Test Isolates

The PCR preparation cocktail consisted of 10 µl of 5x GoTaq colourless reaction, 3 µl of 25mM MgCl2, 1 µl of 10 mM of dNTPs mix, 1 µl of 10 pmol each 27F 5′-AGAGTTTGA TCMTGGCTCAG-3′ and - 1525R, 5′-AAGGAGGTGATCCAGCC-3′ primers of sizes 20 nt and 17 nt respectively and 0.3 units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water 8 µl DNA template. PCR was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) with a PCR profile consisting of an initial denaturation at 94 °C for 5 min; followed by a 30 cycles consisting of 94 °C for 30 seconds, 50 °C for 60 seconds and 72 °C for 1 minute 30 seconds; and a final termination at 72 °C for 10 minutes then chilled at 4 ºC [57][58].

Integrity Test of the Amplified DNA (Agarose Gel Electrophoresis)

The integrity of the amplified DNA about 1.5 Mb gene fragment was checked on a 1 % Agarose gel ran to confirm amplification. The buffer (1XTAE buffer) was prepared and subsequently used to prepare 1.5 % agarose gel. The suspension was boiled in a microwave for 5 minutes. The molten agarose was allowed to cool to 60 °C and stained with 3 µl of 0.5 g/ml ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 minutes to form the wells. The 1XTAE buffer was poured into the gel tank to barely submerge the gel. Two (2) microliter of 10 X blue gel loading dye (which gives colour and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 4µl of each PCR product and loaded into the wells after the 100bp DNA ladder was loaded into well No. 1. The gel was electrophoresed at 120 V for 45 minutes visualized after which it was visualised by ultraviolet trans-illumination and photographed. The sizes of the PCR products were estimated by comparison with the mobility of a 100bp molecular weight ladder that was ran alongside experimental samples in the gel [56].

Purification of Amplified Product

After gel integrity, the amplified fragments were ethanol purified in order to remove the PCR reagents. Briefly, 7.6 µl of Na acetate 3 M and 240 µl of 95 % ethanol were added to each about 40 µl PCR amplified product in a new sterile 1.5 µl eppendorf tube, and was vortexed to mix thoroughly and kept at -20 °C for at least 30 minutes. The mixture was centrifuged for 10 minutes at 13000 G and 4 °C followed by removal of supernatant (invert tube on trash once) after which the pellet were washed by adding 150 µl of 70 % ethanol and was mixed again then centrifuged for 15 minutes at 7500 G and 4 °C. The supernatant were remove then the tubes were inverted on paper tissue and was dried in the

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fume hood at room temperature for 10-15 minutes. After drying it was then resuspended in 20 µl of sterile distilled water and kept at -20 ºC prior to sequencing. The purified fragments were checked on a 1.5 % Agarose gel ran on a voltage of 110 V for about 1hr as previous, to confirm the presence of the purified product and quantified using a nanodrop of model 2000 from thermo scientific.

Sequencing the Amplified Fragments

The amplified fragments were sequenced using a Genetic Analyzer 3130x*l* sequencer from Applied Biosystems and the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit which uses the matrix standard for fragment sequencing as described in the manufacturers' manual.

Following the manufacturers procedure, the fragments so obtained were sequenced by measuring five (5) ml of 10 x Genetic analyser buffer (P/N/402824) and was used to prepare 50 ml of 1x buffer which was properly mixed and stored at 2 to 8 ºC for further usage. The 1x Buffer running in anode cathode were replaced in the buffer reservoir before each sample was run. The oven and instrument doors were properly closed after which the v.3.1 sequencing calibration standards was used. The tubes of the matrix standard was removed from the refrigerator, thoroughly mixed and spinned in a microcentrifuge at 1500 x g for five (5) minutes and Hi-Di formamide was added in ratios then the mixture was thoroughly votexed. The standard tube was heated at 95 ºC to denature the DNA and the tubes cooled on ice for two (2) minutes. The denatured sample was added to the 96 well reaction plates. The chemistry file for the fragment analysis dye was set and the matrix standard was also set to defaults. The oven and front doors were again verified to be closed then the plate records were selected as earlier programmed. The processing dialogue box on the monitor of the computer was navigated to open the dialogue box to click on OK to run the sequencing.

The pass/fail status was then viewed by clicking the tree pane of the data collection software ga 3130x*l* to view instrument status for the dye set then observed for the display of four (4) dyes: red-yellow-blue-green with separate distinct spectral profiles. The auto analysis of the distinct spectrum automatically generate the sequencing by creating analysis record plate presented as sets of distinct nucleotide sequence of nucleic acids for the different fragments using the Bio-Edit software for all genetic analysis. The sequences of nucleotide displayed were then printed out. The sequences were then blasted on the NCBI website to obtain the accession number and other sequence profile [59].

