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Heavy metal resistance genes are associated with *bla*$_{NDM-1}$ and *bla*$_{CTX-M-15}$-Enterobacteriaceae

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Abstract

The occurrence of heavy metal resistance genes in multi-resistant Enterobacteriaceae possessing *bla*$_{NDM-1}$ or *bla*$_{CTX-M-15}$ genes were examined by PCR and S1-PFGE. When compared with clinical susceptible isolates (10.0-30.0%), the *pcoA, merA, silC* and *arsA* genes occurred with higher frequencies in *bla*$_{NDM-1}$ (48.8-71.8%) and *bla*$_{CTX-M-15}$ (19.4-52.8%) positive isolates, and they are mostly located on plasmids. Given the high association of metal resistance genes with multidrug resistant Enterobacteriaceae, the use of heavy metals in hospitals and the environment needs increased vigilance.

Keywords: heavy metal resistance, *bla*$_{NDM-1}$, *bla*$_{CTX-M-15}$, plasmids, co-resistance

The increasing spread of multidrug resistant 'superbugs' within clinical environments has prompted worldwide concern, because antibiotic resistance genes such as $bla_{\text{NDM-1}}$ and $bla_{\text{CTX-M-15}}$ leads to limit treatment options to combat bacterial infections (1-4). It is noteworthy that, in addition to emerging antibiotic resistance, heavy metals represent another major sources of environmental contamination that may select for antibiotic resistance (5). Heavy metal compounds for growth promotion and therapeutic treatment, like zinc and cooper, have been used in pig and poultry production and unlike antibiotic food additives, can accumulate in soil, water, aquacultural and marine antifouling treatments or industrial effluent (6). It has been proposed that antibiotic-resistant bacteria are enriched at locations contaminated with metals, and genes conferring co-selection to heavy metal and antibiotic are often found together in many clinical isolates (7-11). Furthermore, genes conferring heavy metal tolerance may coexist on the same genetic element (e.g. plasmid), which could further promote co-dissemination and resistance (10, 12). Here, we characterize the phenotype and genotype of heavy metals resistance in a collection of 95 clinical Gram-negative isolates including *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella oxytoca* and *Providencia stuanti* isolated from the UK and India.

A total of 95 non-duplicate isolates were tested in this study (Table 1): 39 bla_{NDM-1}-positive isolates originated from human lower respiratory and urinary tract samples from the United Kingdome and Indian cities of Chennai and Haryana, as previously described (13); 36 $bla_{CTX-M-15}$-carrying isolates, from burn, bactareamia and UTI patients from a variety of Indian hospitals (Haryana, Mumbai, Calcutta, Kerala, Delhi and Vellore); and 20 control *E. coli* and *K. pneumoniae* susceptible to all known antibiotic classes as control samples, provided by Specialist Antimicrobial Chemotherapy Unit (SACU), Public Health Wales. Minimal inhibitory concentrations (MICs) of four heavy metals ions; $CuSO_4.5H_2O$ for copper (Cu^{2+}), $HgCl_2$ for mercury (Hg^{2+}), $AgNO_3$ for silver (Ag^{+}), and $AsNaO_2$ for arsenic(As^{3+}) were measured by agar dilution using Müller-Hinton agar (Becton Dickinson, USA). *E. coli* (ATCC 25922) was used as a negative control. MIC levels to Cu^{2+} (≥10 mM), As^{3+} (≥2 mM), Hg^{2+} (≥32 μM) and Ag^{+} (≥128 μM) were regarded as resistance (14-16). High MIC values to Cu^{2+} (10 mM), As^{3+} (20 mM) and Hg^{2+} (128 μM) were obtained in the majority of bla_{NDM-1}-positive isolates, with a high resistance rate of 82.1% (32/39), 76.9% (30/39) and 61.5% (24/39), respectively. Similarity with $bla_{CTX-M-15}$-positive strains, 91.7% (33/36), 63.9% (23/36) and 52.8% (19/36) isolates were resistant to Cu^{2+}, As^{3+} and Hg^{2+}, respectively. High MIC values (128-256 μM) for Ag^{+} were observed for all isolates. Antibiotic susceptible control strains also gave high rates of resistance to Cu^{2+} (90%, 18/20), but remained sensitive to Hg^{2+} (15.0%, 3/20) and As^{3+} (25.0%, 5/20).

