KLEBSIELLA PNEUMONIAE: A CASE REPORT OF PNEUMONIA AND CEPHALOSPORINS RESISTANT CLINICAL ISOLATE

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ABSTRACT

Background: *Klebsiella pneumoniae* (*K. pneumoniae*) is a Gram-negative bacterium that causes respiratory, urinary tract and blood stream infections associated with community and nosocomial infections with serious health implications.

Objective: The study aimed to determine the management response of the patient, from the empirical stage until discharge. Also to identify the aetiology and evaluate antimicrobial susceptibility profile of the isolate.

Case management and methods: A case of a 55-year-old-male patient with pneumonia-like symptoms, who presented to Sheikh Muhammad Jidda Specialist Hospital, Kano, Nigeria. The patient was empirically administered with ceftazidime antibiotic, resulting in mild irritation as a symptom of side effects, with no positive response from the patient. Later, the treatment was reviewed to maxipime (1g/50 mL) infusion, which was parenterally administered twice daily for 10 days. The symptoms completely resolved, and the patient fully recovered and was discharged on day 15. Routine and specific bacteriological investigations were conducted to establish the aetiology. A positive string test was conducted on the isolate for the detection of hypermucoviscosity. A polymerase chain reaction (PCR) assay was performed to detect the *rmpA* gene associated with hypermucoviscosity that causes hypervirulence.

Results: Results showed that *K. pneumoniae* was the causative agent with resistance to generations of cephalosporins (second and third). The therapy was successful using maxipime, a fourth-generation cephalosporin (cefepime). The findings indicated that the isolate is multidrug-resistant.The formation of a viscous filament ≥5 mm, confirmed the hypermucoviscosity of the isolate. PCR gel electrophoresis results of the amplified *rmpA* gene showed ~600 bp band size and was confirmed by DNA sequencing.

Conclusion: The use of maxipime as antibiotic therapy for the treatment of the patient with pneumonia infection was successful. The isolate was resistant to second and third-generation cephalosporins. It is important to achieve accurate and prompt identification of such resistant and virulent strains to avoid community-based spread.

Keywords: *Klebsiella pneumoniae*, cephalosporin, resistance, *rmpA* gene, hypermucoviscosity

INTRODUCTION

Klebsiella pneumoniae (*K. pneumoniae*) is a human bacterial pathogen commonly associated with community and nosocomial infections, mainly bloodstream infections (BSI) and urinary

tract infections (UTI) occurring especially in intensive care units (Cubero *et al.,* 2019; Daehre *et al.,* 2018). *K. pneumoniae* is also associated with respiratory infections including pneumonia and is of critical concern (Ikeda *et al.,* 2018).

Cephalosporins are called βeta-lactam antibiotics because all the class members possess vital and active β-lactam ring in their structural formation. Cephalosporins are broad-spectrum antibiotics of natural origin obtained from fungi *Acremonium.* They work by cell wall biosynthesis inhibition, limiting the growth and spread of susceptible bacteria (Brauer *et al.,* 2021; Zhgun *et al.,* 2020).

Drug-resistant microorganisms are passed among people and animals and from one country to another without being noticed. This caused a worrying global public health issue, as infections are associated with high morbidity and mortality rates (Abayneh *et al.,* 2018). Antimicrobial resistance leads to limited therapeutic options, causing increasing difficulty in treatments (Daehre *et al.,* 2018). Emerging resistance of the cephalosporins generations among the clinical isolates, is on the rise globally, primarily associated with *K. pneumoniae* isolates, with severe consequences on the patients and public health (Surgers *et al.,* 2019). The frequent usage of second or third-generation cephalosporins antibiotics is largely responsible for the emergence of gram-negative bacteria, that are resistant to extended-spectrum cephalosporins.

