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Native wild mammals as *Escherichia albertii* carriers in the Chugoku and Kinki regions of western Japan

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Abstract

Escherichia albertii*, a zoonotic pathogen, is reported to cause frequent food poisoning outbreaks in Japan. Although some studies have identified avian carriers of *E. albertii*, the carrying potential of wild mammals has not been well studied. In this study, we analyzed 147 samples from wild mammals, including 46 raccoon dogs, 32 badgers, 23 deer, 15 martens, 10 wild boars, 9 foxes, 9 masked palm civets, and 3 wild rabbits, which were collected as roadkill in the Chugoku and Kinki regions of western Japan. PCR-based screening revealed *E. albertii* in 26 raccoon dogs (56.5%), 23 badgers (71.9%), 2 deer (8.7%), 8 martens (53.3%), 1 wild boar (10.0%), 3 foxes (33.3%), 5 masked palm civets (55.6%), and 1 wild rabbit (33.3%). A total of 38 strains were isolated, including 15, 10, 6, 3, and 4 strains from 14 raccoon dogs, 8 badgers, 5 martens, 2 foxes, and 4 masked palm civets, respectively. *Escherichia albertii* genotyping assigned 33 of these strains to 15 combinations of O- and H-genotypes (EAOgs:EAHgs), and five O-untypable strains were assigned to three combinations. In terms of phenotype, all strains were assigned to *E. albertii* biogroup 3. Identical or highly similar fragment patterns among the same EAOg:EAHg profiles were observed in *Xba*I-digested genomic DNA during pulsed-field gel electrophoresis, regardless of animal species or sampling prefecture, indicating narrow genetic diversity. Potent novel carriers of *E. albertii* such as raccoon dogs and badgers were identified, and *E. albertii* detection and isolation suggested that some mammals may act as natural reservoirs.

Key Words: carrier, *Escherichia albertii*, reservoir, wild mammal

Introduction

Escherichia albertii is an emerging zoonotic pathogen, first added as a taxon of the genus *Escherichia* in 2003[15]. In Japan, several large-scale food poisoning outbreaks caused by *E. albertii* have been reported[21,27,36,37]. In addition, sporadic infections and small-scale food poisoning events occur every few years[1,5,14,17,19,21,25,33]. *Escherichia albertii* causes fever, abdominal pain, and watery diarrhea in infected humans[21] but is rarely pathogenic to birds[30]. *Escherichia albertii* commonly harbors genes for the virulence factor intimin (*eae*) and for cytolethal distending toxin

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Table 1. Number of samples and isolated *Escherichia albertii* strains, and rate of detection and isolation of *E. albertii*.

Animal species	Raccoon dog	Badger	Deer	Marten	Wild boar	Fox	Masked palm civet	Wild rabbit	Total
Sample	46	32	23	15	10	9	9	3	147
Detection of both *lysP* and *Eacdt* (Positive rate for samples)	26 (56.5%)	23 (71.9%)	2 (8.7%)	8 (53.3%)	1 (10.0%)	3 (33.3%)	5 (55.6%)	1 (33.3%)	69 (46.9%)
E. albertii isolated sample (Isolation rate for positive/total samples)	14 (53.8%/30.4%)	8 (34.8%/25.0%)	0 (0.0%/0.0%)	5 (62.5%/33.3%)	0 (0.0%/0.0%)	2 (66.7%/22.2%)	4 (80.0%/44.4%)	0 (0.0%/0.0%)	33 (47.8%/22.4%)
Isolated *E. albertii* strain	15*[1]	10*[2]	0	6*[1]	0	3*[1]	4	0	38

*[1]Two *E. albertii* strains were isolated from one sample.
*[2]Two *E. albertii* strains were isolated from each of two samples.

(*cdt*)[2,16]; some strains also have Shiga toxin 2 genes such as *stx2a* and *stx2f* (mainly *stx2f*)[7,31]. In Japan, most isolated *E. albertii* strains are assigned to biogroup 3, which is positive for indole production and lysine decarboxylase[26,35]. Because *E. albertii*-related food poisoning often occurs in Japan, identification of the source(s) of this bacterium is urgently needed.

In recent years, advances in the bacteriological characterization of *E. albertii* have allowed for the development of useful isolation, detection, identification, and typing methods. Culture using modified MacConkey agar and deoxycholate-hydrogen sulfide-lactose agar, both supplemented with D-xylose and L-rhamnose, is a widely used isolation technique for *E. albertii*[4,13,26,28]. Several specialized enrichment culture media containing highly selective reagents, including novobiocin, cefixime, and tellurite, have also been used to isolate *E. albertii*, but the preparation of such media is complicated[3,4,28,38]. Moreover, several PCR-based detection and identification methods have been reported, for which the primers developed by Hinenoya et al. and Lindsey et al. are simple and efficient[11,20,24]. Currently, 40 O-genotypes and four H-genotypes can easily be detected using the multiplex PCR-based O- and H-genotyping methods developed by Ooka et al. and Nakae et al., respectively[29,32].