The presence of four heavy metal resistance genes was confirmed by PCR: *merA* for Hg^{2+}, *arsA* for As^{3+}, *pcoA* for $Cu^{2+}$and *silC* for Ag^{+}. Primers were designed by primer 3 (Geneious Pro 5.5.6) and NCBI primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 2) and PCRs were performed with the following condition: initial denaturation at 95^{o}C for 5 min;

followed by 30 cycles of denaturation at 95°C for 45 seconds, annealing at 58-60°C for 45 seconds and extension at 72°C for 45 seconds; final extension at 72°C for 5 min. The purified PCR products were randomly selected for following sequencing analysis (Eurofins Genomics, Germany). The *silC*, *merA*, *pcoA* and *arsA* genes were dispersed throughout our $bla_{\text{NDM-1}}$-positive isolates, with 28/39 (71.8%), 26/39 (66.7%), 25/39 (64.1%) and 19/39 (48.7%), respectively (Fig. 1). Similarly, in $bla_{\text{CTX-M-15}}$ producing isolates, the most prevalent heavy metal resistance gene was *merA* (19/36, 52.8%). The genes of *arsA*, *pcoA* and *silC* were only detected in 7 (19.4%), 15 (41.7%) and 15 (41.7%) isolates, respectively. In contrast, the relative low prevalence of *pcoA*, *silC*, *arsA* and *merA* genes were identified in susceptible isolates with detection rates of 30.0% (6/20), 25.0% (5/20), 20% (4/20) and 10% (2/20), respectively (Fig. 1). In addition, the statistical comparisons with these metal resistance genes in three groups of isolates, were conducted using Chi-square (and fisher's exact) test, where p value equal or less than 0.05 was considered as significant. The prevalence of *silC* (71.8% vs 25.0%, p=0.0009), *merA* (66.7% vs 10.0%, p<0.0001), *pcoA* (64.1% vs 30.0%, p=0.0158) and *arsA* (48.7% vs 20.0%, p=0.0482) genes detected in $bla_{\text{NDM-1}}$-positive isolates, are all markedly higher than those in susceptible isolates. Furthermore, the detection rates of *silC* (71.8% vs 41.7%, p=0.0108) and *arsA* (48.7% vs 19.4%, p=0.0144) in $bla_{\text{NDM-1}}$-positive isolates are also significantly higher, comparing to that in $bla_{\text{CTX-M-15-}}$ producing isolates (Fig. 1).

Previous studies have proposed the role of plasmids in conferring resistance to both antibiotics and heavy metals (7, 17, 18). In this study, the location of the *pcoA*, *merA*, *silC* and *arsA* genes were analysed by Pulsed-field gel electrophoresis (PFGE) with S1 nuclease (Invitrogen Abingdon, UK) (S1-PFGE). In brief, isolates carrying heavy metal resistance genes were randomly selected and genomic DNA in agarose blocks was digested with S1 nuclease and

probed. In-gel hybridisation was performed with *pcoA*, *merA*, *silC* and *arsA* genes probe labelled with ^{32}P with a random primer method (Stratgene, Amsterdam, Netherlands). The results showed that *pcoA, merA, silC* and *arsA* genes are located on a diverse range of plasmids backbones, differing from 50- to 500 kb in size (Fig. 2 and Fig. S1). Heavy metal resistance genes were carried upon more than one plasmid in many strains and chromosomal located genes were also identified (Fig. 2 and Fig. S1), suggesting significant plasticity.

