ORIGINAL ARTICLE

# Prevalence of *Salmonella*, *Yersinia* and *Campylobacter* spp. in Feral Raccoons (*Procyon lotor*) and Masked Palm Civets (*Paguma larvata*) in Japan

K. Lee[1], T. Iwata[1], A. Nakadai[1], T. Kato[2], S. Hayama[2], T. Taniguchi[1] and H. Hayashidani[1]

[1] Division of Animal Life Science, Institute of Agriculture, Tokyo University of Agriculture and Technology, Tokyo, Japan
[2] Laboratory of Wildlife Medicine, Nippon Veterinary and Life Science University, Tokyo, Japan

## Impacts

- This is the first report on the prevalence of *Salmonella*, *Yersinia*, and *Campylobacter* spp. in feral raccoons and masked palm civets in Japan.
- Our results indicate that these animals are potential carriers of these pathogens and that these animals probably acquired their infections from human activities, other wild animals, and the environment.
- As these animals live near human habitations or livestock farms, their carrying the pathogens represents a serious public and animal health risk.

**Keywords:**
Alien species; epidemiology; *Salmonella*; *Yersinia pseudotuberculosis; Campylobacter*

**Correspondence:**
H. Hayashidani. Division of Animal Life Science, Institute of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan.
Tel.: +81 42 367 5775; Fax: +81 42 367 5775;
E-mail: eisei@cc.tuat.ac.jp

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## Summary

To estimate the public and animal health risk that alien species pose, the prevalence of *Salmonella*, *Yersinia*, and *Campylobacter* spp. in feral raccoons (*Procyon lotor*, $n = 459$) and masked palm civets (*Paguma larvata*, $n = 153$), which are abundant alien species in Japan, was investigated in urban and suburban areas of Japan. *Salmonella enterica* was detected from 29 samples [26 raccoons, 5.7%, 95% confidence interval (CI) 7.8–3.5%; three masked palm civets, 2.0%, 95% CI 4.2–0%]. Many of the isolates belonged to serovars that are commonly isolated from human gastroenteritis patients (e.g. *S.* Infantis, *S.* Typhimurium, and *S.* Thompson). The antimicrobial susceptibility test showed that 26.9 % of the isolates from raccoons were resistant to at least one antimicrobial agent, whereas none of the isolates from masked palm civets were resistant. *Yersinia* sp. was detected from 193 samples (177 raccoons, 38.6%, 95% CI 43.0–34.1%; 16 masked palm civets, 10.5%, 95% CI 15.3–5.6%). All virulent *Yersinia* strains belonged to *Yersinia pseudotuberculosis*, which was isolated from seven (1.5%, 95% CI 2.6–0.4%) raccoons and six (3.9%, 95% CI 7.0–0.8%) masked palm civets. According to the detection of virulence factors, all the *Y. pseudotuberculosis* isolates belonged to the Far Eastern systemic pathogenicity type. *Campylobacter* spp. was detected from 17 samples (six raccoons, 1.3%, 95% CI 2.3–0.3%; 11 masked palm civets, 7.2%, 95% CI 11.3–3.1%). Among these, three isolates from raccoons were identified as *C. jejuni*. These results showed that these pathogens can be transmitted by human activities, other wild animals, and the environment to feral raccoons and masked palm civets, and vice versa. As these animals have omnivorous behaviour and a wide range of habitats, they can play an important role in the transmission of the enteric pathogens.

## Introduction

The raccoon (*Procyon lotor*) is a medium-sized mammal that is widely distributed in North America. Raccoons were introduced into Japan in the 1970s and have become naturalized in at least 42 of 47 prefectures (Ikeda et al., 2004). In urban areas, raccoons often use human houses for their dens. Raccoons use the feed stores of domestic animals as nests, and thus also have close contact with such animals (Zeveloff, 2002). It has been reported that the raccoon is a reservoir of various kinds of zoonotic pathogens in its place of origin, including the raccoon