Polymerase chain reaction to identify $^{bla-}$TEM-68 gene

Molecular identification of $^{bla-}$TEM-68-coding gene in the isolated Enterobacteriaceae isolates was by simple PCR on the extracted DNA using $^{bla-}$TEM-68 specific primers unique to $^{bla-}$TEM-68 -coding regions. Primer sequences were as earlier documented by Paterson *et al* (2003). Reaction cocktail used for all PCR per primer set included (Reagent Volume µl) - 5X PCR SYBR green buffer (2.5 µl), $MgCl_2$ (0.75 µl), 10pM DNTP (0.25 µl), 10 pM of each forward 5′-AAACGCTGGTGAAAGTA-3′ of 17 nt and backward primer 3′-AGCGATCTGTCTAT-5′ of 14 nt (0.25 µl), 8000U of taq DNA polymerase (0.06 µl) and made up to 10.5 µl with sterile distilled water to which 2 µl template was added. Buffer control was also added to eliminate any probability of false amplification. PCR reaction profile for all the reactions were as follows; PCR profile consisting of an initial denaturation at 94°C for 5 minutes; followed by a 30 cycles consisting of 94 °C for 30 seconds, 44 °C for 45 seconds and 72 °C for 50 seconds; and a final termination at 72 °C for 10 minutes and was chilled at 4 ºC. PCR amplification was confirmed by gel electrophoresis as previously described.

IV. RESULTS AND DISCUSSION

Results obtained for the isolation and identification of *K. pneumoniae* and *C. freundii* revealed that out of all the one hundred and twenty (120) collected from the selected departments/units and screened, revealed occurrence of enterobacteriaceae. In all, eighty-five (85) samples were positive for different lactose fermenting enterobacteriaceae based on colony morphorlogy (Plate 1 and table 1). MacConkey agar, *K. pneumoniae* appear morphologically different from *C. freundii* macroscopically grown on glycerol MacConkey agar in the sense that the colonies of *K. pneumoniae* appear as large, raised, mucoid and pinkish colonies with an undulated margin and a size of about 4mm with a distinctive yeasty smell (plate1 and table1), while *C. freundii* appear as lower or flat, smooth, shiny, mucoid and pinkish colonies that are convex with a size of about 2-4mm on glycerol MacConkey agar with a characteristic distinct nasty foul smelling odour (plate 1 and table 2).

Plate 1: Cultures of *K. pneumoniae* on MacConkey Agar (MCA) and *C. freundii* on Glycerol MacConkey Agar (GMCA)

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Table 1: Occurrence of lactose and non-lactose and non-lactose fermenters from various waste samples generated in the different departments/units of the Specialist Hospital, Yola

Site of waste sample collection (Depts/units)	No of samples analysed	No of samples positive for only lactose fermenters (%)	No of samples positive for only non-lactose fermenters (%)	No of samples which yielded both lactose and non-lactose fermenters (%)
MBL	20	9(7.50)	3(2.50)	8(6.67)
MWD	20	16(13.35)	2(1.67)	2(1.67)
FWD	20	10(8.33)	5(4.17)	5(4.17)
CPL	20	8(6.67)	10(8.33)	2(1.67)
ICU	20	7(5.83)	9(7.50)	4(3.33)
LDU	20	12(10.00)	6(5.00)	2(1.67)
TOTAL	120	62(51.67)	35(29.17)	23(19.18)

KEYS - MBL; Microbiology Laboratory, MWD; Male Ward, FWD; Female Ward, CPL; Chemical Pathology Laboratory, ICU: Intensive Care Unit, LDU; Laundry Unit

Characterisation of K. pneumoniae and C. freundii

Out of the eighty-five (85) isolates of enterobacteriaceae isolated, sixty-two (62) of the isolates were lactose fermenters while thirty-five (35) were non-lactose fermenting enterobacteriaceae. Biochemical confirmatory test showed that out of the 85 lactose fermenting enterobacteriaceae isolated in this study, fourty three (43) isolates were confirmed to be *K. pneumoniae* and ten (10) were confirmed to be *C. freundii* because these isolates were able to ferment the glycerol in Glycerol-MacConkey agar (GMCA) which confirmed the *C. freundii* isolates from the hospital wastes screened in all the departments (Microbiology unit, male ward, female ward, chemical pathology laboratory, Intensive Care Unit and Laundry unit) sampled for different hospital wastes in the Adamawas State Specialist Hospital, Yola (Table 2).

Table 2: Colony morphology and biochemical properties of isolates of *K. pneumoniae* and *C. freundii* isolated from Adamawa State specialist Hospital, Yola.

Colonial morphology of the isolates	Colony odour	Grams Reaction	KIA Slant	KIA Butt	Indole test	Methyl Red test	Citrate test	Motility test	Test isolates confirmed
			Confirmatory Biochemical Tests						
Appear large raised and mucoid colonies with and undulated margin with a of size of about 4mm.	Distinct yeasty odour	-ve	Y	Y(G)	-ve	-ve	+ve	-ve	*K. pneumoniae*
Appear lower or convex mucoid Colonies that are pinkish in colour, with size of 2 – 4 mm.	Nasty foul smelling odour	-ve	Y	Y(G) (H_2S)	-ve	-ve	+ve	+ve	*C. freundii*

Key: Y=Yellow, G = Gas, KIA= Kligler Iron Agar, H_2S= Hydrogen Sulphide

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Occurrence and Distribution of K. pneumoniae and C. freundii in the Different Waste Samples

Out of the one hundred and twenty (120) different types of samples from the different categories of waste in this study, ten (10) isolates of *K. pneumoniae* were recovered from cotton swabs being the highest of all the waste samples analysed, while the lowest of four (4) was recovered from sharps. Three (3) isolates of *C. freundii* was isolated from bandages and tissue papers each being the highest occurrence, while one (1) isolate of *C. freundii* was recovered from catheters and sharps. Six (6) isolates of *K. pneumonia*e were isolated from waste water but no isolate of *C. freundii* was isolated from all waste water samples from the Adamawa state specialist hospital, Yola (table 3).