Neither the transmission routes of *E. albertii* nor the food products at risk of contamination have been comprehensively identified. In Japan, confirmed sources of food poisoning outbreaks have included vegetables and water (spring and well water)[21]; therefore, the involvement of wildlife is suspected. Although wild birds have been extensively studied as reservoirs of *E. albertii*[9,10,26,28,30,31], wild mammals have not been well investigated; to date, only a few studies have reported the presence of *E. albertii* in raccoons, bats, monkeys, seals, martens, and foxes[8,9,12,16,28]. The prevalence of *E. albertii* in raccoons, a non-native species in Japan, has received particular interest, and studies using PCR-based detection have shown a high prevalence (> 50%) of *E. albertii* in this species[12]. Therefore, raccoons are thought to be one of the most important natural reservoirs

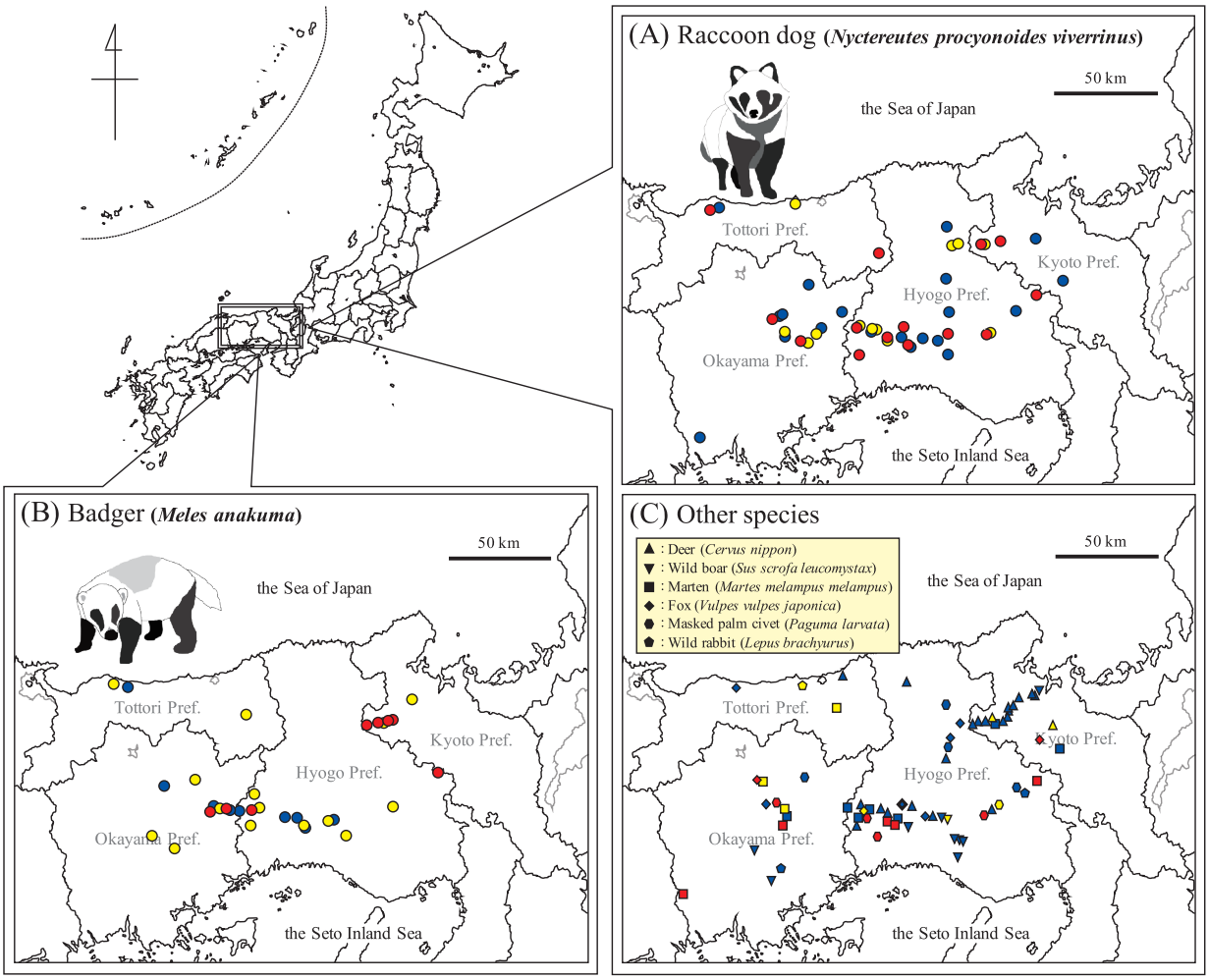


Fig. 1. Map of sampling sites. (A) Raccoon dog (*Nyctereutes procyonoides viverrinus*), (B) badger (*Meles anakuma*), (C) other species. A red mark indicates both isolation of *Escherichia albertii* and detection of its screening genes (both *lysP* and *Eacdt*). A yellow mark indicates detection of the screening genes only. A blue mark indicates only sampling.

of *E. albertii* in Japan. However, although raccoons are widespread in Japan, there are regional differences in their habitat distribution[22]. In Okayama Prefecture, for example, raccoons are not thought to play a significant role in transmitting *E. albertii* because there are a few reports of raccoon sightings, capture, or related crop damage[22,23]. While raccoons are often targeted as carriers of *E. albertii*, these discrepancies regarding distribution range suggest that other mammals are also responsible for *E. albertii* transmission to humans through the environment. Therefore, we hypothesized that some native mammals, including raccoon dogs (*Nyctereutes procyonoides viverrinus*) and badgers (*Meles anakuma*), are also carriers of *E. albertii*.

The aim of this study was to identify novel carriers of *E. albertii* among native wild mammals in the Chugoku and Kinki regions of western Japan in order to broaden our understanding of natural *E. albertii* reservoirs. Furthermore, this investigation was aimed to understand the bacteriological characteristics of their isolates. Our findings from this study will contribute to a better understanding of the natural sources of *E. albertii* contamination and provide more useful bacteriological and epidemiological information in investigations to identify the sources of *E. albertii* infection and prevent future cases of food poisoning.

Table 2. Detected EAOg:EAHg profiles from each animal species and their number of *Escherichia albertii* strains.