 

roundworm (*Baylisascaris procyonis*) (Gavin et al., 2005), rabies virus (Finnegan et al., 2002), *Leptospira* spp. (Hamir et al., 2001), and *Francisella tularensis* (Berrada et al., 2006). Bigler et al. (1975) found that raccoons are so adaptive that they can bridge the gaps among avian, terrestrial, and aquatic environments, and thus they are appropriate as an indicator of the prevalence of various infectious diseases and pollutants. The masked palm civet (*Paguma larvata*) is also a medium-sized carnivore and is distributed widely in Asia. Masked palm civets are thought to have been introduced into Japan, although this contention is controversial (Abe, 2005). Masked palm civets often live in similar places as raccoons and can be carriers of human and animal pathogens, such as severe acute respiratory syndrome (SARS) virus (Tu et al., 2004) and canine distemper virus (Machida et al., 1992).

In Japan, attention has been focused on the role of these highly adaptive mammals in the transmission of zoonotic pathogens, including *Trichinella* T9 (Kobayashi et al., 2007), *Babesia microti*-like parasite (Kawabuchi et al., 2005), *Ehrlichia* spp., *Anaplasma phagocytophilum* (Inokuma et al., 2007), *Strongyloides procyonis* (Sato and Suzuki, 2006), and canine distemper virus (Machida et al., 1992). Pathogens carried by these animals can present serious public and animal health problems in the habitats to which they have been introduced. However, in spite of the fact that these two species often inhabit urban and suburban areas, there have been few reports on the prevalence of enteric pathogens among them.

The objective of this study was to determine the prevalence of the causal agents of enteric diseases including *Salmonella* spp., *Yersinia* spp., and *Campylobacter* spp. in feral raccoons and masked palm civets. We then assessed antimicrobial resistance in the isolates and analysed them using polymerase chain reaction (PCR) to elucidate their virulence factors and transmission routes.

## Materials and Methods

### Sample collection and transport

From March 2006 to May 2007, 459 feral raccoons and 153 feral masked palm civets were captured in Kanagawa, Gunma, and Tokyo Prefectures by each municipality as a part of the local governmental control and eradication programmes. The animals were caught using box traps and were killed by humanitarian methods (Japan Veterinay Medical Association, 2007). Of 459 feral raccoons, 229 (49.9%) were males, 211 (46.0%) were females, and 19 (4.2%) were of unknown sex. Of 153 feral masked palm civets, 72 (47.1%) were males, 79 (51.6%) were females, and two (1.3%) were of unknown sex. The age of the feral raccoons and masked palm civets was determined on the basis of tooth eruption and cranial suture obliteration by the method of Montgomery (1964) and Junge and Hoffmeister (1980). Of 459 feral raccoons, 71 (15.5%) were juveniles (<5 months), 357 (77.8%) were sub-adults or adults, and 31 (6.9%) were of unknown age. Of 153 feral masked palm civets, 47 (30.7%) were juveniles (<6 months), 104 (68.0%) were sub-adults or adults, and 2 (1.3%) were of unknown age. Faecal samples were collected and preserved in Cary and Blair transport medium (Eiken Chemical Co. Ltd., Tokyo, Japan) or sterile centrifuge tubes. The samples were then transported to the laboratory. The samples were suspended in 4 ml of sterile phosphate-buffered saline (PBS; pH 7.2) and tested within a week after collection.

### Isolation and identification of *Salmonella*

One millilitre of each specimen was inoculated into 10 ml of buffered peptone water (BPW; Becton Dickinson, Franklin Lakes, NJ, USA). After incubation at 37°C for 24 h, 1 ml of BPW culture was transferred to 10 ml of Hajna-tetrathionate broth (Eiken Chemical). The broth was incubated at 37°C for 24 h, then one loopful of each tube was inoculated onto a plate of desoxycholate hydrogen sulfide lactose agar (DHL; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) and mannitol lysine crystal violet brilliant green agar (MLCB; Nissui). These plates were incubated at 37°C for 24 h, and at least two suspicious colonies morphologically similar to *Salmonella* spp. from each plate were subcultured for biochemical examinations. Biochemical characteristics were examined on triple sugar iron medium (Nissui) and lysine indole motility medium (Nissui). The subspecies of *Salmonella* isolates were confirmed by biochemical examinations and the multiplex PCR assay (Popoff and Le Minor, 2005; Lee et al., 2009). Serotyping for *Salmonella* isolates was accomplished with commercial O and H antisera (Denka Seiken Co. Ltd., Tokyo, Japan) according to the method of Popoff and Le Minor (2001).

