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Isolation and characterization of *Escherichia albertii* from wild and safeguarded animals in Okayama Prefecture and its prefectural borders, Japan

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ABSTRACT. *Escherichia albertii* has recently been recognized as a zoonotic enteropathogen associated with food poisoning. The reservoirs and transmission routes of this bacterium to humans are still unclear. In this study, we performed a survey of *E. albertii* in fecal specimens of wild and safeguarded animals in Okayama Prefecture and its prefectural borders, Japan to understand its reservoir in the environment. Forty-two *E. albertii* were isolated from 10 and 31 droppings of 59 crows and 125 starlings, respectively. Fifty-two *E. albertii* were isolated from 906 mammal droppings, and out of 52 isolates, origin of 33, 6 and 1 isolates were from martens, foxes, and rabbit, respectively, however, origin of 12 isolates remained unknown. Three *E. albertii* were isolated from two and one feces of 159 dogs and 76 cats, respectively. Pulsed-filed gel electrophoresis analysis grouped 97 *E. albertii* strains into 66 pulsotypes including 36 and 30 pulsotypes of isolates from mammals and birds, respectively. *E. albertii* strains isolated in this study were genetically diverse. Although clonal relationship was not observed between mammal and bird isolates, there were intra- and inter-species relationship in mammalian isolates. All *E. albertii* strains were positive for *eae* and *Eacdt* virulence genes. Furthermore, 20 and 7 strains also carried *Eccdt-I* and *stx2f* genes, respectively. Taken together, the results indicate that genetically diverse and potentially virulent *E. albertii* are distributed among various wild and safeguarded animals in Okayama Prefecture, and the animals could also be reservoirs of *E. albertii*.

KEYWORDS: *Escherichia albertii*, fox, marten, starling, wildlife

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Escherichia albertii is an emerging zoonotic foodborne pathogen causing watery diarrhea, abdominal pain, vomiting and fever [1, 19, 22, 24]. This pathogen has accounted for several outbreaks of food poisoning in humans and epidemic mortal diseases in birds [1, 19, 22, 24]. *E. albertii* is a Gram-negative, facultative anaerobic bacillus and the last member of the genus *Escherichia*. This bacterium has often been misidentified as other bacteria including *Hafnia alvei* [16], *Shigella boydii* [17], enteropathogenic *Escherichia coli* (EPEC), enterohemorrhagic *E. coli* (EHEC) [9, 12, 25] and cytolethal distending toxin (CDT)-II producing *E. coli* [10, 13] since similar biochemical features and the locus of enterocyte effacement (LEE) pathogenicity island encoding type three secretion system were observed in these bacteria. *E. albertii* express CDTs encoded by *Eacdt* (commonly detected) and *Eccdt-I* (less frequently detected) genes, which could be associated with virulence and persistent colonization [28]. Some strains also produce Shiga toxin 2 (Stx2a or Stx2f) [2, 9, 10, 20, 25], which is a primary virulence factor of EHEC including serotype O157:H7.

Although clinical importance of *E. albertii* has increasingly been recognized [10, 16, 24, 25], the epidemiological information, particularly about its prevalence in animals, foods and environment, are still insufficient to know the reservoirs and transmission routes of this pathogen to humans. Among animals used as food, *E. albertii* was found in poultries [5, 11], and was identified in poultry meats and its innards more frequently than other meats (pork, mutton and beef) [29]. Thus, poultries have been considered as one of the important reservoirs of *E. albertii* but not other livestock and their meat products particularly chickens could be the source

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of human infections. On the other hand, *E. albertii* food poisoning outbreaks in Japan might be due to contaminated water (spring and well waters) or vegetables, but not meats [19]. This observation strongly suggest that other warm-blooded animals may play a role as reservoirs of *E. albertii* in the environment since bacteria belonging to the genus *Escherichia* normally reside in intestine of warm-blooded animals. Our recent study unveiled that *E. albertii* is notably prevalent (57.7%) in Japanese raccoons [8]. Since they have omnivorous feeding habits and prefer riparian areas, they might contaminate vegetables and environmental water. Furthermore, *E. albertii* has also been identified in other wild and safeguarded animals such as bat, cat, dog, monkey, penguin, seal, and various birds (bulbul, cormorant, duck, fantail, finch, gyrfalcon, honeyeater, magpie, pigeon, redpoll, short-tailed shearwater, siskin, sparrow, starling, swallow, teal, wagtail, woodpecker, wren) [3, 5, 6, 15, 17, 21–23, 25]. However, the distribution as well as bacteriological characteristics of *E. albertii* in wild and safeguarded animals is still not well understood.