Some strains of *K. pneumoniae* produce mucoid colonies on culture media that could be mucoid or hypermucoviscous phenotypes. The difference between the two phenotypes is determined based on a positive string test. The positive result of the test indicates the formation of the viscous filament (≥5 mm) after stretching colony growth of *Klebsiella* species with a wire loop on an agar plate (Catalán-Nájera *et al.,* 2017). The hypermucoviscous phenomenon highlights hypervirulence invasive infection commonly associated with kidney, prostate, bone, liver, lung abscesses and multidrug resistance (Catalán-Nájera *et al.,* 2017; Walker *et al.,* 2019).

The E-test antimicrobial gradient method combines the principle of both dilution and diffusion methods. It is to determine the minimum inhibitory concentration (MIC) value (Balouiri *et al.,* 2016). The E-test method shares similarity with the disk diffusion method. It differs in using unique plastic strips containing the antibiotic concentration gradient distributed along the strip length instead of antibiogram discs antibiotics commonly used in bacterial AST assays (Balouiri *et al.,* 2016). Extended third-generation cephalosporin (ceftazidime) showed less efficacy in treating the patient.

Case Report

A 55-year-old-male patient was attended to at the emergency unit of the hospital on 29th November 2020 (day 0) with a complaint of fever, chills, abdominal pain, cough (red currant jelly, productive sputum) and mild shortness of breath. The patient had no underline medical condition before pneumonia symptoms emergence. The patient's medical history indicated that the patient had received treatment with penicillin antibiotic elsewhere before coming to the hospital. The patient was empirically administered with ceftazidime, a third-generation cephalosporin, based on presumptive pneumonia due to Enterobacteriaceae infection, pending laboratory investigations.

The clinician requested a sputum culture and sensitivity tests after the examination. The patient's sputum sample was collected and cultured under an aseptic condition. The culture yielded bacterial growth and the Gram stain reaction was performed. The isolate was then subjected to biochemical assays and antimicrobial sensitivity testing (AST) E–test strips (Biomerixeux).

It is based on the comparison of the isolate and *K. pneumoniae* ATCC reference strain (ATCC 700603), according to Clinical Laboratory Standard Institute (CLSI) standard guidelines. Polymerase chain reaction (PCR) was performed and confirmed by DNA sequencing for the presence of the *rmpA* virulent gene. The gene is a positive regulator of extracapsular polysaccharide synthesis that confers a mucoid phenotype. It is a candidate gene causing hypermucoviscosity in *K. pneumoniae*.

Day 4: The patient returned to the hospital for follow-up and clinical evaluation. The clinician observed no marked clinical deterioration, though initial symptoms persist. The clinician, due to lack of resolution of the conditions, decided to change the empiric drug to maxipime, a cefepime-based antibiotic together with analgesic based on the laboratory diagnostic findings. On day 15, the patient returned for further clinical evaluation. All the symptoms disappeared, including the mild irritation and the patient fully recovered and was discharged.

The study aimed to isolate and identify the etiologic agent responsible for the symptoms of pneumonia, presented by the patient and to evaluate the AST profile usingthe E-test methodon the clinical isolate in comparison with ATCC strains using second, third and fourth-generations of cephalosporins antibiotics. The E-test phenotypic method showed potential for easy detection of resistant strains before molecular analysis for early therapy initiation. The study also determined the presence of the *rmpA* as a virulent gene in the *K. pneumoniae* isolate.

MATERIALS AND METHODS

Identification of isolates and their antimicrobial susceptibility testing to cephalosporins

The sputum specimen was aseptically collected from the patient and then cultured onto prepared MacConkey, and cysteine-lactose-electrolyte-deficient (CLED) agar media using a sterile wire loop and incubated at 37°C for 24 h to obtain bacterial colonies. To obtain a pure colony, the isolates were subcultured on a fresh MacConkey and CLED agar media (Ejerssa *et al.,* 2021). The isolate was identified under aerobic conditions using morphological features and biochemical tests (Gram stain reaction, motility test, indole production test and methyl red test). The same tests were also conducted on the ATCC reference strains for confirmation under the same conditions. The ATCC strain [*K. pneumoniae* (ATCC 700603)] was obtained from a supplier ATCC (ATCC, Malaysia) according to CLSI definition and recommendations. Both the test isolate and reference strains were used to evaluate the antimicrobial activity of cephalosporins [cefoxitin (FX_{30}), cefotaxime (CT_{30}), ceftazidime (TZ_{30}), and cefepime (PM_{30})]. In the E-test strip method, the antibioticswere placed face down on the plate with a higher concentration on top. The E-test MIC result was read and determined at the ellipse intersection and the corresponding growth inhibition zone (Balouiri *et al.,* 2016).