### Isolation and identification of *Yersinia*

Specimens were incubated at 4°C for 4 weeks. After alkali treatment (Aulisio et al., 1980), a loopful of sample suspension was spread on virulent *Yersinia enterocolitica* (VYE) agar (Fukushima, 1987) and Irgasan-Novobiocin (IN) agar containing 2.5 mg/l of irgasan and novobiocin in Yersinia Selective Agar Base (Difco Laboratories, Detroit, MI, USA) (Schiemann, 1979). All plates were incubated at 25°C for 48 h. Colonies morphologically similar to *Yersinia* spp. were subcultured for biochemical examination. The identification of yersiniae was performed by the methods of Wauters et al. (1988). All isolates identified as yersiniae were subjected to autoagglutination tests to evaluate their potential pathogenicity (Laird and Cavanaugh, 1980).

Then, virulent isolates were subjected to further analysis. Serotyping of *Y. pseudotuberculosis* was accomplished by slide agglutination with commercial antisera (Denka Seiken). Isolates identified as *Y. pseudotuberculosis* were genotyped by the presence patterns of the gene encoding *Y. pseudotuberculosis*-derived mitogen typeA (YPMa) and high-pathogenicity island (HPI) so as to analyse their geographical origin using PCR assay (Fukushima et al., 2001).

### Isolation and identification of *Campylobacter*

One millilitre of the specimen was inoculated into 5 ml of Preston enrichment broth (OXOID CM0067 + SR0117E + SR0232E) containing 5% defibrinated horse blood. After incubation at 37°C for 24 h, one loopful of each tube was inoculated onto a skirrow blood agar plate (OXOID CM0271 + SR0069E + SR0232E) containing 5% defibrinated horse blood. All skirrow blood agar plates were incubated for 48 h at 37°C under microaerobic conditions and examined for the presence of characteristic colonies of *Campylobacter*. Confirmation and characterization of the isolates were performed on the basis of microscopic morphology, an oxidase test, a catalase test, growth at 25°C and 42°C, and a multiplex PCR assay for *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus* (Wang et al., 2002; Vandamme et al., 2005)

### Antimicrobial susceptibility test

Antimicrobial susceptibility testing was performed according to the disc diffusion method (National Committee for Clinical Laboratory Standards, 2002; Luangtongkum et al., 2007). The following antimicrobial paper discs (Sensi-Disc; Becton Dickinson) were used: ampicillin (10 μg/disk), cefazolin (30 μg/disk), ceftriaxone (30 μg/disk), ciprofloxacin (5 μg/disk), chloramphenicol (30 μg/disk), gentamicin (10 μg/disk), kanamycin (30μg/disk), nalidixic acid (30 μg/disk), oxytetracycline (30 μg/disk), and streptomycin (10 μg/disk) for *Salmonella* and *Y. pseudotuberculosis*; and ampicillin, ciprofloxacin, clindamycin (2 μg/disk), erythromycin (15 μg/disk), gentamicin, kanamycin, nalidixic acid, norfloxacin (10 μg/disk), oxytetracycline, and streptomycin for *Campylobacter*.

### Statistical analysis

Differences of the prevalence were analysed by the chi-squared test in SPSS software (SPSS Inc., Chicago, IL, USA).

## Results

The raccoons and masked palm civets were essentially normal except for various external parasites. No discernible pathological evidence or signs of disease were observed. There were no significant sex- and age-specific differences in *Salmonella* spp., *Yersinia* spp., and *Campylobacter* spp prevalence in both animals (Table 1). There was no sample which was positive for more than two bacterial species or serotypes tested in this study, except non-pathogenic *Yersinia* spp.

