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Research Article
Investigation of Colistin and Polymyxin B on Clinical Extreme Resistant *Enterobacteriaceae* Isolates for Surveillance Purposes

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Abstract

Background and Objective: As bacterial resistance to colistin and polymyxin B escalates, prompt detection of resistant strains is necessary, to control the outbreak. This study evaluates possible extremely colistin resistance *Enterobacteriaceae* clinical isolates from ICU patients and determines their carriage of DNA *mcr-1* resistant gene. Also to compare the resistance pattern between colistin and polymyxin B. **Materials and Methods:** Ninety-one gram-negative bacterial isolates were used, comprising of *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Shigella flexneri*, which were clinical isolates that are part of patient care. Vitek compact 2 automated system was used for bacterial ID confirmation. Disc diffusion, Etest and Broth Microdilution (BMD) were used to assess resistance status. Chromosomal *mcr-1* gene carriage was investigated. **Results:** Vitek compact 2 automated system analysis indicated 96% resistance, disc diffusion detecting 89% and Etest with 96% for the isolates. Comparison between disc diffusion and Etest revealed that very major errors (false sensitivity) were encountered with 5 *E. coli* isolates with zones of inhibition $\geq$14 mm whereas, Etest MIC ranged between 8-20 μg mL^{-1}. None of the *K. pneumoniae* and *P. aeruginosa* isolates were susceptible to colistin by Etest. Four *E. coli* isolates tested positive for the *mcr-1* gene with 309 bp (2), 500 bp and 1 kb, respectively. *Pseudomonas aeruginosa* had genes with more than 2 kb amplicons. **Conclusion:** BMD assay revealed a similar resistance pattern between colistin and polymyxin B. Our findings further confirm the presence of chromosomal *mcr-1* genes in the region of study, suggesting timely surveillance to contend the spread of resistance.

Key words: Polymyxin B, colistin, susceptibility, extremely drug-resistant, *mcr-1* gene, gram-negative bacteria

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Polymyxin antibiotics were widely used in the management of serious infections caused by gram-negative bacilli until they were removed from use because of their toxicity expressed as nephrotoxicity and neurotoxicity[1]. Due to the lack of alternative antibiotics for the treatment of emerging Extremely Drug-Resistant (XDR) gram-negative bacteria, polymyxin antibiotics were brought back into practice, employed as part of the last line antibiotic treatment. Unfortunately, their increased use in clinical settings has also led to resistance emergence among these groups of bacteria[2]. According to the report of Li *et al*.[3], polymyxins' resistance has been found in *P. aeruginosa*, *A. baumannii*, *Campylobacter* species (spp.) including *K. pneumoniae* amongst other *Enterobacteriaceae*. Bacterial resistance to polymyxins has been documented over a decade ago[4,5] and evidence show that it is attributed to chromosomal mutations[6,7]. This phenomenon has been reported as modification of LPS due to alterations to the negative charge of the outer membrane which prevents polymyxin binding[8]. In addition, the study shows that a mechanism involving the inactivation by insertion or deletion in the lipid A biosynthesis cluster gene lead to complete loss of lipid A[9]. The consequent loss of lipid A in the gram-negative bacterial cell membrane prevents the interaction with polymyxins causing resistance with a MIC >128 μg mL^{-1}. Acylation of lipid A has also been suggested as a possible contributor to polymyxin resistance in *K. pneumoniae*, *P. aeruginosa* and *A. baumannii* amongst other *Enterobacteriaceae* species[10]. Furthermore, a resistance gene, *mcr-1* has been identified in a conjugative plasmid *Escherichia coli* isolates responsible for polymyxins' treatment failure[11]. Documented evidence also shows that the plasmid *mcr-1* gene is carried by a diverse group of *Enterobacteriaceae*[12]. In addition, according to Sun *et al*.[13] *E. coli*, strains containing chromosomal *mcr-1* have also been isolated from humans as well as from retail meat products. Meaning that the *mcr-1* gene is diverse as it has been detected in many countries and identified from humans, animals and environments[14]. Therefore, the acquisition of multiple copies of *mcr-1*, especially on the chromosome, will be prone to stabilization of the *mcr-1* genes, facilitating stable persistence of polymyxins resistance in the host strain chromosome[13,15]. Reports indicate that the *mcr-1* gene integrates easily into various regions of the bacteria. This character helps to facilitate its dissemination among bacteria and hence, explains its rapid spread in humans, animals and the environment[16]. A recent finding suggests that a single copy of *mcr-1* could result in modification of Lipopolysaccharide (LPS) which causes polymyxin resistance in different bacteria strains[13]. According to Yamaguchi *et al*.[17] an antibiotic-free environment can exert a significant metabolic burden on the host bacterial strain. Therefore, antibiotic-resistant plasmids may be lost during their multiplication due to transposition and transposon to the chromosome. Thereby initiating the process of stabilizing *mcr-1* due to the loss of the insertion sequences. Then, the chromosomal *mcr-1* transposon can eventually progress into a more stable genotype, hence the detection of chromosomal mediated polymyxin resistance[18]. Therefore, monitoring of polymyxins' resistance in the face of re-emerging XDR underscores the importance of much needed clinical treatments as there are no available alternative last line agents. Continued surveillance is needed to curtail the spread of potential danger of the threat of losing this brand of antibiotics. In the region of the present study, the occurrence of polymyxins' resistance is underreported. To compare colistin and polymyxin B bacterial growth inhibition using Broth Microdilution (BMD) according to the guidelines and recommendations of CLSI[19]. Finally, determine if the isolates DNA carry the *mcr-1* resistant gene.

Therefore, this study aims to investigate possible extremely colistin resistance in clinical isolates of *Enterobacteriaceae* from ICU patients.

MATERIALS AND METHODS

Study area: The study was carried out at the Microbiology Division of the Department of Biomedical Science, College of Medicine, King Faisal University, with samples collected from June, 2019-January, 2021.

Materials: Colistimethate sodium (Hikma Italy), polymyxin B sulfate (Schaumburg IL USA), colistin 10 μL disc (Condalab, Torrejon de Ardoz, Madrid, Spain), Qiagen DNA extraction kit (Qiagen, Germany), colistin Etest strip (AB Biodisk, BioMerieux, Sweden).

Bacteria isolates and confirmation of ID: Ninety-one gram-negative bacterial isolates were used for this study and made up of *Acinetobacter baumannii* (28 strains), *Escherichia coli* (26 strains), *Klebsiella pneumoniae* (23 strains), *Pseudomonas aeruginosa* (13) and *Shigella flexneri* (1), Isolates were from clinical samples, which formed part of patient care and store in -80 microbank in the Laboratory of Microbiology Division, College of Medicine, King Faisal University. Samples from where they had been isolated included urine, sputum, wound swabs, transtracheal aspirates

and blood. Isolates were retrieved from the -80°C microbank by culturing on MacConkey agar, incubated aerobically at 37°C for between 18-24 hrs. The resulting overnight growth was again plated out on MacConkey agar, incubated under the same conditions and used for bacteria ID and antimicrobial susceptibility assay. Vitek compact 2 automated system (BioMerieux, Marcy L'Etoile, France) was used for bacterial ID confirmation according to the guidelines of the manufacturers. Susceptibility assay was also by vitek compact 2 automated system (BioMerieux, Marcy L'Etoile, France) using AST-GN cards against the following antibiotics: Amoxicillin, ampicillin, amoxicillin/clavulanic acid, ampicillin/sulbactam, piperacillin/ tazobactam, cefalotin, cefoxitin, ceftazidime, ceftriaxone, cefepime, imipenem, meropenem, amikacin, gentamicin, ciprofloxacin, tigecycline, nitrofurantoin, trimethoprim/ sulfamethoxazole, aztreonam, tobramycin, levofloxacin, ticarcillin/clavulanic acid, minocycline, colistin. The minimum inhibitory concentrations were given also by the Vitek compact 2 automated system (BioMerieux, Marcy L'Etoile, France). Isolates were defined as Multidrug-Resistant (MDR) when resistant to more than one in three or more categories of antibiotics, Extensively Drug-Resistant (XDR) if non-susceptibility to one agent in all but 1 or 2 categories of antibiotics, Carbapenem-Resistant *Enterobacteriaceae* species (CRE) if resistant to any of the carbapenems according to CDC guidelines.

