National sentinel site surveillance for antimicrobial resistance in *Klebsiella pneumoniae* isolates in South Africa, 2010 - 2012

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Background. The increasing rates of antimicrobial resistance observed in the nosocomial pathogen *Klebsiella pneumoniae* are of major public health concern worldwide.
Objectives. To describe the antibiotic susceptibility profiles of *K. pneumoniae* isolates from bacteraemic patients submitted by sentinel laboratories in five regions of South Africa from mid-2010 to mid-2012. Molecular methods were used to detect the most commonly found extended-spectrum beta-lactamase (ESBL) and carbapenemase resistance genes.
Methods. Thirteen academic centres serving the public healthcare sector in Gauteng, KwaZulu-Natal, Free State, Limpopo and Western Cape provinces submitted *K. pneumoniae* isolates from patients with bloodstream infections. Vitek 2 and MicroScan instruments were used for organism identification and susceptibility testing. Multiplex polymerase chain reactions (PCRs) were used to detect $bla_{\text{CTX-M}}$, bla_{SHV} and bla_{TEM} genes in a proportion of the ESBL isolates. All isolates exhibiting reduced susceptibility to carbapenems were PCR tested for bla_{KPC} and $bla_{\text{NDM-1}}$ resistance genes.
Results. Overall, 68.3% of the 2 774 isolates were ESBL-positive, showing resistance to cefotaxime, ceftazidime and cefepime. Furthermore, 46.5% of all isolates were resistant to ciprofloxacin and 33.1% to piperacillin-tazobactam. The major ESBL genes were abundantly present in the sample analysed. Most isolates (95.5%) were susceptible to the carbapenems tested, and no isolates were positive for bla_{KPC} or $bla_{\text{NDM-1}}$. There was a trend towards a decrease in susceptibility to most antibiotics.
Conclusion. The high proportion of ESBL-producing *K. pneumoniae* isolates observed, and the prevalence of ESBL genes, are of great concern. Our findings represent a baseline for further surveillance in SA, and can be used for policy and treatment decisions.

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The increasing prevalence of serious hospital- and community-acquired infections is of great concern, with patients dying as a result of emerging antimicrobial resistance. Antibiotic-resistant organisms are widespread globally, both in developed and developing countries.[1] These organisms are a major threat to public health, reduce the effectiveness of empiric antimicrobial treatment options, and increase morbidity, mortality and healthcare expenditure. A national surveillance system for antimicrobial resistance is essential to establish a baseline of the extent of the problem, to follow trends of resistance, and to form the basis for recommendations of appropriate antimicrobial use to clinicians and other healthcare providers. A

national system also controls for differences in case selection, data management and demographic descriptions of regional populations. To meet this need, a national laboratory-based antimicrobial resistance surveillance system for nosocomial pathogens was established in 2010, which included *Klebsiella pneumoniae* as a sentinel organism by which to monitor resistance.

K. pneumoniae is an important nosocomial pathogen, with the highest prevalence of resistance to third- and fourth-generation cephalosporins among the Enterobacteriaceae. The spread of class A or group 2be extended-spectrum beta-lactamases (ESBLs) in Enterobacteriaceae is of public health concern. The most frequently detected and clinically important ESBLs belong to the TEM, SHV, and CTX-M families, and *K. pneumoniae* commonly produces all three groups of enzymes.[2] In the past decade, CTX-M enzymes have emerged as the most prevalent type. There are more than 100 different types, which can be broadly divided into five groups based on their amino-acid identities: CTX-M1, CTX-M2, CTX-M8, CTX-M9 and CTX-M25. Enzymes are characterised by epidemiological differences, and some have even been shown to spread beyond the hospital environment into the community.[2] Of further public health concern are the recent emergence of *K. pneumoniae* strains capable of producing carbapenem-hydrolysing enzymes and the apparent ease of spread of resistance mechanisms by mobile genetic elements.[2]

