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Clonal diversity of CTX-M-producing, multidrug-resistant *Escherichia coli* from rodents

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ABSTRACT

This territory-wide study investigated the occurrence of fecal carriage of extended-spectrum β-lactamase (ESBL)-producing *Escherichi coli* among wild rodents from the 18 districts in Hong Kong. Individual rectal swabs were obtained from the trapped animals and cultured in plain and selective media. A total of 965 wild rodents (148 Chestnut Spiny rats [*Niviventer fulvescens*], 326 Indo-Chinese forest rats [*Rattus andamanensis*], 452 brown rats [*R. norvegicus*], 39 black rats [*R. rattus*]) were sampled. ESBL carriage was 0% in Chestnut Spiny rats, 0.6% in Indo-Chinese forest rats, 7.7% in black rats and 13.9% in brown rats. Among brown rats, the prevalence of ESBL carriage differed markedly by geographical locations: absent in two districts, low (7-10%) in six districts, moderate (11-19%) in seven districts and high (21-50%) in three districts. Nonetheless, there was no correlation between prevalence of ESBL in brown rats and human population density in the 18 districts. CTX-M-type enzymes was detected in 92.0% of the ESBL-producing isolates, of which 83.1% were resistant to ≥3 non-β-lactam drugs. The CTX-M producing isolates were genetically diverse but a large proportion (47.8%) were included in six successful clones that are strongly associated with human diseases and CTX-M dissemination viz sequence type complex [STC]10/phylogroup A, STC23/phylogroup B1, STC38/phylogroup D, STC155/phylogroup B1, ST405/phylogroup D and ST131/phylogroup B2. In conlusion, our results show that brown rats often carry potentially zoonotic clones of CTX-M producing,

multidrug-resistant *E. coli*. The potential for rats to be a source of CTX-M producing *E. coli* for human deserves further consideration.

INTRODUCTION

In the past decade, the prevalence of *Enterobacteriaceae* producing extended-spectrum beta-lactamase (ESBL) has increased rapidly, mainly attributed to the successful spread of the CTX-M enzymes (D'Andrea et al., 2013; Woerther et al., 2013). In both humans and animals, the CTX-M enzymes are most often carried by *E. coli* (D'Andrea et al., 2013). Given that *E. coli* is carried by human and a wide range of animals as part of the commensal gut flora, the organism is often adopted as an indicator organism for detection of antimicrobial resistance (Lo *et al.*, 2010). While companion and food-producing animals have been extensively studied as potential sources of ESBL strains and resistance ($bla_{\text{CTX-M}}$) genes (Ho *et al.*, 2012a; Woerther *et al.*, 2013), the potential for pests, in particular urban rodents as a reservoir of this emerging resistance mechanism has received fewer attention (Guenther et al., 2012; Guenther et al., 2013; Ho et al., 2011a). Urban rodents, especially brown rats (*Rattus norvegicus*) and black rats (*R. rattus*) may carry zoonotic pathogens that can be transmitted to human through animal contacts, contamination of food items or the environment (Himsworth et al., 2013). Various antimicrobial-resistant bacteria including ESBL-producing *E. coli* have been described in urban rats (Guenther *et al.*, 2010; Guenther *et al.*, 2012). However, previous studies on this topic are limited by sampling relatively small number of rodents (<100 animals) from one or several districts. Hence, the findings may not accurately reflect the situation in big cities. In addition, only small numbers (<10 isolates) of CTX-M producing *E.*

coli isolates from rodents have been characterized (Guenther *et al.*, 2012; Guenther *et al.*, 2013), thereby limiting the inference that could be made about their clonal distribution and zoonotic risk (Naseer & Sundsfjord, 2011). Here, we studied the prevalence of fecal carriage of ESBL-producing *E. coli* in a large sample of rats collected from all districts in a metropolitan city. The clonal structure of the CTX-M producing *E. coli* isolates were determined.