Conjugation experiments were performed as described previously (13), to investigate co-transfer of heavy metal and antibiotic resistance genes. Conjugations were performed with $bla_{\text{NDM-1}}$ and $bla_{\text{CTX-M-15}}$-positive donors with the rifampin-resistant recipient *E. coli* UAB190. Selection of $bla_{\text{CTX-M-15}}$-positive transconjugants was performed on Brilliance UTI Clarity agar (Oxoid Ltd., Basingstoke, United Kingdom) supplemented with rifampicin (100 mg/L) (Sigma-Aldrich, St. Louis, MO, USA) and cefotaxime (2 mg/L). $bla_{\text{NDM-1}}$-positive transconjugants were selected using rifampicin with meropenem (0.5 mg/L) (AstraZeneca, London, United Kingdom). PCR for $bla_{\text{NDM-1}}$ and $bla_{\text{CTX-M-15}}$ genes were used for further confirmation of gene transfer (13, 19). Plasmid incompatibility groups were characterized by PCR-based replicon typing as previously described (20). A total of 18 and 14 transconjugants were obtained in *E.coli* UAB190 from 39 $bla_{\text{NDM-1}}$ and 36 $bla_{\text{CTX-M-15}}$ isolates, respectively. In 11 of 18 transconjugants, $bla_{\text{NDM-1}}$ was located upon IncA/C-type plasmids, 78.6% (11/14) of plasmids carrying $bla_{\text{CTX-M-15}}$ belonged to IncFII, reflective of global molecular epidemiology (2, 21). Plasmids carrying $bla_{\text{NDM-1}}$ from six transconjugants could not be typed. The heavy metal resistance genes *arsA*, *merA* and *pcoA* were found on two $bla_{\text{NDM-1}}$ and one $bla_{\text{CTX-M-15}}$ positive plasmids, respectively (Table 1).

Our data indicates the abundant and mobility of heavy metals resistance genes (*pcoA*, *merA*, *silC* and *arsA*) that can contribute to antibiotic resistant genes dissemination and maintenance. Furthermore, many of these genes are found on transmissible plasmids. Therefore, our findings suggest that the co-selection of heavy-metal resistance genes in $bla_{\text{NDM-1}}$ and $bla_{\text{CTX-M-15}}$ positive isolates have significant implications for hospital and environmental (industrial waste) contamination with heavy metals.

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Conflict of interest: none declared

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202 Table 1. Phenotypic and genotypic resistances to heavy metals in 95 clinical strains in this study

Strains ID	bacterial organism	Phenotype (MIC)				Genotype
		Ag(uM)	Hg(uM)	Cu(mM)	As(mM)	
39 bla_{NDM-1} strains						
N1	*Klebsiella pneumoniae*	128	128	10	0.625	*merA*, *silC*
N2	*Klebsiella pneumoniae*	128	128	10	2.5	*arsA*, *merA*
N3	*Citrobacter freundii*	128	128	10	2.5	*arsA*, *merA*
N4	*Enterobacter cloacae*	128	16	10	20	*pcoA*, *silC*
N5	*Enterobacter spp*.	128	16	5	1.25	neg.
N6	*Escherichia coli*	128	128	10	20	*arsA*, *merA*, *pcoA*, *silC*
N7	*Klebsiella pneumoniae*	128	128	10	10	*arsA*, *merA*, *pcoA*, *silC*
N8	*Klebsiella pneumoniae*	128	128	10	20	*arsA*, *merA*, *pcoA*, *silC*
N9	*Klebsiella pneumoniae*	128	16	10	0.625	*pcoA*, *silC*
N10	*Klebsiella pneumoniae*	128	16	10	0.625	*silC*
N11	*Klebsiella pneumoniae*	128	16	10	0.625	*silC*
N12	*Klebsiella pneumoniae*	256	128	10	10	*arsA*, *merA*, *pcoA*,*silC*
N13	*Citrobacter freundii*	256	128	10	10	*arsA*, *merA*, *pcoA*, *silC*
N14	*Escherichia coli*	128	128	10	10	*arsA*, *merA*, *pcoA*, *silC*
N15	*Escherichia coli*	128	16	5	1.25	*pcoA*, *silC*
N16	*Klebsiella pneumoniae*	128	128	10	1.25	*arsA*, *merA*, *pcoA*,*silC*
N17	*Klebsiella pneumoniae*	128	128	10	20	*arsA*, *merA*, *pcoA*,*silC*
N18	*Klebsiella pneumoniae*	128	64	10	10	*arsA*, *merA*, *pcoA*, *silC*
N19	*Klebsiella pneumoniae*	128	128	10	20	*arsA*, *merA*, *pcoA*, *silC*
N20	*Escherichia coli*	128	16	5	2.5	*neg*.
N21	*Klebsiella pneumoniae*	128	128	10	2.5	*merA*, *pcoA*,*silC*
N22	*Klebsiella pneumoniae*	128	128	10	2.5	*merA*, *pcoA*,*silC*
N23	*Escherichia coli*	128	128	5	0.625	neg.
N26	*Enterobacter spp*	128	128	10	10	*arsA*, *merA*, *pcoA*
N27	*Klebsiella pneumoniae*	128	128	5	10	*arsA*, *merA*, *pcoA*, *silC*
N28	*Klebsiella oxytoca*	128	16	10	5	*arsA*, *merA*, *pcoA*, *silC*
N29	*Escherichia coli*	128	16	10	10	*arsA*, *silC*
N31	*Enterobacter cloacae*	128	16	10	20	*pcoA*, *arsA*, *silC*
N32	*Enterobacter cloacae*	128	16	10	0.625	*pcoA*, *silC*,*merA*, *arsA*
K15	*Klebsiella pneumoniae*	128	16	10	5	*merA*, *pcoA*, *silC*
K7	*Klebsiella pneumoniae*	128	128	10	2.5	*merA*, *pcoA*, *silC*
IR25	*Klebsiella pneumoniae*	128	128	10	5	*merA*
IR18k	*Klebsiella pneumoniae*	128	128	10	20	*merA*
IR28k	*Klebsiella pneumoniae*	128	128	10	20	*merA*, *pcoA*, *silC*
IR29	*Escherichia coli*	128	128	5	5	*merA*, *pcoA*, *silC*
IR26	*Escherichia coli*	128	128	5	5	neg.
IR22	*Escherichia coli*	128	16	5	5	*neg*.
IR61	*Klebsiella oxytoca*	128	16	10	20	*neg*.
IR5	*Escherichia coli*	128	128	10	20	*arsA*, *merA*, *pcoA*, *silC*

