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Evaluation of a novel epidemiological screening approach for detection of colistin resistant human *Enterobacteriaceae* isolates using a selective SuperPolymyxin medium

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1. Introduction

Polymyxin antibiotics which include polymyxin B and colistin (also known as polymyxin E) were initially discovered after second world war and widely used in human medicine until the 1970s when they were largely replaced by more active and less toxic antibiotics such as beta-lactams and aminoglycosides (Poirel et al., 2017). Increased incidence of multi-drug resistant Gram-negative bacilli (MDRGNB) has revived interest in polymyxins, which retain activity against MDRGNB and are considered one of the last-line antibiotics for treatment of MDRGNB infections in particular against carbapenemase-producing GNB (WHO, 2014). However, with increased use of colistin for treatment of MDRGNB infections, colistin resistance is increasingly being reported. Colistin resistance is most frequently reported in MDR *Klebsiella pneumoniae* where resistance is usually the result of chromosomal mutations (Poirel et al., 2017; Grundmann et al., 2017; Giani et al., 2015). True prevalence of colistin resistance is unknown as colistin susceptibility data is rarely systematically obtained. According to EARS-Net data it was reported for a little over a quarter of invasive *K. pneumoniae* isolates and overall, 8.5% of the tested isolates were resistant to colistin. However these findings should be interpreted with caution as most of the data came from countries with high prevalence of carbapenem-resistant *Enterobacteriaceae* (CRE) and is probably not representative for Europe as a whole (ECDC, 2017a). Less data is available for *Escherichia coli*; according to the SENTRY antimicrobial surveillance data from 2016, low prevalence of resistance to colistin was reported worldwide (< 0.5%) (Castanheira et al., 2016). In 2016, the first isolate harbouring plasmid-mediated colistin resistance *mcr-1* was reported in an *E. coli* isolate in China (Liu et al., 2016). Since then plasmid-mediated resistance has been most frequently reported in *E. coli* as well as *Salmonella* isolates (Skov and Monnet, 2016). Plasmid-mediated resistance has given new momentum to the developing story of colistin resistance. Increasing selective pressure generated by colistin usage, particularly in countries with higher prevalence of MDRGNB, presumably leads to selection of resistant mutants (Giani et al., 2015). This selective pressure not only increases the occurrence and spread of isolates harbouring chromosomal mutations but also creates an advantageous environment that can facilitate the spread of plasmid-mediated resistance. MCR-1 producing *E. coli* have already been reported worldwide, including several European countries (Poirel et al., 2017). There is very limited data on colistin resistance in GNB in Slovenia. Due to its limited use in human medicine, susceptibility to colistin is not routinely tested and is mainly performed for MDRGNB isolates and isolates from cystic fibrosis patients. A single retrospective

Abbreviations: MDRGNB, multi-drug resistant Gram-negative bacilli; AST, antimicrobial susceptibility testing; BMD, broth microdilution; MIC, minimal inhibitory concentration; CRE, carbapenem-resistant *Enterobacteriaceae*; XDR, extensively drug resistant; SPM, SuperPolymyxin medium; PCR, polymerase chain reaction; TP, true positive; TN, true negative; FP, false positive; FN, false negative; NPV, negative predictive value; PPV, positive predictive value

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study on susceptibility of MDRGNB isolates to colistin in Slovenia showed excellent susceptibility to colistin in carbapenem-resistant (CR) *E. coli*, however in CR *K. pneumoniae* colistin resistance was almost 9% (Pirš et al., 2017). No data on overall susceptibility of clinical isolates of *Enterobacteriaceae* to colistin in human isolates in Slovenia is currently available and so far no plasmid-mediated colistin resistance has been found.

The European Surveillance of Antimicrobial Consumption Network (ESAC-Net) data shows a steadily increasing trend of polymyxins consumption (ATC group J01XB) in Slovenia in the past decade – from 0.0001 to 0.0082 DDD/1000 inhabitants and per day (ECDC, 2017b). Regional colistin consumption in both human and veterinary medicine shows considerable variation; higher consumption in both human and veterinary medicine is found for example in neighbouring Italy (EMA, 2016). The increase of colistin consumption in Slovenian hospitals is mainly due to the increased prevalence of MDRGNB, in particular *Acinetobacter baumannii*, where susceptibility to imipenem has decreased from 94% in 2011 to 66% in 2016 and to a lesser degree extensively drug resistant (XDR) *Pseudomonas aeruginosa* (Štrumbelj et al., 2016). With increased use of colistin we can expect an increase in colistin resistance levels.

