〈Original Article〉

Antimicrobial resistance of Enterobacteriaceae in feral pigeons living in the Kanto region of Japan

Akihisa Hata[1], Toshiyuki Shibahara[1], Hiroshi Yamamoto[2] and Noboru Fujitani[1]

Summary This study investigated the antimicrobial drug resistance of Enterobacteriaceae extracted from the feces of feral pigeons inhabiting an urban area (Taito ward, Tokyo, n=20) and rural region (Asahi and Choshi, Chiba, n=20), respectively. In both regions, the most common Enterobacteriaceae were *Escherichia* sp., which comprised 47% and 77% of the total detected bacteria in Tokyo and Chiba, respectively. In the urban area, drug sensitivity tests revealed that most pigeons harbored ampicillin (ABPC)-resistant bacteria (n=10 pigeons), followed by cephalothin- (CET, n=8), tetracycline- (TC, n=4), cefotaxime- (CTX, n=3), ceftazidime- (CAZ, n=3), gentamicin- (GM, n=3), ciprofloxacin- (CPFX, n=3), and chloramphenicol-resistant bacteria (CP, n=1); the remainder harbored no drug-resistant bacteria (n=4). In the rural region, TC-resistant bacteria were found most often (n=16 pigeons), followed by ABPC- (n=13), CP- (n=13), CET- (n=4), GM- (n=3), CPFX- (n=2), and CTX-resistant bacteria (n=1); as above, several pigeons harbored no resistant bacteria (n=4). Thus, the pigeon populations in each area harbor distinctly different patterns of antimicrobial drug-resistant gut bacteria, which may reflect the regional use of particular antimicrobial compounds and/or the potential for an outbreak of specific drug-resistant bacteria.

Key words: AMR, Monitoring, Local environment, Rural area, Urban area

1. Introduction

A global action plan concerning bacterial drug resistance was adopted at the World Health Organization general meeting in 2015[1]. Shortly thereafter (2016), in Japan, the antimicrobial drug-resistant (AMR) action plan (Ministry of Health, Labour and Welfare) was presented. As one of the aims of this action plan, monitoring of AMR and the consumption of antimicrobial drugs, identification of indicators of drug-resistance changes, and expansion of the aforementioned aims were advocated. To accomplish these aims, AMR surveillance in many different fields (e.g. human and veterinary medicine, agriculture, animal husbandry, and wild animal

[1]Graduate school of Risk and Crisis Management, Chiba Institute of Science
[2]Graduate School of Medicine, University of Toyama
Corresponding author: Noboru Fujitani, Graduate School of Risk and Crisis Management, Chiba Institute of Science. 15-8 Shiomi-cho, Choshi-city, Chiba, Japan, 288-0025
e-mail: nfujitani@edu.kake.ac.jp
Tel: +81-479-30-4744
Fax: +81-479-30-4778
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populations) is required. However, AMR surveillance in wild animals and the environment in general in Japan is insufficient for a proper level of alertness to be maintained. AMR in bacteria in wild animal populations is closely related to overall environmental AMR; in particular, AMR in wild birds reflects AMR in water, soil, and agricultural areas[2-5]. Indeed, the usefulness of the AMR index of the local environment has begun to attract attention[6,7]. In this study, we targeted the Enterobacteriaceae inhabiting the gut of the feral pigeon (*Columba livia*). Enterobacteriaceae are gram-negative bacilli that are distributed broadly throughout the environment and gastrointestinal tract of many animals, including humans. Most Enterobacteriaceae are not pathogenic to a healthy person, but are instead opportunistic. The near ubiquity of these bacteria and the chance of opportunistic infection emphasize the importance of surveillance of drug-resistant Enterobacteriaceae; the significance has grown with the discovery that drug-resistant Enterobacteriaceae are already distributed in wild and domestic animals and healthy people[8-10]. Several studies suggest that soil and water environments such as farmland and rivers can act as reservoirs of drug-resistant Enterobacteriaceae[11-14]. The feral pigeon in Japan is an introduced species originally bred for domestication (as carrier pigeons, racing pigeons, etc.); however, it rapidly established feral populations and is now distributed over all of Japan, especially in urban regions, where it remains all year. Therefore, we hypothesize that the feral pigeon would be suitable for local environmental AMR investigation. In this study, we analyzed the antimicrobial drug resistance of Enterobacteriaceae in feral pigeons living in urban and rural environments, to confirm whether the feral pigeon is a useful proxy for the index of environmental AMR.

