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Introduction: Management of infections with carbapenem-resistant Enterobacteriaceae (CRE) is challenging. In recent times, agents such as colistin and fosfomycin have been used in combination with other antibiotics to treat such infections. In this study, we aim to seek frequency of colistin and fosfomycin resistance in CRE from Pakistan.

Methods: This study was conducted at clinical laboratories, Aga Khan University Hospital. In total, 251 CRE were included in the study. Colistin minimum inhibitory concentrations (MICs) were performed using broth microdilution (BMD) method and VITEK® 2 system, whereas fosfomycin susceptibility was performed using Kirby–Bauer method. MIC₅₀ and MIC₉₀ were calculated for colistin and agreement between VITEK and BMD was also calculated.

Results: Out of 251 strains colistin MIC of ≥4 µg/mL was seen in 40 (15.9%). Of these strains 20 (50%) were *Klebsiella pneumoniae*. Colistin MIC₅₀ and MIC₉₀ were found to be 0.5 and 16 µg/mL, respectively. BMD and VITEK 2 showed 100% categorical agreement. Essential agreement was 88.5% with kappa score 0.733 indicating strong agreement between VITEK and BMD. 31 out of 251 (12.3%) CREs were resistant to fosfomycin.

Conclusion: Study shows frequency of colistin and fosfomycin resistance to be 15.9% and 12.3%, respectively. In countries where rate of CREs is high, emerging resistance against these last resort antibiotics is alarming as it leaves clinicians with almost no options to manage such multidrug resistant and extensively drug resistant infections.

Keywords: emerging drug resistance, colistin resistance, fosfomycin resistance, carbapenem resistant enterobacteriaceae, salvage antibiotics

Introduction

Carbapenem-resistant Enterobacteriaceae (CRE) are increasingly being identified worldwide and pose a major threat to the treatment of infectious diseases and to infection control within health care facilities.¹ In 2014, a meta-analysis showed mortality rates attributable to CRE to be 26%–44%.² Frequency of CRE is increasing worldwide.^{3,4} In Pakistan, the rates have been increasing steadily, data from our hospital showed increase in carbapenem-resistant *Escherichia coli*, *Enterobacter* spp. and *Klebsiella pneumoniae* from 5%, 7% and 15% in 2013 to 6%, 23% and 22% by 2015, respectively.⁵

With increasing antimicrobial resistance and given the paucity of new drugs, the focus of management has shifted to older agents.⁶ Currently, such antibiotics in use in Pakistan include colistin and fosfomycin. It has earlier been shown that treatment of CRE with combination of carbapenem and colistin is associated with better outcomes⁷ leading to increased use of colistin in clinical practice.⁸ Fosfomycin has traditionally been used to treat urinary tract and gastrointestinal infections. A meta-

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analysis including 62 studies, showed utility of fosfomycin either alone or in combination with other antibiotics for the treatment of pneumonia, osteomyelitis, meningitis, ear, nose, throat infections and gynecological infections,⁹ hence, identifying fosfomycin as having potential for treatment of invasive infections. In February 2010, intravenous (IV) fosfomycin was prospectively evaluated for the treatment of carbapenem-resistant *K. pneumoniae* wherein all patients had bacteriological clearance with no adverse reactions.¹⁰

Given rising antimicrobial resistance and reports of CRE, this study was designed to evaluate the frequency of colistin and fosfomycin resistance in carbapenem-resistant Gram-negative bacterial infections in our population.

Materials and methods

Study setting

This was a prospective study conducted on archived bacterial isolates from clinical specimens received in the Clinical Microbiology Laboratory, Aga Khan University. The archived strains were stored at -80°C in glycerol phosphate broth and were revived on MacConkey agar before use.

Bacterial isolates

All CRE isolated from January 2015 to August 2016 were included. CREs were defined using CLSI 2015 cut-offs for ertapenem, imipenem or meropenem.¹¹ Identification of strains included in the study was done using API 20E system. Strains with inherent resistance to colistin such as those belonging to genus *Proteus*, *Providencia* and *Serratia* were excluded from the study.

- Susceptibility testing: Carbapenem susceptibilities were performed by disc diffusion (Kirby–Bauer) method on Mueller Hinton agar¹¹ and by VITEK 2 system.¹² Isolates were labeled as CREs by disc diffusion zone diameters and by VITEK minimum inhibitory concentrations (MICs). For interpretation CLSI 2015 breakpoints for imipenem, ertapenem and meropenem, that is, for ertapenem disc diameter ≤ 19 mm or MIC ≥ 1 , for imipenem and meropenem disc diameter ≤ 21 mm or MIC ≥ 2 were used.¹¹
- Colistin susceptibility was performed as follows: broth microdilution using 96-well microtiter plates to perform MICs using cation adjusted Mueller Hinton broth as culture medium. Drug concentration ranging from 0.03 to 16 $\mu\text{g}/\text{mL}$ was used. MICs were performed in batches of 10–20 strains per batch, 5 days a week from July 2016 till August 2016 on archived strains. Colistin sulfate powder was obtained from Sigma-Aldrich Co. (St Louis,

MO, USA). ATCC *E. coli* 25922 and ATCC *P. aeruginosa* 27853 were used as quality control strains. Results were read at 24 hours and interpreted using colistin cut-offs for Enterobacteriaceae in EUCAST 2016, that is, MIC ≤ 2 as sensitive.¹³ Colistin MICs were also determined using automated VITEK 2 system. Results were compared with broth microdilution. Broth microdilution was used as the gold standard. Agreement between broth and VITEK was calculated.