This study was aimed to further understand the distribution of *E. albertii* in wild and safeguarded animals and their bacteriological properties including genomic diversity and virulence potential to human. Therefore, we have extensively surveyed fecal droppings of wild mammals and birds, and feces of safeguarded dogs and cats in the environment, respectively, for *E. albertii* in Okayama Prefecture and its prefectural borders, Japan. *E. albertii* strains were isolated from fecal specimens of the animals, and bacteriological properties of isolated *E. albertii* strains were further examined.

MATERIALS AND METHODS

Sampling of wildlife fecal specimens

Fecal droppings were collected from wild mammals (n=906) and birds (n=184) in Okayama Prefecture and its prefectural borders between 2018 and 2019. Sampling information is shown in Fig. 1 and Table 1. For mammal fecal samples, apparently fresh droppings were collected at 11 sites (a–i, k, l) located mainly in the riparian areas of the mountains and placed into plastic bags. Animal species of the feces were deduced from fecal aspects including sizes, shapes, contents, odors, and places collected. Additionally, fecal samples

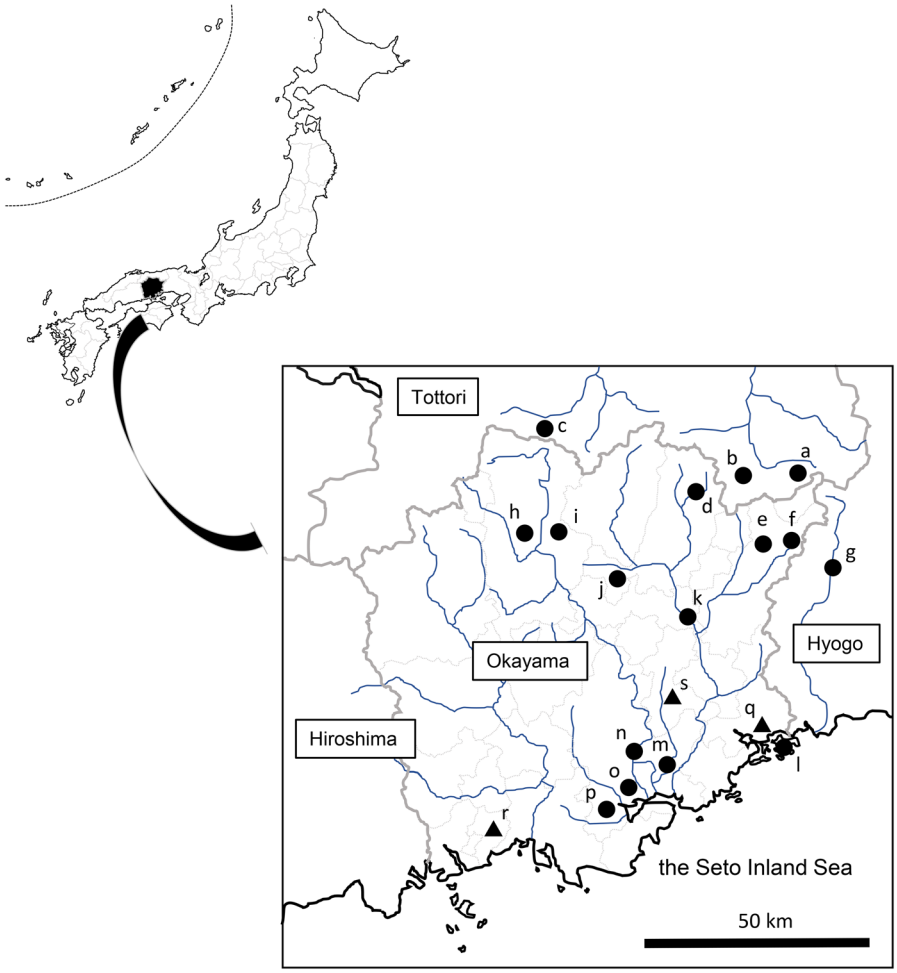


Fig. 1. Map of sampling sites in and border of Okayama Prefecture. Places (16 sites, a to p) where fecal samples were collected from wild and safeguarded animals are indicated by filled circle (●). Filled triangle (▲) indicates the locations (q to s) where *Escherichia albertii*-positive dogs and cat were safeguarded. Rivers, prefectural borders and coastal line are shown by blue, gray and black lines, respectively, in the enlarged view.