2. Materials and methods

2.1. Sampling of feral pigeon feces

In May and June 2014, we collected the feces from 20 pigeons each in an urban region (Taito ward, Tokyo) and a rural region (Asahi and Choshi, Chiba) (Fig. 1). In Taito ward (population, 186,276; region, 10.11 km^2; cultivated region, 0 km^2), 10 samples were taken at two sites, Ueno and Asakusa. Ten samples each were collected in Asahi and Choshi city (population, 68,265 and 64,079; region, 129.9 and 83.9 km^2; cultivated region, 63.7 and 25.4

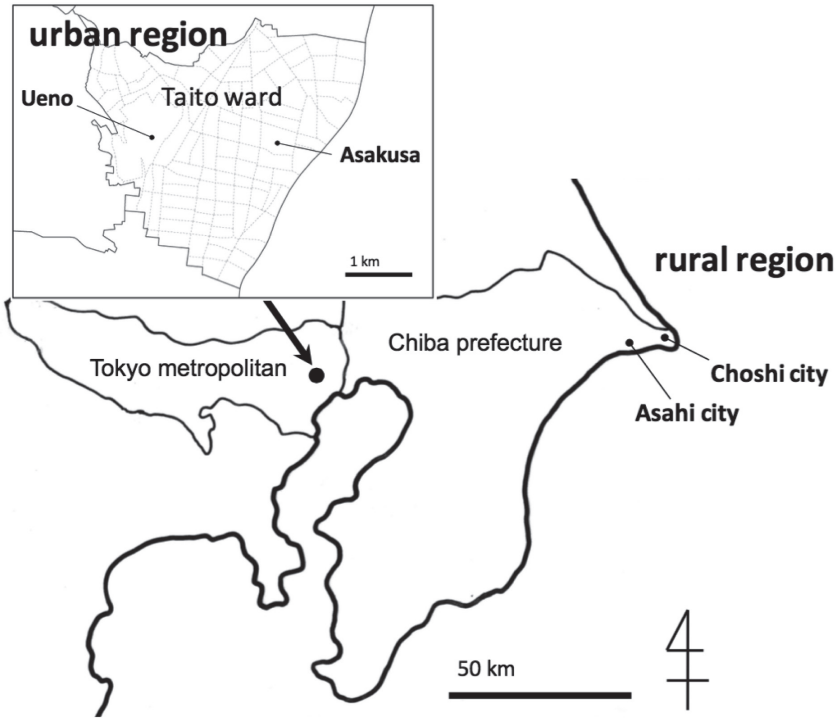

Fig. 1 Map of the sampling points.
The type of area (urban vs. rural/agricultural) is also noted.

km^2), respectively. We collected the feces of the pigeon which did not attach the leg ring that meant breeding pigeon. Feces were collected by verifying the specific feral pigeon visually and collecting the portion which did not contact the ground. A sterilized swab was used for the collection of feces. Carry-Blair transport medium (Nissui Pharmaceutical, Japan) was used for preservation and transportation of the samples. The fecal samples were kept at 4°C and transported to laboratory during the course of the day; Enterobacteriaceae were cultured immediately.