**Table 1.** Prevalence of *Salmonella enterica*, *Yersinia pseudotuberculosis*, and *Campylobacter* sp. in raccoon and masked palm civets in each sex or age groups

| | | | No. positive sample (%) | | |
|---|---|---|---|---|---|
| Origin | Sex/age | Sample size | *Salmonella enterica* | *Y. pseudotubeculosis* | *Campylobacter* spp. |
| Raccoon | Female | 211 | 11 (5.2) | 2 (0.9) | 2 (0.9) |
| | Male | 229 | 15 (6.6) | 5 (2.2) | 3 (1.3) |
| | Unknown | 19 | 0 | 0 | 1 (5.3) |
| | Juvenile | 71 | 2 (2.8) | 1 (1.4) | 0 |
| | Sub-, Adult | 357 | 24 (6.7) | 6 (1.7) | 5 (1.4) |
| | Unknown | 31 | 0 | 0 | 1 (3.2) |
| | Total | 459 | 26 (5.7) | 7 (1.5) | 6 (1.3) |
| Masked palm civet | Female | 79 | 1 (1.3) | 3 (3.8) | 7 (8.9) |
| | Male | 72 | 2 (2.8) | 3 (4.2) | 4 (5.6) |
| | Unknown | 2 | 0 | 0 | 0 |
| | Juvenile | 47 | 1 (2.1) | 3 (6.4) | 5 (10.6) |
| | Sub-, Adult | 104 | 2 (1.9) | 3 (2.9) | 6 (5.8) |
| | Unknown | 2 | 0 | 0 | 0 |
| | Total | 153 | 3 (2.0) | 6 (3.9) | 11 (7.2) |


 

**Table 2.** Serovar and antibiotic resistance of *Salmonella* isolated from raccoons and masked palm civets

| Origin | Species | Subspecies | Serovar | Antibiotic resistance | No. isolates |
|---|---|---|---|---|---|
| Raccoon | *Salmonella enterica* | *enterica* | *S.* Mbandaka | –* | 5 |
| | | | *S.* Infantis | ABPC, NA, OTC | 1 |
| | | | | NA, OTC | 1 |
| | | | | OTC | 1 |
| | | | | – | 1 |
| | | | *S.* Typhimurium | ABPC, NA, OTC | 1 |
| | | | | ABPC, KM, OTC | 1 |
| | | | | – | 2 |
| | | | *S.* Nagoya | – | 2 |
| | | | *S.* Berta | – | 1 |
| | | | *S.* Manhattan | OTC | 1 |
| | | | *S.* Nigeria | OTC | 1 |
| | | | *S.* Rubislaw | – | 1 |
| | | | *S.* Thompson | – | 1 |
| | | *diarizonae* | UT | – | 6 |
| Masked palm civet | *Salmonella enterica* | *enterica* | *S.* Enteritidis | – | 1 |
| | | | *S.* Nagoya | – | 1 |
| | | | 4,12:i:- | – | 1 |

UT, untypable; ABPC, ampicillin; NA, nalidixic acid; OTC, oxytetracycline; KM, kanamycin.
*Susceptible to all antimicrobial agents used in this study.

### *Salmonella*

*Salmonella enterica* was isolated from the faecal samples of 26 of 459 raccoons (5.7%, 95% CI 7.8–3.5%) and three of 153 masked palm civets (2.0%, 95% CI 4.2–0%). Table 2 shows the serovars and antimicrobial resistance patterns of the isolates. Nine and three serovars were identified in the isolates from raccoons and masked palm civets, respectively. Those serovars included common serovars in gastroenteritis patients and domestic animals (e.g. *S.* Infantis, *S.* Typhimurium, *S.* Thompson, and *S.* Enteritidis) (Esaki et al., 2004; Ishihara et al., 2009; National Institute of Infectious Disease, 2009). Seven of 26 isolates (26.9%) from raccoons showed resistance to at least one antimicrobial agent used in this study, whereas all isolates from masked palm civets were susceptible to all of the antimicrobial agents.