There are few published reports on national antimicrobial resistance rates of *K. pneumoniae* in South Africa (SA), with data primarily emanating from the private sector,[3] regional studies with limited numbers of local isolates[1] or certain clinical settings.[4] To date, molecular data on ESBL *K. pneumoniae* in SA are based on detailed studies of small populations. Essack *et al.*[5] examined bla_{SHV} and bla_{TEM} genes in 25 isolates, and found a high degree of diversity in terms of plasmids present, genes detected and the combination of genes within isolates. In a study of ESBL *K. pneumoniae* from seven countries in 2003, bla_{TEM-10}, bla_{TEM-12}, bla_{TEM-63}, various bla_{SHV-2} and bla_{SHV-5} types, bla_{CTX-M2} and bla_{CTX-M3} genes were detected in 27 SA isolates.[6] A more recent study on 53 ESBL clinical pathogens from Pretoria detected bla_{TEM}, bla_{SHV} and $bla_{CTX-M-1}$ genes, although the contribution of the 31 *K. pneumoniae* isolates in this group was not specified.[7] This diversity in enzyme production and the prolific nature of *K. pneumoniae* as a nosocomial pathogen highlight the need for further investigation into the genes responsible for these enzymes in the SA setting.

In this study, we determined the antibiotic susceptibility profiles of *K. pneumoniae* isolates from bacteraemic patients with positive blood cultures from sentinel sites representing five SA regions. We used national laboratory-based surveillance data to characterise the third- and fourth-generation cephalosporin-resistant and carbapenem-resistant phenotypes and genotypes of *K. pneumoniae* from 2010 to 2012.

Methods

Patient selection

Thirteen academic centres serving the public healthcare sector in SA were included, and participation was voluntary. The sites represented regions in Gauteng, KwaZulu-Natal, Free State, Limpopo and Western Cape provinces. Isolates of *K. pneumoniae* from patients with bloodstream infections were submitted, with a 3-week exclusion thereafter to avoid duplicate isolates of the same organism.

Phenotypic methods

K. pneumoniae isolates were submitted on Dorset transport media. Organism identification was confirmed using the Vitek 2 GN card (Biomerieux, France). Susceptibility testing and determination of ESBL phenotype was performed on the MicroScan Walkaway (Siemens Healthcare Diagnostics, USA), using NM37 panels. Categorical results and susceptibility profile of each antimicrobial agent tested were based on 2012 Clinical Laboratory Standards Institute (CLSI) interpretative criteria,[8] the European Committee on Antimicrobial Susceptibility Testing guidelines[9] and/or the MicroScan recommendations. The MIC_{50} and MIC_{90} (minimum inhibitory concentrations needed to inhibit the growth of 50% and 90% of organisms, respectively) were determined. The Agresti-Coull interval method was used to calculate confidence intervals, and a χ^2 test was performed to analyse trends in antibiotic susceptibility. A p-value of <0.05 was deemed statistically significant.

Genotypic methods

Two hundred and seventy ESBL-producing isolates were randomly selected (approximately 14% per region) and screened for the presence of ESBL genes. DNA was extracted from half a loop (~2 mm in diameter) of bacterial culture. This was resuspended in 400 µl tris-ethylenediaminetetraacetic acid buffer (pH 8.0), vortexed briefly, heated at 95°C for 25 minutes and pelleted by centrifugation. The supernatant was aliquoted and stored at –70°C for further use.

The LightCycler 480 instrument (Roche Applied Science, Germany) and LightCycler 480 Probes Master kit (Roche Diagnostics, USA) were used for real-time polymerase chain reaction (PCR). The bla_{TEM} and bla_{SHV} genes were amplified by multiplex real-time PCR using the primers shown in Table 1. The primers were selected for specificity by GenBank comparisons and PCR products from control strains were sequenced. The limit of detection was determined to be 750 colony-forming units (cfu)/ml for bla_{TEM} and 4 000 cfu/ml for bla_{SHV}. The reaction conditions were 0.5 µM primers, 0.2 µM probes, denaturation for 95°C for 5 minutes, and then 45 cycles of 95°C for 10 seconds, 55°C for 30 seconds and 72°C for 1 second. The bla_{CTX-M} PCR was a multiplex assay targeting bla_{CTX-M} groups M1 and M2-9 using primer and probe sequences as described previously.[10] Detection of bla_{NDM-1} and bla_{KPC} genes was performed on isolates with reduced susceptibility to carbapenems as described previously.[11] Reduced susceptibility to carbapenems was defined according to the CLSI guidelines of 2012.[8] Strains from the