Colistin disc diffusion and Etest susceptibility analysis: Colistin 10 µL disc (Condalab, Torrejon de Ardoz, Madrid) was used for disc diffusion susceptibility testing. Plates of Muller-Hinton Agar (MHA) were individually seeded with each bacterial isolate and colistin disc introduced into them. All plates were incubated aerobically at 37 for 24 hrs. Zones of inhibition were measured in millimetres (mm) and the results were interpreted according to CLSI[19] guidelines. For *Enterobacteriaceae*, a diameter zone of inhibition $\geq$14 mm was considered susceptible while that of $\leq$11 mm was taken as resistant. For *Acinetobacter baumannii*, a zone of inhibition $\leq$12 mm was considered resistant and $\geq$14, susceptible[20,21].

Minimum Inhibitory Concentration (MIC) was determined with colistin Etest strip (AB Biodisk, BioMerieux, Sweden). 0.016-256 mcg mL^{-1} range values. MHA plates were seeded individually with bacterial isolates and the surface allowed drying before applying the Etest strip. Plates were incubated for 24 hrs at 37°C. Results interpretation is according to the manufacturer's guidelines with values taken at the point where growth inhibition stopped on the Etest strip. Applying CSLI[9] (https://www.nih.org.pk/wp-content/uploads/2021/02/CLSI-2020.pdf). Breakpoint recommendations, values $\geq$4 mg L^{-1} were considered as resistant for *Acinetobacter baumannii* while those $\leq$2 mg L^{-1} as susceptible. For *Pseudomonas aeruginosa* and the *Enterobacteriaceae*, the CLSI[19] recommendation for colistin resistance test was applied for interpretation of MIC result. Values $\geq$4 mg L^{-1} were considered resistant and $\leq$2 mg L^{-1} were considered intermediate, susceptible was taken as <2.

Genomic DNA extraction and detection of *mcr-1* gene by PCR amplification: Qiagen DNA extraction kit was used for the extraction of bacterial genomic DNA according to the manufacturer's protocol. Briefly, a loopful of each isolate was suspended in 100 µL of TE buffer and boiled at 100 for 10 min. The resultant product was centrifuged at 6000 G for 5 min and supernatant diluted in Tris buffer at 1:10 and used as DNA template. For the detection of colistin resistance *mcr-1* genes, the laboratory protocol by the National Food Institute, Denmark [https://www.eurl-ar.eu/CustomerData/Files/Folders/21-protocols/278_mcr-multiplex-pcr-protocol-v2-oct16.pdf] was used with the primers *mcr-1* (35-343). CLR F 5'-CGGTCAGTCCGTTTGTTC-3', CLR R 5'-CTTGGTCGGTCTGT AGGG-3'[22]. PCR amplification constituted a final volume of 25 µL composed of 3 µL DNA template, 0.5 µL forward and reverse primers each and 21 µL master mix. PCR thermocycling conditions are as earlier described[22,23]. The resulting PCR products were stained with ethidium bromide (10 mg mL^{-1}). Two percent agarose gel electrophoresis was used to analyse the stained amplified products and visualized with a UV transilluminator.

Inhibitory assay of dilutions of colistin and polymyxin B against the isolates: Broth dilution[19] was used to determine the growth inhibitory effects of colistin and polymyxin B on the selected number of isolates. Fresh overnight grew bacterial cultures were used for preparing bacterial suspension in 2 mL Muller-Hinton broth. The method is as described by Badger-Emeka *et al.*[24] according to the guidelines of CLSI 2020. The initial turbidity of each sample was measured with DensiCHEK™ plus for the vitek compact 2 automated system. Bacterial optical density was measured at 580 nm wavelength as recommended by the manufacturers (https://www.accessdata.fda.gov/cdrh_docs/reviews/K083536.pdf). Briefly, the DensiCHEK™ instrument was calibrated to zero 0.0 McF standard with a test tube filled with sterile Muller-Hinton broth. Bacterial inoculum suspension (1 mL) were prepared individually for the isolates using the 3.0 McF standard. Setup control experiment for each bacterial isolate

was a 2 mL bacteria suspension in Muller-Hinton with no drugs added to any of them.

Four dilutions (1, 2, 4 and 8 μg mL^{-1}) of colistin and polymyxin B were each introduced into individually prepared bacterial suspension in glass tubes for macro-dilution assay[25]. Initial bacterial turbidity was before introducing the drugs into the tubes. Prepared macro-dilutions were all incubated aerobically at 37°C for 24 hrs. The bacterial optical density of the resultant 24 hrs post-incubation suspension was (1 mL) was measured individually and the differences between the initial and final turbidity according to McFarland standard[5] (~1×10^8 CFU mL^{-1}) was used to ascertain the effect of both drugs on the isolates.

Statistically analysis: The analysis of data were done using statistical software (SPSS, version 23, USA). The significance of the data was determined using a two-tailed t-test and p-value set at 0.05. Disc diffusion assay and Etest MIC comparison were as earlier described[20]. Results in which isolates were susceptible by disc diffusion zone of inhibition but resistant by Etest MIC were categorised as a very major error. When isolates were resistant by the diameter zone of inhibition (mm) while being sensitive by Etest, results were interpreted as a major error. Intermediate results by a zone of inhibition diameter as against a resistant or susceptible Etest MIC were considered as minor errors.

RESULTS

Distribution, sources of isolates and antimicrobial susceptibility pattern: The study was carried out using a total of clinical 91 isolates with origins from different clinical samples (Fig. 1a). The majority (51%) of the isolates were from urinary tract infections while those from the skin and soft tissue infections represented 21% of the isolates. Other isolates were from the respiratory tract and bloodstream infections (15 and 11%), respectively (Fig. 1a).

Antimicrobial assay by vitek compact 2 automated system, showed that none of the isolates tested against the following antibiotics, colistin, minocycline, ticarcillin/clavulanic acid, ampicillin/sulbactam, amoxicillin and ampicillin was sensitive to them (Fig. 1b). There was also no intermediate susceptibility against these drugs too. Resistance was high for other antibiotics such as levofloxacin (90%) and ciprofloxacin (84%). Sensitivity was high for tigecycline (69%) and amikacin (87%). Generally (Fig. 1b), the majority of the isolates were highly resistant to the tested antibiotics with 56% of them being MDR, 21% of the isolates were Carbapenem-Resistant *Enterobacteriaceae* (CRE). This was followed by 17% of Extensively Drug-resistant (XDR) isolates as well as those susceptible strains (6%) (Fig. 1c).