### *Yersinia*

*Yersinia* spp. were isolated from the faecal samples of 177 of 459 raccoons (38.6%, 95% CI 43.0–34.1%) and 16 of 153 masked palm civets (10.5%, 95% CI 15.3–5.6%). Among the isolates, seven strains from raccoons and six strains from masked palm civets showed positive reactions in autoagglutination tests and were subsequently identified as *Y. pseudotuberculosis*. Therefore, the prevalence of *Y. pseudotuberculosis* in raccoons and masked palm civets was 1.5% (95% CI 2.6–0.4%) and 3.9% (95% CI 7.0–0.8%), respectively. Four, one, and two *Y. pseudotuberculosis* isolates from raccoons belonged to serotypes 1b, 3, and 4b, respectively. Three, two, and one *Y. pseudotuberculosis* isolates from masked palm civets belonged to serotypes 1b, 3, and 4b, respectively. All *Y. pseudotuberculosis* isolates were susceptible to all of the antimicrobial agents used in this study. In all of the *Y. pseudotuberculosis* isolates, the gene of YPMa was detected and the gene of HPI was not detected by PCR assay.

### *Campylobacter*

*Campylobacter* spp. were isolated from the faecal samples of six of 459 raccoons (1.3%, 95% CI 2.3–0.3%) and 11 of 153 masked palm civets (7.2%, 95% CI 11.3–3.1%). Three isolates from raccoons were identified as *C. jejuni* by multiplex PCR assay. The other *Campylobacter* isolates from raccoons and masked palm civets exhibited three different phenotypic patterns (Table 3). All *the Campylobacter* isolates were susceptible to all of the antimicrobial agents used in this study.

**Table 3.** Biochemical characteristics of *Campylobacter* spp. isolated from raccoons and masked palm civets

| Origin | Species | No. isolates | Oxidase | Catalase | Growth at 25°C | Growth at 42°C |
|---|---|---|---|---|---|---|
| Raccoon | *Campylobacter jejuni* | 3 | + | + | – | + |
| | *Campylobacter* spp. | 2 | + | + | – | – |
| | | 1 | + | + | – | + |
| Masked palm civet | *Campylobacter* spp. | 9 | + | + | – | + |
| | | 1 | + | + | + | + |
| | | 1 | + | + | – | – |

## Discussion

### *Salmonella*

In this study, the prevalence of *S. enterica* in raccoons (5.7%) was almost concordant with the prevalence in Western Pennsylvania, USA (7.4%) (Compton et al., 2008). Investigations in other wild mammals also have revealed similar prevalence rates in UK (6.5%) and Spain (7.2%) (Euden, 1990; Millan et al., 2004). However, Morse et al. (1983) reported that *S. enterica* was isolated from 31.1% of feral raccoons. This finding may suggest that raccoons have the potential to harbor *S. enterica* at a high rate. The prevalence of *S. enterica* in masked palm civets was somewhat lower than that in raccoons. Differences in their behaviour could be responsible for the difference in the prevalence. Further analysis of their food habits and habitat choice may verify this hypothesis.

Some of the isolates were indicated to have originated from human activities, because many of the serovars isolated have been common in human gastroenteritis and in domestic animals, and the isolates from raccoons showed a relatively high resistance rate (26.9%). Interestingly, six strains isolated from raccoons were identified as *S. enterica* subsp. *diarizonae*. Because this *Salmonella* subspecies is not common in warm-blooded animals but is common in cold-blooded animals and the environment, it may be associated with their omnivorous behaviour and proclivity for wet habitats (Zeveloff, 2002; Bopp et al., 2003; Haley et al., 2009).

### *Yersinia*

*Yersinia pseudotuberculosis* isolates belonged to serotypes 1b, 3, and 4b, which are predominant serotypes in human patients and wild animals in Japan (Hamasaki et al., 1989; Fukushima and Gomyoda, 1991; Hayashidani et al., 2002). The prevalence of *Y. pseudotuberculosis* in raccoons and masked palm civets was comparable with that of those studies. It is difficult to compare the prevalence with that in its place of origin, because there are few reports on isolation of pathogenic *Yersinia* from raccoons and masked palm civets (Hacking and Sileo, 1974). All of the *Y. pseudotuberculosis* strains showed the same genotypic pattern with the $YPMa^{+}$ $HPI^{-}$ Far Eastern systemic-pathogenicity type. Fukushima et al. (2001) reported that most of the strains isolated in Far East Asia showed such a pattern and differed from the strains isolated in European countries. In addition to the information regarding geographical origin, this virulence characteristic has a clinical implication, because *ypmA* encodes YPMa, which contributes to the virulence of *Y. pseudotuberculosis* in systemic infection (Carnoy et al., 2000).

