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Detection of Colistin Resistant Enterobacteria Isolates from Human Fecal Samples in the Greater Accra Region of Ghana

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ABSTRACT

Background and Objective: Colistin resistance is a major threat to the treatment of multidrug-resistant gram-negative infections. The scarcity of alternative antibiotics and the rapid evolution of resistant bacteria make this a critical problem that needs to be addressed urgently. This study aimed at phenotypic detection of colistin resistance by enterobacteria in humans. **Materials and Methods:** As 135 stool samples were collected from patients presenting with diarrhea, dysentery and typhoid. Bacterial isolation was first done to identify the WHO-priority pathogens involved greatly in bacterial drug resistance. The bacterial isolates were screened against several dilutions of colistin sulfate antibiotics and the minimum inhibitory concentration was determined for each isolate. **Results:** *Klebsiella* species (62%) and *Morganella morgani* (16%) were the most prevalent enterobacteria isolated, with *Escherichia* coli (2%) being one of the least prevalent organisms isolated. As 86% of all isolates were resistant to colistin, while 14% were sensitive. There was no significant difference in the susceptibility pattern of the isolates based on age, gender, or sample collection location. **Conclusion:** The prevalence of colistin resistance amongst isolates from the gastrointestinal tract causing diarrheal infections is high. This study is an early warning sign highlighting the emergence of colistin resistance in Ghana.

KEYWORDS

Colistin, antibiotic resistance (ABR), Antimicrobial Resistance (AMR), enterobacteriaceae, multidrug-resistance (MDR), antimicrobial susceptibility testing (AST), minimum inhibitory concentration (MIC)

INTRODUCTION

Antimicrobial Resistance (AMR) is a serious, cross-cutting global health issue that primarily affects sustainable development, food security and public health. Antimicrobial Resistance (AMR) develops when microorganisms develop defences against the effects of antibiotics. Antibiotic resistance (ABR), a subgroup

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of Antimicrobial Resistance (AMR) that began in the 1970s among gram-negative bacteria can develop naturally through genetic mutation or when one species picks up resistance from another[1]. Random mutations can allow resistance to develop on its own. However, prolonged use of antibiotics seems to promote the selection of mutations that can render antibiotics useless[2]. Each year, millions of people die as a result of clinical diseases brought on by AMR. The treatment of infections brought on by resistant microorganisms is more challenging and sometimes involves using higher doses of antibiotics or riskier alternatives[3]. These strategies might also cost more money. Multidrug-resistant (MDR) microorganisms are those that are resistant to numerous antibiotics[4].

Numerous pathogens, including *Escherichia coli, Salmonella species, Shigella* species, *Klebsiella* species, *Enterobacter* species, *Serratia* species, *Proteus* species, *Morganella* species, *Yersinia* species and other species, are members of the broad gram-negative family of Enterobacteriaceae. These pathogens are a typical component of the gut flora and are prevalent in the human gastrointestinal system and can cause widespread dangerous infectious diseases by moving through the human, animal, agricultural and environmental sectors[5]. Additionally, about three million fatalities and 200 million instances of diarrhea worldwide are caused by *Salmonella*. Similarly, Shigella is to blame for over 650,000 fatalities[6].

Enterobacteriaceae, like other bacteria, can develop resistance to antibiotics, especially those belonging to the carbapenem family, which are typically regarded as the last line of defence against resistant pathogens. Enterobacteriaceae pose a serious global threat, especially in underdeveloped nations like Ghana. Due to their low toxicity and high bactericidal potency, β-lactams are the first-line compound in the treatment of enterobacterial infections[7]. However, a substantial part of the development of enterobacteria resistance can be attributed to the widespread use of antibiotics. The reintroduction of colistin is a result of the growth of extensively drug-resistant (XDR) and multidrug-resistant (MDR) Gram-negative bacteria as well as the paucity of innovative treatments for these infections[8].