Animal species (Total number of strains)	EAOg:EAHg profile	Number of *E. albertii* strains
Raccoon dog (n = 15)	EAOg5:EAHg1	5
	EAOg7:EAHg3	2
	EAOg8:EAHg1	1
	EAOg11:EAHg1	1
	EAOg25:EAHg3	2
	EAOg26:EAHg1	1
	EAOg38:EAHg2	1
	EAOgUT:EAHg2	2
Badger (n = 10)	EAOg1:EAHg4	1
	EAOg5:EAHg1	1
	EAOg10:EAHg4	1
	EAOg18:EAHg3	2
	EAOg19:EAHg4	1
	EAOg21:EAHg1	1
	EAOg30:EAHg3	1
	EAOgUT:EAHg4	2
Marten (n = 6)	EAOg5:EAHg1	1
	EAOg7:EAHg3	1
	EAOg8:EAHg1	1
	EAOg18:EAHg3	1
	EAOg25:EAHg3	1
	EAOg38:EAHg2	1
Fox (n = 3)	EAOg5:EAHg1	1
	EAOg25:EAHg3	1
	EAOg26:EAHg1	1
Masked palm civet (n = 4)	EAOg1:EAHg4	1
	EAOg16:EAHg4	1
	EAOg32:EAHg4	1
	EAOgUT:EAHg3	1

Materials and Methods

Sample collection

Sampling was conducted in the Chugoku (Tottori and Okayama Pref.) and Kinki regions (Hyogo and Kyoto Pref.) from June to November in 2022, and sampling procedures were approved by relevant local administrations. Dead wild mammals that had been hit by moving vehicles were sampled, and a total of 147 samples were collected from 46 raccoon dogs, 32 badgers, 23 deer (*Cervus nippon*), 15 martens (*Martes melampus melampus*), 10 wild boars (*Sus scrofa leucomystax*), 9 foxes (*Vulpes vulpes japonica*), 9 masked palm civets (*Paguma larvata*; treated as a native species in this study), and 3 wild rabbits (*Lepus brachyurus*). Details regarding sampling are provided in Figure 1, Table 1, and Supplementary Table 1. Rectal swabs were taken from these animals, with one sample collected per individual, using Seed Swab γ No. 1 (Eiken Chemical Co., Ltd., Tokyo, Japan). If the carcasses had been badly damaged, the leaked contents from the intestinal tract were swabbed instead of the rectum (Supplementary Table 1). After sampling, dead animals were properly handled according to relevant local road administration management. All samples were chilled in a cooler box and transported to the laboratory, where they were stored at 4 °C and tested within a week. This study was performed using found carcasses only; therefore, no ethical review or approval was required.

PCR-based screening targeting lysP *and* Eacdt

The 147 collected samples were subjected to PCR-based screening for *E. albertii* (Table 1 and Supplementary Table 1). The contents adhering to a swab were suspended in 10 ml of brain heart infusion (BHI) broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), and the suspension was incubated at 37 °C for 18 hr. From these aliquots, template DNA was prepared using the alkaline-boiling method, and PCR assays for *lysP* (*E. albertii*-specific sequences in the *lysP* locus[16]) and *Eacdt* (*E. albertii*-specific *cdt* gene[11]) were conducted (details are provided in the Supplementary Information) using appropriate primers (Supplementary Table 2). The samples in which both genes were detected were considered *E. albertii*-positive and subjected to subsequent isolation.

Isolation and identification of E. albertii

To improve efficiency, biochemical properties that have been reported in the past to be useful for the isolation and identification of *E. albertii*[3,5,10,25,26,28,31,35] were used as selection criteria in this study (Table 3), and a total of 69 samples judged as *E. albertii*-positive from the 147 samples (46.9%) were subjected to selective enrichment culture (Table 1 and Supplementary Table 1). An aliquot (0.5 ml) of the cultured BHI broth was inoculated into 10 ml of Rappaport-

Table 3. Bacteriological characteristics of *Escherichia albertii* strains.

Characteristics tested	38 *E. albertii* strains isolated from wild mammals in this study*[1]	28 *E. albertii* strains isolated from wild birds and mammals in our previous study (28)*[2]	Reference information reported by others (3, 5, 10, 25, 26, 31, 35)*[3]
Virulence genes			
eae	100.0%	100.0%	+
cdt	100.0%*[4]	100.0%	+
stx2f	0.0%	3.6%	v
E. albertii biogroup			
Biogroup 3	100.0%	100.0%	+v
Biochemical properties*[5]			
β-Glucuronidase	0.0%	0.0%	-
Gas from glucose	100.0%	100.0%	+v
Glucose fermentation	100.0%	100.0%	+
H_2S production (TSI)	0.0%	0.0%	-
Lysine decarboxylase	100.0%	100.0%	+v
Motility (37 ℃)	0.0%	0.0%	-
Indole production	100.0%	100.0%	+v
IPA production (SIM)	0.0%	0.0%	N.A.*[6]
Voges-Proskauer	0.0%	0.0%	-
Methyl red (37 ℃)	100.0%	N.T.*[7]	N.A.
Citrate utilization	0.0%	0.0%	-
Acetate utilization	97.4%	78.6%	v
Urease	0.0%	0.0%	-
β-Galactosidase (ONPG)	81.6%	N.T.	+v
Malonate utilization	0.0%	0.0%	-
Arginine dihydrolase	0.0%	0.0%	-v
Ornithine decarboxylase	100.0%	100.0%	+v
Esculin hydrolysis	2.6%	0.0%	N.A.
Acid from carbohydrates			
Lactose	0.0%	0.0%	-v
Sucrose	34.2%	25.0%	v
L-Rhamnose	0.0%	0.0%	-
D-Xylose	0.0%	0.0%	-
D-Melibiose	0.0%	0.0%	-
D-Raffinose	0.0%	0.0%	-
Dulcitol	0.0%	0.0%	-
D-Mannitol	100.0%	100.0%	+
Inositol	0.0%	0.0%	-
D-Sorbitol	15.8%	42.9%	v
D-Cellobiose	0.0%	0.0%	-
D-Trehalose	100.0%	96.4%	+v
L-Arabinose	100.0%	100.0%	+
L-Sorbose	100.0%	89.3%	N.A.
Adonitol	0.0%	0.0%	-
D-Maltose	89.5%	82.1%	+v
Salicin	2.6%	3.6%	-v

