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Factors associated with antimicrobial-resistant *Escherichia coli* in zoo animals

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ABSTRACT

Factors associated with the carriage of antimicrobial-resistant *Escherichia coli* isolates were analysed among zoo animals. An association was observed between selection of amoxicillin as the first-line therapy and a significantly higher percentage of resistance to ampicillin (54.5%) from 11 animals treated with antimicrobials, compared with isolates from 32 untreated animals (9.4%). In addition, the percentage resistance to kanamycin (36.4%), gentamicin (27.3%), trimethoprim (27.3%) and tetracycline (63.6%) from 11 treated animals was significantly higher than those from 32 untreated animals (3.1%, 3.1%, 3.1% and 25%, respectively), although these antimicrobials were rarely used. All kanamycin-, gentamicin- and trimethoprim-resistant isolates and more than half of the tetracycline-resistant isolates from treated animals were also resistant to ampicillin. Co-resistance to other antimicrobials with ampicillin was suggested to contribute to an increasing of resistance towards antimicrobials that were rarely administered. The present investigation revealed an association of antimicrobial treatment with the spread of antimicrobial-resistant bacteria among zoo animals.

1. Introduction

An increase in antimicrobial resistance is one of the biggest current public health problems worldwide. Increases in antimicrobial resistance in *Escherichia coli* due to the use of antimicrobials in food-producing animals have been previously reported (Asai et al., 2005). *Enterobacteriaceae* isolates from zoo animals were reported to harbour antimicrobial resistance genes such as β-lactamase and plasmid-mediated quinolone resistance genes (Ahmed et al., 2007). Baldy-Chudzik and Stosik (2007) reported a significant relationship between prevalence of antimicrobial resistance and phylogenetic background among commensal *E. coli* isolates from various mammals examined in a zoo. Although zoo animals were occasionally treated with antimicrobials, the carriage of antimicrobial-resistant *E. coli* isolates influenced by antimicrobial treatment in zoo animals has not been examined.

In the present investigation, *E. coli* isolates from faecal samples of zoo animals were found to have antimicrobial resistance and were genotyped. The relationship between carriage of antimicrobial-resistant *E. coli* isolates and antimicrobial treatment or feeding habits was examined.

2. Materials and methods

2.1. Samples

Faecal droppings ($n = 99$) were collected from following animal species raised at Sapporo Maruyama Zoo (Sapporo, Japan) between July and September 2008: common chimpanzee (6 samples), Diana monkey (6), eastern grey kangaroo (5), sheep (5), Steller's sea eagle (5), Tokara goat (5), domestic guinea pig (4), miniature horse (4), black-tailed prairie dog (3), polar bear (3), Asian black bear (2), Bennetts wallaby (2), black kite (2), eland (2), giraffe (2), grant's zebra (2), hippopotamus (2), lion (2), ostrich (2), red panda (2), snow leopard (2), American black bear (1), American flamingo (1), Andean condor (1), Bactrian camel (1), blue-eared pheasant (1), bornean orangutan (1), common brushtail possum (1), common raccoon (1), De Brazza's monkey (1), eastern bewick's swan (1), eastern timber wolf (1), Ezo brown bear (1), Ezo sika deer (1), flemish giant (1), Harris's hawk (1), Humboldt penguin (1), Japanese goshawk (1), Japanese macaque (1), llama (1), Malayan sun bear (1), Malayan tapir (1), phasianidae (1), ring-tailed lemur (1), Russian flying squirrel (1), scarlet ibis (1), serval (1), sloth bear (1), snowy owl (1), sparrow hawk (1), sulphur-crested cockatoo (1) and Swinhoe's Pheasant (1).

These faecal samples were collected from each pen before cleaning in the morning. To avoid duplication, faecal samples from

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each pen were collected once. The number of collected samples was limited to the same or less than the number of reared animals in each pen. Moreover, at most five faecal samples were collected from each pen where five or more animals were reared. From three aquariums for largha spotted seals (*Phoca largha*), harbour seals (*P. vitulina*) and a Steller's sea lion (*Eumetopia jubata*), three water samples of approximately 500 ml, instead of faecal samples, were collected before the addition of disinfectant to aquarium water.

Individual animals for 63 samples were identified. The originating animal species of the 39 remaining samples could be identified but not the individual animal because two or more animals were reared together in the pen. The five samples from sheep or Tokara goat (*Capra hircus domestic*) were neither identifiable at the species nor individual level because they were reared together in the same pen.

To analyse influences on carriage of antimicrobial-resistant *E. coli* isolates, the following details regarding target individual animals were gathered: record of antimicrobial treatment in the 6 months prior to sampling, types of feed used, rearing pens and houses utilized and rearers who usually take care each animal. Animals were grouped according to their feeding habits.

2.2. Isolation and identification

The faecal samples were inoculated onto desoxycholate-hydrogen sulphate-lactose (DHL) agar medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) alone or DHL supplemented with 2 μg/ml enrofloxacin (DHL-E; Sigma–Aldrich Japan K.K., Tokyo, Japan) or 2 μg/ml cefpodoxime (DHL-C; Sankyo Co. Ltd., Tokyo, Japan). The water samples were filtered with 0.45 μm filters (Nihon Millipore K.K., Tokyo, Japan) and the filtrate inoculated into Brain Heart Infusion Broth (Becton Dickinson and Co., Sparks, MD, USA) then incubated at 37 °C overnight. The enrichment cultures were inoculated onto DHL, DHL-E and DHL-C.