Colistin, a polymyxin antibiotic, has been classified as a 'highest priority critically important antimicrobial' by the World Health Organization[9]. For the time being, this antibiotic is only used as a last option to treat Enterobacteriaceae that are multidrug-resistant. Polymyxin E, sometimes referred to as colistin, was first identified from the bacteria *Paenibacillus polymyxa* subsp., in 1947. It is a polycationic peptide containing domains that are both lipophilic and hydrophilic. In the outer cell membrane, where the cationic areas interact with bacterial lipopolysaccharide (LPS), magnesium and calcium counter ions are displaced. Lipopolysaccharide (LPS), which coats Gram-negative bacteria, is targeted by the colistin drug[10]. The bacterial membrane becomes more permeable as a result of the colistin's binding to the LPS, which finally causes cell death by allowing the cytoplasm to leak out[11]. Colistin is also used to prevent and treat respiratory tract infections in patients in intensive care units and to decontaminate the digestive tract on a selective basis[12]. Data on colistin resistance surveillance in Ghana are scarce because regular laboratories no longer report colistin resistance. A joint working group on polymyxin breakpoints was formed in March, 2016 by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[13] and it made the recommendation that only broth microdilution (BMD, ISO 20776-1) be used for colistin susceptibility testing. Colistin-resistant enterobacterial infections are becoming more widespread, which is a serious public health issue. This limits the options for treating gram-negative bacterial infections that are multidrug-resistant[14].

The most prevalent covalent resistance mechanism in enterobacteria is the cationic substitution of the lipid. A moiety of lipopolysaccharides (LPS), neutralizes the negative charge of LPS and subsequently lowers the binding affinity of colistin for its target. Loss of lipid A, a decrease in the outer membrane's net negative charge, or efflux pumps can all lead to colistin resistance. The preferred treatment for such infections is carbapenems[15]. However, due to the rising prevalence of carbapenem resistance worldwide, colistin is increasingly frequently used as a last-resort antibiotic to treat enterobacteria that are resistant to carbapenem[16]. Gram-negative rods, particularly Enterobacteriaceae, are thought to be the major agents of enteric pathogens[17].

The most common bacteria strains causing illnesses are *E. coli*, *Salmonella* species and *Shigella* species. Extended-Spectrum Beta-Lactamases (ESBLs), which give resistance to the majority of beta-lactams, have been acquired and propagated, contributing to the rise in resistance of Enterobacteriaceae in particular. Hospitals were the first places where multi-resistant enterobacteriaceae (MRE) were discovered, but today, most commonly in low-and-middle-income nations like Ghana, they are widespread throughout society.

Due to a shortage of substitute antibiotics, colistin sulfate resistance has become a major public health concern[16] as patients have fewer alternatives for treatment, which can result in more hospitalizations, longer hospital stays and occasionally even death. Colistin usage and bacterial evolution are linked to this occurrence. Although colistin-resistant genes are more common in animal and food isolates than in humans, recent reports of colistin-resistant bacteria in humans pose a significant threat[18,19]. The effectiveness of future medicines for bacterial infections is seriously jeopardized by this. There is however little surveillance on colistin resistance globally. More so, the prevalence of colistin resistance in infections caused by Enterobacteriaceae in Ghana has not been calculated and this may be because there are not enough reliable techniques for identification[20]. The detection of colistin resistance has been proposed using a variety of in vitro techniques. For susceptibility testing against colistin, the straightforward antimicrobial susceptibility test (AST) by disc diffusion is not advised. This is because the colistin molecule from the filter paper disc is observed to diffuse poorly across the agar media, thus allowing the bacteria to grow very close to the antibiotic disc and producing a small zone of inhibition leading to a misleading impression that the isolate is resistant to the antibiotic. Hence, disc diffusion and gradient diffusion methods are not recommended for reporting colistin resistance[10]. To enhance monitoring and awareness, the WHO recently developed detailed guidelines[9] for the detection and notification of colistin sulfate resistance. Therefore, it is imperative to track the prevalence of colistin resistance. Thus, this study aimed to phenotypically characterize colistin-resistant *Enterobacteria* in cases of intestinal infections in Ghana.

MATERIALS AND METHODS

This survey was conducted from April to July, 2022.