Table 1. Details about sampling sites, number of wild animal feces and their species, and number of *Escherichia albertii* isolates

Animals	Species	No. of specimens tested (No. of specimens from which *E. albertii* was isolated)																	No. of isolates
		Total	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	
Mammals (n=1,141)	Weasel/Marten*[1]	726 (39)	55 (5)	72 (2*[2])	15 (1)	183 (16*[6])	28 (1)	20 (0)	55 (0)	53 (6)	2 (1)	-	238 (7)	5 (0)	-	-	-	-	40
	Fox*[1]	28 (4)	1 (0)	-	-	12 (0)	-	4 (1)	1 (0)	1 (0)	-	-	9 (3)	-	-	-	-	-	4
	Unknown*[1]	152 (8)	6 (0)	47 (2*[3])	-	38 (1*[4])	-	1 (0)	7 (1)	2 (0)	-	-	51 (4*[5])	-	-	-	-	-	8
	Dog	159 (2)	Not applicable																2
	Cat	76 (1)	Not applicable																1
Birds (n=184)	Crow	59 (10)	-	-	-	-	-	-	-	-	-	3 (3)	-	6 (1)	13 (0)	27 (6)	-	10 (0)	10
	Starling	125 (31)	-	-	-	-	-	-	-	-	-	-	-	-	10 (2)	5 (1)	109 (28*[6])	1 (0)	32

*[1]Animal species were deduced from fecal aspects when feces were collected. *[2]One strain was confirmed to be derived from the dropping of rabbit but not weasel and marten. *[3]One strain was confirmed to be derived from the dropping of marten. *[4]The strain was confirmed to be derived from marten. *[5]Three and one strains were confirmed to be derived from fox, and marten, respectively. *[6]Two strains were isolated from one specimen.

were also collected from dogs (n=159) and cats (n=76), which were safeguarded in the environment by Okayama Prefectural Animal Welfare Center. For bird samples, fresh feces were collected from crows (*Corvus corone* and *C. macrorhynchos*) and starling (*Sturnus cineraceus*) on the ground while they were perching on trees or electric wires at the 6 sites (j, l–p). Plastic sheets were placed under the trees and electric wires, and fresh feces were collected as soon as they dropped on the sheets as described above. All the samples were kept in cool bags, transported to the laboratory, and kept at 4°C in a refrigerator. They were processed within a week for the detection and isolation of *E. albertii*. The fecal specimens collected in 2018 were kept at 4°C after processing for the *E. albertii* screening, and moved to −20°C in 2019, while the specimens collected in 2019 was directly put into freezer at −20°C after the screening.

Isolation and identification of E. albertii

Culture media used in this study, except buffered peptone water (BPW; Thermo Fisher Scientific, Waltham, MA, USA), were prepared in the laboratory using individual medium components and details are given in Supplementary Table 1.

Fecal samples collected from birds (n=52) in the year 2018 were directly streaked onto modified deoxycholate-hydrogen sulfide-lactose (mDHL) agar plates and the plates were incubated at 37°C for 18 hr. Bird samples (n=132) collected in 2019 and all non-bird sample aliquots (0.05 to 0.1 g) were suspended in 10 mL of BPW supplemented with two different concentrations of cefixime trihydrate (0.06 mg or 0.28 mg/L), and the media were incubated at 37°C for 18 hr. One milliliter of the enrichment culture was inoculated into 10 mL of modified *E. coli* (mEC) broth, and the culture was incubated at 42°C for 18 hr. One loopful culture was streaked onto mDHL agar plates and the plates were incubated at 37°C for 18 hr.

Maximum 5 colorless colonies from each specimen were isolated and subsequently examined for their biochemical properties by conventional methods [4] using TSI (triple sugar iron) agar, LIM (lysine indole motility) medium, Simmon's citrate agar, malonate broth, purple agar containing a sugar (0.5% w/v), L-rhamnose or D-xylose. Potential *E. albertii* isolates, which were non-motile and positive for glucose fermentation, negative for hydrogen sulfide production and utility of citrate, malonate, L-rhamnose and D-xylose, were confirmed to be *E. albertii* by a triplex PCR targeting *clpX*, *lysP* and *mdh* genes [17, 22] and the *Eacdt* gene-based PCR [14]. *E. albertii* isolates were also examined for the sugar utility of D-lactose, D-melibiose and D-sucrose using purple agar as basal medium.

Antimicrobial susceptibility of E. albertii isolates

Antimicrobial susceptibility test was done by a disk diffusion method in accordance with the Clinical and Laboratory Standards Institute guidelines (M02-A12, 2015). The strains were tested for 16 antimicrobial agents (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) including ampicillin (10 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefoxitin (30 µg), meropenem (10 µg), imipenem (10 µg), fosfomycin (50 µg, FOF), streptomycin (10 µg, STR), kanamycin (30 µg), gentamicin (10 µg), chloramphenicol (30 µg), tetracycline (30 µg), norfloxacin (10 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), and sulfamethoxazole-trimethoprim (23.75 µg/1.25 µg).

Genotypic characterization of E. albertii isolates

Presence of virulence genes in *E. albertii* isolates were examined by PCR assays using primers targeting *eae*, *Eccdt-I*, *stx1*, *stx2* [*stx2a-stx2e*, *stx2g*], and *stx2f* as described previously [8]. *E. albertii* O-genotypes (EAOgs) of the isolates were examined by PCR assays developed by Ooka *et al.* [26], which can differentiate 40 O-genotypes (EAOg1 to EAOg40). Pulsed-field gel electrophoresis (PFGE) was performed using *Xba*I-digested genomic DNA of *E. albertii* strains isolated from wildlife sources as described previously [15]. DNA fingerprints obtained were analyzed and phylogenetic tree was constructed by a UPGMA method using a BIONUMERICS software (Fingerprints version 8.0; Applied Maths NV, Sint-Marten-Latem, Belgium).