2.2. Detection of Enterobacteriaceae

The feces of the feral pigeon were suspended in sterilized saline solution and smeared via a platinum loop onto a deoxycholate agar (Kanto Chemical, Japan). The plate was cultured under aerobic conditions in an incubator set to 35°C for 18±2 h. After incubation, we randomly picked 5 colonies per pigeon, focusing on lactose-degrading colonies (a biochemical characteristic of many Enterobacteriaceae) that were then purified in nutrient agar. The 200 picked colonies were tested for oxidase activity, with Enterobacteriaceae being demonstrated by negative oxidase activity. The 16S rDNA of the identified oxidase-negative colonies was then sequenced.

2.3. Identification of the genera of sampled Enterobacteriaceae

Partial 16S rDNA sequences (about 0.8 kbp) were determined by Bacterial 16S rDNA PCR Kit Fast (800), Takara Bio, Inc. (Tokyo, Japan). The sequences were analyzed on the basis of their homogeneity using the Basic Local Alignment Search Tool (BLAST) program. The genus of an isolated strain was considered positively identified if homology was ≥97.5% between the 16S rDNA sequence and the compared Enterobacteriaceae strain. The sequences of the 16S rDNA revealed 170 colonies to be Enterobacteriaceae.

2.4. Drug sensitivity profile testing

We performed a drug sensitivity test on the 170 Enterobacteriaceae stocks. We used the agar plate dilution method to test the drug sensitivity of the Enterobacteriaceae strains, according to the performance standards issued by the Clinical and Laboratory Standards Institute (CLSI)[15]. The Blake Point of resistance was based on CLSI M100-S24 criteria. Antimicrobial sensitivity tests were performed using the following antibiotics (the resistance-determining concentration is given in parentheses): ampicillin (ABPC, ≥32 μg/mL), cephalothin (CET, ≥32 μg/mL), cefotaxime (CTX, ≥4 μg/mL), ceftazidime (CAZ, ≥16 μg/mL), imipenem (IPM, ≥4 μg/mL), tetracycline (TC, ≥16 μg/mL), gentamicin (GM, ≥16 μg/mL), amikacin (AMK, ≥64 μg/mL), chloramphenicol (CP, ≥32 μg/mL), and ciprofloxacin (CPFX, ≥4 μg/mL). American Type Culture Collections (ATCC) 25922 (*Escherichia coli*) and ATCC 27853 (*Pseudomonas aeruginosa*) were used as control strains. Combined results of the sensitivity tests revealed the genus of the stock, and the distributions of the genera compared between areas.

3. Results

3.1. The genera of Enterobacteriaceae detected in a feral pigeon (Table 1)

The genus analysis of the bacteria extracted and cultured from the fecal samples from the urban region demonstrated more of the genus *Escherichia* (47% of 100 samples from the cultured colonies) than any other genus; this was followed by *Enterobacter* (27%), *Cronobacter* (7%), *Pantoea* (5%), and *Leclercia* (1%). In addition, 13% of the samples were not Enterobacteriaceae. *Cronobacter* and *Leclercia* were detected only in Asakusa. In the rural region, *Escherichia* (77% of 100 samples from the cultured colonies) was the most common genus, followed by *Cronobacter* (4%), *Klebsiella* (1%), and *Salmonella* (1%). In addition, 17% of the samples were not Enterobacteriaceae. We found that *Klebsiella* and *Salmonella* were detected only in Asahi city. Only the genera *Cronobacter* and *Escherichia* were detected in both urban and rural regions.