Colistin antimicrobial susceptibility testing (AST) must be performed using broth microdilution (BMD) in accordance with the joint CLSI – EUCAST Polymyxin Breakpoints Working Group recommendation issued in July 2016 (CLSI-EUCAST, 2016). This method for minimal inhibitory concentration (MIC) determination is more time consuming and labour intensive compared to gradient diffusion or Vitek2 AST, which were common methods of choice for colistin AST in the past. Addition of colistin BMD into routine AST for *Enterobacteriaceae* in laboratories which primarily use non-BMD methods for AST is not a realistic possibility. Even in cases where BMD is routinely used, colistin is rarely part of routine AST panels. Unless an isolate is MDRGNB (or has been isolated from a cystic fibrosis patient) where colistin MIC is usualy determined for potential clinical application colistin AST serves mainly for epidemiological purposes.

To determine the level of colistin resistance in certain region studies can be performed, however such data is usually limited to certain time period or certain type of isolates (for example invasive isolates, MDRGNB) resulting in only incidental detection of colistin resistance (plasmid-mediated or chromosomal). With such limited isolate targeting the detection of plasmid-mediated resistance particularly among well susceptible isolates is unlikely, nonetheless clinical microbiology laboratories should consider testing for colistin susceptibility more frequently (Skov and Monnet, 2016).

The use of selective culture media is a well-accepted method in clinical microbiology and was recommended as an initial screening tool for colistin resistance (Caniaux et al., 2017). Selective culture based screening requires overnight cultivation, and in case of growth resistance must be confirmed using reference method. Even though the method is simple and can be easily incorporated in laboratory workflow, it still requires a large number of plates of screening medium (one per isolate), thus increasing the cost and workload. We aimed to find a low-cost approach to screen a large number of *Enterobacteriaceae* isolates in a setting where low prevalence of colistin resistance was expected with minimum hands-on time and costs.

Here we describe a novel epidemiological screening protocol for detection of colistin resistant human *Enterobacteriaceae* isolates regardless of resistance mechanisms. The aim of our study was to screen a large number of isolates for colistin resistance, assess the burden of resistance in isolates regardless of overall susceptibility profile and to determine whether plasmid-mediated colistin resistance is present among resistant isolates.

2. Materials and methods

Prospective study was conducted at the Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Slovenia, which performs microbiological diagnostics for health care institutions in the Central Slovenian region including a tertiary centre and a specialized oncology hospital. A total of 700 *Enterobacteriaceae* isolates (364 *Escherichia coli*, 176 *K. pneumoniae*, 31 *Klebsiella oxytoca*, 88 *Enterobacter* spp., 41 *Citrobacter* spp) were randomly prospectively collected from clinical and surveillance samples. For all these isolates epidemiological screening for colistin resistance was performed as well as MIC of colistin determined. Among them, 583 (83.3%) were isolated from clinical samples (42.9% urine, 42.0% blood culture, 10.4% lower respiratory tract, 4.7% other samples) and 117 (16.7%) from surveillance samples (mainly stool samples or rectal swabs).

2.1. Bacterial identification

Bacteria were isolated from clinical and surveillance samples following the standard laboratory protocol. Colonies were identified using matrix-assisted laser desorption/ionization time-of-flight mass-spectrometry (MALDI TOF MS) (MBT COMPASS 4.1, Microflex, Bruker Daltonics, Bremen, Germany).

2.2. Antimicrobial susceptibility testing and determination of MIC

AST was performed using disk diffusion or the Vitek2 Compact system (BioMérieux, Marcy-l'Étoile, France). Routinely tested antimicrobial agents included ampicillin, amoxicillin-clavulanic acid, piperacillin-tazobactam, cefuroxime, cefotaxime, ceftriaxone, ceftazidime, cefepime, imipenem, ertapenem, gentamicin, amikacin, ciprofloxacin, levofloxacin and trimethoprim-sulphametoxazole. Results were interpreted to the EUCAST guidelines (EUCAST, 2017a). Extended-spectrum beta-lactamase (ESBL) production and carbapenemase production was tested according to EUCAST recommendations (EUCAST, 2017b).

Colistin MIC was determined from the growth control plate (MacConkey medium), results were interpreted according to the EUCAST guidelines (EUCAST, 2017a). Colistin MIC was determined in duplicates in accordance with the joint CLSI-EUCAST Polymyxin Breakpoints Working Group recommendation using BMD without polysorbate following ISO-20776 standards (CLSI-EUCAST, 2016). Colistin (sulphate salt; Sigma-Aldrich, France) was used in a serial two-fold dilution ranging from 0.12 mg/L to 128 mg/L. Non-treated polystyrene 96-microtiter plates were used (CLSI-EUCAST, 2016). MICs of ≤ 2 mg/L were interpreted as susceptible and > 2 mg/L as resistant (EUCAST, 2017a).