203
204 Table 1 continued.
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Strains ID	bactrial organism	Phenotype (MIC)				Genotype
		Ag(uM)	Hg(uM)	Cu(mM)	As(mM)	
36 bla$C_{TX\text{-}M\text{-}15}$ strains						
A5/3	*Klebsiella pneumoniae*	128	16	10	5	*arsA, pcoA, silC*
A5/7	*Klebsiella pneumoniae*	128	128	10	20	*arsA, merA, pcoA, silC*
A5/4	*Klebsiella pneumoniae*	128	128	5	5	*pcoA, silC*
C5/8	*Klebsiella pneumoniae*			10	0.625	*arsA, merA*
C5/7	*Klebsiella pneumoniae*	128	128	10	10	*arsA, merA, pcoA, silC*
C5/5	*Klebsiella pneumoniae*	128	16	10	5	*neg.*
D5/12	*Klebsiella pneumoniae*	128	128	10	0.15	*merA*
D5/4	*Klebsiella pneumoniae*	128	16	10	0.625	*pcoA, arsA*
E5/14	*Klebsiella pneumoniae*	128	16	10	5	*merA, pcoA, silC*
E5/17	*Klebsiella pneumoniae*	128	128	10	2.5	*arsA, merA, pcoA, silC*
G5/2	*Klebsiella pneumoniae*	128	16	10	5	*arsA, pcoA, silC*
G5/6	*Klebsiella pneumoniae*	128	128	10	0.3	*merA*
G5/11	*Klebsiella pneumoniae*	128	128	10	0.3	*merA, pcoA, silC*
I5/5	*Klebsiella pneumoniae*	128	128	10	20	*merA, pcoA, silC*
F5/6	*Klebsiella pneumoniae*	128	16	10	0.3	neg.
E5/19	*Klebsiella pneumoniae*	128	128	10	5	*merA, pcoA, silC*
A4/8	*Escherichia coli*	128	16	10	0.3	*neg.*
F4/3	*Escherichia coli*	128	16	10	5	*neg.*
B4/6	*Escherichia coli*	128	16	10	2.5	*neg.*
A4/11	*Escherichia coli*	128	16	10	5	*neg.*
C4/3	*Escherichia coli*	128	128	10	2.5	*merA*
E4/4	*Escherichia coli*	128	128	10	2.5	*neg.*
D4/12	*Escherichia coli*	128	16	10	2.5	*merA*
C4/12	*Escherichia coli*	128	64	10	2.5	*merA*
G4/12	*Escherichia coli*	128	16	10	2.5	*neg.*
I4/9	*Escherichia coli*	128	128	10	2.5	*merA*
I4/3	*Escherichia coli*	128	16	10	0.3	*neg.*
I4/13	*Escherichia coli*	128	16	5	2.5	*merA, pcoA,silC*
H4/5	*Escherichia coli*	128	16	10	0.3	*neg.*
H6/20	*Salmonella spp.*	128	128	10	0.15	*neg.*
G6/9	*Salmonella spp.*	128	16	10	0.625	*merA, pcoA,silC*
G6/13	*Salmonella spp.*	128	64	10	0.15	*merA, silC*
I2/5	*Enterobacter spp.*	128	128	10	20	*pcoA, silC*
I2/2	*Enterobacter spp.*	128	128	10	20	*pcoA, silC*
F2/6	*Enterobacter spp.*	128	128	0.625	0.15	*merA*
B1/10	*Providencia stuanti*	128	128	10	20	*merA*

Table 1 continued.