3.2. Results of antimicrobial drug sensitivity testing (Table 2)

In the urban cultures, AMR of the *Escherichia* sp. comprised resistance to ABPC (34% of 47 strains), TC (26%), GM (19%), CPFX (19%), and CET (13%). In the *Enterobacter* sp., AMR comprised resistance to CET (93% of 27 strains), ABPC (78%), CTX (7%), CAZ (7%), TC (4%), CP (4%), GM (4%), and CPFX (4%). In the *Cronobacter* sp., AMR comprised resistance to ABPC (43% of 7 strains), TC (43%), GM (43%), and CPFX (29%). In the *Pantoea* sp.,

Table 1. The genera of Enterobacteriaceae detected in a feral pigeon.

	urban region		rural region	
genus	species	n	species	n
Cronobacter	*Cronobacter sakazakii*	7	*Cronobacter sakazakii*	3
			Cronobacter sp.	1
Enterobacter	*Enterobacter asburiae*	1		
	Enterobacter cloacae	2		
	Enterobacter kobei	1		
	Enterobacter ludwigii	1		
	Enterobacter sp.	22		
Escherichia	*Escherichia coli*	44	*Escherichia coli*	65
	Escherichia fergusonii	1	*Escherichia fergusonii*	11
	Escherichia sp.	2	*Escherichia sp.*	1
Leclercia	*Leclercia adecarboxylata*	1		
Pantoea	*Pantoea agglomerans*	1		
	Pantoea eucalypti	1		
	Pantoea sp.	3		
Klebsiella			*Klebsiella variicola*	1
Salmonella			*Salmonella enterica*	1
gram-negative bacilli except Enterobacteriaceae		13		17
total number		100		100

Table 2. Results of antimicrobial drug sensitivity testing.

genus	n		resistance rate (%)																			
			ABPC		CET		CTX		CAZ		IPM		TC		CP		GM		AMK		CPFX	
	urban	rural	u	r	u	r	u	r	u	r	u	r	u	r	u	r	u	r	u	r	u	r
Cronobacter	7	4	43	50	0	0	0	0	0	0	0	0	43	50	0	50	43	0	0	0	29	0
Enterobacter	27	0	78	-	93	-	7	-	7	-	0	-	4	-	4	-	4	-	0	-	4	-
Escherichia	47	77	34	52	13	8	0	3	0	0	0	0	26	71	0	51	19	6	0	0	19	6
Klebsiella	0	1	-	100	-	0	-	0	-	0	-	0	-	100	-	100	-	0	-	0	-	0
Leclercia	1	0	100	-	0	-	0	-	0	-	0	-	100	-	0	-	100	-	0	-	100	-
Pantoea	5	0	0	-	0	-	40	-	40	-	0	-	0	-	0	-	0	-	0	-	0	-
Salmonella	0	1	-	100	-	0	-	0	-	0	-	0	-	100	-	100	-	0	-	0	-	0

Number of tested Enterobacteriaceae strains: urban region, 87; rural region, 83

Abbreviations: ampicillin, ABPC; cephalothin, CET; cefotaxime, CTX; ceftazidime, CAZ; imipenem, IPM; tetracycline, TC; gentamicin, GM; amikacin, AMK; chloramphenicol, CP; ciprofloxacin, CPFX

CTX-resistance was the most common (40% of 5 strains), followed by CAZ (40%). Finally, in the *Leclercia* sp. identified (1 strain), we found resistance to ABPC, TC, GM, and CPFX. No identified strains were resistant to IPM or AMK.

In the rural region, the *Escherichia* sp. were resistant to TC (71% of 77 strains) most often, followed by ABPC (52%), CP (51%), CET (8%), GM (6%), CPFX (6%), and CTX (3%). In the *Cronobacter* sp., ABPC resistance was the most common (50% of 4 strains), followed by TC (50%), CP (50%). Finally, in the 1 strain of *Klebsiella* sp. we identified, we found resistance to ABPC, TC, and CP. No genera were resistant to CAZ, IPM and AMK.