Table 3: Occurrence of *K. pneumoniae* and *C. freundii* confirmed from different waste samples in Specialist Hospital, Yola.

Types of waste samples analysed	No. of waste samples analysed (%)	No. of lactose fermenting enterobacteriaceae recovered (%)	No. of isolates of *K. pneumoniae* confirmed (%)	No. of isolates of *C. freundii* Confirmed (%)
Cotton swabs	20	17(14.17)	10(10.83)	2(1.67)
Bandages	20	18(15.00)	7(5.83)	3(2.50)
Tissue papers	20	15(12.50)	9(7.50)	3(2.50)
Catheters	20	10(8.33)	8(6.67)	1(0.83)
Sharps	20	11(9.17)	4(3.33)	1(0.83)
Waste water	20	14(11.67)	6(4.17)	--
Total	120	85(70.84)	43(35.83)	10(8.33)

Molecular Identification of Isolates Using the 16S rDNA

The PCR results using 16s rDNA amplification confirmed the presence of *K. pneumoniae* and *C. freundii*. Samples from the Female Ward of the hospital recorded the highest number of isolates of *K. pneumoniae,* while the male ward had the highest isolates of *C. freundii* of four (4).

The Molecular identification ascession number deduced from sequencing of fragments of PCR products revealed that the *K. pneumoniae* isolate (RKPS2) were 93 % identical to *Klebsiella pneumoniae* strain KPNIH48, while the *C. freundii* (RCFS2) was found to be 99 % Identical to *Citrobacter freundii* complex sp. CFNIH4 (Plate 2; table 4a and b).

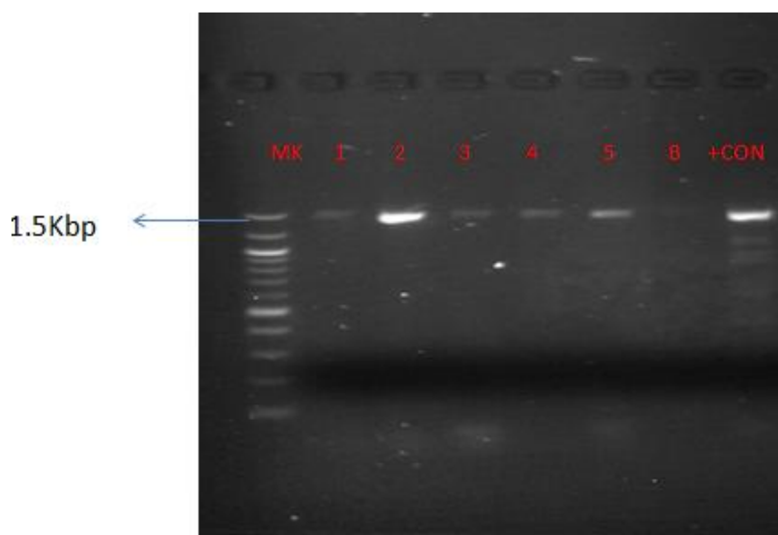

Plate 2: Agarose gel electrophoresis of the Purified PCR products amplified from bacteria isolates using the 16S rDNA universal primers. Gel image indicates a positive amplification in all samples (Mk 100bp marker), RKPS2, RPMS1, RPMS2, RECS1 and RCFS2.

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Table 4a: Target pathogen and its sequence profile

Test isolates	Sequence blast	Ascension no.	Sequence identity (%)	Query cover (%)	Score Bit
RKPS2	Identical to *Klebsiella pneumoniae* strain KPNIH48 TGGGGGCTTCCATGCAGTCGAGCGGTAACACGTATAG CTTGCTTTCCGGTGACGAGCGGCGGACGGGTGAGTAA TGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACT GGAAACGGTAGCTAATACCGCATAATGTCTCAAGACC AAAGAGGGGGACCTTCTGTCCTCATGCCATCGGATGT GCCCAAATGGGATTAGCTAGTAGGTGGGGTAATGGCT CACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATG ACCAGCCACACTGGAACTGAGACACGGTCCAGACTCC TACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG CGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAA GGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAA GGCGTTAAGGTTAATAACCTTGGCGATTGACGTTACC AGCAGAACAAGCACCGGCTAACTCCGTGCCAGCAGCC GCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTA CTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTTG GATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATT TGAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAG AATTCCAGGTGTAGCGGTGAAATGCGTAGAGATGTGG AGGAATACCGGTGGCGAACGCGGCCCCCTGGACAAG ACTGACGCTCAAGTGCGAAAGCGTGGGGAGCAAACA GGATTACATACCCTGGTAGTCCACGCCGAAACGATGT CGATTTGGAGGTTGTGCCCATGAGCGTGCTTCCAGAG CTAACGCGTTAAATCGACCGCCTGGGGGAGTACGGCC GCAAGCTCAAAACTCATATGAATTGACCGGGGGCCCC GCACAAGCGTGAGCATGTGGTTTAATTCGATGCAAC AGGAAAAACCCTAACTGATCTTGACATCCCAGTAACT TACAAAGATGGCTCGATGCATCAGGAAACTCTGAAAC AGGTCCTGCCATGCGGTCGTCAGCTCTGTTAGTAATGT AATCAATCCCGACAGAGCCCTAACCATTACC	MN12417 2	93	100	93