2.3. Control strains

Control strains used for PCR and BMD were ATCC 25922 *E. coli*, NCTC 13864 *mcr-1* positive *E. coli* strain and *mcr-2* positive *E. coli* that was kindly provided by the University of Antwerp, Belgium (Xavier et al., 2016).

A *mcr-1* positive *E. coli* strains from three laboratory collections used for evaluation of effectiveness of the protocol were kindly provided by Dr. Poirel and Prof Nordmann (5 strains), Prof Sambri (5 strains) and Austrian National Reference Centre for Nosocomial Infections and Antimicrobial Resistance (2 strains) (Nordmann et al., 2016; Del Bianco et al., 2018; Hartl et al., 2017).

2.4. Epidemiological screening protocol for colistin resistance using selective SuperPolymyxin medium

Epidemiological screening was performed using in-house prepared SuperPolymyxin medium (SPM) containing 3.5 mg/L of colistin as described previously (Nordmann et al., 2016). Isolates were screened for colistin resistance from the same 0.5 McFarland bacterial suspension that was used to perform routine AST to reduce workload and cost.

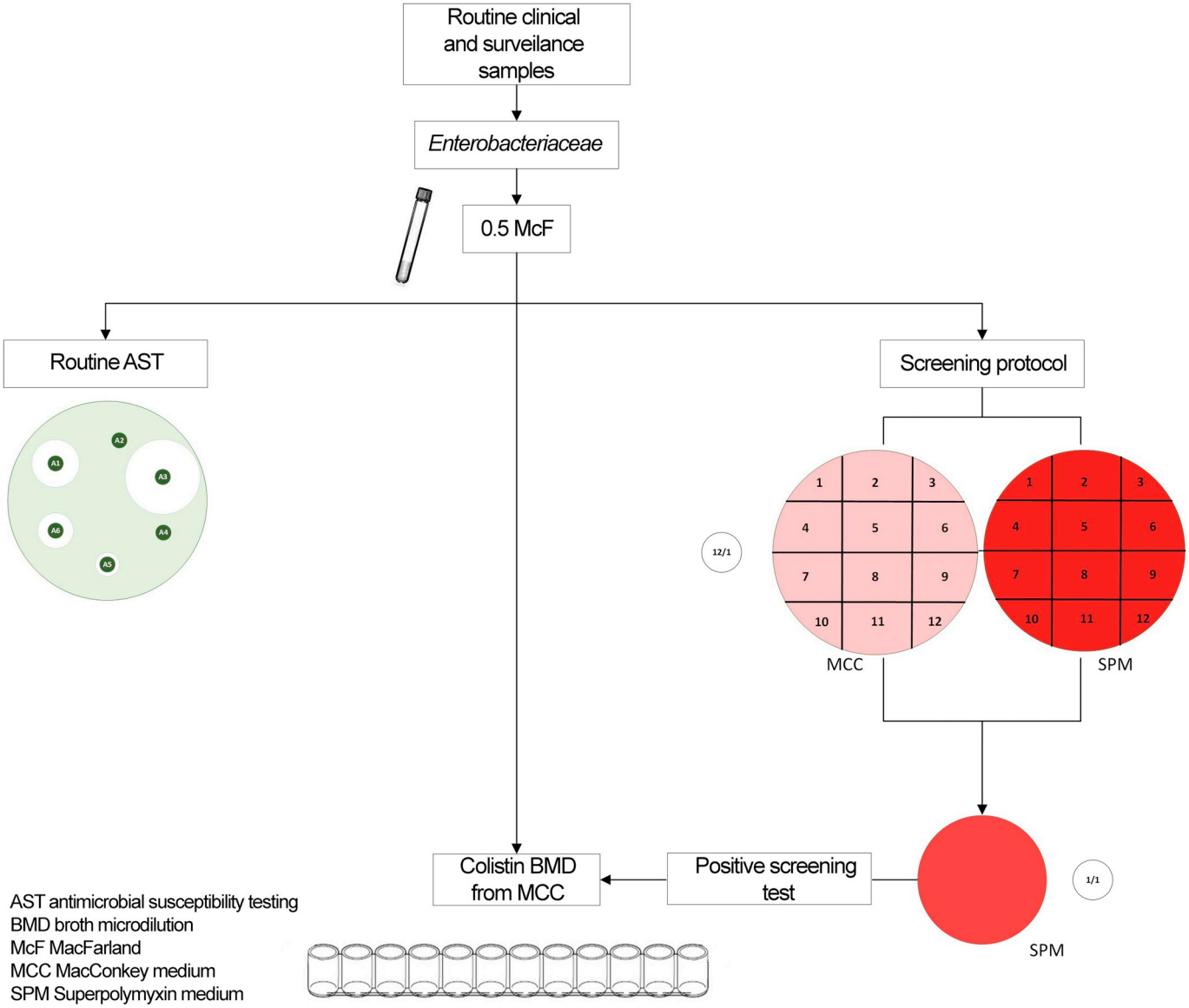

Fig. 1. Epidemiological screening protocol using a selective SuperPolymyxin medium to detect *Enterobacteriaceae* isolates resistant to colistin.

Instead of using one plate per isolate as originally described we used a modified protocol (Nordmann et al., 2016). In brief, a wood-cotton swab was immersed into a 0.5 McFarland bacterial suspension, excess fluid was removed by pressing and turning the swab against the inside of the tube. The bacterial suspension was spot inoculated with a wood-cotton swab onto SPM and MacConkey medium used as a growth control plate. One SPM plate, previously divided into 12 sectors, was inoculated with up to 12 bacterial strains (12/1 SPM). All media were incubated at 35 °C overnight aerobically. In case of growth on 12/1 SPM the same isolate from the growth control plate was inoculated on a single SPM plate (1/1 SPM) by preparing a 0.5 McFarland bacterial suspension followed by streaking 10 µL of suspension using the four quadrant streak method, as shown in Fig. 1 (Nordmann et al., 2016).

The effectiveness of the protocol to detect colistin resistant isolates was evaluated using colistin resistant MDRGNB *Enterobacteriaceae* (2 *E. coli*, 3 *K. pneumoniae*, 3 *Enterobacter cloacae complex*), 5 intrisically resistant *Enterobacteriaceae* (3 *Proteus mirabilis*, 2 *Serratia marcescens*) from laboratory strain collection (colistin MIC 4–64 mg/L) which were all *mcr(1–5)* negative as well as the two *mcr-1* and *mcr-2* positive *E. coli* control strains as well as 12 *mcr-1* positive *E. coli* strains from three laboratory strain collections (colistin MIC 4 mg/L).