The isolation agar media were incubated at 37 °C overnight. Two colonies of suspected *E. coli* per sample were selected and sub-cultured on nutrient agar (Nissui Pharmaceutical Co. Ltd.). One isolate per sample identified by the API 20E kit (Sysmex Co. Ltd., Kobe, Japan) as *E. coli* was selected for further tests. The isolates were stored in a Microbank (Pro-Lab Diagnostics Inc., Richmond Hill, Canada) at −80 °C.

2.3. Phylogenetic classification

The DNA of isolates was extracted from cultures with an InstaGene Matrix (Bio-Rad Laboratories Inc., Tokyo, Japan). All *E. coli* isolates were classified into one of the four main phylogenetic groups: A, B1, B2 and D, as previously described (Clermont et al., 2000). All isolates were tested for the presence of the *chuA* gene. On the basis of the first polymerase chain reaction (PCR), *chuA*-positive isolates were tested for *yjaA* and *chuA*-negative isolates were tested for TspE4.C2.

2.4. Antimicrobial susceptibility testing

We performed minimal inhibitory concentration (MIC) determinations using the broth micro-dilution method with an Eiken frozen plate (Eiken Chemistry Co. Ltd., Tokyo, Japan) according to the Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2008). The following antimicrobial agents were tested: ampicillin, cefazolin, cefpodoxime, cefmetazole, ceftazidime, streptomycin, kanamycin, gentamicin, chloramphenicol, tetracycline, nalidixic acid, ciprofloxacin, trimethoprim, cefpodoxime/clavulanic acid and ceftazidime/clavulanic acid. The breakpoints are shown in Table 3. These breakpoints were in accordance with the CLSI guidelines (CLSI, 2008) or a previous study (Kojima et al., 2009). In the case of streptomycin, an intermediate MIC of bi-modal distribution was defined as the breakpoint in this study.

2.5. β-lactamase gene detection

Presence of the genes bla_{TEM}, bla_{SHV}, $bla_{\mathrm{CTX\text{-}M\text{-}1}}$, $bla_{\mathrm{CTX\text{-}M\text{-}2}}$, $bla_{\mathrm{CTX\text{-}M\text{-}8}}$, $bla_{\mathrm{CTX\text{-}M\text{-}9}}$ and $bla_{\mathrm{CMY\text{-}2}}$ were determined in cefpodoxime -resistant isolates (Arlet et al., 1995; Saladin et al., 2002; Ishii et al., 2005; Kojima et al., 2005). The seven sets of PCR primers used for β-lactamase genes are shown in Table 1. Nucleotide sequences were determined for both strands, directly from PCR products of class A β-lactamase. The DNA alignments and deduced amino acid sequences were compared with DDBJ database using the BLAST program (http://blast.ddbj.nig.ac.jp/top-j.html) (Altschul et al., 1997).

2.6. Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) analysis was performed according to the Pulse Net CDC protocol (Centers for Disease Control and Prevention, 2004). Genomic DNA in each agarose plug was digested with *Xba*I (Takara Bio Ltd., Shiga, Japan). PFGE was performed using a CHEF-DRIII system (Bio-Rad Laboratories Inc.), under the following conditions: switch time of 2.2–54.2 s, running time of 18 h, 120° included angle, 6 V/cm and 14 °C.

2.7. Conjugation assay

To determine transfer of antimicrobial resistance genes, conjugation experiments were carried out by the broth mating method. The *E. coli* strain ML4909 (resistant to rifampicin) was used as a recipient. Cultures of recipient and donor were mixed with each other at 9:1 ratio (recipient:donor) and incubated on shaking for 1 or 3 h at 37 °C. The transconjugants were selected and sub -cultured by Mueller–Hinton agar (Oxoid Ltd., Hampshire, UK) supplemented with rifampicin (50 μg/ml; Sigma–Aldrich Japan K.K.) and ampicillin (50 μg/ml; Sigma–Aldrich Japan K.K.). Antimicrobial resistance genes of donors and transconjugants were confirmed by PCR (Chen et al., 2004; Ishihara et al., 2011; Kozak et al., 2009; Lanz et al., 2003; Sengeløv et al., 2003).

2.8. Statistical analysis

The proportions of antimicrobial-resistant *E. coli* isolates were compared among different preparations of agar isolation media, antimicrobial treatments or feeding habits using a Chi-square test. When at least one expected frequency was less than five, Fisher's exact test was used for comparison between two groups.

3. Results

3.1. Feeding habits, isolation and phylogenetic groups

Seventy-one samples were from carnivores, which were fed only horse, chicken or fish. Twenty-seven samples were classified as herbivores, fed on vegetables, fruit, grass and pellets. The remaining four samples were grouped as omnivores.

Using DHL alone, *E. coli* isolates were obtained from 66 out of 102 samples (64.7%). *E. coli* isolates belonging to the phylogenetic group B1 were predominant (Table 2).

Using DHL-E, fluoroquinolone (FQ)-resistant *E. coli* isolates were obtained from 34 samples (33.3%), which belonged to phylogenetic groups A or B1 (Table 2). Animals carrying FQ-resistant *E. coli* were reared in a tropical animal house, petting zoo area, bear house,