Table 4b: Target pathogen and its sequence profile

Test isolates	Sequence blast	Ascension no.	Sequence identity (%)	Query cover (%)	Score Bit
RCFS2	Identical to *Citrobacter freundii* complex sp. CFNIH4 CGGGCCCAGCCCTAACCCAAGCAAGTCGAACCGTAGC ACAGAGGAGCTTGCTCCTTGGGTGACGAGTGGCGGAC GGGTGAGTAATGTCTGGGAAACTGCCCGATGGAGGGG GATAACTACTGGAAACGGTAGCTAATACCGCATAACG TCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGC CATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGG GGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCT GAGAGGATGACCAGCCACACTGGAACTGAGACACGGT CCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGC ACAATGGGCCGCAAGCCTGATGCAGCCATGCCGCGTG TATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCG AGGAGGAAGGCGTTGTGGTTAATAACCGCAGCGATTG ACGTTACTCGCAGAAGAAGCACCGGCTAACTCCTGCC AGCAGCCGCGGTAAAACGGAGGGTGCAAGCGTTAATC GGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGT CAAGTCGGATGTGAAATCCCCGGGCTCAACCCTGGGA ACTGCATCCGAAACTGGCAGGCTAGAGTCTTGTAGAG GGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAG	MN124171 5	99	100	99

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AGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCT
GGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGA
GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
AACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTG
GCTTCCGGAGCTAACGCGTTAGTCGACCGCCTGGGGA
GTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGG
GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG
ATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAG
AGAACTTAGCAGAGATGCTTTGGTGCCTTCGGGAACTC
TGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTG
TGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TTATCCTTTGTTGCCAGCGRTTCGGYCGGGAACTCAAA
GGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGAT
GACGTCAAGTCATCATGGCCCTTACGAGTAGGCTACA
CACGTGCTACAATGGCATATACAAAGAGAAGCGACCT
CGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGT
CCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGG
AATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAA
TACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA
TGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTACCCT
TCCGGA

Molecular Identification of bla-TEM-68 Gene for TEM-68 ESBLs

The outcome of the molecular identification of *bla-*TEM-68-coding gene five suspected Enterobacteriaceae isolates screened by simple PCR on the extracted DNA using *bla-*TEM-68 specific primers unique to *bla-*TEM-68 -coding regions with primer sequences as earlier documented by [60] revealed that upon completion of 30 cycles, the Agarose gel electrophoresis of the PCR products of *bla-*TEM-68 gene amplified from five Enterobacteriaceae isolates indicates Gel images of positive amplification with band size of approximately 750bp in only *K. pneumoniae* (amplicon1) and *E. coli* (amplicon4) are samples indicating the presence of *bla-*TEM-68 gene. Mk 100bp marker, RKPS2, RCFS1, RCFS3, RKPS1 and RCFS2 i.e Amplicon1=RKPS2 (Resistant *K. pneumoniae* strain 2), Amplicon2=RCFS1 (Resistant *C. freundii* strain 1), Amplicon3=RCFS3 (Resistant *C. frenudii* strain 3), Amplicon4=RECS1 (Resistant *E. coli* strain1), Amplicon5=RCFS2 (Resistant *C. frenudii* 2) as shown in plate. 3.

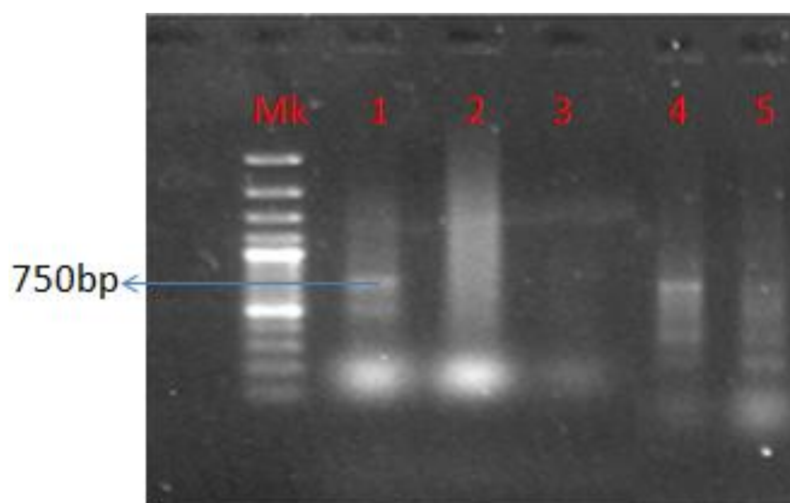

Plate 3: Agarose gel electrophoresis of the PCR products of *bla-*TEM-68 gene amplified from five Enterobacteriaceae isolates. The (Band size approximately 750bp) as shown in the Gel image indicates a positive amplification in only *K. pneumoniae* and *E. coli* samples indicating the presence of *bla-*TEM-68 gene. Mk 100bp marker, RKPS2, RCFS1, RCFS3, RECS1 and RCFS2.