Table 1. Primer and probe sequences for bla_{TEM} and bla_{SHV} gene detection

SHV Forward Primer	5′-CAG CAG GAT CTG GTG GAC TAC T-3′
SHV Reverse Primer	5′-GTC AAG GCG GGT GAC GTT-3′
SHV A Primer	5′-AAG GCG GGT GAC GTT GTC-3′
SHV S Primer	5′-CCG GTC AGC GAA AAA CAC-3′
SHV Probe	5′-Cy5-TCT GGC GCA AAA AGG CAG TCA-BBQ-3′
TEM Forward Primer	5′-AAG TTC TGC TAT GTG GTG CGG TA-3′
TEM Reverse Primer	5′-TGT TAT CAC TCA TGG TTA TGG CAG C-3′
TEM A Primer	5’-GTA AGA TGC TTT TCT GTG ACT GGT GA-3′
TEM S Primer	5′-AGT TCT GCT ATG TGG TGC GGT ATT A-3′
TEM Probe	5′-FAM-TGC GGC GAC CGA GTT GCT CTT-BBQ-3′

American Type Culture Collection (ATCC) or the National Culture Collection Laboratory of the National Institute for Communicable Diseases were used as positive controls.

Results

A total of 2 774 discrete *K. pneumoniae* isolates were received and included in the antimicrobial susceptibility testing over the 3-year period. The majority of isolates demonstrated an ESBL phenotype (68.3%) with marked resistance to third- and fourth-generation cephalosporins. The distribution of isolates according to province, and ESBL rates, are shown in Table 2. A breakdown of susceptibility by agent for the 3-year period is presented in Fig. 1.

Reduced carbapenem susceptibility was noted in only 124 (4.5%) of isolates (Table 2). The carbapenemase genes bla_{NDM-1} and bla_{KPC} were not detected in any of the 124 isolates and the presence of other carbapenemase-producing genes was not investigated.

Susceptibility to aminoglycosides over the 3-year period was variable, with 95.3% susceptible to amikacin but only 31.1% and 31.3% susceptible to tobramycin and gentamicin, respectively (Fig. 1). Susceptibility to levofloxacin (75.2%) was higher than to ciprofloxacin (53.5%). Sixty-seven (66.9%) per cent of all isolates were susceptible to piperacillin-tazobactam (MIC_{50} = 8 µg/ml) for the 3 years, and susceptibility to cefoxitin was high at 87.4% (Table 3).

Trend analysis was performed on the isolates, comparing the susceptibility rates between 2010, 2011 and 2012 (Table 4). Small but statistically significant declines in susceptibility rates were noted for many antibiotics, including amoxicillin/clavulanate, tobramycin, gentamicin, ciprofloxacin, ceftazidime and cefotaxime.

Molecular characterisation was performed on 270 ESBL-positive isolates. All phenotypically ESBL-positive isolates were confirmed to possess one or more of the bla_{CTX-M}, bla_{SHV} and bla_{TEM} genes, with 93.0% of the isolates tested expressing more than one resistance gene (Fig. 2).

Table 2. Provinces of submission of *K. pneumoniae* isolates 2010 - 2012 and proportion of isolates found to be ESBL-positive or to have reduced susceptibility to carbapenems

Province	Isolates submitted 2010 - 2012, *N*	ESBL-positive isolates 2010 - 2012, *n* (%)	Reduced carbapenem susceptibility, *n* (%)
Gauteng	1 737	1 207 (69.5)	75 (4.3)
Western Cape	620	414 (66.8)	25 (4.0)
KwaZulu-Natal	268	203 (75.7)	15 (5.6)
Free State	134	59 (44.0)	7 (5.2)
Limpopo	15	12 (80.0)	2 (13.3)
Totals	2 774	1 895 (68.3)	124 (4.5)

ESBL = extended-spectrum beta-lactamase.