Positive string test for hypermucoviscosity detection

It is a qualitative method for the detection of hypermucoviscosity of the isolate (Walker *et al.,* 2019). Single pure colonies were cultured on prepared blood agar and Mac Conkey agar plates (Oxoid, UK). The produced colonies were studied for their ability to form viscous strings. The hypermucoviscosity of the isolate was determined based on the formation of a viscous string filament of ≥ 5mm length according to Khaertynov *et al.* (2018).

Determination of MIC using E-test method

According to the manufacturer's guidelines, the MIC of the cephalosporins E-test (Biome'rieux, Sweden) listed below was performed on unsupplemented Mueller-Hinton agar (MHA) plates.

The dried MHA plates were inoculated with 0.5 Mc Farland prepared suspensions (~1 x 10^8 CFU/ml) of the isolates and the reference strains before placing the E-test strips incubated at 35°C for 18 h. MIC concentration gradient was in µg/mL scale for FX_{30} with MIC range: 0.016 – 256 µg/ml, CT_{30} with MIC range: 0.02 – 32µg/ml, TZ_{30} with MIC range: 0.016 – 256 µg/ml and PM_{30} with MIC range: 0.016 – 256 µg/ml cephalosporins were used for AST. The MIC is where the zone of inhibition intersects the strip's edge and this was determined the following day. Gentamicin disk was used as the control antibiotic for *K. pneumoniae* isolate seeded on dried plates. The plates were incubated under the aerobic condition at 35°C for 24 h. The zone of inhibition result was read on a millimetre (mm) scale according to CLSI M100-S24.

Genomic DNA extraction

The genomic DNA extraction of *K. pneumoniae* isolate and the corresponding reference strains ATCC 700603 grown on overnight lysogenic broth cultures was conducted using QIAamp® genomic DNA mini kit according to the manufacturer's guidelines. The DNA extract concentrations were measured using a NanoDrop spectrophotometer (ThermoScientific, U.S.A) and kept at – 20°C until used.

Polymerase Chain Reaction (PCR) amplification

The PCR amplification was performed in a 200 µL PCR tube for *rmpA* gene detection in three distinct steps (denaturation, annealing and extension). The PCR reaction comprised 0.75µL (0.3 mM) of each primer, 0.75µL of Kapa 10 mM of dNTPs mixture components suspended in a 5 µL volume of Kapa 5X Hifi buffer with 1x final concentration in a total volume of 25µL of nuclease-free water per reaction. The DNA was amplified by 0.5µL (0.5 U) Kapa HiFi HotStart DNA polymerase (kit code: 2502) (Kapa Biosystem, U.S.A). Standardized extracted DNA concentrations (75 ng/µL) of the isolate' and the ATCC reference strain 700603) were used as templates for test and positive controls respectively. RNase-free water served as a negative control for the reaction. BioRad Gradient PCR machine (USA) was used for the PCR amplification. The primers were designed using SnapGene Viewer 5.3.2 software with sequences and label as: KpF 5' GGATGAATATTGATGGAGC 3' and KpR 5' TAGATGTCATAATCACACCC 3'. The primers were purchased from Bio Basic (Canada) with high-affinity purification (HAP). The cycling condition started with an initial denaturation at 94°C for 5 min; denaturation at 94°C for 1 min and annealing at 44°C for 1 min. The extension was set at 72°C for 1 min and the final extension at 72°C for 5 min. with a total number of 30 cycles. The resultant amplicons were separated on a 1.5% agarose gel using a BioRad electrophoresis machine (BioRad Laboratories, USA). Moreover, they were visualized by UV illumination light using AlphaImager® 2200 gel documentation system (Protein Simple, U.S.A).