KEY: Amplicon1 (*bla-*TEM-68 gene) =RKPS2 (Resistant *K. pneumoniae* strain 2), Amplicon2=RCFS1 (Resistant *C. freundii* strain 1), Amplicon3=RCFS3 (Resistant *C. frenudii* strain 3), Amplicon4 (*bla-*TEM-68 gene) =RECS1 (Resistant *E. coli* strain1), Amplicon5=RCFS2 (Resistant *C. frenudii* 2)

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V. DISCUSSION

Most of the resistant *K. pneumoniae* isolated from the hospital wastes were ESBLs positive this is in agreement with the report by [61]. Although one isolates of *C. freundii* was found to be phenotypically ESBLs positive but was confirmed using the DDST to be negative for ESBLs which agrees with the findings of [32] which suggested that these new strains of *C. freundii* carries a set of chromosome encoding AmpC celphalosporinase which are only phenotypically ESBLs positive and such AmpC genes are encoded in the chromosomes and are not transferred to other enterobacteriaceae as reported by [33].

The molecular screening only demonstrated the presence of $^{bla-}$TEM-68 gene in *K. pneumoniae* and findings in this work has shown that *C. freundii* those not carry or habour the complex novel gene as result turned negative for the gene amplification as can be seen from the PCR image in Plate 3.

At molecular level, the isolates of *K. Pneumoniae* which is of interest in this study habour the complex $^{bla-}$TEM-68 gene after being subjected to PCR for the specific gene amplification but interestingly some enterobacteriaceae isolated in this also carried the $^{bla-}$TEM-68 this agrees with the study by [62] where $^{bla-}$TEM-68 gene was demonstrated in *K. pneumoniae* by demonstrating ESBLs activity in transformed clinical isolate of *E. coli* carrying $^{bla-}$TEM-68 gene which proved that clinical isolate of *E. coli* construct carrying the novel ESBLs was obtained as a product of transconjugation with *K. pneumoniae*, through a deliberate attempt which led to the transfer of the $^{bla-}$TEM-68 gene from *K. pneumoniae* to the clinical isolate of *E. coli*, this further confirmed that although the $^{bla-}$TEM-68 Producing isolate 31511/98 was the first reported strain of *K. pneumoniae* expressing a complex mutant β-lactamase and one of the examples of non- *E. coli* isolate that produces a class A β-lactamase with IR(Inhibitor Resistance) activity, this therefore proves that *E. coli* isolate carrying $^{bla-}$TEM-68 gene in this current study may have been transformed from its wild type to a complex resistant ESBLs producing strain by simple gene transfer among the enterobacteriaceae. Therefore *K. pneumoniae* carrying this complex gene is capable of spreading it among enterobacteriaceae cohabiting together in the different wards and units of the Adamawa Sate Specialist Hospital, Yola.

Plasmid curing of the resistant isolates in this current study also further confirmed the fact that $^{bla-}$TEM-68 gene can also be located in the chromosomes as some previously confirmed ESBLs positive isolates turned out to be ESBLs negative upon treatment with 10 % SDS in an over-night culture, which is strongly in line with the findings that genes encoding the (*bla* genes) are located on either bacteria chromosomes or plasmid, transpires or intercrops [63][64][65]. The SDS plasmid curing confirmed that the ESBLs genes in ESBLs positive *K. pneumoniae* in the hospital waste are plasmid borne thereby posing the challenge of possible transfers of ESBLs gene among organisms in the hospital wastes which could lead to more chances of increased health burden, financial surge and selection pressure in the presence of residues of antibiotics and disinfectants in the hospital environment.

There are possibilities of Nosocomial infections in Adamawa State Specialist Hospital, Yola since the waste generated in the hospital setting habour *K. pneumonia*e with $^{bla-}$TEM-68 gene and beta-lactamase is the most frequently encountered mechanism of resistance among Gram negative bacteria such as *K. pneumomiae*, *E. coli* and *C. freundii* implicated in the cause of nosocomial infection as reported by [66][67].

VI. CONCLUSION

In conclusion, the indiscriminate use of antibacterial and agents may disrupt the microbial balance in favour of resistant bacteria which may habour and transfer plasmid borne $^{bla-}$TEM-68 genes (a complex mutant) in the Adamawa state specialist hospital, Yola.

Findings in this study revealed that isolates of *K. pneumoniae* and *E. coli* habouring the novel mutant ESBLs coding gene are present in the Adamawa state specialist hospital, Yola which translates that there is a danger of increased multidrug resistance among the pointer organisms and hence an indicator of possible increase in nosocomial infections and comobidity in the hospital setting due to possible spread of multidrug resistant and biocide resistant *K. pneumoniae* and ESBLs producing *E. coli* isolates by health workers, out door, patients and patients admitted for a prolonged period of time.