Control strains ATCC 25922 as negative and *mcr-1* and *mcr-2* positive *E. coli* as positive controls were used for agar quality control for every batch of SPM.

In order to evaluate the performance of the modified epidemiological screening protocol colistin MIC was determined for 700 randomly selected *Enterobacteriaceae* isolates that were screened on SPM. True positive (TP) isolates were defined as growth on 12/1 and 1/1 SPM plates and MIC > 2 mg/L. True negative (TN) isolates were defined as no growth on 12/1 SPM plates and MIC ≤ 2 mg/L. False positive (FP) isolates were defined as growth on 12/1 and 1/1 SPM plates and MIC ≤ 2 mg/L. False negative (FN) isolates were defined as no growth on 12/1 SPM plates and MIC > 2 mg/L.

2.5. Nucleic acid extraction

Nucleic acid from isolates with colistin MIC value > 2 mg/L was extracted from colonies grown on media without colistin (purity plates from suspension used for MIC determination or MacConkey medium). Genomic DNA was isolated using the Instant Gene Matrix (Bio-Rad Laboratories, Hercules, USA) following the manufacturer's instructions.

2.6. Detection of mcr genes

Specific detection of *mcr-1* and *mcr-2* genes was performed on isolates with colistin MIC ≥ 2 mg/L using PCR as previously described (3). The presence of specific *mcr* genes was verified by sequencing PCR products and comparing sequences with known sequences listed in the GenBank. Additionally, multiplex PCR for detection of *mcr-1, mcr-2, mcr-3, mcr-4* and *mcr-5* was also performed (Rebelo et al., 2018).

2.7. Beta-lactamase genes detection

Beta-lactamase producers were identified phenotypically and molecularly characterized with the DNA array 'Check-MDR CT103 XL' (Check-Points, Wageningen, the Netherlands) for detection of β-lactamase genes of ESBLs, plasmid AmpCs and carbapenemases. The full gene list is available at http://www.check-points.eu/products/check-mdr-ct103xl.html. The test was performed according to the manufacturer's instructions. Briefly, following DNA extraction, ligation with tagged probes that recognize beta-lactamase genes occurs. The tagged probes are amplified in a multiplex PCR. The resulting products are detected with a tube microarray.