RESULTS

Susceptibility of *K. pneumoniae* was evaluated against selected cephalosporins: one second-generation (cefoxitin), two third-generation (cefotaxime and ceftazidime) and one fourth-generation (cefepime) (**Table 1**) using the E-test method according to CLSI guidelines. *K. pneumoniae* ATCC 700603 was used as a quality control strain (positive control). It was also tested with standard gentamicin (10 µg) antibiotic.

Antimicrobial susceptibility testing (AST) of the isolate

In this study, one clinical isolate of *K. pneumoniae* obtained from the sputum sample was used for the study. The resistance breakpoints of the four cephalosporins antibiotics (cefoxitin, cefotaxime, ceftazidime and cefepime) were determined in both the clinical isolate and the ATCC reference strain for comparison.

The results indicated less activity of cefoxitin, cefotaxime, and ceftazidime on the clinical isolate with MIC values of ≥ 96 µg/ml, ≥ 32 µg/ml, and ≥ 64 µg/ml respectively. However, the cefepime indicated a slightly better activity with a MIC value of ≥ 16.0 µg/ml that falls within the CLSI breakpoints (**Figure 1**) (**Table 1**).

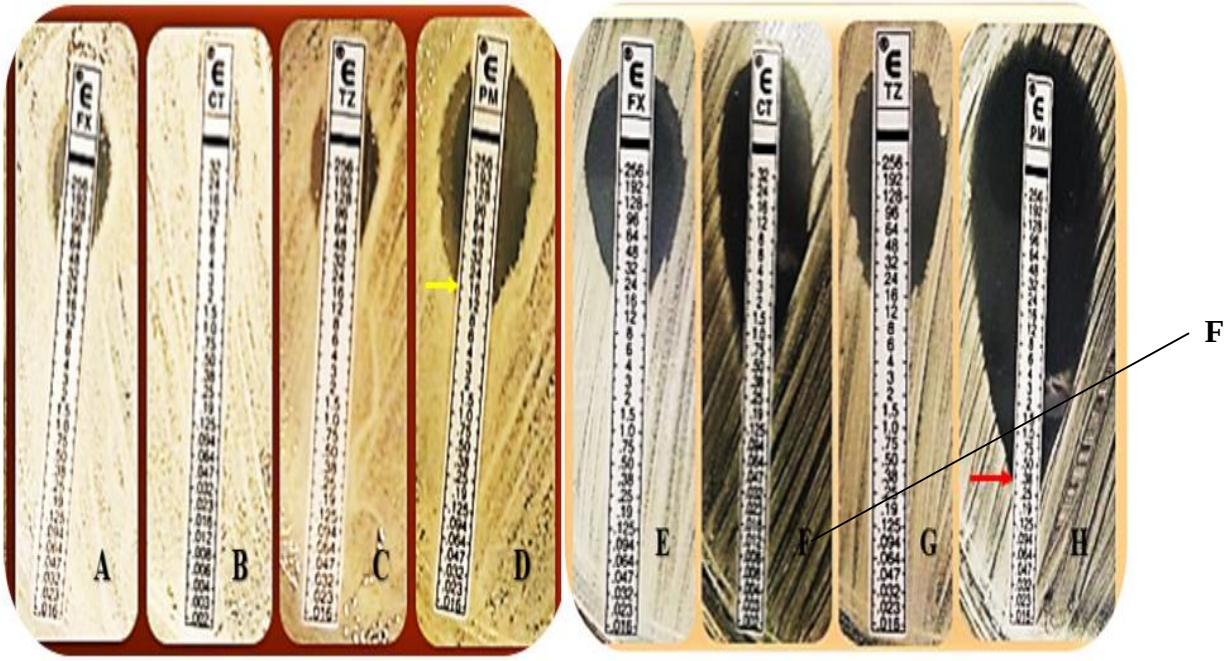

Figure 1: E-test susceptibility assay of *Klebsiella pneumoniae* clinical isolate (A – B) andATCC 700603 reference strain (C – D) using cephalosporins generations. A and E: cefoxitin; B and F: cefotaxime; C and G: ceftazidime; D and H: cefepime. Legend: Yellow and red arrowsindicated cefepime MICs for isolate and ATCC reference strain respectively.