*[1] The details are shown in Supplementary Table 6.
*[2] The details are shown in Supplementary Table 4.
*[3] The only results with culture-based methods were used for biochemical properties. Each symbol means as follows: + for positive; - for negative; v for variable; +v for variable but most positive; and -v for variable but most negative.
*[4] Detected as *Eacdt* (*E. albertii*-specific *cdt* gene)
*[5] Gray bands indicate the results in the assays for biochemical properties of the criteria for isolation and identification in this study, and yellow bands indicate those for classification into *E. albertii* biogroup 3.
*[6] No data available
*[7] Not tested

Vassiliadis (RV) broth (Nissui Pharmaceutical Co., Ltd.), and the resulting medium was incubated at 42 ºC for 18 hr. After incubation, a loopful (approximately 10 µl) of cultured medium was streaked both onto a MacConkey agar plate supplemented with lactose, D-raffinose, and salicin (LRS-MAC agar; details are provided in the Supplementary Information) and onto a CHROMagar™ E.coli (CHROMagar, Paris, France) plate, and the media were incubated at 37 ºC for 20–24 hr. A maximum of 10 colorless colonies on an LRS-MAC agar plate and 10 white colonies on a CHROMagar™ E.coli plate were isolated and subsequently inoculated onto a MacConkey agar plate supplemented with D-xylose and L-rhamnose (XR-MAC agar; details are provided in the Supplementary Information) linearly. The colorless lines were then examined for their biochemical properties using conventional methods with triple sugar iron (TSI) agar, lysine indole motility (LIM) medium, Simmon's citrate (SC) agar, Ewing modified malonate (EM) broth, and Voges-Proskauer (VP) medium (sources of these media are provided in the Supplementary Information). After incubation at 37 ºC for 24 hr, the putative isolates, which yielded negative results for H_2S production (TSI agar), motility (LIM medium), utilization of citrate (SC agar) and malonate (EM broth), and in the VP test (VP medium) and positive results for gas from glucose and glucose fermentation (both TSI agar), were confirmed to be *E. albertii* using triplex PCR targeting *clpX* (sequences common in the *clpX* locus of *E. albertii*, *E. coli*, and *Shigella* as positive controls[16,30]), *lysP*, and *mdh* (*E. albertii*-specific sequences in the *mdh* locus[16]) and additional PCR targeting *Eacdt* (details are provided in the Supplementary Information) using appropriate primers (Supplementary Table 2). The isolates in which all genes were detected were identified as *E. albertii*. If *E. albertii* was not isolated successfully via the selective enrichment culture using RV broth, isolation from the positive samples was attempted again directly from the cultured BHI broths within a week of storage at 4 ºC.

Selection of representative* E. albertii *strains and genetic characterization

According to the multiplex PCR-based O-genotyping method developed by Ooka et al.[32], *E. albertii* O-genotypes (EAOgs) of 143 isolates (data not shown), which were successfully isolated from 33 of the 69 positive samples identified in *Isolation and identification of* E. albertii (47.8%), were determined to select representative strains in this study (Table 1 and Supplementary Table 1). For each sample, one isolate representing the EAOg of the sample was selected as a representative strain; if multiple EAOgs were detected in one sample, one of them was selected from each genotype. If no matching EAOgs were obtained, one isolate exhibiting a single set of biochemical properties (in the isolation step) was selected as a representative EAOg-untypable (EAOgUT) strain for each sample. As a result, a total of 38 representative strains were selected (Table 1). Next, *E. albertii* H-genotypes (EAHgs) of the 38 strains were determined according to the PCR method developed by Nakae et al.[29] In addition, the strains were confirmed to harbor *eae*, *stx1* (*stx1a*, *stx1c*, and *stx1d*), and *stx2* (*stx2a*–*stx2g*) using appropriate primers[18,34] (Supplementary Table 2). The fragment patterns of *Xba*I-digested genomic DNA of the 38 strains were confirmed using pulsed-field gel electrophoresis (PFGE; details are provided in the Supplementary Information). The *Xba*I-digested genomic DNA of *Salmonella* Braenderup H9812 was used as a size marker. Images were visualized using a Dual-Intensity Transilluminator (Analytik Jena GmbH+Co. KG, Jena, Germany) after staining with ethidium bromide solution and were post-adjusted for sharpness, contrast, and background color. Unnecessary patterns in the pictures were removed, and the remaining parts were moved horizontally. Each picture that was processed in the same manner was arranged using the marker strains as a standard.

Biochemical characterization

The biochemical properties of the 38 strains selected above were determined using culture-based methods. Commercially available media were used (sources of these media are provided

in the Supplementary Information) in addition to the following homemade media: acetate (AT) agar, Christensen's urea (CU) agar, esculin (EL) agar, Moeller's decarboxylase (MD) broth, and purple agar for carbohydrate fermentation (PC agar) (Supplementary Table 3). After incubation at 37 ºC, the following biochemical assays were performed: β-glucuronidase (24 hr, cellobiose lactose indole β-D-glucuronidase [CLIG] agar); gas from glucose and glucose fermentation (both 24 hr, TSI agar); motility (24 hr, LIM and sulfide indole motility [SIM] media); production of H_2S (TSI agar), indole (LIM and SIM media), and indole pyruvic acid (IPA) (SIM medium) (all 24 hr); VP test (24 hr, VP medium); methyl red (MR) test (48 hr, VP-MR broth); utilization of citrate (SC agar) and acetate (AT agar) (both 96 hr, not at the same time as the isolation step with SC agar); utilization of malonate (48 hr, not at the same time as the isolation step, EM broth); urease (96 hr, CU agar); β-galactosidase (24 hr, o-nitrophenyl-β-D-galactopyranoside [ONPG] disc); lysine decarboxylase (24 hr, LIM medium); arginine dihydrolase and ornithine decarboxylase (both 48 hr, MD broth); esculin hydrolysis (48 hr, EL agar); and acid production from lactose, sucrose, L-rhamnose, D-xylose, D-melibiose, D-raffinose, dulcitol, D-mannitol, inositol, D-sorbitol, D-cellobiose, D-trehalose, L-arabinose, L-sorbose, adonitol, D-maltose, and salicin (all 48 hr, PC agar). Finally, all the strains were assigned to *E. albertii* biogroups based on the results of lysine decarboxylase and indole production assays[26].