- Fosfomycin susceptibility was tested by disc diffusion method using fosfomycin trometamol disc (200 μg) (Thermo Fisher Scientific, Waltham, MA, USA) containing 50 μg G6PD on Muller Hinton agar. Results were interpreted using CLSI 2015 disc diffusion cut-offs for *E. coli* in urinary tract isolates, that is, ≥ 16 as sensitive, 13–15 intermediate and ≤ 12 as resistant.¹¹

Ethical approval

As this study was conducted on archived bacterial isolates and not on patients' samples, it was given exemption by the ethical review committee Aga Khan University Hospital (ERC# 3992-PAT-ERC-16).

Statistical analysis

Data entered in Microsoft Excel 2010[®] and imported to Stata SE (version 12) was used to calculate MIC₅₀ and MIC₉₀. MIC₅₀ and MIC₉₀ were defined as the lowest concentration of the antibiotic at which 50% and 90% of the tested strains were inhibited. Categorical and essential agreement between VITEK and broth microdilution was calculated using Kappa scores.

Results

The study included 251 strains. These included *E. coli* 39.04% (98/251) followed by *K. pneumoniae* 31% (78/251), *Raoultella* spp. 15.5% (*Raoultella terrigena* and *Raoultella ornithinolytica*) (39/251), *Enterobacter* spp. 7.5% (19/251), *Klebsiella oxytoca* 5.1% (13/251) and *Citrobacter* spp. 1.1% (3/251). Details regarding source and characteristics of patients from whom these isolates were obtained are provided in Table S1.

MIC₅₀ and MIC₉₀

Colistin MICs by broth microdilution was performed on all strains. MIC₅₀ value was calculated to be 0.5 $\mu\text{g}/\text{mL}$, whereas MIC₉₀ was 16 $\mu\text{g}/\text{mL}$. MIC₅₀ and MIC₉₀ values among different bacterial species was separately calculated (Figure 1).

Colistin MICs was ≥ 4 $\mu\text{g}/\text{mL}$ in 40 out of 251 (15.9%) study strains which were identified as being colistin resistant.

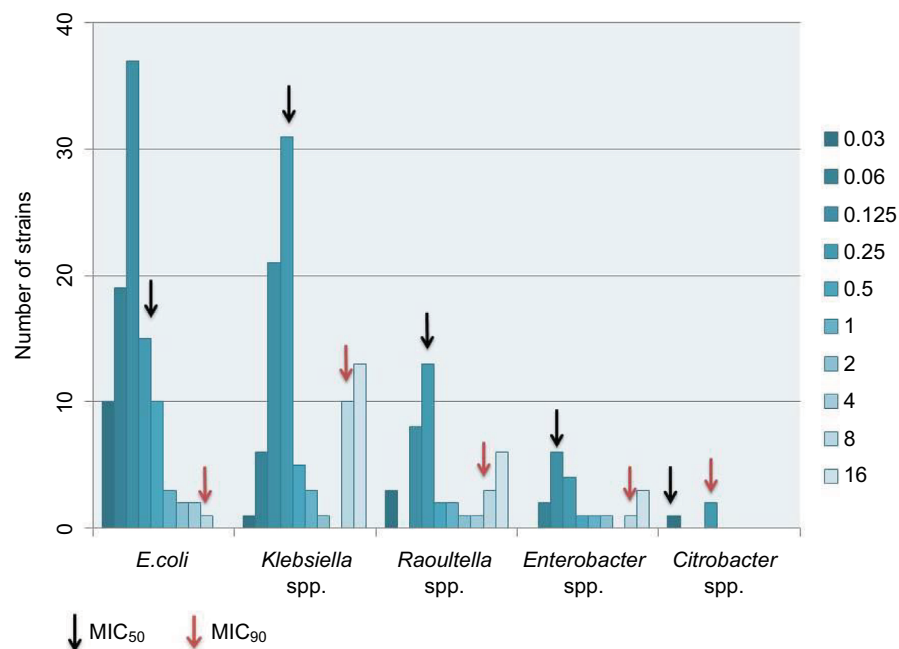


Figure 1 Groups of Enterobacteriaceae with MIC ranges from 0.03 to 16 µg/mL.

Out of these 40 strains 50% were *K. pneumoniae* (n: 20), 22.5% *R. terrigena* (n:10), 10% *Enterobacter* spp. (n:4), 7.5% *E. coli* (n:3) and 7.5% *K. oxytoca* (n:3).

Agreement between VITEK and broth

VITEK MICs were available for 149 strains and agreement was calculated. Broth and VITEK showed 100% categorical agreement with kappa score of 1, CI (0.84–1.16). Essential agreement was calculated to be 88.59% with kappa score of 0.7334, CI (0.6–0.798).