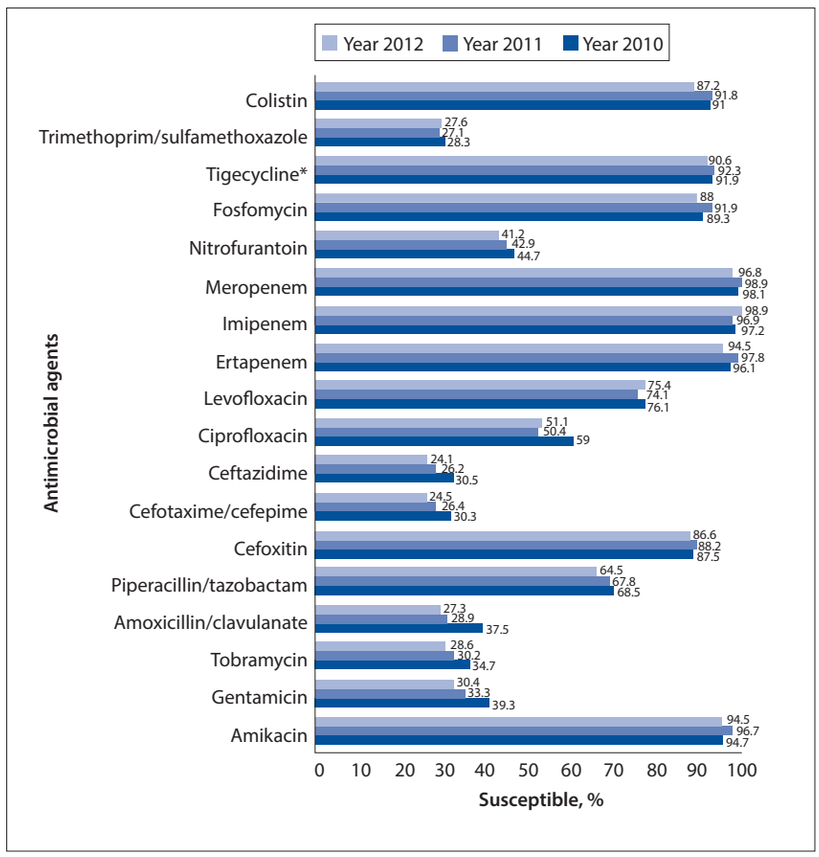

*Fig. 1. Percentage susceptibility to antimicrobial agents, 2010 - 2012. Confidence intervals were a maximum of ±2% for all antibiotics for each year. Susceptibility percentages for cefotaxime and cefepime were identical. (*European Committee on Antimicrobial Susceptibility Testing guidelines.[9])*

Discussion

Gauteng contributed the majority of isolates in the study (62.6%), probably because it is the most populous province with the largest academic centres. The high percentage of bacteraemic *K. pneumoniae* that were resistant to third-generation cephalosporins (68.3%) is of serious public health concern. Rates of resistance in a 2006 study from SA private laboratories showed 52% resistance to cefuroxime, 46% to ceftriaxone and 44% to cefepime.[3] A study of resistance at seven public sector hospitals between 2010 and 2012 reported an overall level of ESBL detection of 65%.[12] Our study shows a similar rate (68.3%) of resistance to extended-spectrum cephalosporins. The difference between the private and public sectors may indicate a dramatic increase in development of resistance in the interim, and/or a difference in sampling and resistance patterns between these sectors. Also, because they are referral centres, the academic centres that submitted isolates in this study potentially contributed a disproportionate number of patients who were more likely to harbour ESBL isolates. Globally, reports show a trend towards an increase in resistance of *K. pneumoniae* to third-generation cephalosporins.[13] While rates of resistance are low in some countries,

Table 3. Antibiotic MIC_{50}, MIC_{90} and breakpoints for 2 774 *K. pneumoniae* isolates

	2010		2011		2012		MIC interpretive breakpoints (μg/ml)*	
Antibiotics	**MIC_{50}**	**MIC_{90}**	**MIC_{50}**	**MIC_{90}**	**MIC_{50}**	**MIC_{90}**	**S**	**R**
Amikacin	≤8	16	≤8	16	≤8	16	≤16	≥64
Gentamicin	>8	>8	>8	>8	>8	>8	≤4	≥16
Tobramycin	>8	>8	>8	>8	>8	>8	≤4	≥16
Amoxicillin/ clavulanate	16/8	>16/8	16/8	>16/8	16/8	>16/8	≤8/4	≥32/16
Piperacillin/ tazobactam	≤8	>64	≤8	>64	≤8	>64	≤16/4	≥128/4
Cefoxitin	≤8	16	≤8	16	≤8	16	≤8	≥32
Cefotaxime	>32	>32	>32	>32	>32	>32	≤1	≥4
Ceftazidime	16	>16	16	>16	>16	>16	≤4	≥16
Cefepime	>16	>16	>16	>16	>16	>16	≤8	≥32
Ciprofloxacin	≤0.5	>2	≤0.5	>2	1	>2	≤1	≥4
Levofloxacin	≤1	>4	≤1	>4	≤1	>4	≤2	≥8
Ertapenem	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≥2
Imipenem	≤2	≤2	≤2	≤2	≤2	≤2	≤1	≥4
Meropenem	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≥4
Nitrofurantoin	64	>64	64	>64	64	>64	≤32	≥128
Fosfomycin	≤32	>32	≤32	>32	≤32	>32	≤64	≥256
Tigecycline†	≤1	≤1	≤1	≤1	≤1	≤1	≤1	>2
Trimethoprim/ sulfamethoxazole	>4/76	>4/76	>4/76	>4/76	>4/76	>4/76	≤2/38	≥4/76
Colistin†	≤2	≤2	≤2	≤2	≤2	4	≤2	≥2