These results lead to the conclusion that raccoons and masked palm civets probably have acquired their infections in Japan and play a similar role to other indigenous animals on the ecology of *Y. pseudotuberculosis*. In previous studies, natural reservoirs of *Y. pseudotuberculosis* in Japan have been suggested to be wild mammals and birds, especially rodents and raccoon dogs (*Nyctereutes procyonoides*) (Hamasaki et al., 1989; Fukushima and Gomyoda, 1991; Hayashidani et al., 2002). The major transmission routes of the pathogen in wildlife are suggested as preying upon infected animals or ingesting environmental substances contaminated with *Y. pseudotuberculosis* rather than contact with human activities (Fukushima and Gomyoda, 1991). It is likely that raccoons and masked palm civets acquired their infection, as a result of sharing habitats with other reservoirs.

### *Campylobacter*

The principal reservoirs of *Campylobacter* in the environment are wild mammals and birds (Mörner, 2001), with the occurrence of enteric *Campylobacter* higher in birds than in wild mammals. This tendency is consistent with our results. The prevalence rates in raccoons and masked palm civets were 1.3% and 7.2%, respectively, whereas the rate is often more than 10% in wild birds (Kapperud and Rosef, 1983; Matsusaki et al., 1986; Ito et al., 1988). However, wild mammals, especially species that have direct or indirect contact with human activities, are still important reservoirs of the pathogen. Workman et al. (2005) demonstrated that dogs were one of the most likely sources of human campylobacteriosis. Domestic animals, including dogs, are more likely to have opportunities to acquire infections from excretory substances or from environments contaminated by raccoons and masked palm civets.

Among the isolates, three strains were identified as *C. jejuni*. However, the other 14 isolates from both raccoons and masked palm civets could not be identified by the multiplex PCR assay. Additional biochemical tests suggested that these strains belonged to uncommon species of *Campylobacter* (Table 3). As such species (e.g. *C. hyointestinalis*, *C. larienae* or *C. rectus*) are isolated from healthy animals and enteritis patients, their pathogenicity and epidemiology have not been well known (Vandamme et al., 2005). Because there is little information about the carriage of uncommon *Campylobacter* species in wild mammals, further investigations are required to elucidate the real impact of these species in wild animals on public and animal health.

From these results, we concluded that raccoons and masked palm civets could be potential reservoirs of enteropathogenic *Campylobacter* (*C. jejuni*) and are more


 

likely to possess uncommon *Campylobacter* species. To assess the risk and potential source, wild birds in the same habitat and the environment where the animals live should be investigated.

## Conclusion

In the present study, we investigated the prevalence of three important enteric pathogens, including *Salmonella*, *Yersinia*, and *Campylobacter* spp. in feral raccoons and masked palm civets. These results lead to the conclusion that these animals are potential reservoirs of the pathogens. The characteristics of the isolates showed that these animals probably acquired the pathogens from human activities, other wild animals, and the environment. The presence of human-associated serovars and the antimicrobial resistance of the *Salmonella* isolates revealed the effect of human activities on these animals. This represents a typical spill-over of pathogens from human activities to wildlife (Daszak et al., 2000). Meanwhile, the carrying of pathogens which are usually isolated from wildlife or from the environment (e.g. *S. enterica* subsp. *diarizonae* and *Y. pseudotuberculosis*) indicated that these animals could play an important role in the life cycles of those bacteria in their habitats. These findings are in concordance with their omnivore behaviour and their wide range of habitats from forests to urban areas (Zeveloff, 2002; Abe, 2005).

Our results revealed that raccoons and masked palm civets play an important role on the spreading of human-related pathogens. Moreover, carriage of wildlife- and environment-related pathogens in these animals showed the possibility of the transmission of these pathogens to humans and domestic animals. Raccoons and masked palm civets live near areas of human habitation and often nest in attics or in the feed stores of livestock. The enteric pathogens that we investigated can be transmitted to humans and domestic animals via feces, contaminated water and soil (Fukushima et al., 1988; Humphrey and Bygrave, 1988; Handeland et al., 2002). Thus, not only the ecological threats but also the public and animal health risks presented by these animals should be assessed in detail.

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