REFERENCES

[1] World Health Organization (1999). Unsafe injection practices and transmission of blood borne pathogens. Bull. World Health Organization.77:787-819

[2] Baquero, F., Martinez, J.L. and Canton, R. (2008). Antibiotics and antibiotic resistance in water environments. *Current Opinion in Biotechnology*. 19:260-5

[3] Program for Appropriate Technology in Health (PATH), (2009). Achieving effective sharp waste management in GAVI host countries. A proposed approach with estimates of cost 2006. Available at http:/www.path.org. Accessed 23rd march, 2016.

[4] Bailey, J.K., Pinyon, J.L., Anantham, S. and Hall, R.M. (2010). Commensal *Eschirichia coli* of healthy humans: a reservoir for antibiotic-resistance determinants. *Journal of Medical Microbiology*. 59, 1331-1339

[5] Szmolka, A. and Nagy, B. (2013). Multidrug resistant commensal *Escherichia coli* in animals and its

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impact for public health. *Frontiers in Microbiology* 4:258.
[6] Alanis, A.J. (2005). Antibiotics susceptibility pattern of Enterobacteriaceae isolated from hospitals. *Archive of Medical Research.* 36, 697
[7] Washington State Department of Health (2014). CDC guidlines and recommendations on the surveillance and control and carbapenem resistant enterobacteriaceae. p 420-97.
[8] World Health Organization (2002). W.H.O Report on the emerging new cases of antibacterial resistance in Gram-negative bacteria.
[9] World Health Organization (2018). Epidemiology of Antibacterial resistance: global report on surveillance. Geneva: World Health Organization.
[10] World Health Organization (2014). Antibacterial resistance: global report on surveillance. Geneva: World Health Organization.
[11] Neidell, M.J., Cohen, B., Furuya, Y, Hill, J., Jeon, C.Y., Glied, S. and Larson, E.L. (2012). Costs of healthcare- and community-associated infections with antibacterial-resistant versus antibacterial-susceptible organisms. *Clinical Infectious Diseases 55*: 807–815.
[12] Martin-Loeches, I, Torres, A., Rinaudo, M., Terraneo, S., de Rosa, F., Ramirez, P., Diaz, E, Fernandez-Barat, L., Li Bassi, G.L and Ferrer, M. (2015). Resistance patterns and outcomes in intensive care unit (ICU)-acquired pneumonia. Validation of European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC) classification of multidrug resistant organisms. *Journal of Infections,* 70: 213-222.
[13] Lim, C.J., Cheng, A.C., Kong, D.C and Peleg, A.Y. (2014). Community-onset bloodstream infection with multidrug-resistant organisms: a matched case-control study. *Biomed Central Infectious Diseases* 14: 126
[14] Stefaniuk, E., Suchocka, U., Bosacka, K and Hryniewicz, W. (2016). Etiology and antibiotic susceptibility of bacterial pathogens responsible for community-acquired urinary tract infections in Poland. *European Journal of Clinical Microbiology and Infectious Diseases, 35*: 1363–1369.
[15] Pandey, S. (2006). Weter pollution and health-review articles. *Kathmandu University Medical Journal* 4:128-13.
[16] Sosa, A.J, de, J., Byarugaba, D.K., Amabile, C., Hsueh, P.R., Kariuki, S. and Okeke, I.N. (Eds). (2010). Antibacterial Resistance in developing countries. New York: ISBN: 978-0-38789369-3. *Springer Science,* 23:554.
[17] Russo, T.A. and Johnson J.R. (2003). Medical and Economic impact of Extra-intestinal infections due to *Escherichia coli*: focus on an increasingly important endemic problem. *Microbes and Infections* 5:449-456.
[18] World Health Organization (2002). W.H.O Report on the emerging new cases of antibacterial resistance in Gram-negative bacteria.
[19] Shears, P. (2000). Atimicrobial resistance in the tropics. *Tropical Doctors.* 30:114- 116
[20] Shears, P. (2001). Antibiotic resistance in the tropics. Epidemiology and surveillance of Antibacterial resistance in the Tropics. Trans. R. Soc. *Journal of Tropical Medicine and Hygiene,* 95:127-130
[21] Howard, D., Cordell, R., McGown, J.E. Jnr., Packard, R.M. and Scott, R.D.II. and Solomon, S.L (2001). Measuring the economic costs of antibacterial resistance in hospital settings: the summary of the Centre for Desease Control and Prevention-Emory workshop. *Clinical Infectious Diseases* 33:1573-1578.
[22] Paladino, J.A, Joseph, A., SunderlinConnie, S., Price, J. and Schentag, J. (2002). Economic consequences of antibacterial resistance. *Surgical Infections Reviews* (Larchmt) 3:3.
[23] Cosgrove, S.E. and Carmeli, Y. (2003). The impact of antibacterial resistance on health and economic outcomes. *Journal of Clinical Infectious Disease*; 36:1433-1437.
[24] Byarugaba, D.K. (2004). A view on antibacterial resistance in developing Countries and responsible risk factors. *International Journal of Antibacterial Agents* 24(2):105-10
[25] Parimi, N., Pinto Pereira L.M. and Prabhakar, P. (2002). The general public perceptions and the use of antibacterials Trinidad and Tobago. *Pan American Journal of Public Health* 12:11-18.
[26] Lipstitch, M, and Samoure, M.H. (2002). Antibacterial use and antibacterial resistance: a population perspective. *Emerging Infectious Diseases.* 8:347-354
[27] Hossain, M.M., Glass, R.I. and Khan, M.R (1982). Antibiotic use in a rural community in Bangladesh. *International Journal of Epidemiology* 11:402- 405.
[28] Kumarasamy, K. K., Toleman, M. A and Walsh, T. R. (2010). Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *The Lancet Infectious Diseases* 10(9):597-602.
[29] Bush K., Jacoby, G. A. and Medeiros A. A (1995). A functional classification scheme for β-lactamases and its correlation with molecular structure. *Antibacterial Agents and Chemotherapy*. 1995; 39(6):1211–1233
[30] Queenan, A. M and Bush K. (2007). Carbapenemases: the versatile β-lactamases. *Clinical Microbiology Reviews*. 20(3):440–458.
[31] Kim, P.W., Harris, A.D., Roghmann, M.C., Morris, J.G. Jnr., Strinvasan, A. and Perencevich, E.N. (2003). Epidemiologic risk factors for isolation of ceftri- axone-resistant versus-susceptible *C. freundii* in hospitalized patients. *Antibacterial Agents in Chemotherapy* 47(9): 2882:2887.
[32] Nada, T., Baba, H., Kawamura, K., Ohkura, T., Torii, K. and Ohta, M. (2004). A small outbreak of third generation cephalosporin-resistant *Citrobacter freundii* infection on surgical ward. *Japanese Journal of Infectious Diseases.* 57, 181-182.