Strains ID	bactrial organism	Phenotype (MIC)				Genotype
		Ag(uM)	Hg(uM)	Cu(mM)	As(mM)	
20 Susceptible strains						
Kp ff160	*Klebsiella pneumoniae*	128	128	10	10	*arsA*, *merA*, *pcoA*, *silC*
Kpff217	*Klebsiella pneumoniae*	128	16	10	0.3	*pcoA*, *silC*
KpFF11	*Klebsiella pneumoniae*	128	128	10	5	*arsA*, *merA*, *pcoA*,*silC*
KpFF197	*Klebsiella pneumoniae*	128	16	10	0.625	*silC*
KpFF177	*Klebsiella pneumoniae*	128	16	10	0.3	*pcoA*
KpFF296	*Klebsiella pneumoniae*	128	16	10	10	*arsA*, *pcoA*, *silC*
KpFF101	*Klebsiella pneumoniae*	256	16	10	10	*neg*.
KpFF264	*Klebsiella pneumoniae*	128	16	10	0.15	*neg*.
KpFF267	*Klebsiella pneumoniae*	128	16	10	0.15	*neg*.
KpFF153	*Klebsiella pneumoniae*	128	16	10	0.3	*pcoA*
Ec66	*Escherichia coli*	128	8	10	0.15	*neg*.
Ec9	*Escherichia coli*	128	16	10	0.15	*neg*.
Ec63	*Escherichia coli*	128	8	10	0.15	*neg*.
Ec59	*Escherichia coli*	128	8	5	0.15	*neg*.
Ec60	*Escherichia coli*	128	16	5	0.15	*neg*.
Ec166	*Escherichia coli*	128	8	10	0.15	*neg*.
Ec284	*Escherichia coli*	128	8	10	0.625	*neg*.
Ec61	*Escherichia coli*	128	128	10	5	*neg*.
Ec141	*Escherichia coli*	128	16	10	0.15	*neg*.
Ec98	*Escherichia coli*	128	16	10	0.15	*neg*.
Transconjugants and control strains						
25922	*Escherichia coli*	64	16	5	0.15	*neg*.
GFP	*Escherichia coli*	64	16	5	1.25	*neg*.
TCE5/19	*Escherichia coli*	64	16	5	2.5	*pcoA*
TCN12	*Escherichia coli*	128	64	5	10	*arsA*, *pcoA*, *merA*
TCN22	*Escherichia coli*	128	8	5	2.5	*pcoA*

215

216 Table 2. Details of primers used for heavy metal resistance genes detection in this study

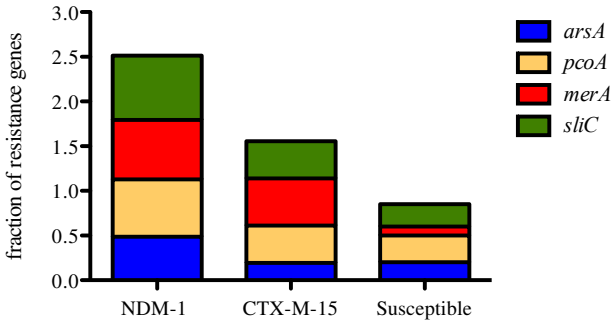
metal ions	primers	sequence (5'-3')	Tm	size(bp)	Genbank ID or GI number
Hg^{2+} (mercury)	*merA*_F1	CTGCGCCGGGAAAGTCCGTT	58°C	1035	DQ126685
	*merA*_R1	GCCGATGAGCCGTCCGCTAC			
	*merA*_F2	GAGCTTCAACCCTTCGACCA	60°C	849	575669924
	*merA*_R2	AGCGAGACGATTCCTAAGCG			
As^{3+} (arsenic)	*arsA*_F1	CAGTACCGACCCGGCCTCCA	58°C	861	CP000648
	*arsA*_R1	AGGCCGTGTTCACTGCGAGC			
	*arsA*_F2	GGCTGGAAAAACAGCGTGAG	58°C	1002	387605479
	*arsA*_R2	CCTGCAAATTAGCCGCTTCC			
Cu^{2+} (copper)	*pcoA*_F	CGGCCAGGTTCACGTCCGTC	58°C	1371	NC_009649
	*pcoA*_R	TGCCAGTTGCCGCATCCCTG			
Ag^{+} (silver)	*silC*_F1	CGTAGCGCAAGCGTGTCGGA	58°C	1090	NC_009649
	*silC*_R1	ATATCAGCGGCCCGCAGCAC			
	*silC*_F2	TTCAACGTCACGGATGCAGA	60°C	872	157412014
	*silC*_R2	AGCGTGTCGGAAACATCCTT			