METHODS

Sampling. From September 2008 to August 2013, rats were cage trapped from sampling locations covering all the 18 administrative districts in Hong Kong by trained staff at the Food and Environmental Hygiene Department (FHED) and the Agriculture, Fisheries and Conservation Department (AFCD) of the Hong Kong government. Brown rats (*R. norvegicus*) and black rats (*R. rattus*) were captured during pest controls. Captured animals were collected and euthanized at designated FHED collection centres. The areas targeted for pest control included markets, buildings, hawker bazaars, lanes close to food premises and other problematic spots with rodent infestations. Chestnut Spiny rats [*Niviventer fulvescens*] and Indo-Chinese forest rats [*Rattus andamanensis*] were trapped as part of the AFCD's regular nature conservtion program in the countryside areas at Lantau island, Robin's Nest, Shek O, Shing Mun, Tai Lam and Tai Mo Shan. Swabs were taken directly at the place of capturing

and the animals were released afterwards. Trained staff identified the species, sex and maturity of the rats according to morphological features. This study was approved by the University of Hong Kong's Committee on Animal Ethics (CULATR).

Microbiological methods. Individual faecal materials (~0.1 g) were collected with sterile swabs (TRANSWAB, Medical Wire and Equipment Co. Ltd., Corsham, Wilts, England), transported in Amies media and seeded onto three MacConkey agars (one plain and two supplemented with 2 mg/L cefotaxime or 2 mg/L ceftazidime, Sigma Chemical Co., St Louis, MO, USA). For each agar plate, at least one and up to five colonies were investigated (Ho *et al.*, 2011a; Ho *et al.*, 2013). Bacteria were identified as *E. coli* by Vitek GNI system (BioMerieux, Durham, NC, USA). and their antibiotic susceptibilities were determined by the disc diffusion method and results interpreted according to the Clinical and Laboratory Standards Institute M100-S23 document (CLSI, 2013). The double-disk synergy test was used for detection of ESBL (Ho et al., 2000). Isolates from the same animal were considered to be unique if the resistance profiles for cefotaxime, amikacin, tetracycline, co-trimoxazole, ciprofloxacin, gentamicin, chloramphenicol and nitrofurantoin differed by at least one drug. One isolate from each MacConkey plate was included and up to three isolates per animal were included in the final analysis. The levels of resistance were graded according to the European Food Safety Authority (European Food Safety Authority, 2013): low (1-10%),

98 moderate (11-20%), high (21-50%), very high (51-70%) and extremely high (≥71%).

99

100 **Molecular studies.** The *bla*$_{\text{CTX-M}}$ genes were detected by PCR using primers with specificity
101 for the CTX-M subgroups (*bla*$_{\text{CTX-M1G,}}$ *bla*$_{\text{CTX-M2G,}}$ *bla*$_{\text{CTX-M8G,}}$ *bla*$_{\text{CTX-M9G,}}$ and *bla*$_{\text{CTX-M25G}}$)
102 (D'Andrea *et al.*, 2013; Ho *et al.*, 2011b; Ho *et al.*, 2012a). PCR assays were used to assign
103 the *E. coli* isolates to phylogroups A, B1, B2, C, D, E and F (Clermont et al., 2013).
104 Multilocus sequence typing (MLST) was carried out and results analysed using the
105 University of Warwick scheme (http://mlst.warwick.ac.uk/mlst/). PCR and sequencing were
106 used to determine the serotype and *fimH* subtype of ST131 isolates (Banerjee *et al.*, 2013; Ho
107 *et al.*, 2012b).

108

109 **Statistical analysess.** Categorical variables were compared by Chi square or Fisher exact test.
110 The *P* values obtained from multiple comparisons were adjusted with Bonferroni correction.
111 Correlation analyses were used to investigate the relationship between prevalence of fecal
112 carriage of ESBL-producing *E. coli*, density of human popluation and number of sewage
113 treatment plants in the districts. A *P* value of <0.05 was used to indicate statistical
114 significance. All statistical analyses were performed with SPSS Software. Maps were
115 produced using ArcMap, 9.2 (ESRI, Redlands, CA, USA) for graphical ilustration.