3. Results

3.1. The effectiveness of epidemiological screening protocol for detection of colistin resistant isolates

All *mcr(1–5)* negative and intrinsically colistin resistant *Enterobacteriaceae* isolates from laboratory strain collection (colistin MIC 4–64 mg/L) as well as the 13 *mcr-1* and *mcr-2* positive *E. coli* strains showed significant growth on the 12/1 SPM as well as growth on the 1/1 SPM plate and were thus detected using the epidemiological screening protocol.

3.2. Epidemiological screening for colistin resistance using a selective medium SPM

In total 90 of 700 isolates showed growth on the 12/1 SPM plate and 52 isolates showed further growth also on the 1/1 SPM plate. Three out of these 52 isolates were susceptible to colistin (FP isolates; one *E. coli* with MIC 0.5 mg/L and two *K. pneumoniae* isolates with MICs 0.25 and 2 mg/L). In the remaining 49 isolates colistin resistance was confirmed with MICs 4 mg/L or higher (TP isolates) (Table 1). MICs of the 38 isolates with significant growth on the 12/1 SPM plate and no growth on the 1/1 SPM plate ranged from 0.25 to 2 mg/L. Seven FN isolates were detected with colistin MIC > 2 mg/L and did not present growth on SPA 12/1; two *K. pneumoniae* (MICs 8 and 16 mg/L) and five *Enterobacter* spp. isolates, with MICs ranging from 64 to 128 mg/L. MIC_{50} for colistin among 700 *Enterobacteriaceae* was 0.5 mg/L, MIC_{90} was 1 mg/L. Overall sensitivity and negative predictive value (NPV) of the epidemiological screening protocol were 87.5% and 98.9%, with 99.5% specificity and 94.2% positive predictive value (PPV). Detailed results and performance evaluation are presented in Table 1.

3.3. Characteristics of colistin resistant Enterobacteriaceae strains

A total of 56 colistin resistant isolates were found during the study of which 13 were *E. coli*, 20 *K. pneumoniae*, 22 *Enterobacter* spp. and a single *Citrobacter* spp. isolate. Fourty colistin resistant isolates were isolated from clinical samples (eight from primary sterile sites, 22 from urine samples, 10 from lower respiratory tract) and 16 from surveillance samples. Colistin MICs ranged from 4 to 256 mg/L. Among the resistant isolates 30 were also resistant to 3rd generation cefalosporins (16 were confirmed ESBL producers, ten plasmid AmpC betalactamases, four derepressed chromosomal AmpC betalactamases) and a single isolate resistant to carbapenems (not a carbapenemase producer) (Table 2).

A single *E. coli* harbouring *mcr-1* gene was detected and no strains with *mcr-2, mcr-3, mcr-4* or *mcr-5* genes were found. The *mcr-1* positive *E. coli* strain was isolated from a urine sample. It was resistant to ampicillin, gentamicin and trimethoprim/sulfamethoxazole, and susceptible to all other tested antibiotics. Colistin MIC was 4 mg/L (Table 2).

4. Discussion

Here we present the results of the evaluation of novel epidemiological screening approach for detection of colistin resistant *Enterobacteriaceae* isolates regardless of resistance mechanisms.

The use of colistin has been increasing in recent years mainly due to the increased incidence of MDRGNB (WHO, 2014). Routine MIC determination of colistin in *Enterobacteriaceae* is problematic because of its limited usefulness in human medicine, higher cost and workload associated with BMD. When systematic surveillance of resistance trends are available for clinicaly relevant antibiotics, evolving trends can be detected which can be useful when a novel, in particularly plasmid-mediated resistance mechanism emerge, as its emergence and overall impact can be assessed on this data (Cornaglia et al., 2004). As colistin is rarely a part of routine antibiotic panels, the data available is limited mainly to MDRGNB making a risk assessment of colistin resistance and in particularly plasmid-mediated colistin resistance difficult. The use of MIC determination solely for epidemiological purposes is not realistic or practical due to increased workload and cost.

In order to screen a large number of isolates to assess the scope of colistin resistance and to determine whether plasmid-mediated colistin resistance is present in Slovenian human *Enterobacteriaceae* isolates we have developed an epidemiological screening protocol to screen *Enterobacteriaceae* isolates for colistin resistance using a selective medium SPM (Nordmann et al., 2016).