Study site, design and sampling: The study followed a cross-sectional design pattern and involved some selected hospitals within Accra Municipal namely Claron Health International, Medifem Hospital and Ussher Hospital. Stool samples (Fig. 1) were obtained from the hospitals after written informed consent to partake in the study had been obtained from its management. The laboratory investigation was carried out at the Microbiology laboratory of Radford University College, East Legon, Accra, Ghana. The study

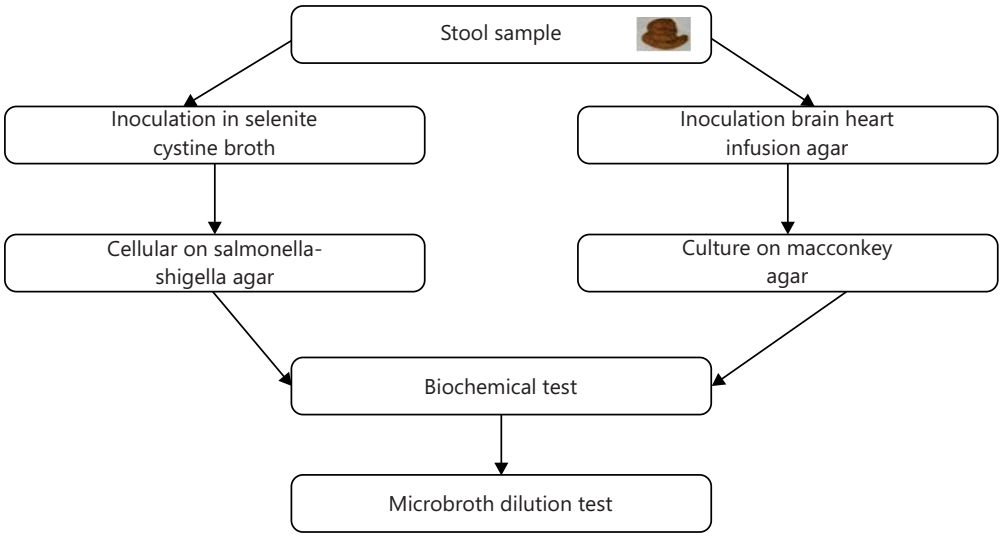

Fig. 1: Sample processing flowchart

population consisted of patients who visited these facilities with gastrointestinal tract infections such as diarrhea, dysentery and vomiting.

Sample collection, handling and storage: As 135 fresh stool samples were collected in clean stool containers after participants consented to partake in the study. The demographic characteristics of the participants were gathered using a well-structured close-ended questionnaire. The samples received were transported to the laboratory within two hours after collection for analysis. Samples were labelled using unique codes generated for the study. All samples were stored at -20°C for at least one year after the study. This initial extended storage period was to ensure sample availability for repeat or to verify experiments.

Laboratory analysis: Samples were collected and species of Enterobacteria were isolated and identified using conventional methods described by Dione *et al*.[21] Briefly, a loopful of stool sample was inoculated into 15 mL of Selenite cysteine broth (Thermo Scientific™ TV5018E) (for selective isolation of *Salmonella* spp. and *Shigella* spp.) and incubated at 37°C for 8 hrs. Similarly, a loopful of stool was inoculated into brain heart infusion broth (Oxoid, UK-for general enrichment) and incubated overnight at 37°C. Subsequently, a loopful of the liquid culture was inoculated into Salmonella-Shigella agar (Oxoid, UK) and MacConkay agar (Oxoid, UK) respectively and incubated overnight at 37°C under aerobic conditions for 18-24 hrs. Bacterial isolates were identified using standard biochemical methods and microscopy[22].

Minimum inhibition concentration for colistin: The micro broth dilution method of susceptibility testing described by Yusuf *et al*.[23], was used as it has been shown to be more reliable in determining colistin resistance[24]. Susceptibility results were interpreted in accordance with European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria[13].

Quality control: *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (NCTC 13442) and *Pseudomonas aeruginosa* (ATCC 27853) were used as quality control strains in this study.

Statistical analysis: Using SPSS (version 23.0), the data were analyzed with a 95 % confidence interval and a p-value of 0.05 being considered statistically significant. The percentage distribution in each category was used to emphasize the results and the Chi-square Test of association was used to examine significant associations.