116

RESULTS

Prevalence of ESBL carriage by rat species and rat characteristics

A total of 965 wild rodents (148 Chestnut Spiny rats, 326 Indo-Chinese forest rats, 452 brown rats, 39 black rats) were sampled. The annual numbers of animals sampled ranged 12-50 for Chestnut Spiny rats, 47-80 for Indo-Chinese forest rats, 75-106 for brown rats and 5-13 for black rats. Overall, 7.0% (68/965) of the animals were were found to carry ESBL-producing *E. coli*. The prevalence of ESBL-producing *E. coli* by animal species was as follows: 0% (0/148 animals) in Chestnut Spiny rats, 0.6% (2/326 animals) in Indo-Chinese forest rats, 7.7% (3/39 animals) in black rats and 13.9% (63/452 animals) in brown rats ($P<0.001$, chi square). There were some variations in the annual proportions of Indo-Chinese forest rats (0%-1.7%), black rats (0%-33.3%) and brown rats (5.7%-19.4%) tested positive for ESBL-producing *E. coli* during the five year period but no significant temporal trends were found. Presence of ESBL carriage was not associated with the sex (male 7.6%, female 5.9%, $P=0.301$) and maturity (juvenile 7.9%, adult 6.3%, $P=0.471$) of the animals.

Prevalence of ESBL carriage by geographical districts

The prevalence of ESBL in brown rats was further analyzed by districts (Fig. 1). The mean ± SD number of brown rats tested in each district was 25 ± 13 and the range was 11-60. The prevalence differed markedly by districts (range 0-50%, $P=0.004$): no ESBL carriage was

found in two districts, low levels (1-10%) were found in six districts, moderate levels (11-20%) were found in seven districts and high levels (21-50%) were found in three districts. There was no correlation between prevalence of ESBL in brown rats and human population density in the 18 districts (Fig. 2). A total of 67 sewage treatment plants were located in 16 of the 18 districts. The two districts with no sewage treatment plants were Wong Tai Sin (#13 in Fig. 2) and Yau Tsim Mong (#14 in Fig. 2). The average number of plants in the 16 districts with such facilities was four (range 1-17). Again, there was no correlation between prevalence of ESBL in brown rats and the number of sewage treatment plants in the districts (R square = 0.007, P=0.746).

Antimicrobial susceptibilities

The cultures yielded 281 unique *E. coli* isolates of which 77 were ESBL producers. The ESBL-producing isolates were recovered from brown rats (71 isolates from 63 animals), black rats (4 isolates from 3 animals) and Indo-Chinese forest rats (2 isolates from 2 animals). The antimicrobial resistance rates stratified by animal species are summarized in Table 1. Significantly different resistance rates were found among isolates from the four rat species for ampicillin, cefotaxime, nalidixic acid, tetracycline, cotrimoxazole, chloramphenicol and gentamicin. In general, resistance rates were highest for isolates from brown rats and lowest for those from Chestnut Spiny rats. The multidrug resistance rate was highest isolates from

brown rats (33.0%), following by black rats (16.7%) and Indo-Chinese forest rats (5.1%). ESBL-producing isolates were significantly more likely to have co-resistance to ≥3 classes of non-β-lactam agents than non-ESBL-producing isolates (83.1% vs. 3.9%, $P<0.001$)

Genotypic characteristics of the ESBL-producing isolates

Seventy-five of the 77 ESBL-producing isolates were investigated further because two isolates were lost during the experiments. About thirty percent of the ESBL-producing isolates were allocated to the phylogroups B2 (4.0%, 3/75) and D (16.0%, 12/75) and group F (10.7%, 8/75) which are associated with extraintestinal pathogenicity. The frequencies of the other phylogroups were as follows: A, 25.3% (19/75); B1, 38.7% (29/75); C, 1.3% (1/75) and nontypeable, 4.0% (3/75). PCR showed that 92.0% (69/75) of the ESBL-producing isolates were *bla*$_{\text{CTX-M}}$-positive, including *bla*$_{\text{CTX-M1G}}$ in 36 isolates, *bla*$_{\text{CTX-M9G}}$ in 31 isolates, and both *bla*$_{\text{CTX-M9G}}$ and *bla*$_{\text{CTX-M1G}}$ in two isolates (Table 2). MLST analysis revealed 43 different sequence types (STs) under 12 ST complexes (STC) and 18 singletons, including overrepresentation of STC10/phylogroup A (18.9%, 13/69) and STC155/phylogroup B1 (11.6%, 8/69). The two ST131/phylogroup B2 isolates (Table 2) from brown rats had serotype/fimH subtype, O25b/fimH30 and O16/fimH41, respectively.