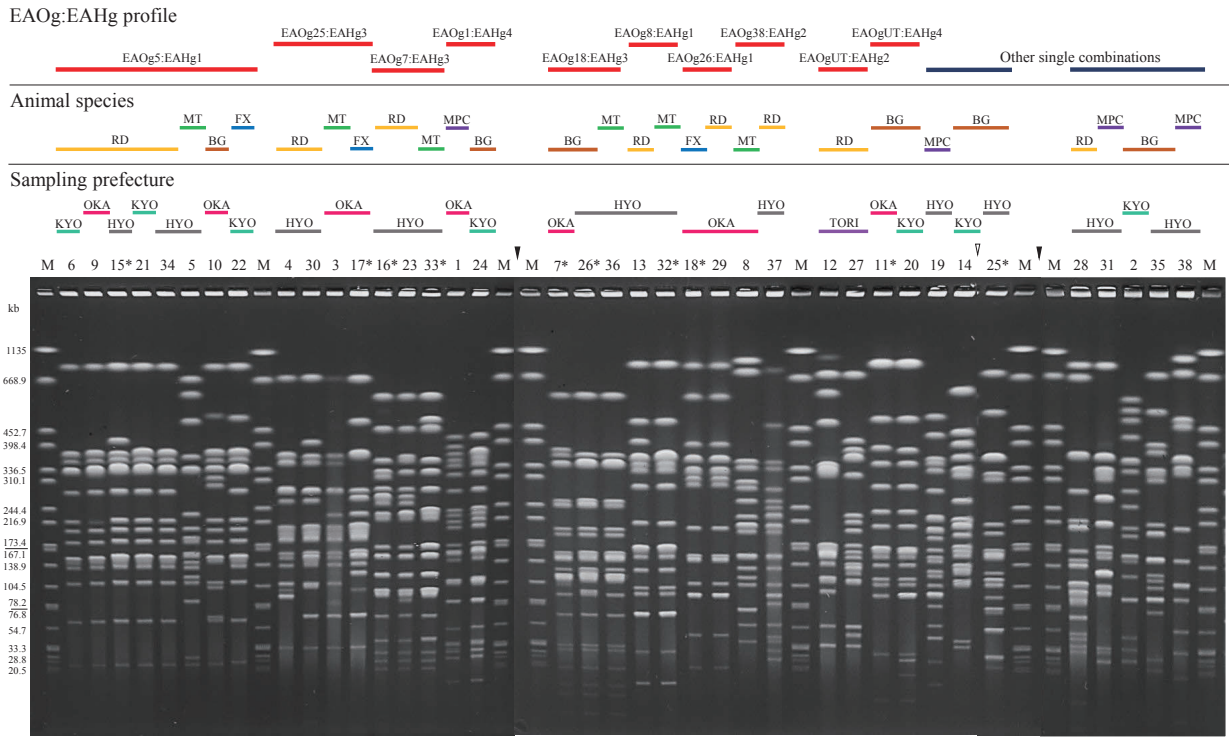
Data from previously isolated strains

In this study, data regarding 32 bacterial strains previously isolated from wild animals and identified as *E. albertii*[28] were used to compare their phenotypic and genotypic features with those of the present strains. Detailed information on the past strains is provided in Supplementary Tables 4 and 5. The data from 28 out of the 32 previously isolated strains were used to compare the bacteriological characteristics (Table 3 and Supplementary Table 4). The data from eight out of the 32 past strains were used to compare the PFGE fragment patterns (Fig. 3 and Supplementary Table 5). With the exception of one strain isolated from a masked palm civet sampled in Hyogo Pref. in 2022, all the strains (name: EAB/EAW/EAD) were isolated from samples collected in Hyogo, Okayama, and Tottori Pref. in 2018–2020[28] and identified as *E. albertii* by several laboratories using different methods. Almost all the data, including the results of PFGE, were taken again using methods similar to those in the present study in 2019 and 2020. However, the previous strains were not subjected to O- and/or H-genotyping, nor to the MR test or β-galactosidase assays, so these data were not available for comparison.

Results

Escherichia albertii *strains isolated from wild mammals*

The results regarding the detection and isolation of *E. albertii* are shown in Table 1 and Supplementary Table 1. According to the results of the PCR-based screening used in this study, both *lysP* and *Eacdt* were detected in 26 raccoon dogs (56.5%), 23 badgers (71.9%), 2 deer (8.7%), 8 martens (53.3%), 1 wild boar (10.0%), 3 foxes (33.3%), 5 masked palm civets (55.6%), and 1 wild rabbit (33.3%), and these samples were considered *E. albertii*-positive. Among these 69 positive samples, *E. albertii* was successfully isolated from 14 raccoon dogs (53.8%), 8 badgers (34.8%), 5 martens (62.5%), 2 foxes (66.7%), and 4 masked palm civets (80.0%) but not from the deer, wild boar, or wild rabbit. The isolation rate for total samples was 30.4%, 25.0%, 33.3%, 22.2%, and 44.4% for raccoon dogs, badgers, martens, foxes, and masked palm civets, respectively. Then, a total of 143 isolates (data not shown) were collected from 33 samples. During representative strain selection, two strains were selected from one sample for raccoon dogs, martens, and foxes, and two strains were selected from each of two samples for badgers, resulting in a total of 38 strains. Of these strains, 15, 10, 6, 3, and 4 were found in raccoon dogs, badgers, martens, foxes, and masked palm civets, respectively.



