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Antimicrobial susceptibility in *Enterobacteriaceae* recovered from Okinawa least horseshoe bat *Rhinolophus pumilus*

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Susceptibility to antimicrobial agents among *Enterobacteriaceae* recovered from feces of the Okinawa least horseshoe bat *Rhinolophus pumilus* (OLHB) in Japan was investigated in 78 isolates. Of these, one isolate was resistant to chlortetracycline and streptomycin, and nine were resistant to sulfadimethoxine (SDMX). Half of these (n = 5) resistant isolates had the transmissible R plasmid for antimicrobials suggesting that OLHB is a species that hardly harbors antimicrobial-resistant *Enterobacteriaceae*. One possible reason for this low prevalence of antimicrobial resistance is that the bat lives in a clean environment with no or only very low artificial drug contaminations due to low contact with such as livestock that frequently carries antibiotic-resistant bacteria. This study suggests that it's important to monitor antimicrobial resistance of *Enterobacteriaceae* in OLHB as a model for natural R plasmid transfer in a natural environment without specific pathological bacteria and artificial drugs.

The Okinawa least horseshoe bat (OLHB) *Rhinolophus pumilus* is a cave-dwelling species endemic to the central and southern Ryukyus, Japan (Yoshino et al. 2006). It is now designated as vulnerable by the Ministry of the Environment of Japan.

Since high correlations between the use of veterinary chemotherapeutic antimicrobial agents and resistance in *Escherichia coli* isolated from the feces of food-producing animals have been reported (Asai et al. 2005), concern has been raised about the potential impact of antibiotic-resistant bacteria on public health (Gilliver et al. 1999, Abiodun et al. 2009). Attention has also been focused on the fact that antibiotic-resistant *E. coli* has been detected the wild animals that have not been exposed to artificial drugs (Piddock 1996, Costa et al. 2008). Prevalence of antimicrobial-resistant *E. coli* has been shown to be widespread among geographic locations and animal species (Gilliver et al. 1999, Gopee et al. 2000, Österblad et al. 2001). We have previously reported that antimicrobial-resistant *Salmonella* spp. isolates were rarely found in the wild cranes, hooded crane *Grus monacha* and white naped crane *Gurus vipio* (Kitadai et al. 2010) whereas a high prevalence of antimicrobial-resistant *E. coli* was demonstrated in Japanese weasels *Mustela itatsi*, and a barely detectable incidence was found in the java mongoose *Herpestes javanicus* (Nakamura et al. 2011). Furthermore,

studies reported that prevalence of antimicrobial-resistant bacteria and R plasmids is associated with the food habits of specific animal species (Kanai et al. 1981). These findings suggest that further studies of antimicrobial-resistance in wild animals might lead to improved knowledge of the species-specific biology of wildlife. Kanai et al. (1981) have suggested that wild birds and bats might become carriers of resistant bacterial strains, and thus spread the R plasmid over wide areas. Bats are one of the most widely distributed mammals in the world, and are reservoirs or carriers of several zoonoses (Kobayashi et al. 2006, Abiodun et al. 2009).

Enterobacteriaceae are a common component of the intestinal tract flora in a wide variety of animals and also humans (Skurnik et al. 2006). These intestinal bacteria can be easily disseminated in different ecosystems through water, soil, food and other media and as, for *E. coli*; they are considered to be an important indicator bacterium used for tracking the evolution of antibiotic resistance in different ecosystems (Skurnik et al. 2006).

In the present study, we investigate the antimicrobial susceptibility of *Enterobacteriaceae* recovered from the feces of the OLHB, and also whether they contained any of the transmissible plasmids responsible for the resistance; in the context of the OLHB biology.

Table 1. Resistance patterns and transmissible R plasmids of *Enterobacteriaceae* isolated from Okinawa least horseshoe bat.

R plasmids (after transmission)		Original strains (before transmission)	
Resistance patterns	Number of strains	Resistance patterns	Number of strains
SDMX	4	SDMX	9
CTC, SM,	1	CTC, SM	1
Total	5	Total	10

SDMX: sulfadimethoxine; CTC: chlortetracycline; SM: streptomycine.

Material and methods

We collected a total of 20 samples of fresh feces randomly found in a cave inhabited by the bats in the southern part of Okinawa Island, and carried out the procedure at room temperature within 12 h as described by (Nakamura et al. 2011). Eventually, 26 isolates (16.3%) of *Escherichia coli*, 38 isolates (23.8%) of *Klebsiella pneumoniae* and 14 isolates (8.8%) of *Citrobacter freundii* were recovered from the 160 colonies showing typical *E. coli* morphology on MacCon key agar plate. By using these 78 *Enterobacteriaceae* isolates, we made antimicrobial susceptibility test. In vitro susceptibility test was made by the agar dilution method recommended in the guideline of the Japanese Society of Chemotherapy using relevant concentrations for the seven antimicrobials: ampicillin (ABPC), chloramphenicol (CP), chlortetracycline (CTC), kanamycin (KM), streptomycin (SM), nalidixic acid (NAL), sulfadimethoxine (SDMX) (Mitsuhashi et al. 1981, Kojima et al. 2009). The results were recorded using terms of susceptible (S), resistant (R) or intermediate (I) which indicates high none and low susceptibility (Sandeep et al. 2013), respectively. Conjugation test was conducted as described in (Kinjo et al. 1992) and ML1410 (NAL resistant); was used as the recipient of conjugative R plasmids.