Control strains analysis using Disc Diffusion assay

The *K. pneumoniae* ATCC 700603 strain was used as the positive control and tested using gentamicin based on the CLSI standard. Gentamicin (10 µg) was selected and purchased from Oxoid Company (Thermo Scientific™, U.S.A) for the assay. The gentamicin AST results for*K. pneumoniae* clinical isolate and reference strain were determined based onthe zone of inhibition sizes as 20- and 12mm, respectively (**Table 1**) (**Figure 2**).

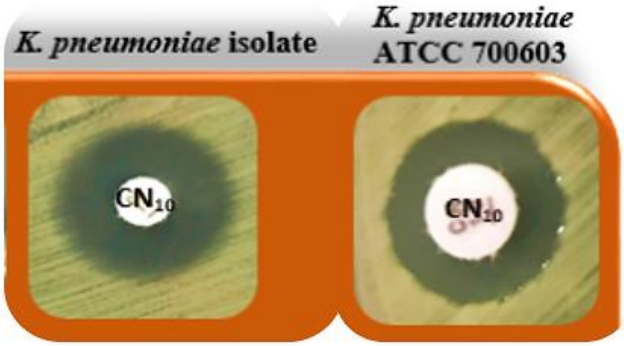

Figure 2: Control disc diffusion test on *K. pneumoniae* clinical isolate and ATCC 700603 reference strain using gentamicin (10 µg) antibiotic.

Table 1: Comparison of E-test MICs for *K. pneumoniae* clinical isolate and ATCC reference strains (CLSI 2017).

Cephalosporin generation	Drug tested	Minimal inhibitory concentration (µg mL^{-1})	
		***K. pneumoniae* isolate**	***K. pneumoniae* ATCC 700603**
2nd	Cefoxitin (FX_{30})	96	24
	Range		≤ 8 – ≥32
3rd	Cefotaxime (CT_{30})	≥ 32	1.0
	Range		≤ 1 – ≥4
3rd	Ceftazidime (TZ_{30})	64	≥24
	Range		≤ 4 – ≥ 16
4th	Cefepime (PM_{30})	16	0.38
	Range		≤ 4 – 16
Control	Gentamicin (CN_{30}) (mm)	20	12
	Range		≤ 4 – ≥15

Key: ATCC = American type culture collection; **E-test =** Epsilon test

Molecular analysis

All the extracted DNAs from both clinical isolates and reference bacterial strains were subjected to PCR assays using gene-specific primers. The resultant amplicons were visualized on 1.5% agarose gel as described above. The *rmpA* gene was also confirmed in both *K. pneumoniae* clinical isolate and *K. pneumoniae* ATCC 700603 reference strain (positive control). It is a hypermucoid virulent gene that regulates extracapsular polysaccharide synthesis and can positively control the mucoid phenotype of *K. pneumoniae* (Yu *et al.*, 2006). The gene size according to the gel was ~600 bp. The actual size of the *rmpA* gene (595 bp) was confirmed by DNA sequencing analysis.