There were also observed differences species wise in their susceptibility to the antibiotics (Fig. 2). Of the 15 antibiotics against which *Acinetobacter baumannii* isolates were tested, there was 100% resistance to 9 of them (ampicillin/sulbactam, ticarcillin/clavulanic acid, piperacillin/tazobactam, ceftazidime, imipenem, meropenem, ciprofloxacin, levofloxacin, colistin. Some isolates (AC 4, AC 42 and AC 82) were sensitive to one of the tested drugs. One isolate (AC 30) was resistant to all the tested antibiotics (Fig. 2a). For *Klebsiella pneumoniae*, of the 17 antibiotics against which the isolates were tested, there was 100% resistance to 2 (amoxicillin and ampicillin). None of the *K. pneumoniae* isolates was resistant to all tested drugs, however, 2 isolates (KP96, KP97) were sensitive to only one antimicrobial, tigecycline. In addition, isolate KP 97 showed intermediate susceptibility to imipenem and tigecycline while the remaining KP isolates were sensitive to this antibiotic (Fig. 2b). A similar pattern of antimicrobial susceptibility is seen with *Escherichia coli* isolates showing 100% resistance to amoxicillin, ampicillin. One isolate (EC100) was sensitive only to one of the tested antibiotics (tigecycline) as against 17 tested antibiotics (Fig. 2c). Three *Pseudomonas aeruginosa* isolates (PS84, PS90 and PS95) were resistant to the 12 antibiotics against which they had been tested. Also, all *P. aeruginosa* isolates were resistant to tigecycline (Fig. 2d).

Twenty-six percent of *Acinetobacter baumannii* isolates were XDR while 59% were MDR (Table 1). Of the *Escherichia coli* isolates, 7.7% of them were CRE and the remaining isolates were MDR. The *Klebsiella pneumoniae* isolates displayed a high percentage (73.9%) of CRE while results showed *Pseudomonas aeruginosa* to be either XDR (53.8%) or MDR (30.8%).

Disc diffusion assay and colistin Etest evaluation: By disc 10 μL diffusion assay, susceptibility between the isolates also varied. Four (14%) isolates of *A. baumannii* were susceptible with disc diameter values of 15, 16 and 18 mm. Two (7%) of *A. baumannii* with 13 mm inhibition zones were shown to be intermediate susceptibility. Five (19%) of the *E. coli* isolates were susceptible with zones of inhibition diameters that ranged between 14 and 15 mm while 3 isolates (12%) with 13 mm diameter zone of inhibition were listed as intermediate susceptibility (Table 1). One (4%) *K. pneumoniae* isolate with a 14 mm diameter zone of inhibition was susceptible while three (13%) isolates with a zone inhibition diameter of 13 mm were intermediate in susceptibility. By disc diffusion assay, there was no susceptibility to *Pseudomonas*

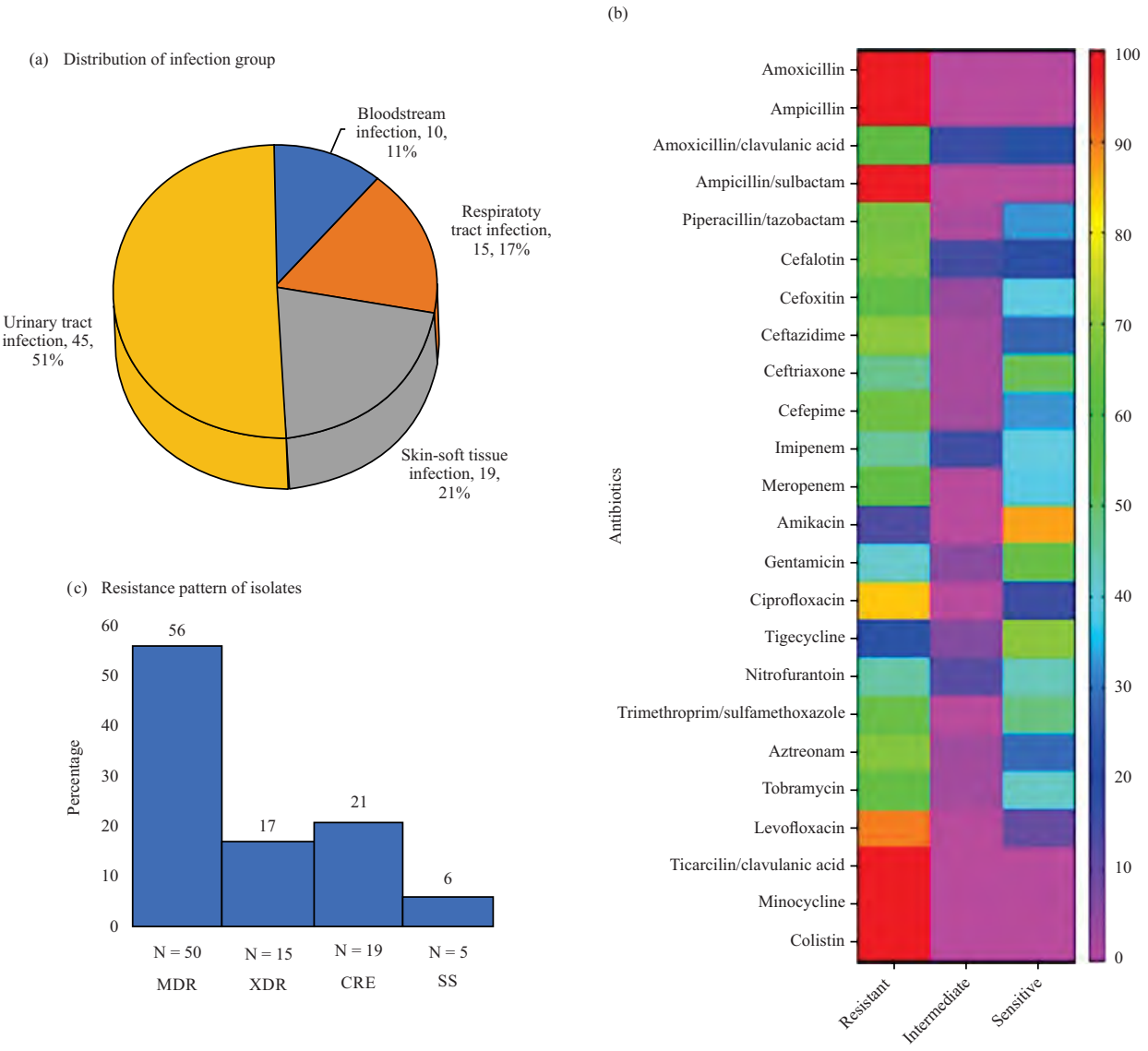

Fig. 1(a-c): Samples distribution based on types of infection with isolates resistance characteristics and percentage antimicrobial resistance

MDR: Multidrug-resistant, XDR: Extensively drug-resistant, SS: Sensitive strain, CRE: Carbapenem-resistant *Enterobacteriaceae*, ESBL: Extended-spectrum beta-lactamases and for the tested isolates, there were none sensitive to the following antibiotics: Colistin, minocycline, ticarcillin/clavulanic acid, ampicillin/sulbactam, amoxicillin and ampicillin. There was also no intermediate susceptibility against them too

species while one (8%) with a 13 mm diameter inhibition zone showed intermediate susceptibility (Table 1). Colistin Etest results are presented in Table 2 based on CSLI interpretation guidelines on, susceptibility and resistance. Only one isolate each for *A. baumannii* and *E. coli* were susceptible with MIC values of 2 μg mL^{-1}. For both bacterial species, 3 μg mL^{-1} were taken as intermediate values (Table 2). None of the *K. pneumoniae* and Pseudomonal isolates as susceptible to colistin by Etest assay. Images of some Etest results are shown in Fig. 3.