To reduce the workload and cost of screening we spot-inoculated suspensions of 12 isolates that were originally prepared for routine AST on the same SPM plate (12/1). In case of growth on this SPM plate the same isolate was inoculated onto a single SPM (1/1) plate by four

Table 1
Performance evaluation of epidemiological screening protocol using a selective SuperPolymyxin medium to detect *Enterobacteriaceae* isolates resistant to colistin.

	E. coli	*K. pneumoniae*	*K. oxytoca*	*Enterobacter* spp.	*Citrobacter* spp.	*Enterobacteriaceae*
Isolates tested (Number)	364	176	31	88	41	700
True positive	13	18	0	17	1	49
False positive	1	2	0	0	0	3
True negative	350	154	31	66	40	641
False negative	0	2	0	5	0	7
Sensitivity (%)	100	90.0	not applicable	77.3	100	87.5
Specificity (%)	99.7	98.7	100	100	100	99.5
Positive predictive value (%)	92.9	90.0	not applicable	100	100	94.2
Negative predictive value (%)	100	98.7	100	93.0	100	98.9

Table 2
Characteristics of the 56 colistin resistant *Enterobacteriaceae*.

Isolate	Protocol number	Specimen	SPM screening	Colistin MIC (mg/L)	*mcr-1-5*	Additional antimicrobial resistance	Beta-lactamase genes
E. coli	42/12	urine	TP	16	NEG	AM AMC	NA
E. coli	74/2	urine	TP	16	NEG	AM AMC CTX/CAZ FEP CIP	*ctx-m-15-like*
E. coli	132/6	rectal swab	TP	8	NEG	AM AMC TZP CTX/CAZ FEP CIP SXT GEN	*ctx-m-15-like*
E. coli	186/9	urine	TP	4	NEG	ND	NA
E. coli	191/10	urine	TP	16	NEG	AM AMC	NA
E. coli	209/4	urine	TP	4	NEG	ND	NA
E. coli	HK 6/32	blood culture	TP	4	NEG	SXT	NA
E. coli	245/8	urine	TP	8	NEG	AM AMC TZP CIP SXT	NA
E. coli	284/11	urine	TP	4	*mcr-1*	AM SXT GEN	NA
E. coli	HK 11/21	blood culture	TP	4	NEG	AM CTX/CAZ FEP	*ctx-m-15-like*
E. coli	328/3	urine	TP	8	NEG	AM CIP GEN	NA
E. coli	367/1	urine	TP	8	NEG	AM AMC TZP CTX/CAZ FEP CIP AN	*ctx-m-15-like*
E. coli	442/10	urine	TP	4	NEG	ND	NA
K. pneumoniae	26/2	rectal swab	FN	8	NEG	AM AMC TZP CTX/CAZ FEP CIP SXT	*ctx-m-15-like, tem-164*
K. pneumoniae	39/5	urine	TP	4	NEG	AM AMC	NA
K. pneumoniae	43/3	urine	TP	8	NEG	AM	NA
K. pneumoniae	46/10	urine	TP	32	NEG	AM	NA
K. pneumoniae	41/3	rectal swab	TP	8	NEG	AM AMC TZP CTX/CAZ FEP CIP SXT GEN	*ctx-m-1, shv-238, shv-240, tem-140, tem-164, tem-238*
K. pneumoniae	61/1	abdominal fluid	TP	32	NEG	AM AMC TZP CTX/CAZ FEP CIP SXT	*ctx-m-15-like*
K. pneumoniae	69/12	rectal swab	TP	16	NEG	AM AMC CTX/CAZ FEP CIP SXT GEN	*ctx-m-15-like, tem-164, shv-238*
K. pneumoniae	84/8	rectal swab	TP	64	NEG	AM AMC TZP CTX/CAZ FEP CIP SXT GEN	*ctx-m-15-like, tem-164, shv-238*
K. pneumoniae	88/10	sputum	TP	64	NEG	AM	NA
K. pneumoniae	136/6	urine	TP	4	NEG	AM	NA
K. pneumoniae	138/7	urine	TP	16	NEG	AM	NA
K. pneumoniae	137/2	TA/BAL	TP	32	NEG	AM AMC TZP CTX/CAZ FEP CIP SXT GEN	*ctx-m15-like*
K. pneumoniae	158/11	urine	TP	16	NEG	AM AMC CTX/CAZ FEP CIP SXT GEN	*ctx-m-1, tem-164*
K. pneumoniae	156/9	TA/BAL	TP	4	NEG	AM SXT	NA
K. pneumoniae	215/8	TA/BAL	FN	16	NEG	AM	NA
K. pneumoniae	219/5	urine	TP	32	NEG	AM AMC TZP CTX/CAZ FEP CIP SXT GEN	*ctx-m-1*
K. pneumoniae	226/2	rectal swab	TP	32	NEG	AM AMC TZP CTX/CAZ FEP ETP IPM/MEM CIP SXT	*ctx-m-3-like*
K. pneumoniae	229/6	rectal swab	TP	32	NEG	AM AMC TZP CTX/CAZ FEP ETP IPM/MEM CIP SXT	*ctx-m-1*
K. pneumoniae	275/1	urine	TP	32	NEG	AM AMC TZP CTX/CAZ FEP CIP SXT GEN	*ctx-m-15-like, tem-164*