*Strains 7 and 11, 15 and 16, 17 and 18, 25 and 26, and 32 and 33 were isolated from one sample each.

Fig. 2. Pulsed-field gel electrophoresis fragment patterns of *Xba*I-digested genomic DNA of *Escherichia albertii* strains isolated in this study. For EAOg:EAHg profile, a red bar indicates the same combination, while a blue bar indicates other single combinations. For animal species, RD indicates raccoon dog; MT, marten; BG, badger; FX, fox; and MPC, masked palm civet. For sampling prefecture, KYO indicates Kyoto Pref.; OKA, Okayama Pref.; HYO, Hyogo Pref.; and TORI, Tottori Pref. The number on each lane indicates the strain number. M on a lane indicates the marker strain, *Salmonella* Braenderup H9812. A black triangle indicates a seam of pictures. A white triangle indicates where an unnecessary strain was cut out.

Bacteriological characterization of E. albertii *strains*

Among the 38 strains, 33 were associated with 15 distinct EAOg:EAHg profiles, and the details are provided in Table 2 and Supplementary Table 6. Specifically, eight strains were identified as EAOg5:EAHg1; four as EAOg25:EAHg3; three each as EAOg7:EAHg3 and EAOg18:EAHg3; two each as EAOg1:EAHg4, EAOg8:EAHg1, EAOg26:EAHg1, and EAOg38:EAHg2; and one each as EAOg10:EAHg4, EAOg11:EAHg1, EAOg16:EAHg4, EAOg19:EAHg4, EAOg21:EAHg1, EAOg30:EAHg3, and EAOg32:EAHg4. The remaining five strains were assigned to three types of EAOgUT combinations, including two for EAOgUT:EAHg2, two for EAOgUT:EAHg4, and one for EAOgUT:EAHg3. For raccoon dogs, the 15 identified strains were distributed as follows: five for EAOg5:EAHg1; two each for EAOg7:EAHg3, EAOg25:EAHg3, and EAOgUT:EAHg2; and one each for EAOg8:EAHg1, EAOg11:EAHg1, EAOg26:EAHg1, and EAOg38:EAHg2. For badgers, the 10 identified strains included two instances each of EAOg18:EAHg3 and EAOgUT:EAHg4, plus one each for EAOg1:EAHg4, EAOg5:EAHg1, EAOg10:EAHg4, EAOg19:EAHg4, EAOg21:EAHg1, and EAOg30:EAHg3. For martens, the six identified strains were assigned to EAOg5:EAHg1, EAOg7:EAHg3, EAOg8:EAHg1, EAOg18:EAHg3, EAOg25:EAHg3, and EAOg38:EAHg2. For foxes, the three identified strains were assigned to EAOg5:EAHg1, EAOg25:EAHg3, and EAOg26:EAHg1. For masked palm civets, the four identified strains were assigned to EAOg1:EAHg4, EAOg16:EAHg4, EAOg32:EAHg4, and EAOgUT:EAHg3.

As for bacteriological characteristics (Table

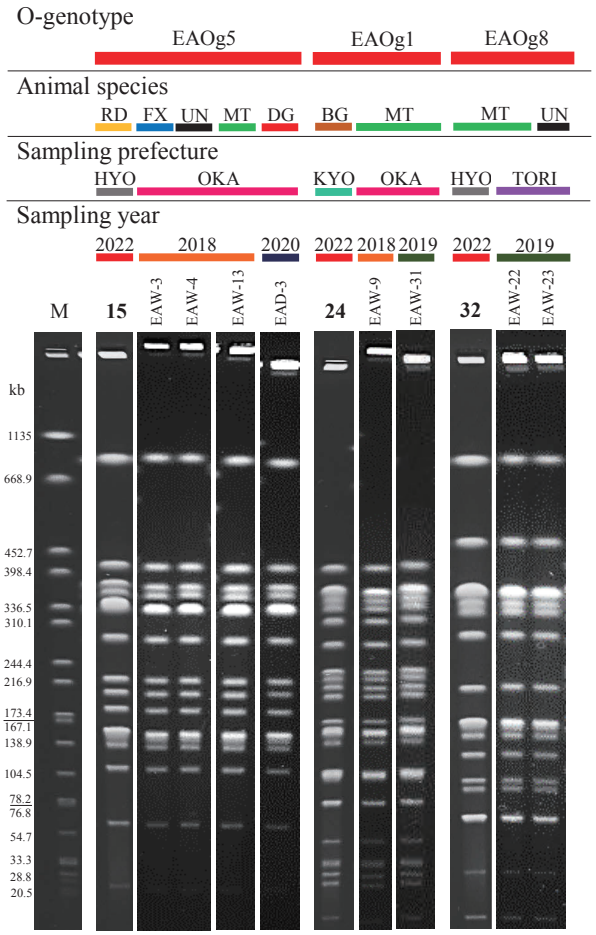


Fig. 3. Comparison of pulsed-field gel electrophoresis fragment patterns of *Xba*I-digested genomic DNA of *Escherichia albertii* strains isolated in this study and those isolated in our previous study. For animal species, RD indicates raccoon dog; FX, fox; UN, wild mammal, species unknown; MT, marten; DG, rescued wild dog; and BG, badger. For sampling prefecture, HYO indicates Hyogo Pref.; OKA, Okayama Pref.; KYO, Kyoto Pref.; and TORI, Tottori Pref. The number on each lane indicates the strain in this study, and EAW/EAD indicates the strain in our previous study (28) (details are shown in Supplementary Table 5). M on a lane indicates the marker strain, *Salmonella* Braenderup H9812.