RESULTS AND DISCUSSION

Pathogen distribution: The study participants consisted mostly of youths with the majority of them being within the age bracket of 21-30 years of age (Table 1). A total of 135 stool samples were incorporated into this study out of which 9 aerobic gram-negative bacteria were isolated 168 times. Unlike other studies[25,26] that reported *E. coli* as the most prevalent enterobacteria involved in gastrointestinal infections, *Klebsiella oxytoca* and *Morgenella morgani* were the most prevalent (62 and 16%, respectively) organisms isolated from this study. The least encountered pathogens were *Salmonella* spp., *Serratia marcesces*, *Shigella sonnei* and *Pseudomonas aeruginosa* all recording a 2% prevalence each (Fig. 2). As 86% of the pathogens isolated were identified to be resistant with MIC results $\geq$2 μg mL^{-1} while the remaining 14% were sensitive to colistin. No significant correlation was found to exist between the isolates and the gender or age of the participants. The World Health Organization (WHO) produced a list of 12 bacterial species with critical, high and medium levels of antibiotic resistance in 2017 known as the Global Priority Pathogens[27]. *Escherichia coli*, *Morganella* spp., *Serratia* spp., *Proteus* species, *Providencia* spp. and *Klebsiella pneumonia* are on the WHO's priority pathogen list of critical importance[27]. The study has significant implications for public health and antibiotic management. The identification of *Klebsiella oxytoca* as the most prevalent bacteria causing intestinal infections challenges the previous findings that *Escherichia coli* is the primary

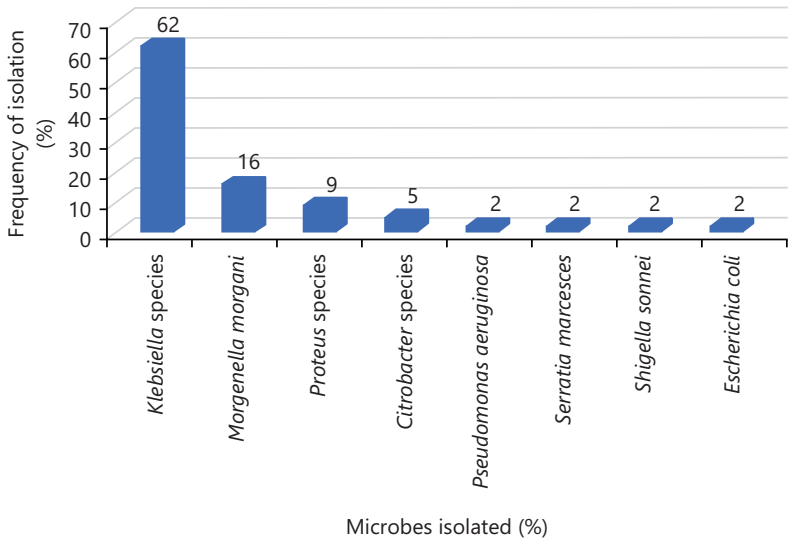

Fig 2: Prevalence of pathogens isolated

Table 1: Age groupings of study participants

	Frequency	Percent
11-20	6	4.4
21-30	66	48.9
31-40	42	31.1
41-50	9	6.7
51-60	12	8.9
Total	135	100.0

culprit. The high resistance of these intestinal pathogens to colistin, a last resort antibiotic, continues to pose a global health threat as emphasized in earlier research works[28,29] and raises concerns about the effectiveness of treatment options. The study also highlights the potential role of horizontal gene transfer and co-evolution in the development of antibiotic resistance thus confirming earlier findings[30-32]. The study's results can guide clinical practices by informing healthcare professionals about the changing landscape of intestinal infections and the prevalence of antibiotic-resistant strains. The identification of *Klebsiella oxytoca* as a major pathogen suggests that diagnostic tests and treatment protocols should be adjusted accordingly.

The study underscores the importance of judicious use of antibiotics, especially colistin, to preserve its efficacy as a last resort treatment. Based on the findings of this study, the authors recommend the development of more precise diagnostic tools to accurately identify *Klebsiella oxytoca* infections, allowing for effective treatment and strict antibiotic management stewardship to control the use of colistin and other critical antibiotics in clinical, agricultural and veterinary settings, comprehensive surveillance system to track antibiotic resistance patterns and the emergence of new resistant strains in both clinical and non-clinical settings and further research to investigate the molecular basis of colistin resistance and the mechanisms driving it in *Klebsiella oxytoca* and related bacteria.

Females accounted for 64.4% of the study participants while 35.6% of the participants were males. The mean age of the participants was 33 years although the majority (48.9%) of the stool samples were obtained from participants within the age bracket of 21-30 years (Table 1).