Species identification of wild and safeguarded animals shedding E. albertii

The remaining mammalian fecal samples from which *E. albertii* strains were isolated excluding dogs and cats kept at 4 or −20°C were subjected to D-loop mitochondrial DNA sequence analysis in order to determine the species of the host animal. The D-loop mitochondrial DNA sequencing was carried out following the protocol developed by Hayashi *et al.* [7] with some modifications. Briefly, DNA was isolated from fecal specimens by a QIAamp Fast DNA Stool kit (QIAGEN, Hilden, Germany) following a protocol for Human DNA analysis. Subsequently, partial sequence (~400 bp) of mitochondrial DNA D-loop region was amplified by PCR using TaKaRa Taq™ (Takara Bio Inc., Kusatsu, Japan) and primer pairs of −21-tanaD-F and Rev-tanaD-R, and m(−21-tanaD-F) and Rev-tanaD-R, respectively. m(−21-tanaD-F) is the modified primer of Rev-tanaD-R, from which adaptor sequence was deleted. PCR was conducted with initial denaturation at 94°C for 2 min, followed by 35 cycles of 98°C for 10 sec, 60°C for 30 sec, 72°C for 30 sec, and final extension at 72°C for 2 min. PCR products were purified by a QIAquick PCR Purification Kit (QIAGEN) and subjected to nucleotide sequence analysis as described previously [13] using BigDye Terminator v1.1 (Thermo Fisher Scientific) and a primer of M13-21. The sequence was assembled and the primer sequence was trimmed with the SeqMan software (Lasergene; DNASTAR, Madison, WI, USA). The resulting DNA sequences were applied to homology search through BLAST program using a blastn method. Primer sequences used in this analysis are given in Supplementary Table 2.

RESULTS

Isolation of E. albertii

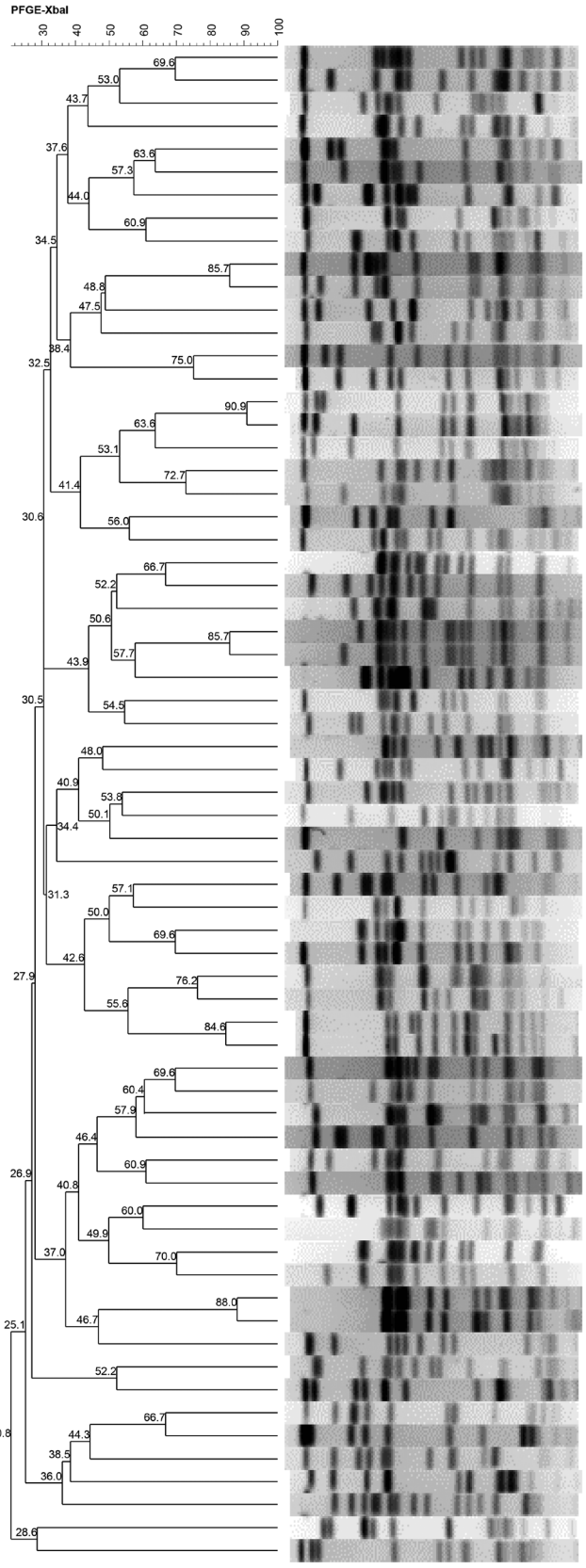
In addition to fecal droppings from wild birds (59 crows and 125 starlings), a total of 906 droppings from wild mammals were collected at 11 different sites in Okayama Prefecture and its prefectural borders (Fig. 1) to examine if *E. albertii* are present or not (Table 1). Possible animal species of the mammal droppings were deduced as weasel/marten (weasel or marten; n=726) and fox (n=28) but 152 samples remained unknown to deduce the species from their fecal aspects as described in Materials and Methods. Feces collected from safeguarded animals (159 dogs and 76 cats) in Okayama Prefecture were also examined in this study. *E. albertii*-like colonies screened by biochemical tests were obtained from 39 weasels/martens, 4 from foxes, 8 from unknown sources, 2 from dogs, 1 from a cat, 10 from crows and 31 from starlings samples. The isolates were finally confirmed to be *E. albertii* by PCR assays. When *E. albertii* isolates from a sample showed identical biochemical properties to each other, one isolate from each sample was selected as a representative isolate. Accordingly, a total of 97 *E. albertii* isolates (40 from weasels/martens, 4 from foxes, 8 from unknown sources, 2 from dogs, 1 from a cat, 10 from crows and 32 from starlings) were subjected to subsequent analyses.