3 and Supplementary Tables 4 and 6), all the 38 strains harbored both *eae* and *cdt*, as did the strains from our past study[28]. However, *stx2* was not confirmed in the present strains. All the current strains were assigned to *E. albertii* biogroup 3, similar to the previous strains. The present strains yielded negative results in the assays for IPA production, urease, arginine dihydrolase, and acid production from D-melibiose, dulcitol, inositol, D-cellobiose, and adonitol. These results were consistent with those seen in our previous strains. Regarding positive assay results, the present strains were similar to the past strains for ornithine decarboxylase and acid production from D-mannitol and L-arabinose, but not for acid production from D-trehalose or L-sorbose. The results, including those for utilization of acetate and acid production from sucrose, D-sorbitol, and D-maltose, were various and so were the results of the previous strains. In particular, the D-sorbitol degradation was reduced by less than half in the current strain (15.8%) compared with the previous strains (42.9%; 9 of 15 wild-bird-derived isolates [60.0%] and three of 13 mammalian isolates [23.1%] were positive). Notably, one strain was positive for esculin hydrolysis, although all our past strains were negative for this property.

Genetic diversity of E. albertii *strains in* Xba*I-digested genomic DNA*

The PFGE fragment patterns of *Xba*I-digested genomic DNA of the 38 strains are shown in Figure 2. Among those with the EAOg5:EAHg1 profile, strains 6, 21, and 34 showed an identical pattern, whereas strains 5, 9, 10, 15, and 22 showed different patterns. Strains 9, 15, and 22 exhibited only one-band differences from strains 6, 21, and 34. The three strains with the identical pattern (strains 6, 21, and 34) were all isolated from raccoon dogs; however, their sampling prefectures were different, as strains 6 and 21 were collected from Kyoto Pref. and strain 34 was collected from Hyogo Pref. The strains with one-band differences (strains 9, 15, and 22) did not have exhibit any fixed relationship between animal species and sampling location either. Strains 9, 15, and 22 were isolated from a raccoon dog sampled in Okayama Pref., a raccoon dog in Hyogo Pref., and a fox in Kyoto Pref., respectively. For the EAOg26:EAHg1 profile, strains 18 and 29, which also showed an identical pattern, were isolated from different animal species, a fox and a raccoon dog, but were both sampled in Okayama Pref. Slight differences were confirmed between strains 13 and 32 (EAOg8:EAHg1), strains 16 and 23 (EAOg7:EAHg3), strains 1 and 24 (EAOg1:EAHg4), strains 7, 26, and

36 (EAOg18:EAHg3), and strains 11 and 20 (EAOgUT:EAHg4). Although strains 16 and 23 (EAOg7:EAHg3) were both from raccoon dogs in Hyogo Pref., the other strains did not show consistency between species and location. Various patterns were confirmed in the single EAOg:EAHg profiles with only one representative strain each (strains 2, 14, 19, 25, 28, 31, 35, and 38).

Some strains isolated in this study exhibited PFGE fragment patterns of *Xba*I-digested genomic DNA identical to those of previously isolated strains (EAHgs were not typed; details are provided in Supplementary Table 5) from samples that had been collected in Okayama and Tottori Pref. in 2018–2020[28] (Fig. 3). Strain 15 (raccoon dog, Hyogo Pref., 2022) had the same EAOg5 PFGE fragment pattern as strains EAW-3 (fox, Okayama Pref., 2018), EAW-4 (wild mammal, species unknown, Okayama Pref., 2018), EAW-13 (marten, Okayama Pref., 2018), and EAD-3 (rescued wild dog, Okayama Pref., 2020) from the previous dataset. Strain 24 (badger, Kyoto Pref., 2022) had the same EAOg1 PFGE fragment pattern as strains EAW-9 (marten, Okayama Pref., 2018) and EAW-31 (marten, Okayama Pref., 2019). Strain 32 (marten, Hyogo Pref. 2022) had the same EAOg8 PFGE fragment pattern as strains EAW-22 (marten, Tottori Pref., 2019) and EAW-23 (wild mammal, species unknown, Tottori Pref., 2019).

Discussion

In Japan, research on *E. albertii* reservoirs in wild mammals has only recently become of interest. The lack of studies on wild mammals is due to the difficulty in obtaining samples from these animals, as only the species targeted for feeding, hunting, and extermination are commonly available for sampling. In a past study, we carried out experiments using fecal samples from Japanese wild mammals but found that identifying carriers of *E. albertii* was cumbersome, and it was difficult to understand the prevalence of *E. albertii* in each animal[28]. Therefore, we conducted the present study of native wild mammals as *E. albertii* carriers by obtaining samples directly from dead wild mammals that had been hit by moving vehicles in the Chugoku and Kinki regions of western Japan.

This is the first report to focus on the potential role of Japanese native wild mammals in *E. albertii* transmission. Notably, more than 50% of raccoon dogs and badgers harbored both *lysP* and *Eacdt* and yielded successful *E. albertii* isolations, suggesting that these animals represent potent *E. albertii* carriers. This result is particularly reliable due to the considerable sample sizes obtained for both animals. The detection rate of *E. albertii* from both raccoon dogs and badgers in the PCR-based screening was comparable to that of raccoons in the previous report[12]. Notably, although we could not sufficiently isolate *E. albertii* from badgers due to interference by other bacteria, our obtained *E. albertii*-positive rate of 71.9% is the highest value reported for any wild mammal to date. Considering this, badgers were the most potent *E. albertii* carriers among the species examined in this study. In terms of bacteriological profiles, EAOg5:EAHg1 was more commonly distributed in raccoon dogs than other mammals, suggesting that raccoon dogs are a major reservoir of this genotype; no specific distribution of EAOg:EAHg profiles was observed in badgers. Raccoon dogs, badgers, and their subspecies have been widely distributed in Japan since ancient times, and their habitat distribution is more even than that of raccoons[6,22]. In addition, our results confirmed the widespread presence of *E. albertii* in these animals across sampling prefectures. Thus, raccoon dogs and badgers may contribute more than raccoons to the transmission of *E. albertii* to humans via the environment throughout Japan.