Results

We observed only one isolate resistant to CTC and SM in *E. coli*; and (Table 1) while we detected nine isolates resistant to SDMX. These species were composed of four of *Klebsiella pneumoniae* and five *Citrobacter freundii* (Table 2). The prevalence of antimicrobial-resistant *Enterobacteriaceae* was 12.8% and this multiple antimicrobial resistant isolate had the transmissible R plasmid for CTC and SM (Table 1); and four of the nine isolates resistant to SDMX

also harbored the transmissible R plasmid for SDMX. In total, five of the ten resistant isolates exhibited the transmissible R plasmid (Table 1). The isolates indicating intermediate (I) resistance to ABPC (26.5%) and CTC (35.9%) towards *Klebsiella pneumoniae* (Table 2).

Discussion

The investigation of antimicrobial resistant *Enterobacteriaceae* in wildlife reflects history of the administration of artificial drugs, degree of contact with livestock and characterization of the species. This study suggests that OLHB is a species that only minimally harbors antimicrobial-resistant *Enterobacteriaceae*. Since the prevalence of antimicrobial-resistant *Enterobacteriaceae* in OLHB was only 12.8%. This frequency was considerably lower than that in other wildlife. Frequencies of more than 30% have been reported in a bird of prey (Tsubokura et al. 1995), the whistling swan (Tsubokura et al. 1995), and Japanese weasels (Nakamura et al. 2011). Furthermore, Abiodun et al. (2009) have reported that among 49 isolates of *E. coli* recovered from chiroptera bats in Trinidad, 40 isolates (82%) exhibited resistance to one or more antimicrobial agents (Abiodun et al. 2009). The reason for such a large difference in the same chiroptera order is unknown. However, the chiroptera in that study did not include *Rhinolophus* genus. In our previous (Nakamura et al. 2011) study, we found a large difference in the prevalence of antimicrobial-resistant *E. coli* between invasive alien mammals, Java mongoose and the Japanese weasel. Since we cannot find detailed reasons for such a difference, we can only conclude that it may be species-differences. Despite similar general food habits, differences in particular food items may contribute to the prevalence of antimicrobial-resistant bacteria among wild animals.

Table 2. The frequencies of antimicrobial *Enterobacteriaceae* in Okinawa least horseshoe bat.

Antimicrobials	Species of <i>Enterobacteriaceae</i> (number of strains)											
	<i>Escherichia coli</i> (26)			<i>Klebsiella pneumoniae</i> (38)			<i>Citrobacter freundii</i> (14)			Total (78)		
	S	I	R	S	I	R	S	I	R	S	I	R
ABPC	26	0	0	26	12	0	10	4	0	62	16	0
CP	26	0	0	38	0	0	14	0	0	78	0	0
CTC	25	0	1	14	24	0	10	4	0	38	28	1
KM	26	0	0	38	0	0	14	0	0	78	0	0
SM	25	0	1	38	0	0	14	0	0	78	0	1
NAL	26	0	0	38	0	0	14	0	0	78	0	0
SDMX	18	8	0	33	0	5	10	0	4	61	8	9

ABPC: ampicillin; CP: chloramphenicol; CTC: chlortetracycline; KM: kanamycin; SM: streptomycin; NA: nalidixic acid; SDMX: sulfadimethoxine S: susceptibility; I: intermediate (low susceptibility); R: resistance.

Abiodun et al. (2009) reported that *E. coli* was detectable in chiroptera feces at a rate of 13% i.e. prevalence similar to that of the present study. In the present study, we recovered 26 *E. coli* isolates from 160 colonies with typical *E. coli* morphology, and the prevalence of *E. coli* in intestinal tract of chiroptera was 16.3%, which is similar to the report by Abiodun et al. (2009). However, the prevalence of *E. coli* (13% and 16.3%) was considerably lower than the figures of 25–78% reported for other mammal species (Moreno et al. 1975, Costa et al. 2008).

The antimicrobial agents responsible for the resistances detected in the present study were chlortetracycline, streptomycin and sulfadimetoxine. Since isolates resistant to these antimicrobials have also been observed frequently in livestock (Asai et al. 2005), the spread of R plasmids appears to involve both livestock and wild animals. In the resistant isolates detected in the present study, about half harbored the transmissible R plasmid for each of the antimicrobial agents involved. Therefore, OLHB may become a carrier of antimicrobial resistance. However, since the prevalence of antimicrobial resistance in OLHB was very low, dissemination of antimicrobial-resistant organisms by OLHB does not seem to be a matter of concern. OLHB inhabits caves, and its low prevalence of antimicrobial-resistant organisms may reflect a lack of exposure due to their dietary habits and limited contact with livestock and humans on Okinawa Island.

On the other hand, intermediate bacteria-types resistant to ABPC (20.5%) and CTC (35.9%) were detected somewhat frequently as frequently reported in livestock (Asai et al. 2005). The present results suggest that although OLHB has little contact with livestock, it may yet be influenced by livestock. Therefore continuous investigation of drug-resistant bacteria in OLBH may be necessary.

In our previous study using wild crane which migrates to Kagoshima prefecture in Japan; for wintering, we have reported that there was almost no change in their ownership rate of *Salmonella* spp. from 2002 to 2008 nor in, their prevalence of antimicrobial resistant *Escherichia Coli*, which suggests this crane has lived under a constant environment without artificial drug and pathological bacteria (Kitadai et al. 2010, 2012). Thus, continuous investigation of antimicrobial susceptibility in *Enterobacteriaceae* of wildlife is useful for understanding their contact with livestock and humans that frequently harbor antimicrobial-resistant bacteria. We conclude that OLHB is a species that only minimally harbors antimicrobial-resistant *Enterobacteriaceae* due to low frequency of contact with artificial drugs and other wild animals that frequently harbor antimicrobial-resistant organisms. By continuous examination, it can be confirmed if OLHB lives under a constant environment without a-specific antimicrobial resistant pathological bacteria and artificial drugs.

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