3.3. Multi-drug resistance in the genera of Enterobacteriaceae (Table 3)

Multi-drug resistant Enterobacteriaceae were detected in samples from urban pigeons, with resistance to as many as 4 drugs detected. The patterns of multi-drug resistance are presented below, with the number of strains detected and genus in parentheses. Patterns included the following: ABPC-TC-GM-CPFX (13 strains, *Cronobacter, Enterobacter, Escherichia*, *Leclercia*); ABPC-CET-CTX-CAZ (1 strain, *Enterobacter*); ABPC-CET-CTX-CP (1 strain, *Enterobacter*); ABPC-CET-CAZ (1 strain, *Enterobacter*); ABPC-CET (22 strains, *Enterobacter*, *Escherichia*); ABPC-GM (1 strain, *Cronobacter*); CET-TC (1 strain, *Escherichia*); and CTX-CAZ (1 strain, *Pantoea*).

Patterns for samples from the rural region included the following: ABPC-CET-CTX-TC-CP (1 strain, *Escherichia*); ABPC-CET-TC-CP-GM (1 strain, *Escherichia*); ABPC-CET-TC-CP-CPFX (1 strain, *Escherichia*); ABPC-TC-CP-GM (8 strains, *Escherichia*); ABPC-TC-CP-CPFX (5 strains, *Escherichia*); ABPC-CET-TC-CP (3 strains, *Escherichia*); ABPC-TC-CP (24 strains, *Escherichia*, *Cronobacter*, *Salmonella*, *Klebsiella*); ABPC-CP (1 strain, *Escherichia*).

3.4. Quantity of feral pigeons carrying AMR Enterobacteriaceae

Figure 2 provides the number of pigeons carrying AMR Enterobacteriaceae. In urban pigeons, carriers of ABPC-resistant Enterobacteriaceae were

Table 3. Patterns of multi drug-resistance by number of strains and pigeons.

number of drugs*	urban region				n of strain	n of pigeon	rural region					n of strain	n of pigeon
							ABPC	CET	CTX	TC	CP	1	1
5					0	0	ABPC	CET	TC	CP	GM	1	1
							ABPC	CET	TC	CP	CPFX	1	1
	ABPC	TC	GM	CPFX	13	3	ABPC	TC	CP	GM		8	2
4	ABPC	CET	CTX	CAZ	1	1	ABPC	TC	CP	CPFX		5	1
	ABPC	CET	CTX	CP	1	1	ABPC	CET	TC	CP		3	2
3	ABPC	CET	CAZ		1	1	ABPC	TC	CP			24	9
	ABPC	CET			22	5	ABPC	CP				1	1
2	ABPC	GM			1	1							
	CET	TC			1	1							
	CTX	CAZ			1	1							
	ABPC				6	2	TC					16	5
1	CET				6	2							
	TC				3	2							

*Number of drugs to which a given strain/pigeon has shown resistance.

Number of tested Enterobacteriaceae strains: urban region, 87; rural region, 83.

Number of pigeons with detected Enterobacteriaceae: urban region, 18; rural region, 20.

Abbreviations: ampicillin, ABPC; cephalothin, CET; cefotaxime, CTX; ceftazidime, CAZ; imipenem, IPM; tetracycline, TC; gentamicin, GM; amikacin, AMK; chloramphenicol, CP; ciprofloxacin, CPFX