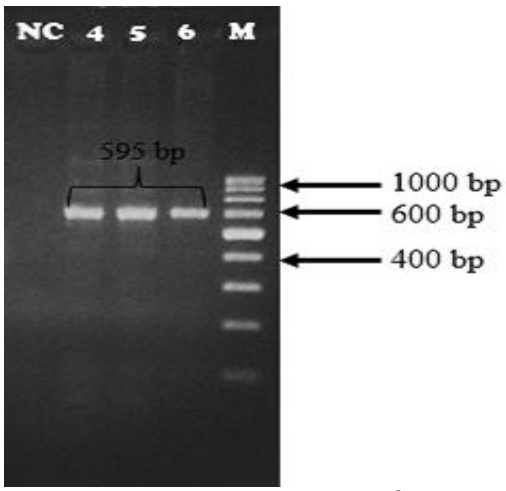

Figure 3: PCR gel electrophoresis to detect *rmpA* gene from *K. pneumoniae.* Legend: Lane NC: negative control; Lanes: 4 and 5: *K. pneumoniae* clinical isolate*;* Lane 6: *K. pneumoniae* ATCC 700603 (positive control): Lanes M: 1 kb ladder (100 bp).

DISCUSSION

The recent increasing detection of β-lactamase-producing Gram-negative bacilli has raised the alarm over resistance to β-lactam antibiotics (Saha and Jhora, 2018). Antimicrobial resistance due to multidrug resistance strains is emerging primarily from the genera: *Mycobacterium tuberculosis, Enterococcus faecium, Enterobacter cloacae, K. pneumoniae, S. aureus, Acinetobacter baumanii,* and *Pseudomonas aeruginosa* (Gashe *et al.,* 2018). It has been documented that treatment using higher-generation cephalosporins is more efficacious and often shows less resistance, particularly in *Enterobacteriaceae* (Dolinsky, 2017). Early detection of such isolates is critical for preventing its outbreak in affected facilities (Saha and Jhora, 2018).

Therefore, in this study, we determined cephalosporin resistance, hypermucoviscosity, and the *rmpA* virulence gene in *K. pneumoniae* clinical isolate from a sputum sample of a patient.

Our findings on *K. pneumoniae* isolate indicated less activity of cefoxitin, cefotaxime, and ceftazidime on the clinical isolate with high MIC values respectively. Thus, *K. pneumoniae* was resistant to all the second and third generations of cephalosporins except the fourth generation cefepime. In comparison with the ATCC reference strain (*K. pneumoniae* 700603), cefepime and cefotaxime had the lowest MIC values of 0.38 µg/ml and ≤ 1.0 µg/ml respectively. They corresponded to a sensitivity of established MIC breakpoints provided by CLSI (Dolinsky, 2017). These findings corroborated Abayneh *et al.* (2018) study, that isolates demonstrating resistance to cefepime showed resistance to all other cephalosporins generations. Our findings showed increasing resistance rates to cephalosporins generations, particularly third and fourth cephalosporins by the *K. pneumoniae* isolates. These findings are in concordance with Kim *et al.* (2017) that reported increasing resistance from 2013 to 2015 in Korea. Accordingly, their findings differed from ours by observing a decrease in cefoxitin resistance.

The resistance rates among *Enterobacteriaceae,* particularly *K. pneumoniae,* to most antimicrobial agents, comprising third-and fourth-generation cephalosporins and fluoroquinolone, are alarming (Kim *et al.,* 2017; Latifpour *et al.,* 2016). Extended-Spectrum β-lactamases (ESBLs) is an enzyme that facilitates resistance to narrow-spectrum antibiotics. It acts on penicillin and first and second cephalosporins via antibiotics hydrolysis (El-Jade *et al.,* 2016). The ESBLs production is the common mechanism of resistance to third-generation cephalosporins among *Enterobacteriaceae* including *K. pneumoniae* and may cause therapeutic challenges in managing infections (Enyinnaya *et al.,* 2021). Our findings revealed that the isolate may likely be an ESBL producer (cefotaxime:≥ 32 µg/ml, and ceftazidime: ≥ 64 µg/ml) since the ESBL results as a presumptive diagnosis were considered positive if the isolates had a MIC (µg/ml) of ≥0.5 for cefotaxime and ≥1 for ceftazidime as reported by Enyinnaya *et al.* (2021). However, confirmatory analysis was not conducted on the isolate to ascertain the ESBL production. According to Enyinnaya *et al.* (2021) findings, 114 (100%) *K. pneumoniae* isolates were resistant to cefotaxime and 105(92.1%) were resistant to ceftazidime and they were ESBL producers. Moreover, the variability of ESBL enzymes results in the difference in the degree of resistance among *Enterobacteriaceae* species, causing difficulty in the antibiotic selection and delaying treatment initiation (Saha and Jhora, 2018). Perhaps that could be the failure of the third generation cephalosporins (ceftazidime) as empiric treatment of the infection administered to the patient.