MIC_{50} = minimum inhibitory concentrations needed to inhibit the growth of 50% of organisms; MIC_{90} = minimum inhibitory concentrations needed to inhibit the growth of 90% of organisms; S = susceptible; R = resistant.
*Clinical Laboratory Standards Institute guidelines, 2012.[8]
†European Committee on Antimicrobial Susceptibility Testing guidelines.[9]

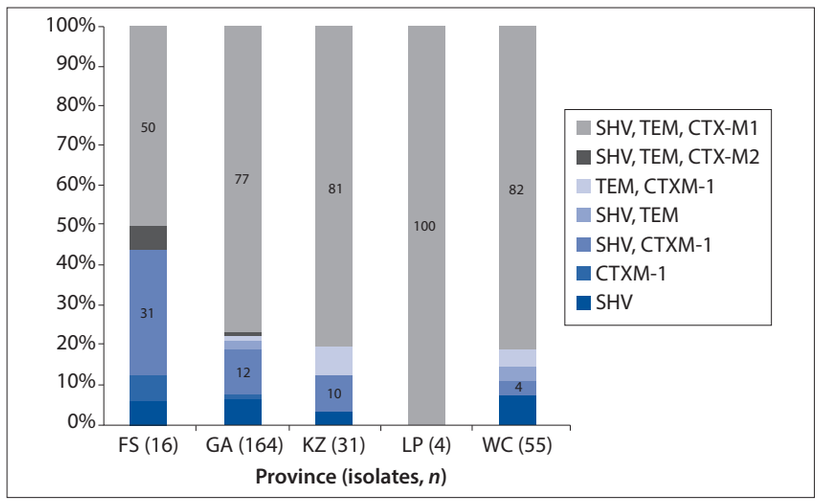

Fig. 2. Distribution of ESBL genes as percentages in 270 isolates tested. Percentages of the most frequent combinations are shown as data labels. (ESBL = extended-spectrum beta-lactamase; FS = Free State; GA = Gauteng; KZ = KwaZulu-Natal; LP = Limpopo; WC = Western Cape.)

e.g. 11.5% in 2010 in the USA,[13] higher rates have been observed in other regions.[14]

This laboratory-based surveillance also underlines the endemic distribution of all three major groups of ESBL genes throughout five regions of SA. The almost identical susceptibility pattern to cefotaxime, ceftazidime and cefepime suggests that there are multiple copies of several ESBL genes in the sample, which is supported by the genotypic results. A limitation of the study is that the genotypic data represent only 14% of *K. pneumoniae* isolates from selected academic or referral laboratories, and may not be fully representative at a national level. The phenotypic data support the genotypic data, however, being fairly representative of all isolates sampled in this survey. These results are also a first indication of the extensive prevalence of bla_{SHV}, bla_{TEM} and bla_{CTX-M} genes in geographically distinct regions of SA.

Susceptibility to carbapenems was high, with only 4.5% of isolates showing reduced susceptibility. However, carbapenem-resistant Enterobacteriaceae have been described in SA,[15] and levels of carbapenem resistance need to be monitored closely. It must also be noted that the breakpoints for ertapenem

have since been revised by the CLSI.[8] A limitation of this study is that we only tested for bla_{NDM-1} and bla_{KPC} genes and did not investigate for other potential mechanisms of carbapenem resistance.