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Volume-8, Issue-5 (September 2021)
https://doi.org/10.31033/ijrasb.8.5.6

[33] Chuang, Y., Tseng, S., Teng, L., Ho, Y and Hsueh, P. (2006). Emergence of cefotaxime resistance in Citrobactor freundii causing necrotizing fasciitis and osteomyelitis" *Journal of Infections* 53:161-163.
[34] Ciğerci, I.H., Korcan, S.E., Konuk., M and Oztürk, S. (2007). Comparison of ALAD activities of Citrobacter and Pseudomonas strains and their usage as biomarker for Pb contamination. *Environmental Monitoring and Assessment* 139(1-3):41-8.
[35] Walsh, C. (2003) Antibiotics: action, origins, resistance Washington DC. ASM press.
[36] Bradford, P.A (2001). Extended spectrum beta-lactamases in the 21st century: Characterization, epidemiology and detection of the important resistance Threat. *Clinical microbiology Reviews* 14:933-951
[37] Paterson, D.L. and Bonomo, R.A. (2005). Extended spectrum beta-lactamase-producing *Escherichia coli* causing bacteremia in centralized Canadian Region. *Journal of Clinical Microbiology* 50(2):294-9.
[38] Poirel, K., Bonnin, R.A. and Nordman, P. (2012). Genetic support and diversity of acquired extended-spectrum beta-lactamase in Gram-negative rods. *Infection Genetics and Evolution Journal* 12(5):883-93
[39] Kitzis, M.D., Billlout- Klein, D., Goldstein, F.W., Williamson, R., Tran Van Nhieu, G., Carlet J., Acar, J.F. and Gutmann, L. (1988). Dissemination of the novel Plasmid-mediated beta-lactamase CTX-1, which confers resistance to Broad - spectrum cephalosporins, and its inhibition by beta-lactamase inhibitors. *Antibacterial Agents and Chemotherapy* 32, 9-14.
[40] Radice, M., Power, P. Di Conza, J. and Gebriel, G. (2002). Early, dissemination of CTX-M- derived enzymes in south America. *Antimicrob. Agents Chemother* 46:602-604
[41] Canton, R. and Coque, T.M. (2006). The CTX-M beta-lactamase pandemic. *Current Opinion in Microbiology*. 9:466-475.
[42] Bonnet, R. (2004). Growing group of extended spectrum beta-lactamases: the CTX-M enzymes. *Antibacterial Agents and chemotherapy.* 48:4-14
[43] Pitout, J.D. (2010). Infections with extended-spectrum Beta-Lactamase-Producing enterbacteriaceae: Changing epidemiology and drugs treatment choices. *Drugs journal* 70(3):313-33
[44] Arpin, C., Dubbois V. Maugein, J., Jullin, J., Dutilh, B., Brochet, J., Larribet, G., Fischer, I. and Quentin, C (2005). Clinical and molecular analysis of extended spectrum beta-lactamase-producing enterobacteria in the community setting. *Journal of Clinical Microbiology*. 43:5048-5054
[45] Lewis, J.S., Herrera, M., Wickes, B., Patterson, J.E. and Jorgensen, J.H. (2007). First report of the Emergence of CTX-M-type extended – spectrum beta – lactamase (ESBLSs) as the predominant ESBLS isolated in a U.S. Health care system. *Antibacterial Agents and Chemotheapyr* 51, 4015-4021.
[46] Pitout, J.D. and Laupland, K.B. (2005). Extended-spectrum beta-lactamase-producing Enterobacteriaceae: an emerging public health concern. *Lancet infectious Diseases* 8:159-166.
[47] Wieler, L.H., Ewers, C. Guenthers, S, Walther, B. and Lubke-Becker. A. (2011). Methicilin-resistant *staphylococci* (MRS) and extended-spectrum-beta lactamases (ESBL)-producing Enterobacteriaceae in companion animals: nosocomial infections are one reason for the rising prevalence of these potential zoonotic pathogens in clinical samples. *International Journal of Medical Microbiology,* 301:635-641.
[48] Mshana, S.E., Imirzalioglu, C., Hamid, H.C., Hein, T., Dormann, E. and Chakraborty, T (2009). Conjugative IncFI plasmid carrying CTX-M-15 among *Escherichia coli* ESBLS producing isolates at a university hospital in Germany. *Biomed Central Infecious Diseases* 9:97.
[49] Livermore, D.M., Canton, R., Gniadkowski, M, Nordmann, P., Rossolini, G.M., Alert, G, Ayala, J., Coque, T.M., Kern-ZdNanowicz, I., Luuzzaro, F., Poirel, L.L. and Woodford, N. (2007). CTX-M: changing the face of ESBLs in *European Journal of Antibacterial Chemotherapy,* 59, 165-174.
[50] Adebayo, A.A. & Tukur, A.L. (1999). Adamawa State in maps. First edition. Paraclete Publishers, Yola.
[51] American Public Health Association (APHA) (1999). Standard methods for examination of water and waste water, Washington. American Public Health Association (APHA), America water works Association (AWWA), Water Environment Federation (WEF). pp 1120
[52] Fawole, M.O. and Oso, B.A. (2001). Laboratory manual of Microbiology. Spectrum books L.t.d. p77.
[53] Cheesbrough, M. (2006). *District Laboratory Practice in Tropical countries*. 2nd Edition, Cambridge University Press, Cambridge, UK., ISBN-13: pp 46
[54] Schreckenberger, P.C., Janda, J.M., Wong, J.D. and Barone, E.J. (1999). *Algorithm for identification of Gram-negative bacteria. In: Manual of clinical Microbiology.* Washington: ASM press. pp. 438-441.
[55] Engelkirk, P.G., and Duben-Engelkirk, J. (2008). *Laboratory diagnosis of Infectious Diseases.* Essentials of Diagnostic Microbiology. Balti Lippincott William and Wilkins.
[56] Trindade, L.C., Marques, E., Lopes, Lopes, D.B. and Ferreira, M.A.S.V (2007). Development of a molecular method for detection and identification of *Xanthomonas compestris, Plasmopara viticola. The Official Journal of Sao Paulo State Plant Pathology Association,* 33(1):16-23.
[57] Mark, G. D. and Murrell, C.J. (2005). Stable isotope probing-linking microbial identity to function. *Nature Review Microbiology* 3(6), 499.
[58] Frank, J.A., Reich, C.I., Sharma, S., Weisbaum, J.S., Wilson, B.A and Olsen, G.J. (2008) *Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA Genes*. Applied and Environmental Microbiology. Vol. 74. pp. 2461-2470