The majority of the samples were obtained from individuals receiving health care at the Ussher Hospital located in Bukom while the least number of samples were received from the Accra metropolis (Fig. 3). The high sampling recorded from Bukom may be a result of the unhygienic nature of the environment as those areas are prone to most gastrointestinal infections.

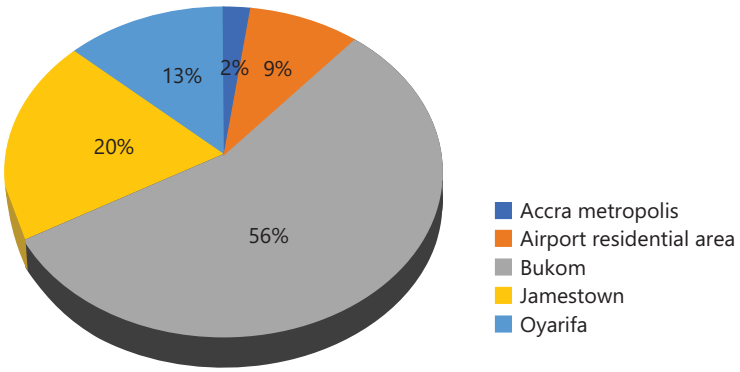

Fig. 3: Frequency of samples per location

Table 2: Statistical associations between age groups, MIC results, organisms isolated, gender and sample origin

	Age group	Mic result	Organisms isolated	Gender	Sample origin
Age group					
Pearson correlation	1	-0.165	-0.081	0.110	0.000
significant (2-tailed)		0.278	0.596	0.474	1.000
N	45	45	45	45	45
Mic result					
Pearson correlation	-0.165	1	-0.033	-0.063	0.251
significant (2-tailed)	0.278		0.830	0.683	0.096
N	45	45	45	45	45
Organisms isolated					
Pearson correlation	-0.081	-0.033	1	-0.079	-0.014
significant (2-tailed)	0.596	0.830		0.605	0.928
N	45	45	45	45	45
Gender					
Pearson correlation	0.110	-0.063	-0.079	1	-0.017
significant (2-tailed)	0.474	0.683	0.605		0.910
N	45	45	45	45	45
Sample origin					
Pearson correlation	0.000	0.251	-0.014	-0.017	1
significant (2-tailed)	1.000	0.096	0.928	0.910	
N	45	45	45	45	45

*Correlation is significant at the 0.05 level (2-tailed) and **Correlation is significant at the 0.01 level (2-tailed)

Minimum inhibitory concentration: All isolates identified in the study were screened for phenotypic resistance to the colistin sulfate antibiotic of which the majority (86%) displayed resistance to colistin using the micro broth dilution method.

No significant association (Table 2) was found to exist between the organisms isolated and factors such as the age group, gender, origin of sample and susceptibility pattern ($p>0.05$).

CONCLUSION

The findings of this study indicate that *klebsiella oxytoca* is the most prevalent bacteria responsible for intestinal infections as against *E. coli* which has been reported by some other studies as the most implicated causative agent of intestinal infections. The results of the minimum inhibitory concentrations indicate that these intestinal pathogens facilitating gut infections are greatly resistant to the last resort antibiotic, colistin. This affirms to literature that colistin usage in Ghana has gained root and these gut microbiomes may have developed resistance to it through horizontal gene transfer or co-evolution. Thus, in order to preserve colistin as a last resort antibiotic, an antibiotic management approach must be used to limit its use. The molecular determination of colistin resistance and mechanisms mediating colistin resistance amongst this enterobacteria should be investigated using samples from the environment, livestock and clinical settings all gathered at the same time to understand the source of resistance in humans.

SIGNIFICANCE STATEMENT

Due to their relatively recent return to clinical procedures, the overall incidence of colistin resistance in human clinical isolates is still very low. However, there has been a pronounced increase in colistin-resistant bacteria in recent years as a result of the rapidly expanding colistin usage in hospitals and in environments where animals are cared for, such as poultry, which has increased selection pressure for resistance. This made it necessary for this study to check for colistin-resistant bacteria in humans. It was discovered that there were a lot of phenotypically identified colistin-resistant bacteria in stools. Therefore, molecular characterization of colistin-resistant bacteria in feces and research into their mode of resistance is required.

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