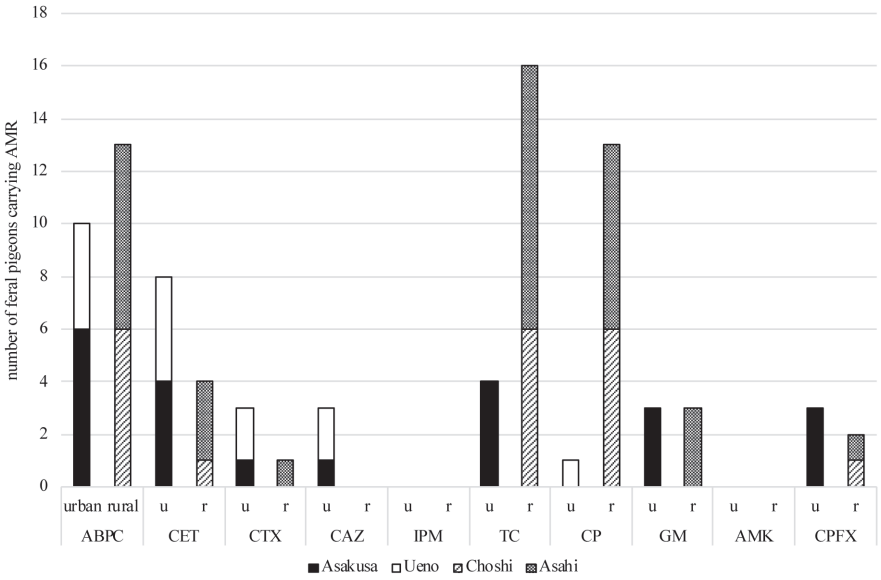

Fig. 2 Number of feral pigeons carrying antimicrobial drug resistance (AMR). Abbreviations: ampicillin, ABPC; cephalothin, CET; cefotaxime, CTX; ceftazidime, CAZ; imipenem, IPM; tetracycline, TC; gentamicin, GM; amikacin, AMK; chloramphenicol, CP; ciprofloxacin, CPFX

most common (10 pigeons), followed by carriers of CET- (8), TC- (4), CTX- (3), CAZ- (3), GM- (3), CPFX- (3), and CP-resistant Enterobacteriaceae (1). Four pigeons had none. By sampling site, pigeons carrying bacteria resistant to TC, GM and CPFX were found only in Asakusa; those with CP-resistant bacteria were detected only in Ueno. There were more pigeons carrying bacteria resistant to ABPC and CET in Asakusa than in Ueno; and more whose samples displayed CTX and CAZ resistance in Ueno than in Asakusa.

In the rural pigeons, carriers of TC-resistant Enterobacteriaceae were most common (16 pigeons), followed by carriers of ABPC- (13), CP- (13), CET- (4), GM- (3), CPFX- (2), and CTX-resistant Enterobacteriaceae (1). Four pigeons in the rural group did not have AMR Enterobacteriaceae. Pigeons carrying CTX- and GM-resistant Enterobacteriaceae were detected only in Asahi; more pigeons carrying ABPC-, CET-, TC-, and CP-resistant Enterobacteriaceae were detected in Asahi than in Choshi.

3.5. Quantification of pigeons carrying multi-drug resistant Enterobacteriaceae (Table 3)

In the urban region, the patterns of multi-drug resistant Enterobacteriaceae are described below, with the number of pigeons carrying that pattern in parentheses. Patterns identified included the following: ABPC-TC-GM-CPFX (3 pigeons); ABPC-CET-CTX-CAZ (1 pigeon); ABPC-CET-CTX-CP (1 pigeon); ABPC-CET-CAZ (1 pigeon); ABPC-CET (5 pigeons); ABPC-GM (1 pigeon); CET-TC (1 pigeon); and CTX-CAZ (1 pigeon).

In the rural area samples, patterns included the following: ABPC-CET-CTX-TC-CP (1 pigeon); ABPC-CET-TC-CP-GM (1 pigeon); ABPC-CET-TC-CP-CPFX (1 pigeon); ABPC-TC-CP-CPFX (1 pigeon); ABPC-TC-CP-GM (2 pigeons); ABPC-CET-TC-CP (2 pigeons); ABPC-TC-CP (9 pigeons); and ABPC-CP (1 pigeon).

4. Discussion

Enterobacteriaceae detected in the feces of feral pigeons included *Escherichia* sp., *Enterobacter* sp., *Pantoea* sp., *Klebsiella* sp., *Leclercia* sp., and *Salmonella* sp., all of which are known intestinal bacteria in birds[16-20]. These results suggest that the detection method (i.e. using feral pigeon feces) is a valid technique. Notably, there are few reports of the detection of *Cronobacter* sp. in bird feces; however,

they are commonly found in the cereals that are used as bait[21]. Thus, the *Cronobacter* sp. we detected were likely from the pigeons also. In this investigation, we found *Enterobacter* sp. only in urban pigeons. The above results indicate that while the technique is valid, there exists the possibility that the environment and the bait impacted the composition of the enterobacterial flora.