K. pneumoniae	HK 16/19	blood culture	TP	8	NEG	AM	NA
Citrobacter spp.	261/12	sputum	TP	32	NEG	AM AMC	NA
E. aerogenes	85/6	urine	TP	32	NEG	AM AMC TZP CTX/CAZ ETP	*act/mir*
E. aerogenes	301/8	TA/BAL	TP	32	NEG	AM AMC TZP CTX/CAZ ETP	NEG, pressumed derepressed chromosomal AmpC
E. cloacae complex	HK 1/43	blood culture	TP	16	NEG	AM AMC	NA
E. cloacae complex	41/8	urine	TP	8	NEG	AM AMC	NA
E. cloacae complex	67/7	rectal swab	TP	16	NEG	AM AMC CTX/CAZ FEP CIP SXT GEN	*ctx-m-15-like, tem-164, shv-238*
E. cloacae complex	81/3	rectal swab	TP	32	NEG	AM AMC TZP CTX/CAZ FEP ETP	*act/mir*
E. cloacae complex	95/3	TA/BAL	TP	64	NEG	AM AMC	NA
E. cloacae complex	94/6	urine	TP	32	NEG	AM AMC TZP CTX/CAZ FEP ETP	*act/mir*
E. cloacae complex	106/10	rectal swab	TP	64	NEG	AM AMC TZP CTX/CAZ ETP	*act/mir*
E. cloacae complex	104/9	sputum	FN	64	NEG	AM AMC CTX/CAZ ETP	*act/mir*
E. cloacae complex	140/7	urine	TP	16	NEG	AM AMC CTX/CAZ	*act/mir*
E. cloacae complex	153/4	sputum	TP	16	NEG	AM AMC CTX/CAZ ETP	NEG, pressumed derepressed chromosomal AmpC
E. cloacae complex	165/3	sputum	TP	64	NEG	AM AMC	NA
E. cloacae complex	206/8	rectal swab	FN	32	NEG	AM AMC TZP CTX/CAZ ETP	NEG, pressumed derepressed chromosomal AmpC
E. cloacae complex	278/12	rectal swab	FN	32	NEG	AM AMC TZP CTX/CAZ ETP	*act/mir*
E. cloacae complex	286/4	rectal swab	FN	32	NEG	AM AMC TZP CTX/CAZ ETP	*act/mir*
E. cloacae complex	305/2	rectal swab	TP	64	NEG	AM AMC TZP CTX/CAZ ETP	*act/mir*
E. cloacae complex	342/4	rectal swab	TP	128	NEG	AM AMC TZP CTX/CAZ ETP	*act/mir*
E. cloacae complex	393/6	rectal swab	TP	8	NEG	AM AMC TZP CTX/CAZ FEP ETP	NEG, pressumed derepressed chromosomal AmpC
E. cloacae complex	485/10	sputum	TP	32	NEG	AM AMC	NA
E. cloacae complex	HK 5/51	blood culture	FN	64	NEG	AM AMC	NA
E. cloacae complex	HK 21/6	blood culture	TP	64	NEG	AM AMC	NA

SPM Superpolymyxin medium, MIC minimal inhibitory concentration, TP true positive, FN false negative, TA tracheal aspirate, BAL bronchoalveolar lavage, AM ampicillin, AMC amoxicillin/clavulanic acid, CTX cefotaxime, CAZ ceftazidime, FEP cefepime, CIP ciprofloxacine, TZP piperacillin/tazobactam, SXT trimethoprim/sulfamethoxazole, GEN gentamicin, ETP ertapenem, NA not applicable, ND not detected, NEG negative, beta-lactamase genes: extended-spectrum betalactamase (*ctx-m, shv, tem*) and AmpC betalactamase (*act/mir*)-

quadrant streak pattern. This two-step approach is mandatory because spot inoculation followed by BMD for MIC determination in case of bacterial growth results in a large number of FP results due to high bacterial inoculum. Considering only 12/1 SPM method sensitivity and specificity and NPV for *Enterobacteriaceae* were 87.5%, 93.6% and 98.9%, respectively, however PPV was as low as 54.4%. The number of FP is reduced with the second step. This two-step screening protocol showed excelent overall specificity (99.5%), NPV (98.9%) and PPV (94.2%), with good sensitivity (87.5%) for *Enterobacteriaceae*. We have noted significant differences between the species, the protocol is particularly useful for *E. coli* with 100% sensitivity, sensitivity is also good for *K. pneumoniae* (90.0%), while *Enterobacter* spp. is more challenging with sensitivity of only 77.3%.