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[59] www.appliedbiosystems.com accessed on 29th June, 2019.

[60] Paterson, D. L., Hujer, K. M., Hujer, A. M. Yeiser, B, Bonomo, M. D, Rice, L. B. and Bonomo, R.A. (2003). Extended Spectrum Beta-Lactamases in *Klebsiella pneumoniae* bloodstream isolates from seven countries: dominance and widespread prevalence of SHV- and CTX-M-type -lactamases. *Antibacterial Agents and Chemotheapy* 47:3554–3560.

[61] Khanfar, H.S., Bindayna, K.M., Senok, A.C. and Botta, G.A. (2009). Extended spectrum beta-lactamase (ESBLS) in *Escherichia coli* and *Klebsiella pneumoniae*: trend in the hospital and community settings. *Journal of infection in Developing countries* 3(4):295-299.

[62] Jarosz, D. F., Godoy, V.G., Delaney, J.C., Essigmann, J.M. and Walker, G.C. (2009). A DinB variant reveals diverse physiological consequences of incomplete TLS extension by a Y-familiy DNA polymerase. *Proceedings of the National Academy of Science United States of America.* 106, 21137-21142.

[63] Wright, G.D. (2005). Bacterial resistance to antibiotics: enzymatic degradation and modification. *Advanced Drug Delivery Reviews*, 57:1451-1470.

[64] Babic, M., Hujer, A.M. and Bonomo, R.A. (2006). What's new in antibiotic resistance? Focus on beta-lactamases. *Drug Resistance update* 9:142-156

[65] Drawz, S.M. and Bonomo, R.A (2010). Review of three decades of beta-lactamase inhibition. *Clinical Microbiology Review* 23(1):160-201

[66] Faith, M., Villaneuva, E., Thelma, D., Tupaci, G., Abiad, M.D., Benida Q., Baello, C., Rowa, C. and Crnado, D. (2003). Extended spectrum beta-lactamase production among *Escherichia coli* and *Klebsiella* spp. *Philippines Journal Microbiological Infectious Diseases* 32: 3,103-108.

[67] Meatherall, B.L., Gregson, D., Ross, T., Pitout, J.D. and Laupland, K.B. (2009). Incidence, risk factors, and outcomes of *Klebsiella pneumomiae* bacteria. *American Journal of Medicine 122*(9):866-873

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