We compared the AMR between urban and rural pigeons. Cephalosporin (CET, CTX, CAZ)-resistant Enterobacteriaceae were more common in urban regions than in rural pigeons, whereas ABPC-, TC-, and CP-resistant Enterobacteriaceae were more common in the rural pigeons (Fig. 2). The abundance of cephalosporin-resistant bacteria in the urban region is likely related to the finding that *Enterobacter* sp., which naturally produce AmpC beta-lactamase and are thus resistant to penicillins and cephalosporins, were detected only in the urban region. In fact, AmpC-overexpressing strains show resistance to the second-generation cephalosporins such as CTX and CAZ[22,23]. In the urban region, 81% of the CET-resistant strains, and 50% of the CTX and CAZ-resistant strains were *Enterobacter* sp. If *Enterobacter* sp. are excluded from the analysis, the difference between urban and rural regions is much narrower. The pigeons from the rural region carried mostly ABPC-, TC- and CP-resistant bacteria, with CP-resistant bacteria being particularly different (13 rural vs. 1 urban) (Fig. 2). This may reflect regionality in the use of antimicrobial drugs, and/or their use in an outbreak. ABPC and TC are authorized as pharmaceutical products for use in animals, and TC is authorized as a feed additive and pesticide. The administration of CP to animals has been stopped in Japan. However, it has been reported that resistant bacteria are still detected in domestic animals and ranch soil[24]. In fact, ABPC-, TC-, and CP-resistant bacteria were detected in rural Japan in compost made from animal excrement[25]. A pigeon-based survey in Hokkaido noted that the use of the above-mentioned antimicrobials in the stock-raising (animal husbandry) industry is a factor in resistance[26,27].

We also compared the AMR patterns between sampling sites within each region. In the Taito ward, Asakusa is only approximately 2 km away from Ueno. However, TC-, GM-, and CPFX-resistant Enterobacteriaceae were detected only in Asakusa. CPFX is an important antibacterial agent in human medical care. We found that each CPFX-resistant strain had the same multidrug-resistance pattern (ABPC-TC-GM-CPFX), which is intriguingly similar to the combination utilized for prevention of infection in racing pigeons (amoxicillin, doxycycline, gentamicin, norfloxacin) that is recommended by the Japan Racing Pigeon Association. Thus, it is possible that the three pigeons carrying CPFX-resistant bacteria were originally racing pigeons. When these three pigeons are excluded, the differences between Ueno and Asakusa are much narrower.

In the rural region, Asahi is approximately 15 km away from Choshi. In both areas, ABPC-, CTX-, TC- and CP-resistant bacteria were detected in both sample sites; however, more pigeons carried these resistances in Asahi than in Choshi. Furthermore, pigeons carrying CTX- and GM-resistant bacteria were detected only in Asahi. Asahi has more extensive cultivated areas and livestock such as pig and poultry than Choshi (compiled by the Ministry of Agriculture, Forestry and Fisheries, 2015[28]). These regional differences may reflect differences in antimicrobial resistance in the native pigeon populations of the two regions.

In conclusion, the wide distribution and non-migratory behavior of pigeons may allow their use in monitoring local environmental AMR, as well as in identifying areas at high-risk of an outbreak of AMR bacteria. This investigation was limited by the number of pigeons and the investigation area that were both insufficient to perform statistical analyses. However, as a preliminary study, the data herein indicate that pigeons may be successfully used to index the AMR in a local region. Therefore, we believe that further studies accumulating data and validating this study statistically in a larger population are important needs to be met.

Conflicts of Interest

The authors declare no conflicts of interest associated with this manuscript.

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