DISCUSSION

174 This study found that there were striking differences in the prevalences of ESBL carriage and
175 of multidrug-resistant *E. coli* among the four rats species. A previous study found that wild
176 rodents (bank voles and wood mice) from woodland sites in close proximity to human
177 inhabitations in North West England showed high antimicrobial resistance (Gilliver et al.,
178 1999). However, our data revealed that antimicrobial resistance was rare among Chestnut
179 Spiny rats and Indo-Chinese forest rats, indicating that wild animals living in close proximity
180 to humans may not always acquire antimicorbial-resistant bacteria. Other than physical
181 proximity to human inhabitations, the eating habits of these rodents might also affect
182 acquisition of antimicrobial resistance. Depending on access, brown rats and black rats with
183 dwellings in urban areas consume almost everyting including fruit, meat, fish, vegatables and
184 rotten garbage. By comparison, Chestnut Spiny rats and Indo-Chinese forest rats which tend
185 to live at the hillside have dietary preference for plants such as seeds, grass and flowers; and
186 insects, like beetles and termites (Gomez Cano *et al.*, 2013).
187
188 As far as we know, this study revealed for the first time that there could be marked variations
189 in the prevelance of ESBL carriage among brown rats trapped within different areas in the
190 same city. The higher rates obtained for rats with habits for indoor dwelling and underground
191 sewers (brown rats and black rats) than in rats which prefer outdoor dwelling (Chestnut Spiny
192 rats and Indo-Chinese Forest rats) suggests that human feces or sewage may be a source of

193 such resistant bacteria. In Hong Kong, similar sewage collection networks exist in all the
194 geographical districts and the infrastructure is proportionate to population density. We
195 explored whether variations in resistance rates are in some way related to sewage treatment
196 plants. However, the prevalence of ESBL in brown rats did not correlate with the number of
197 sewage treatment plants in the districts. It could be argued that contact with human feces or
198 sewage is unlikely to be a major factor for acquisition of resistance. In contrast to previous
199 studies (Skurnik et al., 2006), we did not found any correlation between human inhabitation
200 density and the level of antimicrobial resistance in the districts. Previous studies assessed
201 changes in human population density over range of <1/km^2, <50/km^2 and 200/km^2 (Skurnik
202 et al., 2006), while the 18 districts in the present study had densities from 800-55200/km^2.
203 Besides sewage, variations in the resistance rates may be explained by other possibilities such
204 as better access of some rats to farms, wet markets, grocery stores, hospitals, human food
205 sources and garbage disposal areas (Guenther *et al.*, 2013; Ho *et al.*, 2011a; Kola *et al.*, 2012).
206 In Hong Kong, farms producing food animals (poultry and pigs) are almost exclusively
207 located in the Yuen Long district (#9 in Fig. 2) where the level of resistance among brown
208 rats was also high. It should be pointed out that live poultry are being sold in wet markets in
209 16 of the 18 districts. Our previous work found that ESBL-producing *E. coli* was carried by
210 eight out of ten live poultry (Ho *et al.*, 2011a). It is possible that better access of some rodents
211 to fecal materials of food animals might have contributed to the variations in resistance rates.

As the street locations where the rats were trapped were not available to us, we were not able to analyze whether proximity of the captive location to farms, wet markets, live poultry stalls, restaurants and garbage disposal areas constitute risk factors for carriage of antimicrobial-resistant bacteria.

In the present study, isolates from brown rats were found to have moderate to high levels of resistance to many antimicrobial agents, of which ampicillin, cefotaxime, ciprofloxacin and nalidixic acid are classified as "critically important" for human medicine by the World Heath Organization (Collignon et al., 2009). While the spectrum of rodent-borne diseases and their risks for public health are well recognized and comprehensively reviewed, information on the role played by rats in the transmission and exchange of antimicrobial resistance among human, pets, livestock animals and the environment is unclear (Himsworth *et al.*, 2013). It may be assumed that increases in urban rat populations, poverty, unhygienic food processing practices and poor access to clean water would facilitate the direct and indirect transmission of rat-associated bacteria including antimicribial-resistant isolates from rats to humans (Meerburg *et al.*, 2009).