Apart from highlighting the carrying potential of raccoon dogs and badgers, this study also provides a useful corroboration of animal species previously proposed as reservoirs and indicates the possible prevalence of *E. albertii* among other unreported animals. We previously revealed martens and foxes as carriers of *E. albertii* by investigating their feces[28], and these results were confirmed in the present study, as we successfully detected and isolated *E. albertii* from these animals. In the past, it was not possible to

determine the positive rate of *E. albertii* in each animal, but the fact that over 30% of martens and foxes in the present study were *E. albertii*-positive suggests that these species may be potent *E. albertii* carriers. For wild rabbits, only one sample could be collected, and only one strain was isolated in the previous study[28]. While no strains could be isolated in the present study, the *E. albertii*-positive result supports that wild rabbits may harbor *E. albertii*. Moreover, we also confirmed the presence of *E. albertii* in masked palm civets with a detection rate of 55.6%, and the relative isolation success among positive and total samples for this species was high at 80.0% and 44.4%, respectively, suggesting that this species may be a potential carrier of *E. albertii*. However, as the results were from a small number of samples, they need to be corroborated by further research. Although the detection rate of *E. albertii* was low and its isolation was not successful from deer and wild boars, these animals may still act as carriers, and further studies with additional samples are needed to comprehensively investigate the role of these animals in the transmission of *E. albertii*.

The comparison of PFGE fragment patterns in *Xba*I-digested genomic DNA is an established genotyping method for *E. albertii*. In this study, isolates associated with the same EAOg:EAHg profiles were found to represent clones or highly similar strains, as identical fragment patterns or those with only slight differences were observed in the *Xba*I-digested genomic DNA. Interestingly, this trend was confirmed regardless of animal species or sampling prefecture, indicating narrow genetic diversity among the *Xba*I-digested genomic DNA of *E. albertii* in wild mammals. In addition, the fact that the same fragmentation pattern as that of the present strains has been observed in previous isolated strains[28] suggests that these clones may be highly conserved in nature. This narrow genetic diversity in *Xba*I-digested genomic DNA in wild mammals may be useful for tracing specific strains in cases of food poisoning outbreaks and other public health risks.

The various bacteriological characteristics of *E. albertii* isolates are not yet fully understood. All isolates obtained in this study were classified as biogroup 3, as in our previous study[28], confirming that the wild mammal isolates may be predominantly of that phenotype. In the past study, one of the most frequently detected EAOgs in mammalian strains, including those from martens and foxes, was EAOg5[28], and similar results were obtained in this study. This suggests that EAOg5 might be the major genotype seen in native wild mammals. The results of the present study disagreed somewhat with those of previous studies in that no strains positive for *stx2f* were identified, despite such strains being isolated from wild mammals in the past[12,28]. The relatively small sample size used in this study was likely responsible for this discrepancy. Regarding biochemical properties, D-sorbitol degradation and esculin hydrolysis were observed as notable differences between the current and previous strains[28]. Focusing on the mammalian isolates, D-sorbitol degradability of present (15.8%) and previous (23.1%) strains is markedly lower than that in wild-bird-derived isolates (60.0%) suggesting that this property may be characteristic of mammalian isolates. Notably, the existence of esculin-degrading strains, which has not been explained in the previous study[28], indicated the presence of strains with unreported biochemical properties. Finally, it is possible that the biochemical features used as criteria for the isolation and identification in this study could have prevented the collection of strains that exhibit different characteristics. This is a challenge for all studies involving the isolation and identification of *E. albertii* strains and requires investigation using a variety of methods for their comprehensive characterization.

Standardized methods for the selection and isolation of *E. albertii* are still under development. RV broth, which is available commercially and easy to prepare, was used for selective enrichment culture in this study for the first time and was effective in the selection of *E. albertii* from samples with an abundance of non-target bacteria; however, the mechanism of selection is unknown. Conversely, depending on the degree of contamination of the cultured BHI broth, this selective enrichment sometimes worked in an inhibitory manner, which means that it may

not be a universal method and requires more experimentation. Moreover, this was the first study using salicin in the isolation of *E. albertii*. The addition of this carbohydrate to the LRS-MAC agar was found to have the secondary effect of facilitating the separation of *E. albertii* from non-target bacteria and not to affect the colony color (colorless) of salicin-degrading *E. albertii* strains due to its slow degradation, indicating that it may be useful. Further investigation is needed to elucidate the significance of these observations regarding selective enrichment and isolation media.

In conclusion, in this study, several *E. albertii* strains were successfully isolated from dead wild mammals that had been hit by moving vehicles in the Chugoku and Kinki regions of western Japan. To the best of our knowledge, this study is the first to suggest the role of raccoon dogs, badgers, and other mammals as carriers of *E. albertii*, and our results show that several Japanese wild mammals, not exclusively raccoons, as previously suspected, may act as potent natural reservoirs of *E. albertii*. However, because the number of samples was limited due to the sampling technique employed, further studies are required to identify which wild mammals act as carriers of *E. albertii* in Japan. This study highlights the role of various wild mammals as carriers of *E. albertii*, providing a comprehensive basis for future research on this bacterium. The findings presented herein will help public health officials to identify the potential sources of *E. albertii* infection and take action to mitigate risks, thereby reducing the frequency of food poisoning outbreaks.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Acknowledgments

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