Confirmation of mammal species by fecal DNA sequencing

To identify the host species of wild mammal-derived fecal droppings from which *E. albertii* were isolated, PCR-based mitochondrial DNA D-loop region sequencing was done using fecal DNA isolated from the feces (Supplementary Table 3). Among 51 fecal specimens collected, 5 (EAW-4, EAW-11, EAW-12, EAW-19 and EAW-29) were used up for *E. albertii* screening, therefore, remaining 46 were available for PCR analysis. Expected size (~400 bp) of PCR amplicons was obtained from 39 out of the 46 fecal specimens examined. Nucleotide sequencing of each of the 39 PCR amplicons was carried out followed by homology search to determine species (Supplementary Table 3). Nucleotide sequences of the DNA isolated from 3 fecal samples were identical to those of red fox (*Vulpes vulpes*). Thus, the red fox (*V. vulpes*) was confirmed as the host species of those 3 fecal samples. Similarly, nucleotide sequences of the DNA derived from 29 feces, except for the specimen EAW-17, were most homologous (>98% identity) to that of Japanese marten (*Martes melampus*) rather than other martens of the genus *Martes* (≤97% identity) and weasels (≤90% identity; *Mustela richardsonii* and *M. itatsi*). Thus, the host species of those 29 feces were considered to be of martens (*M. melampus*) and most likely not of weasels. Unexpectedly, the nucleotide sequence of the DNA obtained from the fecal sample EAW-17 showed homology to that of rabbit belonging to the genus *Lepus* (91% identity) and *Pentalagus* (85% identity), which suggests that the host species might be a rabbit of the genus *Lepus*. Similarly, 6 feces, which were not able to deduce the host species during sampling, were found to be derived from martens (*M. melampus*, n=3) and foxes (*V. vulpes*, n=3). Finally, host animal species of 52 *E. albertii* positive fecal samples of wild mammals were confirmed as martens (n=32), foxes (n=6) and rabbit (n=1) and 12 samples remained unidentified (Supplementary Tables 3 and 4).

Phenotypic and genotypic characterization of E. albertii isolates

E. albertii strains (total 97 isolates), which were isolated from wildlife fecal samples, were examined further for their bacteriological characteristics (Supplementary Tables 4 and 5). In addition to biochemical properties applied to the criteria in isolating *E. albertii*, the isolates also showed similar characteristics to *E. albertii* strains reported previously [13, 25], such as no fermentation of D-melibiose and variation in sucrose utility. All the strains were positive for lysine decarboxylase and indole production, indicating that they were assigned to biogroup 3 [21]. Antimicrobial susceptibility testing revealed resistance to FOF and STR by 2 (EAB-8 and EAB-12) and 1 (EAB-22) *E. albertii* strains, respectively, isolated from fecal samples of birds, while none of the other isolates (n=94) showed resistance to 16 different antimicrobials tested in this study (Fig. 2, Supplementary Tables 4 and 5). By EAO genotyping PCR assays (Fig. 2, Supplementary Tables 4 and 5), 39 *E. albertii* strains of mammalian origin were assigned to 14 different EAO genotypes including 6 strains each belonging to EAOg1 and EAOg5, 5 strains each to EAOg11 and EAOg12, 4 strains in EAOg8, 2 strains each of EAOg7, EAOg28, EAOg34 and EAOg40, and 1 strain each of EAOg9, EAOg16, EAOg18, EAOg23 and EAOg26, while remaining 16 strains were untypable. Thirty *E. albertii* strains isolated from birds were assigned to 15 different EAO genotypes including 6 strains of EAOg2, 4 of EAOg29, 3 strains of EAOg1, 2 strains each of EAOg5, EAOg12, EAOg31, EAOg35 and EAOg37, 1 strain each of