According to our evaluation results growth of a single bacterial colony in 12/1 can be neglected as our cases had low MIC and can be explained with an inoculum effect. Some variables in growth pattern were noticed for different *Enterobacteriaceae* species. In colistin resistant *E. coli* and *K. pneumoniae* bacterial growth over entire spot was presented. However in *Enterobacter* spp. even growth of two colonies within the spot have resulted in a colistin resistant strain. *Enterobacter* spp. has been described as a demanding genus for determination of colistin resistance using methods other than BMD. FN results were observed in the past because of its tendency towards forming heterogenous subpopulations (Simar et al., 2017).

Our epidemiological screening protocol is not intended for clinical decisions regarding colistin treatment as the turnaround time is up to three days. For clinically significant isolates BMD must be performed immediately and when a rapid result for clinically significant isolates is required, alternative methods (e.g. rapid polymyxin NP test) could also be considered (Poirel et al., 2017). For epidemiological purposes our protocol can be easily incorporated into the routine laboratory workflow. This approach has been fully evaluated only for *Enterobacteriaceae*, non-fermenting Gram negative isolates are out of the scope of this study and preliminary results showed unacceptable proportion of FP results by our screening protocol (data not shown).

In total seven isolates tested FN by our screening protocol; five of them belonged to genus *Enterobacter*, that has been already described as chalenging for detection of colistin resistance (Simar et al., 2017). However, interestingly colistin MICs of all isolates which tested FN by screening protocol were not close to breakpoint MIC (4 mg/L); one *K. pneumoniae* isolate had colistin MIC 8 mg/L, the rest had higher MICs. All isolates with colistin MIC 4 mg/L were detected by our screening protocol. One possible explanation would be that perhaps these are heteroresistant isolates with only a small number of bacterial cells displaying colistin resistance that were not picked up by spot inoculation, but were detected in BMD where a larger amount of bacterial suspension is inoculated.

Following our routine laboratory protocol only ten carbapenem resistant isolates out of the 700 tested would have undergone the colistin susceptibility testing and only one isolate among those ten isolates was resistant to colistin. During our study a total of 56 colistin resistant *Enterobacteriaceae* isolates were found including one harbouring plasmid-mediated resistance gene. Were it not for our epidemiological screening protocol these isolates would not have been found except for a single CR-*K. pneumoniae* isolate. A single *E. coli* isolate harbouring *mcr-1* gene was found which would not have been detected as it was well susceptible to majority of the tested antibiotics. To our knowledge this is so far the only *mcr-1* positive *E. coli* isolate detected in Slovenia.

Presence of plasmid-mediated colistin resistance is of major concern particularly in countries with a higher prevalence of MDRGNB as increasing selective pressure is generated by colistin usage with potential selection of resistant mutants which is also creating favourable conditions for the spread of plasmid-mediated resistance (Giani et al., 2015; ECDC, 2017a; Castanheira et al., 2016). The study enabled us to decide if more rigorous methods are needed for colistin resistance surveillance, however due to low prevalence we will continue to determine MIC for clinical isolates where colistin is a treatement option and perform a weekly epidemiological screening for surveillance purpose.

5. Conclusions

Our study has shown that our novel two-step epidemiological screening approach for colistin resistance is useful and that it allows for screening of large number of *Enterobacteriaceae* isolates thus improving surveillance with reduced workload and cost. The protocol is particularly useful for *E. coli* with 100% sensitivity, sensitivity is also good for *K. pneumoniae* (90.0%), while *Enterobacter* spp. is more challenging with sensitivity of only 77.3%. It has enabled us to detect the first Slovenian *mcr-1*-positive colistin resistant *E. coli* isolate which would have otherwise been missed as colistin susceptibility testing is limited mainly to MDRGNB.

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Conflict of interests

The authors declare no conflicts of interests.

Ethical approval

The study was approved by a Medical Ethics Committee of the Republic of Slovenia (ref: 0120-709/2017/6).

Informed consent

Not needed.

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