Fosfomycin susceptibility

Among this CRE collection, rate of fosfomycin resistance was 12.3% (31/251). In the colistin-resistant CRE subgroup (n:40) 35 out of 40 remained susceptible to fosfomycin while 5 stains were found resistant to both colistin and fosfomycin simultaneously.

Discussion

Multidrug resistant (MDR) and extensively drug resistant (XDR) bacteria are a global threat. In countries with limited resources, over-stretched health systems make it difficult to manage such infections. Furthermore, inadequate implementation of infection control policies contribute toward dissemination of both nosocomial infections as well as of antimicrobial resistance.

Our study showed 15.9% colistin and 12.3% fosfomycin resistance among CREs. Both colistin and fosfomycin are salvage antibiotics for treating MDR and XDR infections. Hence emerging resistance against these agents significantly reduces available treatment options. Despite their potential side effects and high cost, in the absence of alternatives, clinicians have had no option but to use these antibiotics.

Aga Khan University hospital is a 680-bedded tertiary care hospital which caters to thousands of patients all year round. Our laboratory has more than 200 collection units and participates in external quality assurance surveys from College of American Pathologist on regular basis. Since rate of CRE in the hospital has been increasing over time, colistin or fosfomycin are only prescribed for patients on clinician's advice and their use is closely monitored by the pharmacy service. As per Antibiotic policy of the hospital, IV fosfomycin and colistin have been labeled as restricted and controlled antibiotics which require mandatory approval from the infectious diseases pharmacist or ID physician for dispense.

Colistin resistance among CRE has been previously reported. Most of these studies have been case reports or outbreaks in hospital settings. An outbreak in three institutions in Detroit, MI, USA reported a cluster of colistin-resistant, carbapenem-resistant *K. pneumoniae* infection in 2009.¹⁴ Another study from Italy also reported emergence of colistin resistance in carbapenem-resistant *K. pneumoniae* strains

collected from 21 hospital laboratories during November 2013 to April 2014.¹⁵ Goel et al reported emergence of a cluster of 24 cases of colistin resistant *K. pneumoniae* in oncology unit in India.¹⁶

Earlier, colistin resistance was known to be mediated by chromosomal mutations which altered the lipopolysaccharide of the bacterial outer membrane. These include *mgrB*, *phoP/phoQ*, *pmrA*, *pmrB*, *pmrC*, and *errABC*.¹⁷ In 2015, a study from China reported emergence of plasmid mediated colistin resistance (*mcr-1*) in *E. coli* and *K. pneumoniae* from human and animals.¹⁸ A variant of *mcr-1* (*mcr-1.2*) and *mcr-2* were also detected in Italy and Belgium.^{19,20} Rapid rise of resistance in such a brief period is alarming and poses a major threat in countries with high CRE prevalence such as Pakistan, further minimizing available antibiotic options for treatment of CRE infections.

Due to excessive and inappropriate use of colistin, resistance has not only emerged among CRE but other bacterial species as well. In 2002, an outbreak of colistin-resistant *Pseudomonas aeruginosa* in a pulmonology unit in the UK created an infection control concern,²¹ over the years this was followed with different reports from Australia, India and Iran where colistin resistance was documented in *Acinetobacter baumannii* as well.^{22–24}

With limited available options for treating CRE infections, fosfomycin held promise. An earlier study on 152 MDR (including 85 XDR) Enterobacteriaceae isolates, reported overall susceptibility against fosfomycin of 92.8% (141/152).²⁵ These findings concurred with another study published in the same year, 2009, reporting 7% fosfomycin resistance in prospectively collected 68 *K. pneumoniae* isolates of which 23 were also nonsusceptible to tigecycline and/or colistin.²⁶ A recent study from Germany in 2013 tested fosfomycin against CRE and reported 78% of the tested strain as fosfomycin sensitive.²⁷ Hence, our data reporting 12.3% resistance in CRE suggests increasing resistance against this antibiotic.

Our study has few limitations. Due to financial constrain test strains were only identified on basis of biochemical identification using API 20E. Neither molecular identification of the isolates, nor resistance gene detection was performed for *mcr-1*, *mrgB* or other mutations. Hence additional studies are required to identify genetic mutations associated with colistin resistance in this part of the world. As we tested colistin and fosfomycin using two different methods (broth microdilution and disc diffusion) we could not look for synergism and, therefore, further studies are needed to evaluate synergy between different antibiotics combination for the treatment of CRE.

Conclusion

Our data suggest emerging in vitro resistance against colistin and fosfomycin in CREs from Pakistan. Our findings suggest a need for regular monitoring of antimicrobial resistance in the country including for colistin and fosfomycin. Our data further highlights the urgency for developing and implementing a national policy toward control of antimicrobial resistance in Pakistan.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

Table SI General characteristics of the bacterial isolates included in the study

General characteristics of the study	N=251
Mean, age	50.9 years
Gender	
Male (%)	56.8
Female (%)	43.13
Site of organism recovery	
Urine	143
Blood	61
Cerebrospinal fluid	4
Pus	11
Tissue	7
Respiratory specimen	14
Other body fluids	11

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