Pulso type	Strain and origin	EAOg	Remarks
1	EAW6(MA,h)	34	-
2	EAW11(d)	34	-
3	EAB42(ST,o), EAB46(ST,o), EAB52(ST,o)	29	-
4	EAW14(MA,k)	8	-
5	EAW5(MA,h)	UT	-
6	EAW22(MA,b), EAW23(b)	8	-
7	EAW12(k)	26	-
8	EAB21(ST,o)	34	-
9	EAB23(ST,o), EAB28(ST,o)	UT	*stx2f*
10	EAW26(MA,b)	UT	-
11	EAW33(MA,a)	UT	-
12	EAB25(ST,o)	18	-
13	EAB30(ST,o)	UT	-
14	EAW29(c)	UT	-
15	EAW56(MA,d)	UT	-
16	EAB19(ST,o), EAB20(ST,o), EAB36(ST,o)	UT	-
17	EAB18(ST,o), EAB54(ST,m)	UT	-
18	EAB39(ST,o)	UT	-
19	EAB29(ST,o)	16	-
20	EAW48(FO,k), EAW49(FO,k), EAW50(FO,k), EAW51(FO,k)	UT	-
21	EAB13(CR,n)	1	-
22	EAB44(ST,o)	31	-
23	EAW16(MA,a)	UT	-
24	EAW32(MA,h)	UT	-
25	EAW34(MA,k)	8	*Eccdt-I*
26	EAW9(MA,e), EAW27(MA,d), EAW28(MA,d), EAW31(MA,d)	1	-
27	EAW30(d)	1	-
28	EAB9(ST,n)	5	-
29	EAB40(ST,o)	UT	-
30	EAB43(ST,o)	12	-
31	EAB5(CR,n)	15	-
32	EAW55(MA,d)	16	-
33	EAB22(ST,o)	29	STR^R
34	EAW54(MA,h)	12	-
35	EAW19(a)	23	-
36	EAW42(MA,d), EAW43(MA,d)	7	-
37	EAB10(CR,n), EAB11(CR,n), EAB14(CR,n)	2	-
38	EAW36(MA,d), EAW40(MA,d)	5	*Eccdt-I*
39	EAB41(ST,o)	5	-
40	EAW3(FO,k), EAW4(d), EAW13(MA,k), EAD3(DO,s)	5	*Eccdt-I*
41	EAW35(MA,d), EAW37(MA,d), EAW41(MA,d)	12	-
42	EAW39(FO,f)	12	-
43	EAD1(DO,q)	UT	-
44	EAD2(CA,r)	UT	-
45	EAW25(MA,k)	28	*Eccdt-I*, *stx2f*
46	EAW47(MA,i)	28	*Eccdt-I*, *stx2f*
47	EAW1(g), EAW44(MA,h), EAW45(MA,h)	11	*Eccdt-I*
48	EAW24(MA,k)	9	-
49	EAB53(ST,m)	36	-
50	EAW20(a), EAW21(a)	11	*Eccdt-I*
51	EAB55(ST,o)	40	*stx2f*
52	EAW52(MA,k), EAW53(MA,k), EAW58(MA,k)	UT	*Eccdt-I*
53	EAW17(RA,b)	40	-
54	EAW38(MA,d)	40	-
55	EAB8(CR,l)	35	*Eccdt-I*, FOF^R
56	EAB12(CR,n)	35	*Eccdt-I*, FOF^R
57	EAB24(ST,o)	4	-
58	EAB47(ST,o)	31	-
59	EAW2(d)	18	*Eccdt-I*, *stx2f*
60	EAB35(ST,o), EAB38(ST,o)	1	-
61	EAW10(d)	1	-
62	EAB32(CR,j)), EAB33(CR,j)), EAB34(CR,j)	2	-
63	EAB17(ST,o)	UT	-
64	EAB50(ST,o), EAB51(ST,o)	37	-
65	EAB37(ST,o)	12	*stx2f*
66	EAB45(ST,o)	UT	-

Fig. 2. Pulsotype, EAO (*Escherichia albertii* O)-genotype, virulence gene profile and antibiogram of *Escherichia albertii* isolated from wild and safeguarded animals. Genomic DNAs of *E. albertii* strains were digested by the restriction enzyme *Xba*I and the digests were separated by PFGE (pulsed-field gel electrophoresis). Non-rooted phylogenetic tree was constructed by a method of UPGMA (unweighted pair group method with arithmetic mean) using PFGE-based DNA fingerprints of *E. albertii* isolates. Fingerprints of representative strains are included in this view. Numbers on the tree indicate the similarity (left). Animal species identified by fecal DNA sequencing are indicated by MA, marten; ST, starling; FO, fox; CR, crow; DO, dog; CA, cat; and RA, rabbit. Pulsotypes (1 to 66), strain ID, origin (a to s), EAO genotypes and other remarks (virulence genes other than *eae* and *Eacdt* and antimicrobial resistance) are also described in the center and right of the figure.