Comparison of colistin disc diffusion assay and Etest MIC values: There were no very major, nor major errors (no false susceptibility nor false resistance) seen in the *Acinetobacter baumannii* isolates. Of the 4 isolates susceptible by disc diffusion, one was intermediated by Etest MIC and this was taken as a minor error (Fig. 4a). However, every major error (false sensitivity) were detected in the *Escherichia coli* isolates. Five of the isolates with zones of inhibition diameter ≥14 mm were resistant by MIC results that ranged between 8-20 μg mL^{-1} (Fig. 4b). There were also major

Table 1: Description of an antimicrobial pattern of susceptibility of the bacterial isolates, colistin disc diffusion and interpretation of results

Acinetobacter baumannii			*Escherichia coli*			*Klebsiella pneumoniae*			*Pseudomonas aeruginosa*		
Lab ID	Resistance pattern	Disc diffusion (mm)	Lab ID	Resistance pattern	Disc diffusion (mm)	Lab ID	Resistance pattern	Disc diffusion (mm)	Lab ID	Resistance pattern	Disc diffusion (mm)
AC 4	XDR	18 (S)	EC 3	MDR	13 (I)	KP 1	CRE	9 (R)	PS 6	SS	8 (R)
AC 14	MDR	10 (R)	EC 7	SS	15 (S)	KP 2	SS	11 (R)	PS 12	SS	10 (R)
AC 16	MDR	0 (R)	EC 8	SS	7 (R)	KP 5	SS	10 (R)	PS 24	XDR	0 (R)
AC 17	XDR	8 (R)	EC 11	SS	9 (R)	KP 13	SS	10 (R)	PS 25	MDR	13 (I)
AC 20	MDR	2 (R)	EC 19	MDR	15 (S)	KP 15	MDR	11 (R)	PS 33	MDR	0 (R)
AC 23	MDR	13 (I)	EC 22	MDR	6 (R)	KP 18	CRE	11 (R)	PS 51	XDR	2 (R)
AC 29	MDR	10 (R)	EC 26	ESBL/MDR	11 (R)	KP 21	CRE	4 (R)	PS 60	XDR	0 (R)
AC 30	XDR	13 (I)	EC 28	MDR	9 (R)	KP 27	CRE	14 (S)	PS 65	MDR	2 (R)
AC 34	MDR	16 (S)	EC 32	MDR	13 (I)	KP 31	CRE	12 (R)	PS 73	XDR	6 (R)
AC 36	XDR	16 (S)	EC 35	MDR	11 (R)	KP 52	CRE	13 (I)	PS 75	MDR	8 (R)
AC 40	MDR	0 (R)	EC 37	MDR	10 (R)	KP 59	CRE	10 (R)	PS 84	XDR	4 (R)
AC 42	XDR	0 (R)	EC 38	MDR	8 (R)	KP 64	CRE	10 (R)	PS 90	XDR	10 (R)
AC 44	MDR	0 (R)	EC 41	MDR	15 (S)	KP 67	CRE	11 (R)	PS 92	XDR	2 (R)
AC 45	MDR	0 (R)	EC 47	MDR	11 (R)	KP 69	CRE	13 (I)	-	-	-
AC 46	MDR	0 (R)	EC 48	MDR	5 (R)	KP 70	CRE	2 (R)	-	-	-
AC 50	MDR	15 (S)	EC 49	MDR	1 (R)	KP 76	CRE	13 (I)	-	-	-
AC 55	MDR	0 (R)	EC 54	MDR	1 (R)	KP 85	MDR	11 (R)	-	-	-
AC 57	MDR	0 (R)	EC 61	MDR	8 (R)	KP 86	CRE	2 (R)	-	-	-
AC 58	MDR	0 (R)	EC 71	MDR	14 (S)	KP 87	MDR	10 (R)	-	-	-
AC 66	MDR	0 (R)	EC 74	MDR	15 (S)	KP 93	CRE	10 (R)	-	-	-
AC 68	XDR	0 (R)	EC 77	MDR	9 (R)	KP 95	CRE	2 (R)	-	-	-
AC 72	MDR	8 (R)	EC 79	CRE	9.5 (R)	KP 96	CRE	4 (R)	-	-	-
AC 78	MDR	9 (R)	EC 94	MDR	5 (R)	KP 97	CRE	12 (R)	-	-	-
AC 82	XDR	6 (R)	EC 98	MDR	9 (R)	-	-	-	-	-	-
AC 83	XDR	6 (R)	EC 99	MDR	7 (R)	-	-	-			
AC 88	MDR	0 (R)	EC 100	CRE	13 (I)	-	-	-	-	-	-
AC 89	MDR	0 (R)	-	-	-	-	-	-	-	-	-
AC 91	MDR	0 (R)									

S: Sensitive, I: Intermediate, R: Resistant, MDR: Multidrug resistant, XDR: Extensively drug resistant, SS: Sensitive strain, CRE: Carbapenem-resistant *Enterobacteriaceae*, ESBL: Extended-spectrum beta-lactamases and for the 10 µL colistin disc, for *Enterobacteriaceae*, resistance (R) was taken at $\leq$10 mm diameter with those $\geq$11 mm were considered as being susceptible (S)