Our findings showed that a large proportion (47.8%, 33/69, Table 2) of the CTX-M producing isolates were included in six successful bacterial clones (STC10/phylogroup A,

231 STC23/phylogroup B1, STC38/phylogroup D, STC155/phylogroup B1, ST405/phylogroup D
232 and ST131/phylogroup B2) with a strong linkage to CTX-M dissemination (Naseer &
233 Sundsfjord, 2011). ST131 and ST405 are virulent clones that have been found globally
234 among disease isolates from human (Banerjee *et al.*, 2013; Ho *et al.*, 2012b). STC10, STC38
235 and STC155 isolates have been commonly found as part of the normal flora in humans, pigs,
236 cattle and poultry (Lo *et al.*, 2010; Lo *et al.*, 2014; Manges & Johnson, 2012), and to cause
237 human urinary tract and bacteremic infections (Ho *et al.*, 2011b; Ho *et al.*, 2012a).
238
239 The strengths of this study include its territory-wide sampling strategy, full species
240 identifcaiton of the animals, a large sample size and molecular characterization of all CTX-M
241 producing strains. However, as each animal was only sampled once, it would not be possible
242 to discern whether the *E. coli* clones represent transient colonization or resident flora.
243
244 In conclusion, our findings showed a high prevelence of CTX-M type resistance among
245 indicator *E. coli* from brown rats and black rats. Many of the CTX-M producing isolates
246 belonged to successful *E. coli* clones which are also commonly found to cause colonization
247 and disease in human. The potential for rats to be a source of CTX-M producing *E. coli* for
248 human deserves further investigation.
249

Conflict of interest statement

All authors have no competing interests

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340

341 **Table 1.** Antimicrobial resistance of 281 *E. coli* isolates

	Chestnut Spiny rats	Indo-Chinese forest rats	Brown rats	Black rats	*P*
No. of isolates	21	39	203	18	
Agent (% resistant)					
Ampicillin	9.5	15.4	42.4	22.2	<0.001
Cefotaxime	0	7.7	35.0	22.2	<0.001
Nalidixic acid	0	7.7	35.0	22.2	<0.001
Tetracycline	0	5.1	30.5	11.1	<0.001
Cotrimoxazole	0	5.1	18.7	11.1	0.028
Chloramphenicol	0	5.1	16.3	5.6	0.043
Ciprofloxacin	0	2.6	13.8	5.6	0.051
Gentamicin	0	0	12.3	5.6	0.034
Nitrofurantoin	0	0	2.0	5.6	0.458
Amikacin	0	0	1.0	0	0.856
ESBL (%)	0	5.1	35.0	22.2	<0.001
Multidrug resistance (%)[a]	0	5.1	33.0	16.7	<0.001

342 [a]Multidrug resistance was defined by resistance to ≥ agent in ≥3 antimicrobial classes:
343 penicllins (ampicillin), cephalosporins (cefotaxime), aminoglycosides (amikacin, gentamicin),
344 chloramphenicol, quinolones (ciprofloxacin, nalidixic acid), cotrimoxazole, , nitrofurantoin
345 and tetracycline. All the isolates were sensitive to imipenem and ertapenem.