EAOg4, EAOg15, EAOg16, EAOg18, EAOg34, EAOg36 and EAOg40, while remaining 12 strains were untypable. Virulence gene profiling of 97 *E. albertii* strains revealed presence of *eae* gene similar to other *E. albertii* strains (Supplementary Tables 4 and 5). Furthermore, 18 *E. albertii* strains isolated from mammalian fecal samples (EAW-1, EAW-2, EAW-3, EAW-4, EAW-13, EAW-20, EAW-21, EAW-25, EAW-34, EAW-36, EAW-40, EAW-44, EAW-45, EAW-47, EAW-52, EAW-53, EAW-58 and EAD-3) and 2 strains of bird origin (EAB-8 and EAB-12) harbored *Eccdt-I* genes, 4 strains isolated from bird fecal samples (EAB-23, EAB-28, EAB-37 and EAB-55) carried *stx2f* genes, and 3 strain of mammalian origin (EAW-2, EAW-25 and EAW-47) harbored both *stx2f* and *Eccdt-I* genes.

Genetic diversity of E. albertii strains

To know the phylogenetic relationships among the *E. albertii* strains, PFGE was performed using *Xba*I-digested genomic DNA (Fig. 2). The 97 *E. albertii* strains showed 66 different pulsotypes including 36 and 30 pulsotypes of strains isolated from mammals and birds, respectively.

Among the mammalian strains, 33 strains from martens were grouped into 23 different pulsotypes. *E. albertii* strains grouped into pulsotypes 20, 26, 36, 38, 40, 41, 47 and 52 included two or more strains with identical PFGE patterns and it is in accordance with their EAO genotypes and virulence gene profiles. *E. albertii* strains isolated from martens in each sampling site were multiclonal and thirteen, eight and six *E. albertii* strains obtained from martens in sites d, k and h were classified into 7, 6 and 5 pulsotypes, respectively. On the other hand, among *E. albertii* strains, 4 strains of pulsotype 26 showed identical PFGE pattern although they were isolated from fecal samples of martens at 2 different sites (d and e in Figs. 1 and 2). Six *E. albertii* strains of fox origin (EAW-3, EAW-39, EAW-48, EAW-49, EAW-50 and EAW-51) were classified into 3 different pulsotypes such as 20, 40, 42 (Fig. 2, and Supplementary Table 4), in which 4 isolates showed identical PFGE pattern (pulsotype 20). Moreover, the strains EAW-3 (from fox, at the site k in Fig. 1), EAW-13 (from marten, site k), and EAD-3 (from dog, site s) showed identical PFGE pattern (pulsotype 40) although they were isolated from different host species at different sites.

Among *E. albertii* strains of bird origin, 10 strains from crows and 32 from starlings were grouped into 6 and 24 pulsotypes, respectively (Fig. 2, and Supplementary Table 5). The strains of pulsotypes 3, 9, 16, 17, 37, 60, 62 and 64 included two or more *E. albertii* strains with identical PFGE patterns, respectively, in accordance with other bacteriological characteristics. *E. albertii* strains isolated from starlings were also multiclonal even among strains which were isolated from fecal specimens collected at same site and same day (Fig. 2, Supplementary Table 5; 4 each pulsotype on 2019/07/08, 2019/07/09, and 2019/07/11, 5 on 2019/07/25, 3 on 2019/07/10, 2 on 2019/07/24, respectively). The *E. albertii* strains EAB-18 and EAB-54, which were isolated from starling feces of different sites (o and m in Fig. 1), showed identical PFGE pattern (pulsotype 17).

DISCUSSION

Importance of *E. albertii* as a food poisoning bacterial pathogen has increasingly been recognized in the world. *E. albertii* has been isolated from various meats, poultries, birds etc., however, the natural reservoir of this bacterium remains unclear. Recently, high prevalence of *E. albertii* in Japanese raccoons has been reported [8] and raccoon is thought to be one of the most important reservoirs of *E. albertii* at least in Japan. However, little is known about distribution and bacteriological properties including genomic diversity of this bacterium in other wild and safeguarded animals, which is important to understand the reservoirs of *E. albertii*. Therefore, we performed a survey of fecal *E. albertii* in various wild and safeguarded mammals and birds to further understand about animal reservoirs of *E. albertii* in Japan. To the best of our knowledge, this is the first report showing the isolation of this bacterium from martens and foxes including the bacteriological characteristics of the isolates.