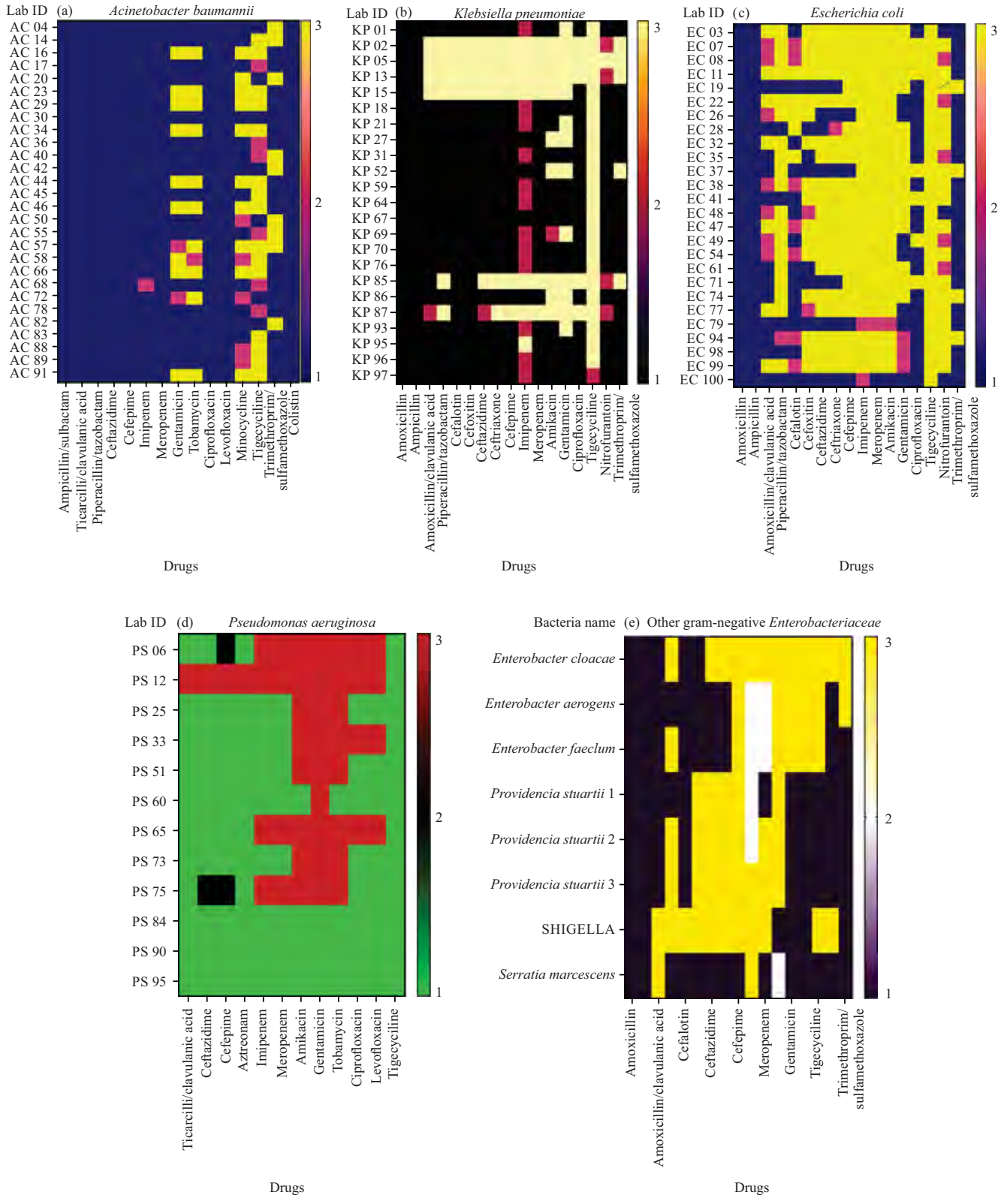

Fig. 2(a-e): Heat map of the antimicrobial pattern of individual bacterial isolates against the tested antibiotics

1: Resistant, 2: Intermediate, 3: Sensitive.

Table 2: Minimum inhibitory concentration colistin Etest values and the source of isolates

Bacterial Isolates											
Acinetobacter baumannii			*Escherichia coli*			*Klebsiella pneumoniae*			*Pseudomonas aeruginosa*		
Lab ID	Source	Etest (mcg mL^{-1})	Lab ID	Source	Etest (mcg mL^{-1})	Lab ID	Source	Etest (mcg mL^{-1})	Lab ID	Source	Etest (mcg mL^{-1})
AC 4	TT. ASP	3 (I)	EC 8	Urine	3 (I)	KP 1	TT.ASP	8 (R)	PS 6	Urine	96 (R)
AC 14	Drainage	3 (I)	EC 11	Urine	6 (R)	KP 2	WS	16 (R)	PS 12	Skin swab	64 (R)
AC 16	WS	4 (R)	EC 19	Urine	16 (R)	KP 5	Urine	32 (R)	PS 24	Skin swab	48 (R)
AC 17	Urine	24 (R)	EC 22	Urine	2 (S)	KP 13	WS	16 (R)	PS 25	WS	256 (R)
AC 20	Sputum	256 (R)	EC 26	WS	32 (R)	KP 15	WS	48 (R)	PS 33	Urine	64 (R)
AC 23	WS	16 (R)	EC 28	ABS. DR	64 (R)	KP 18	WS	8 (R)	PS 51	Sputum	192 (R)
AC 29	TT. ASP	4 (R)	EC 32	Urine	32 (R)	KP 21	TT.ASP	6 (R)	PS 60	Urine	256 (R)
AC 30	NG	2 (S)	EC 35	Urine	4 (R)	KP 27	Urine	8 (R)	PS 65	Urine	48 (R)
AC 34	WS	8 (R)	EC 37	WS	8 (R)	KP 31	Blood	6 (R)	PS 73	WS	256 (R)
AC 36	Urine	16 (R)	EC 38	ABS. DR	24 (R)	KP 52	Urine	56 (R)	PS 75	Urine	64 (R)
AC 40	TT. ASP	8 (R)	EC 41	Urine	28 (R)	KP 59	Drainage	64 (R)	PS 84	TT.ASP	32 (R)
AC 44	TT. ASP	48 (R)	EC 47	Urine	6 (R)	KP 64	Urine	8 (R)	PS 90	WS	64 (R)
AC 45	WS	256 (R)	EC 48	Urine	2 (S)	KP 67	WS	256 (R)	PS 92	ABS. DR	16 (R)
AC 46	WS	64 (R)	EC 49	Urine	8 (R)	KP 69	WS	256 (R)	-	-	-
AC 50	WS	256 (R)	EC 54	Urine	12 (R)	KP 70	WS	12 (R)	-	-	-
AC 55	TT. ASP	6 (R)	EC 61	Urine	4 (R)	KP 85	Urine	16 (R)	-	-	-
AC 57	Urine	256 (R)	EC 71	Blood	64 (R)	KP 86	Urine	12 (R)	-	-	-
AC 68	Urine	256 (R)	EC 74	Urine	24 (R)	KP 87	Urine	48 (R)	-	-	-
AC 72	TT. ASP	256 (R)	EC 77	Urine	18 (R)	KP 93	Urine	64 (R)	-	-	-
AC 82	TT. ASP	256 (R)	EC 79	Peritoneal fluid	48 (R)	KP 95	TT.ASP	16 (R)	-	-	-
AC 83	Sputum	256 (R)	EC 94	Urine	3 (I)	KP 96	WS	12 (R)	-	-	-
AC 88	WS	256 (R)	EC 98	Urine	8 (R)	KP 97	Sputum	48 (R)	-	-	-
AC 89	Sputum	64 (R)	EC 99	Urine	16 (R)	-	-	-	-	-	-
AC 91	TT. ASP	3 (I)	EC 100	Urine	24 (R)		-	-	-	-	-

TT. ASP: Transtracheal aspirate, WS: Wound swab, ABS. DR: Abscess drainage, -No isolate. According to CLSI (2020) guidelines, for *Acinetobacter baumannii*, values $\leq$2 μg mL^{-1} were taken as susceptible (S) and those $\geq$4 μg mL^{-1} were considered as resistant and for *Pseudomonas aeruginosa* and other *Enterobacteriaceae* susceptibilities (S) were values $\leq$2 μg mL^{-1}, resistance were valued $\geq$4 μg mL^{-1} and intermediate I = 3 μg mL^{-1}