346

Table 2. Clonal structure for 69 *bla*$_{CTX-M}$ positive *E. coli* isolates

E coli clones	*n*	MLST[a]	CTX-M subgroup			No. of isolates		
			M1	M9	M1+M9	Brown rats	Black rats	Indo-Chinese forest rats
STC10	13	8 different STs[b]	11	2	0	13	0	0
STC155	8	ST155 (n=6), ST58 (n=2)	5	3	0	7	1	0
STC117	5	ST117 (n=5)	2	3	0	5	0	0
STC38	5	ST38 (n=4), ST2003 (n=1)	1	4	0	5	0	0
STC224	4	ST224 (n=4)	1	1	2	4	0	0
STC23	3	ST88 (n=1), ST366 (n=1), ST410 (n=1)	1	2	0	1	2	0
STC69	3	ST69 (n=3)	1	2	0	3	0	0
STC131	2	ST131 (n=2)	0	2	0	2	0	0
STC206	2	ST2913 (n=1), ST4535 (n=1)	2	0	0	2	0	0
STC405	2	ST405 (n=2)	1	1	0	2	0	0
STC533	2	ST1081 (n=1), ST4531 (n=1)	2	0	0	2	0	0
STC648	2	ST648 (n=2)	0	2	0	2	0	0
Others	18	18 different STs[c]	9	9	0	15	1	2
Total	69		36	31	2	63	4	2

ST, sequence type; STC, ST complex; PG, phylogroup

[a] Including 7 new STs, assigned for the first time in this study (ST4531, ST4532, ST4533, ST4534, ST4535, ST4536 and ST4537).

[b] Including ST10 (*n*=6), ST34 (*n*=1), ST48 (*n*=1), ST617 (*n*=1), ST3489 (*n*=1), ST4375 (*n*=1), ST4534 (*n*=1) and ST4537 (*n*=1)

[c]The following 18 STs had one isolate each: ST12, ST75, ST156, ST162, ST226, ST414, ST453, ST602, ST641, ST994, ST1380, ST2165, ST2178, ST2253, ST3894, ST4532, ST4533 and ST5436.

Fig 1. Prevalence of ESBL-producing *E. coli* in brown rodents by districts of origin in Hong Kong.

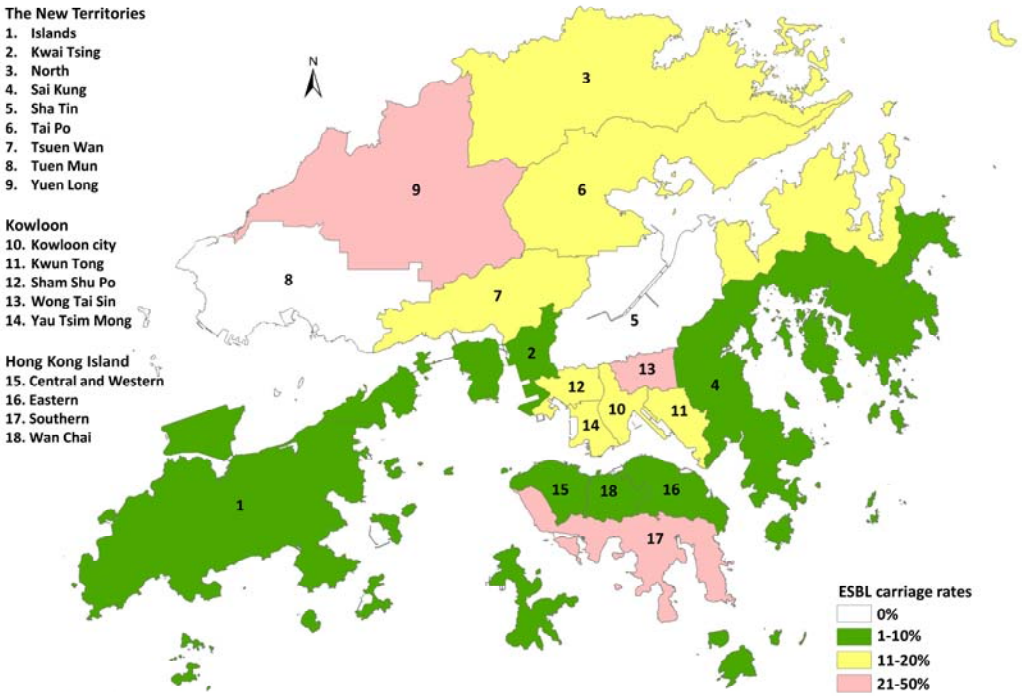

Fig 2. Relationship between human population density and the prevalence of ESBL carriage among brown rats in the 18 districts. The two variables were examined by correlation analysis.