The current resistance pattern extends to third-generation cephalosporin antibiotics (e.g., cefotaxime, ceftriaxone and ceftazidime) and monobactams (aztreonam).

Our findings are in good agreement with Wikaningtyas and Sukandar (2016) that the isolate was resistant to cefoxitin, (cefotaxime and ceftazidime) being second and third-generation cephalosporins respectively. However, our finding indicated total resistance to cefoxitin with a MIC value of ≥ 96 µg/ml was contrary to the findings of Wikaningtyas and Sukandar, (2016) that reported negligible effect on cephamycins (cefotetan and cefoxitin) or carbapenems (imipenem and meropenem) of their isolates. Treatment of infections caused by *K. pneumoniae* is increasingly becoming a problem due to resistance emergence through ESBL production (Abayneh *et al.,* 2018).

The presence of two transcriptional regulators associated with plasmid mediation, known as a regulator of mucoid phenotype A (*rmpA*) and *rmpA2*, can induce capsule production in *K. pneumoniae* PHO (Paczosa & Mecsas, 2016). According to Walker *et al.* (2019), the *rmpA* gene detected was linked to hypermucoviscosity in hypervirulent *K. pneumoniae* as well as capsule gene expression. This is in agreement with our findings that confirmed the presence of the *rmpA* gene as a hypervirulent factor in the isolate. The presence of this gene in *K. pneumoniae* strains in a patient suggests virulence that needs a prompt response for proper management of patients (Ikeda *et al.,* 2018).

The E-test has the advantage of having a stable gradient even in the higher inoculum of bacteria, enabling precise MIC values reading (Giuliano *et al.,* 2019). Saha and Jhora (2018) viewed that E-test is a technically straightforward, versatile and rapid method for ESBL detection and is a suitable alternative to the conventional agar dilution test. This finding is in agreement with the findings of the study conducted by Saleem *et al.* (2022) that out of the seven sputum samples examined, the E-test differentiated three ESBL isolates from the four ESBL negative isolates. Although E-test is user-friendly and easy to perform, it is expensive when many drugs are to be tested since each strip costs about $2 – 3 per antibiotic as in concordance with a report by Balouiri *et al.*(2016); Giuliano *et al.* (2019). The study aimed to assess the case management response and to determine the clinical isolates' susceptibility to the supposedly effective cephalosporins generations. The study also observed the E-test strips' relevance as a potential alternative for easy detection of resistant bacterial strains in hospital settings.

CONCLUSION

In conclusion, the patient harboured the cephalosporins-resistant *K. pneumoniae* clinical isolate. Despite the relatively high MIC value (16 µg/ml) associated with cefepime, the patient's treatment with maxipime was successful. The isolate was resistant to the second and third cephalosporins antibiotics tested. The detection ofthe *rmpA* hypermucoviscosity virulent gene in *K. pneumoniae* isolate may likely contribute to the observed cephalosporins resistance. The presence of a positive string test represents the greatest preliminary approach to identifying hypervirulent clinical isolates. The PCR *rmpA* gene identification confirmed the diagnosis of the virulent strains. The E-test method served as a rapid alternative test for susceptibility evaluation of clinical isolates for clinical decisions in the management of patients. The rapid global emergence and spread of hypervirulent and multidrug-resistant strains require a quick response in the appropriate detection and treatment for public health safety. Improved health and infection control measures are critical for public health safety.

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