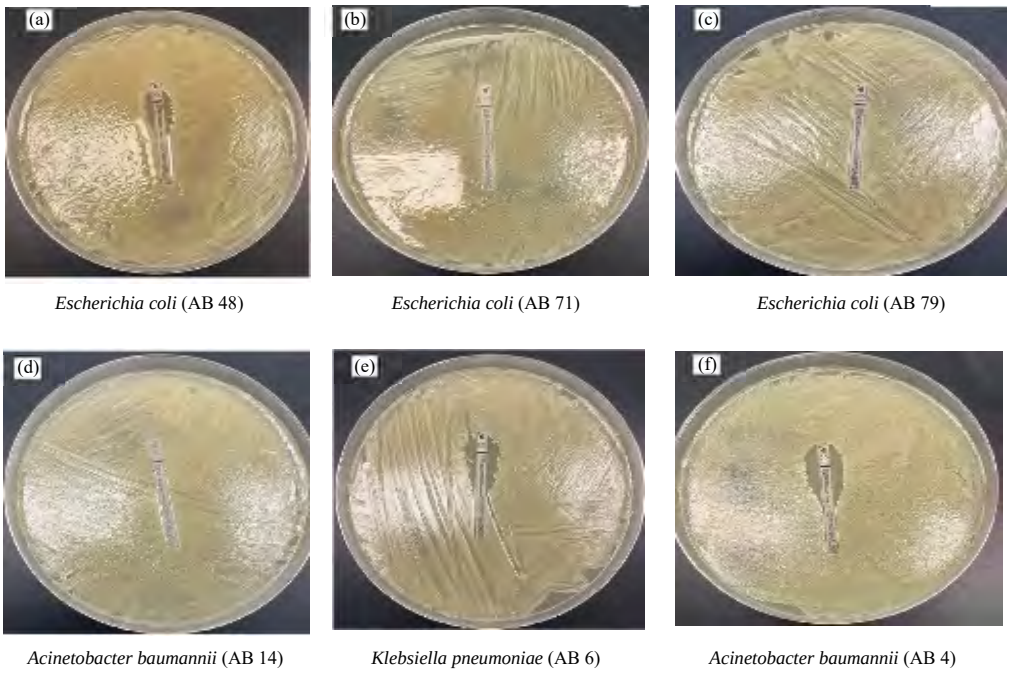

Fig. 3(a-f): Agar plates showing samples of colistin Etest strips results showing susceptible and resistant bacterial strains *Escherichia coli* isolate no. AB45, AB71, AB79, *Acinetobacter baumannii* isolates no. AB4, AB14 and *Klebsiella pneumoniae* no. AB6

errors detected with 2 of the isolates susceptible by MIC values of 2 μg mL^{-1} but resistant by disc diffusion zone of inhibition diameters of 5 and 6 mm. Minor errors detected in *E. coli* results were 3 isolates with intermediate susceptibility (13 mm each) by disc diffusion zone of inhibition diameter with MIC values of 4, 32 and 24 μg mL^{-1}, respectively (Fig. 4b)

A minor error was detected with the one *Klebsiella pneumoniae* isolate that was susceptible to disc diffusion and resistant by Etest MIC (Fig. 4c). There were no very major (false sensitivity) nor major (false resistance) with this group of isolates.

In the case of *Pseudomonas* isolates, all the isolates that were resistant by disc diffusion assay were also resistant by Etest MIC results. Thus, there was neither false susceptible nor false resistance in this group of isolates. However, the one intermediate isolate resistant by Etest MIC was categorised as a minor error (Fig. 4d). Overall results showed that vitek compact 2 automated system analysis indicated 96% resistant bacterial strains while disc diffusion detecting 89% and Etest with 96% for the isolates. These results did not show any statistical significance.

Broth microdilution (BMD) for colistin and polymyxin B MIC determination: The results of BMD MIC determination for colistin and polymyxin B for 14 randomly selected isolates are presented in Table 3. It indicates that MIC range between 1-2 μg mL^{-1} for Colistin and 2-4 μg mL^{-1} for polymyxin B. Isolates of *Acinetobacter baumannii* represented as isolated ID AC20, AC42, AC57 and AC82 showed MIC for colistin as $\geq$2, $\geq$8, $\geq$1 and $\geq$8 μg mL^{-1}, respectively. Polymyxin B MIC for same isolates were $\geq$8, $\geq$2, $\geq$2 and $\geq$8 μg mL^{-1} consecutively. *Pseudomonas aeruginosa* MICs obtained for colistin from BMD were $\geq$1and >8, for PS25 and PS92. Whereas, for the same isolates polymyxin B MICs were $\geq$2 and $\geq$8 μg mL^{-1}.

BMD for *Klebsiella pneumoniae* for colistin gave MICs of $\geq$1, $\geq$2 and >8 μg mL^{-1} for KP67, KP67 and KP97, respectively. The same isolates for polymyxin B gave >8, >2 and >8 μg mL^{-1} consecutively. For *Escherichia coli* isolates, MICs obtained for colistin were $\geq$8, $\geq$8 and >8 μg mL^{-1} (EC61, EC71 and EC79). However, polymyxin B gave similar MICs for the same isolates as >8 μg mL^{-1}. Therefore, BMD MICs determination did not show any specific pattern for *Acinetobacter baumannii* and *Klebsiella pneumoniae*, however, for showed similar MICs for both colistin and polymyxin B.

Detection of DNA-*mcr-1* gene: Of the 91 MDR clinical isolates investigated for the chromosomally encoded *mcr-1* gene, the results presented in Fig. 5a-b showed amplified amplicons in 10 *P. aeruginosa* isolates and 4 *Escherichia coli* isolates. The four *Escherichia coli* isolates were seen to have *mcr-1* gene with 309 bp (2), 500 bp (1) and 1 kb (1) for isolates EC54,