In this study, mammal samples except for dogs and cats were collected as fecal droppings. As fecal characteristics are affected by what the animals feed as well as their own zoological features, the host animal species cannot be determined properly only from fecal aspects. To avoid the misidentifications, we attempted to identify the host animal species of feces by PCR-based sequencing of mitochondrial DNA D-loop region, which is a commonly used technique to identify the mammalian species. Indeed, the sequencing analysis of fecal DNA enabled us to determine successfully the animal species (Supplementary Table 3). Especially, weasel and marten feces are quite similar in their fecal aspects, but here the weasel/marten-like feces could be clearly differentiated except for one sample, which was unexpectedly identified to be of rabbit. Furthermore, 6 fecal specimens could not be deduced the animal species from the fecal characteristics but the host animal species could be determined by the sequencing. However, we were unable to determine the species of 12 fecal specimens because 5 of them were exhausted during detection of *E. albertii* while remaining 7 fecal specimens did not yield any PCR product. This might be due to damage of DNA during storage of fecal samples. This could be the reason because feces collected from 2019 were kept at −20°C after screening but fecal samples collected before that period were stored at 4°C for several months prior to moving to −20°C. Thus, it is unfortunate that seven fecal specimens collected before September 2019 did not yield any PCR product.

As discussed above that *E. albertii* has been identified in various wild mammals and birds [3, 5, 6, 15, 17, 21–23, 25], however, at present less information is available about characteristics and genomic diversity of *E. albertii* isolates from wild and safeguarded animals/birds. In the present study, PFGE analysis clearly indicated presence of genetically diverse *E. albertii* strains among various wild animals even fecal samples collected from them within a limited geographically area (Fig. 2). In fact, multiclonal *E. albertii* strains were isolated from martens in each sampling site. *E. albertii* strains isolated from starlings were also multiclonal even among strains collected at same site and same day, suggesting the possible presence of intra-flock diversity of *E. albertii*. It should be noted that clonally related strains were obtained from feces of different animals and/or areas. The starling and marten strains of pulsotypes 17 and 26, respectively, were obtained from feces collected in different areas. Moreover, the strains belonging to pulsotype 40 were

obtained from 3 different animal feces such as marten, fox and dog (Fig. 2). Therefore, no clonal relationship has been detected between the isolates from mammals and birds in this study, but we believe that certain clonal *E. albertii* strains might be distributed in various wild and safeguarded animals in Okayama Prefecture of Japan. Several *E. albertii* strains showing identical PFGE patterns were seen among those isolated from same sampling sites (12 pulsotypes including 3, 9, 16, 20, 26, 38, 41, 47, 52, 60, 62 and 64), suggesting the distribution of certain *E. albertii* clones in animals residing in the areas. However, since fecal dropping was collected without differentiating animal individuals in the present study, the possibility of droppings collected repeatedly from a same individual cannot be ruled out. Thus, geographical relation of *E. albertii* strains is not discussed in this study.

It is important to consider the possible virulence of *E. albertii* isolated from wild and safeguarded animals. *E. albertii* strains isolated in this study carry the virulence genes *Eacdt* and *eae* as previously reported in clinical *E. albertii* strains [18]. Twenty strains additionally carried the *Eccdt-I* genes, the product of which has been shown to be associated with the severity of disease caused by *E. coli* [27]. Moreover, seven *E. albertii* strains found to carry the *stx2f* genes, which codes for the Shiga toxin 2f, a primary virulence factor of EHEC. Since the *stx2* genes have been identified in *E. albertii* isolates from patients with bloody diarrhea [2] and hemolytic uremic syndrome [18], thus, an *E. albertii* strain carrying a functional *stx2* genes could be highly virulent. Although our knowledge about EA 'O' genotypes and their association with pathogenicity in human remains unclear still EAO genotypes 1, 2, 4, 5, 7, 8, 9, 11, 12, 15, 16, 18, 35, 37 and 40 identified in strains from wild and safeguarded animals were also detected in human clinical strains of *E. albertii* [26]. Taken together, the *E. albertii* strains isolated from wild animals in this study might have the virulence potential to humans although the expression status of each of these virulence factors has not been examined.

In conclusion, different *E. albertii* strains with virulence potential have successfully been isolated from wild animals/birds in Okayama Prefecture and its prefectural borders of Japan. To our knowledge this is first report about the presence *E. albertii* in wild martens and foxes. However, the occurrence and prevalence of this bacterium in wild and safeguarded animals are not fully understood. Further epidemiological studies are required to comprehend the reservoirs and dynamics of *E. albertii* in the environment to know further about its transmission routes for prevention of food poisoning in humans.

POTENTIAL CONFLICT OF INTEREST. The authors have nothing to disclose.

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