For piperacillin-tazobactam, the MIC_{50} was within the susceptible breakpoint while the MIC_{90} was above the resistant breakpoint. Overall susceptibility was 66.9%, and piperacillin-tazobactam may therefore be a potentially useful agent for treatment. However, there is still debate concerning the use of piperacillin-tazobactam to treat infections with ESBL-producing Enterobacteriaceae. There is some evidence that outcomes may be worse if the piperacillin-tazobactam MIC is 8 - 16 mg/l, with suggestions that if piperacillin-tazobactam is used to treat organisms with these MICs, prolonged infusions or more frequent dosing may be needed.[16]

The 87.4% susceptibility to cefoxitin suggests that plasmid-mediated AmpC enzymes are not particularly prevalent in this sample. The disparity in ciprofloxacin and levofloxacin resistance (21.7% difference) is intriguing and although we do not have genotypic data to support it, it may represent an increase in quinolone resistance mechanisms (e.g. plasmid-mediated mechanisms; enzymatic modification) other than the conventional target site mutations, where one would expect cross-resistance[17] as 61.5% ESBL of *K. pneumoniae* isolates were resistant to ciprofloxacin.

Among all aminoglycosides, amikacin exhibited the best activity, although this must be interpreted with caution as the stipulated breakpoints are high considering that this is a concentration-dependent agent. The MIC_{50} and MIC_{90} of 8 μg/ml and 16 μg/ml, respectively, indicate that for many isolates the probability of achieving adequate serum levels is quite low.

Overall, the MIC_{50} and MIC_{90} to most agents have not changed over the 3-year period, although the ability to detect smaller differences may have been limited by the methodological system employed for susceptibility testing. The one exception was colistin, for which a significant increase in MIC_{90} was noted in 2012 (p=0.001) (Table 4). This is alarming, as colistin represents the mainstay of the treatment of most of our extensively drug-resistant Gram-negative pathogens (including carbapenem-resistant *K. pneumoniae).*[18] Trends in susceptibility showed a significant decrease over the study period for most antimicrobial agents, with a few exceptions, e.g. piperacillin-tazobactam, levofloxacin and tigecycline.

Table 4. Statistical significance of antibiotic susceptibility rates for *K. pneumoniae*, 2010 - 2012

	p-value*		
Antibiotics	**2010 v. 2011**	**2011 v. 2012**	**2010 v. 2012**
Amikacin	**0.041**	**0.012**	0.908
Gentamicin	**0.013**	0.145	**<0.001**
Tobramycin	0.056	0.428	**0.015**
Amoxicillin/clavulanate	**<0.001**	0.397	**<0.001**
Piperacillin/tazobactam	0.713	0.105	0.103
Cefepime	0.087	0.295	**0.014**
Cefoxitin	0.668	0.252	0.616
Ceftazidime	0.058	0.272	**0.007**
Ciprofloxacin	**0.001**	0.749	**0.003**
Levofloxacin	0.37	0.481	0.771
Ertapenem	**0.05**	**<0.001**	0.166
Imipenem	0.774	**0.002**	0.016
Meropenem	0.14	**<0.001**	0.141
Nitrofurantoin	0.488	0.42	0.194
Fosfomycin	0.076	**0.003**	0.468
Tigecycline	0.736	0.15	0.417
Trimethroprim/sulfamethoxazole	0.602	0.768	0.796
Colistin	0.591	**<0.001**	**0.028**

*Statistically significant values in bold.

A limitation of this study was that comprehensive patient demographic details were unavailable, and it was not possible to determine accurate trends in patient age, ward and gender. This information will be obtained in future surveys, allowing for more detailed analyses of antimicrobial resistance patterns, and how they change over time.

These surveillance results should be used together with hospital-specific data as tools in antimicrobial stewardship programmes and for the development of empiric therapy guidelines for healthcare-associated infections. Ongoing antimicrobial resistance surveillance, performed in a systematic and standardised manner, could be used as a tool to monitor the effectiveness of antimicrobial stewardship programmes that have been implemented in various centres across the country. Moreover, it provides a foundation for the systematic surveillance of important hospital pathogens that could be expanded and enhanced over time.

Conclusion

SA appears to have a relatively high percentage of ESBL-producing *K. pneumoniae* isolates in comparison with other geographical regions, which is of great concern, and a significant increase in ertapenem resistance over the surveillance period. This study presents the antibiotic resistance patterns of invasive *K. pneumoniae* isolates and gives an indication of the prevalence of resistance genes. Our findings provide important baseline data for further site-specific analysis of *K. pneumoniae* isolates, as well as a platform for enhanced surveillance of *K. pneumoniae* antimicrobial resistance in the country. Additionally, when analysed in conjunction with patient demographic and clinical details these data are important for the development of empiric therapy guidelines for management of sepsis in SA healthcare institutions.

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