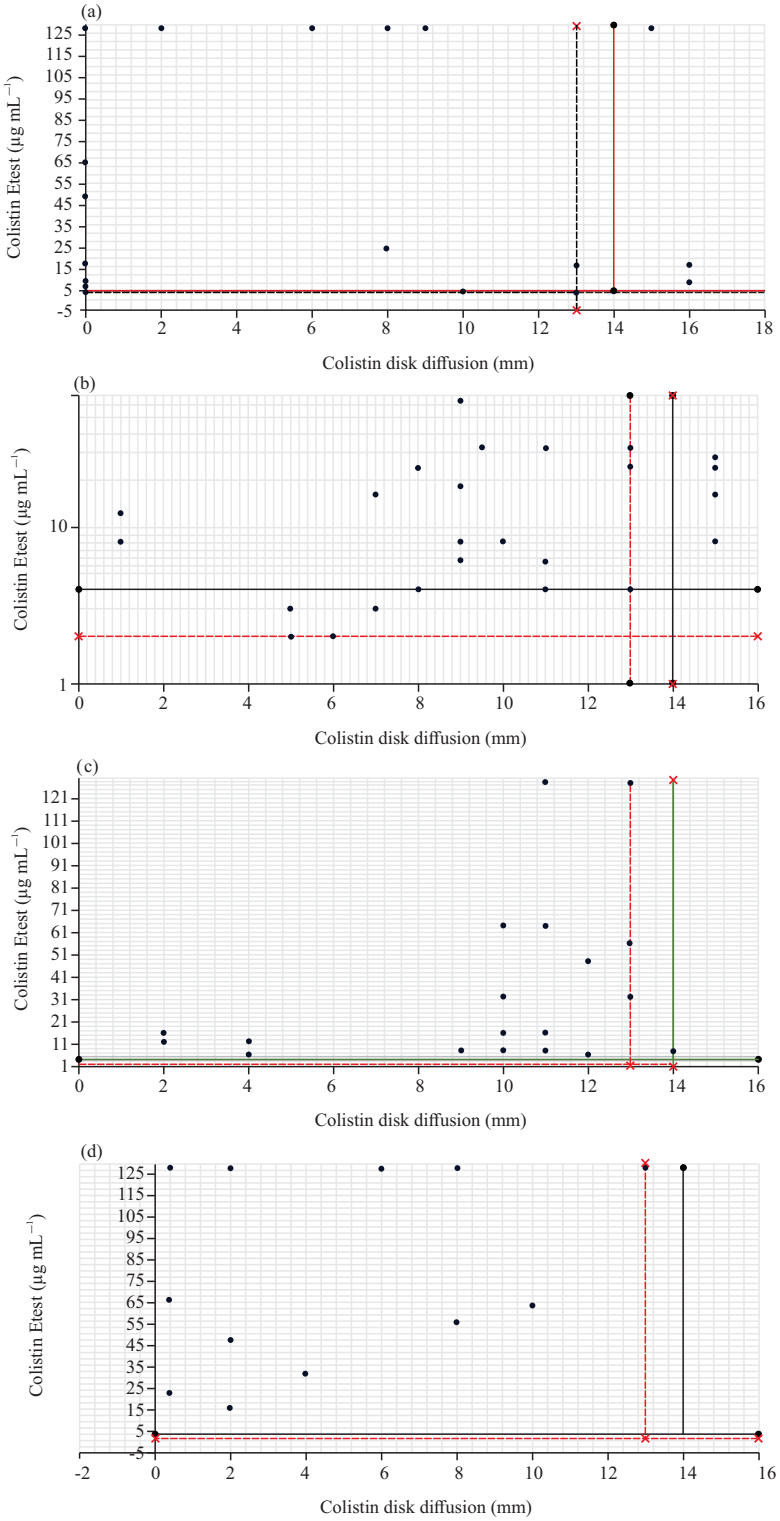

Fig. 4(a-d): Scattergram comparison of Etest minimum inhibitory concentration (MIC) and diameter zone of inhibition (mm) on 10 µg colistin disc diffusion, (a) 28 MDR *Acinetobacter baumannii* isolates, (b) 26 *Escherichia coli* isolates, (c) 23 *Klebsiella pneumoniae* isolates and (d) 13 *Pseudomonas aeruginosa* isolates

Coloured solid lines are CSLI breakpoint values, 10 µg disc zone of inhibition diameter ≤12 mm was considered resistant and ≥14, broken lines represent Intermediate susceptibility (13 mm disc diameter and 3 mg L^{-1} MIC)

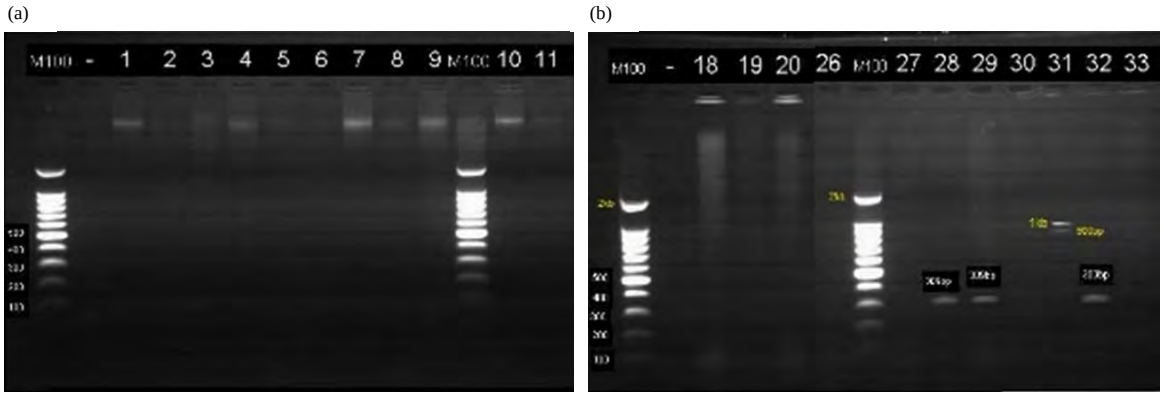

Fig. 5(a-b): Multiplex PCR gel electrophoresis for the detection of *mcr-1* gene, (a) Lanes 1-11 are *Pseudomonas aeruginosa*, isolates codes PS6, PS12, PS33, PS25, PS51, PS60, PS65, PS73, PS75, PS90, PS84, respectively and (b) Lanes 18-33 are *Escherichia coli* isolates EC26, EC28, EC32, EC48, EC49, EC54, EC61, EC71, EC74, EC77 and EC79, *Mcr-1* gene with 309 bp was detected in lanes 28 (EC54), 29 (EC61) and 32 (EC77)

Table 3: Comparing bacterial growth inhibitions in different colistin and polymyxin B concentrations in 14 randomly selected bacterial isolates

		Colistin (μg mL^{-1})					Polymyxin B (μg mL^{-1})				
Lab ID	Bacterial isolates	0	1	2	4	8	0	1	2	4	8
AC 20	*A. baumannii*	2.92	3.5	2.43*	3.74	3.19	2.26	3.04	3.47	3.84	3.57
PS 25	*P. aeruginosa*	3.31	2.87*	3.55	3.5	2.9	3.29	3.58	2.81*	2.95	3.47
AC 42	*A. baumannii*	3.12	3.24	3.19	3.19	3.04	3.6	3.54	3.41*	3.67	3.76
PS 33	*P. aeruginosa*	2.22	2.77	2.89	2.29	2.23	3.21	3.52	2.83*	2.4	2.22
AB 56	*Shigella flexneri*	0.05	0.23	0.66	1.57	0.21	0.8	1.59	1.32	0.49	0.69
AC 57	*A. baumannii*	3.43	3.09*	3.18	3.60	3.65	3.34	3.58	3.1*	2.85	2.64
KP 67	*K. pneumoniae*	1.95	1.92	2.67	1.88	2.41	1.23	2.03	2.05	1.26	1.17
KP 69	*K. pneumoniae*	3.01	3.03	2.79*	3.43	3.18	2.97	3.62	3.34	2.94	3.03
AC 82	*A. baumannii*	2.65	3.6	3.58	3.04	3.37	2.5	2.77	3.4	3.37	3.14
PS 92	*P. aeruginosa*	2.53	3.64	3.07	3.34	3.39	1.6	2.57	3.1	2.42	2.02
EC 61	*Escherichia coli*	3.34	3.48	3.67	3.7	3.77	3.32	3.4	3.34	3.8	3.76
KP 97	*K. pneumoniae*	0.91	2.42	1.7	1.83	1.92	1.16	2.82	2.13	1.24	1.48
EC 71	*E. coli*	3.01	3.45	3.34	3.6	3.32	2.8	3.2	3.4	3.3	2.87
EC 79	*E. coli*	3.5	3.65	3.77	3.82	3.65	3.0	3.4	3.45	3.6	3.1

*Represents minimum growth inhibition by either colistin or polymyxin B compared to 0 μg mL^{-1} drugs concentrations, respectively. However, there were no statistically significant differences observed between them

EC61, EC74 and EC77. In addition, PCR results revealed an amplicon with more than 2 kb for *Pseudomonas aeruginosa* isolates (isolates codes PS6, PS 12, PS33, PS25, PS51, PS60, PS65, PS73, PS75, PS90, PS84) in lanes 1-11. These also revealed the presence of resistant genes as all *Pseudomonas aeruginosa* isolates tested were resistant with all the assay procedures used in this study.

DISCUSSION

The enormous global health challenges resulting from difficult to treat bacterial isolates is again highlighted in this report. The polymyxins (colistin and polymyxin B) are used as the last resort in the treatment of MDR Gram-Negative Bacterial (GNB) isolates[13] and resistance to these drugs is exhibited by the isolates in this investigation. The antimicrobial susceptibility pattern seen among the GNB isolates in this study is not unusual. Saudi Arabia is reported to be in a strategic position with high antimicrobial resistance which could further escalate the spread of resistance to antibiotics globally[26]. This could be due to annual visits into the Kingdom from other regions of the world[27].