217

218
219

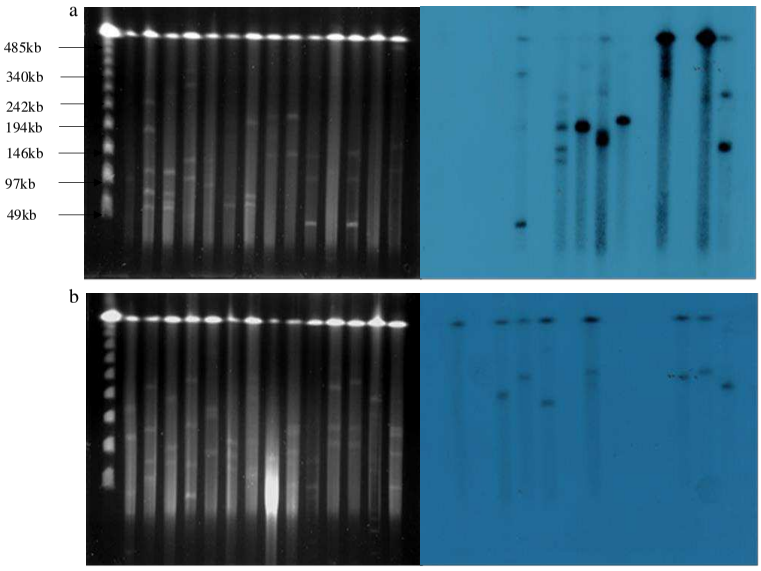
Fig.1 occurrence of heavy metal resistance genes in 95 clinical isolates. *p* values were calculated using Chi-square (and fisher's exact) test. *, ** and *** indicate $0.01 < p$ value ≤ 0.05; $0.001 < p$ value ≤ 0.01; *** indicates *p* value ≤ 0.001, respectively. 'ns' indicates not significant difference.

Fig. 2. PFGE analysis of bla_{NDM-1}-positive strains digested with S1 nuclease, and hybridization with *pcoA* gene probe (a), *silC* gene probe (b), respectively.
Isolates order of lanes 1-14 in A: N1, N2, N3, N4, N5, N6, N7, N8, N9, N10, N11, N12, N13 and N14.
Isolates order of lanes 1-14 in B: N16; N17; N18; N19; N20; N21; N22; N23; N3; 26; N27; N28; N29; N31.



three groups of clinical isolates

Chi-square (Fisher's exact)test	Comparison of detection rates (*p* value)		
	bla_{NDM-1} vs susceptible	$bla_{CTX-M-15}$ vs susceptible	bla_{NDM-1} vs $bla_{CTX-M-15}$
arsA	48.7% vs 20% (*p*=0.0482*)	19.4% vs 20% (*p*=1.0_ns)	48.7% vs 19.4%, (*p*=0.0144*)
pcoA	64.1% vs 30% (*p*=0.0158*)	41.7% vs 30% (*p*=0.5653_ns)	64.1% vs 41.7% (*p*=0.0657_ns)
merA	66.7% vs 10% (*p*<0.0001***)	52.8% vs 10% (*p*=0.0016**)	66.7% vs 52.8% (*p*=0.2463(ns)
sliC	71.8% vs 25% (*p*=0.0009***	41.7% vs 25% (*p*=0.2555_ns)	71.8% vs 41.7% (*p*=0.0108*)


a
485kb
340kb
242kb
194kb
146kb
97kb
49kb
b