Resistance to colistin and polymyxin B is seen in the 4 different GNB isolates investigated in this research when interpreted by CLSI[19] Minimum Inhibitory Concentration values (MIC) with all the isolates differing in the levels of resistance. Resistance to colistin by *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* have been reported globally by other researchers[28] and within the Kingdom by others as well. For *A. baumannii*

Azim *et al.*[29], Ibrahim[30], for *E. coli* Al-Agamy *et al.*[31], Ibrahim[30], Alghoribi *et al.*[32], *K. pneumoniae* Garbati *et al.*[33] and for *P. aeruginosa*, Bandy and Almaeen[34].

Eighty-two percentage colistin resistance by disc diffusion assay was seen in this investigation. Though disc diffusion interpretation for colistin resistance is considered generally unreliable[20], with a recommendation that MIC is used in severe clinical cases. In this investigation, they were used for preliminary tests and further investigated with Etest. With a 91% MIC resistance rate to colistin by Etest did not show any significant (p-value 0.65) difference with that obtained by disc diffusion. Therefore, results here show a high percentage of colistin-resistant GNB indicating that values from both methods are in concordance. Similar findings on good result harmony between disc diffusion and MIC assay had been reported previously[20]. Worthy of note is the absence of both very major and major errors in the comparison of disc diffusion and MIC results for *A. baumannii*, *K. pneumoniae* and *P. aeruginosa* here, findings that are like those of a recent report[35]. In this investigation, the AST results by vitek compact 2 automated system for *A. baumannii* showed all the isolates were resistant to colistin (MIC $\geq$16). Also, with Etest MIC, values ranged between 3 $\geq$128 mg L^{-1} for 96.4% of the isolates, resistance to colistin is high here for this bacterium. There are global reported resistance to colistin by *A. baumannii*[36] as well as local reports in the region of this investigation[37]. In Saudi Arabia, resistance to colistin by *A. baumannii* vary from completely resistant[37] to low[30,38]. However, that *mcr-1* was not detected from *A. baumannii* isolates used in this study could be attributed to the fact that this gene was not being carried in their DNA and hence could either be plasmid-mediated or due to other mcr resistance determinants. The occurrence of chromosomally encoded *mcr-1* though suggested to be rare according to Li *et al.*[39] is now increasingly being reported[17,40,41]. Also, the amplicon size of 550 bp seen in one of the isolates of *E. coli* had been reported previously[42].

With *E. coli* isolates, very major errors (false sensitivity) were encountered as five of the isolates with zones of inhibition $\geq$14 mm with resistant MIC ranging between 8-20 μg mL^{-1}. Major errors (false resistance) were additionally associated with *E. coli* as two of the isolates with susceptible MIC were found to be resistant by disc diffusion. Also, minor errors were encountered in three of the twenty-three *E. coli* isolates investigated. It, therefore, suggests discrepancies that might need to be investigated particularly as *mcr-1* genes were detected in four of the isolates. All four isolates were from urine samples, resistant to colistin by both disc diffusion and Etest MIC results. Similar findings on chromosomally encoded *mcr-1* in *E. coli* strains had previously been reported[17], while plasmid transferable ExPEC colistin-resistant *mcr-1* was 1st reported in Saudi Arabia in 2016[43] as well as in a recent study by Alghoribi *et al.*[32].

The remaining 2 GNB isolates (*K. pneumoniae* and *P. aeruginosa*) were also found to be resistant to colistin according to CLSI[21] [2020] criteria. For *K. pneumoniae*, DNA *mcr-1* was not detected and this might be plasmid-mediated or due to the enormous clonal diversity shown by colistin-resistant *K. pneumoniae* as had earlier been suggested by other reports[28,44] as well as the limited number of colistin-resistant molecular determinants investigated here. Related reported studies in the Kingdom to vary in their observations regarding *K. pneumoniae* resistance to colistin[45] and other antibiotics[33,44]. For *P. aeruginosa*, PCR amplification detected amplicons that were more than 2 kb. However, that the bands were detected in 11 of the 13 investigated isolates would suggest the possibility of other colistin-resistant determinants that would need to be investigated. Also, with *P. aeruginosa*, there are varying reports regarding colistin susceptibility and resistance in Saudi Arabia[29,30]. Worthy of note also is that there were no very major, major or minor errors encountered in *P. aeruginosa* findings as all the 92% isolates found to be resistant by disc diffusion, had resistant MIC (by Etest) while the one intermediate susceptibility by disc diffusion had a resistant MIC as well.

For all the GNB isolates there will be a need for a more detailed investigation as there is the postulation that detecting mcr-positive bacteria early could help prevent the spread of the strains as well as help in providing an appropriate and timely antimicrobial therapy.

Based on the results of the present study, the performance of colistin and polymyxin B using BMD assay, showed significant similarities. Therefore, following CLSI 2020 recommendations, isolates used in this study can be classified as both colistin and polymyxin B resistant bacteria considering that MIC $\leq$2 μg mL^{-1} as sensitive and >2 μg mL^{-1} as resistant. These findings were corroborated by recent documented studies of Zhu *et al.*[46] and Chew *et al.*[35].

Although colistin produced nonsignificant growth reductions at MIC 1 μg mL^{-1} for 2 isolates (*P. aeruginosa* PS25 and *A. baumannii* AC57), there was a subsequent growth increase with higher concentrations. These similarities in activity according to Pogue *et al.*[47] show cross-susceptibility between colistin and polymyxin B as the report indicated a similarity range of 99.52-99.99% for all species. They also indicated that false-nonsusceptibility relating to colistin resistance to polymyxin B susceptibility or Vis vasa accounts for <1% generally. Hence, in our findings, we agree that colistin susceptibility could be a surrogate for polymyxin B susceptibility[47].

This study further confirms that plasmid carrying *mcr-1* gene could likely be transposed into the chromosome, which could eventually progress into a more stable genotype. Hence, the detection of chromosomal *mcr-1* genes in the present study. The phenomenon is postulated to remodel the bacterial chromosome facilitating the emergence of polymyxins resistance. Part of the limitation of this study was that it is lacking in genomic sequence.

CONCLUSION

Resistance to colistin and polymyxin B is increasingly being documented among MDR clinical isolates globally. This study examined MDR clinical isolates and found that they exhibited MDR, XDR or CRE characteristics. Using Vitek compact 2 automated system, disc diffusion, Etest and BMD (also for comparison), isolates were found to be 96, 89, 96 and 100% resistant, respectively. The study also showed high concordance between colistin and polymyxin B with BMD tests. Resistant DNA *mcr-1* genes were detected among four *E. coli* isolates and also, other resistant genes with more than 2 kb were detected with *P. aeruginosa* isolates confirming their 100% resistance seen in this study. The present study further highlights polymyxins bacterial resistance undercutting their present use in the treatment of MDR gram-negative bacterial infections. Therefore, results obtained from this investigation could be contributory in guiding decision-making for the management of hard to treat MDR gram-negative bacterial infections and institute surveillance to monitor this scourge.

SIGNIFICANCE STATEMENT

This study further contributes to the global growing knowledge of polymyxins resistance which is now being used as last resort antibiotics for MDR gram-negative bacterial infections. It also highlights the fact that plasmid carrying *mcr-1* resistant gene could be transferred to chromosomes and becomes more problematic to or hard to treat infections. The present study, therefore, shows the need for prompt detection and surveillance in other to contain the spread of resistant strains and ensure proper antibiotic usage.

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