Prevalence of *Escherichia albertii* in Raccoons (*Procyon lotor*), Japan

Appendix

Sample Collection

We collected rectal specimens using cotton swabs (SEEDSWAB γ 1, Eiken Chemical Co., http://www.eiken.co.jp) from 430 wild raccoons (*Procyon lotor*) in Osaka, Japan, during June 2016–March 2017 (n = 182) and July–September 2017 (n = 248). All the raccoons seemed to be asymptomatic. Raccoons were captured to exterminate them throughout the year in Osaka. The samples were transported to the laboratory at ambient temperature and processed within 6 hours of collection. Fecal sampling in the present study was approved by Osaka Prefectural Government and performed according to the Guidelines for Animal Experimentation of Osaka Prefectural Animal Protection and Livestock Division.

Detection of Eacdt Genes by PCR

We suspended rectal swabs in 1 mL of sterilized Dulbecco's phosphate-buffered saline (PBS). An aliquot (300 μ L) of the suspension was inoculated into 3 mL of tryptic soy broth (Becton Dickinson, www.bd.com), and enriched them at 37°C for 14–16 h with shaking. We centrifuged 100 μ L of the culture at 10,000 *g* at 4°C for 3 min. We suspended the resulting pellet in 85 μ L of 50 mM NaOH, boiled it at 100°C for 10 min, and neutralized it by adding 15 μ L of 1 M Tris-HCl buffer (pH 7.0). After centrifugation at 10,000 *g* at 4°C for 10 min, we subjected the supernatant to PCR analysis using a pair of *E. albertii* specific primers targeting *Eacdt* genes (Appendix Table 1).

Isolation and identification of E. albertii

We serially diluted the swab suspensions from PCR-positive specimens in PBS and spread 100 μ L of each dilution on XRM-MacConkey agar, an *E. albertii*-selective medium (*1*), with composition of MacConkey agar base (Becton Dickinson) supplemented with 1% (w/v) each of xylose, rhamnose, and melibiose, and incubated them at 37°C for 20–24 hours. We examined colorless colonies (maximum 8 colonies) on the medium, which are typical feature of *E. albertii*, by PCR, targeting *Eacdt* genes. *Eacdt* gene-positive colonies were determined to be *E. albertii* by another *E. albertii*-specific PCR assay using a primer pair targeting *yejH* and *yejK* in *E. albertii*, which was developed by Ooka et al. (*2*).

Detection of Virulence Genes

We analyzed the presence of virulence genes by colony hybridization assay using ³²Plabeled DNA probes targeting *eae*, *stx1*, *stx2a*, *stx2f*, *Eccdt-IB*, and *Eccdt-IVB* under high stringent conditions, as described previously (3). When *stx2* and *cdt* genes were detected by the colony hybridization assay, subtype-specific PCRs for *stx2* (4), *Eccdt-I* and *Eccdt-IV* were carried out to determine their subtypes (Appendix Table 1). The entire nucleotide sequence of *stx2f* genes was determined as described previously (5). PCR amplification was done by Veriti Thermal cycler (Thermo Fisher Scientific, https://www.thermofisher.com) using TaKaRa Taq DNA polymerase (Takara Bio, https://www.takarabio.com). We sequenced the PCR products by cycle sequencing method using BigDye Terminator v1.1 and ABI 3130 Genetic Analyzer (Thermo Fisher Scientific).

To determine each intimin subtype, we determined the entire *eae* nucleotide sequence, as previously described (5). Predicted amino acid sequences of *eae* genes were aligned with those of the reference intimin subtypes by the Clustal W program of MEGA6 (https://www.megasoftware.net). The reference intimin subtypes used were from Hinenoya et al. (5). If intimin subtypes of *E. albertii* raccoon strains were determined to be untypable, the putative amino acid sequences were subjected to BLAST homology search using the tblastn module (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Pulsed-Field Gel Electrophoresis (PFGE)

We performed PFGE as described previously (6). Briefly, fresh bacterial cells were embedded in agarose plug and in situ lysis was carried out to isolate total genomic DNA. The genomic DNA embedded plug was subjected to restriction enzyme digestion with 30 U of *XbaI* (Takara Bio), and electrophoretic separation of the DNA fragments was done in 1% pulsed-field certified agarose (Bio-Rad Laboratories, https://www.bio-rad.com) on a CHEF Mapper PFGE (Bio-Rad) using 0.5x TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA [pH 8.0]). Run conditions were generated by the autoalgorithm mode of the CHEF Mapper PFGE system for the sizes ranging between 20 and 300 kb, and the running time was 26.93 hours. *XbaI*-digested genomic DNA of *Salmonella* Braenderup strain H98121 was used as a molecular size marker. DNA fingerprints of *E. albertii* strains were interpreted based on Tenover's criteria (7) and analyzed using Fingerprinting II Software (Bio-Rad) to know their phylogenetic relationships.

Detection of Stx2f Production in stx2f Gene-Positive E. albertii

Production of Stx2f by *stx2f* gene-positive *E. albertii* strains was determined by Vero cells cytotoxicity assay, as previously described (*3*). We prepared crude toxin samples as follows: *E. albertii* was cultured in 3 mL of lysogenic broth (LB broth, Becton Dickinson) at 37°C for 14 hours. An aliquot of the culture was inoculated into 3 mL of fresh lysogenic broth

and cultured until early log phase (≈ 0.2 optical density at 600 nm). Mitomycin C (Kyowa Hakko Kirin, https://www.kyowakirin.com) was added to the culture at the final concentration of 0.5 μ g/mL and further incubated at 37°C for 4 hours aerobically. Culture supernatant was passed through a sterile filter with 0.22- μ m pore size (Merck Millipore,

https://www.emdmillipore.com), and the filtrate was subjected to cytotoxicity assay. Neutralization assay of the toxin activity was also carried out using anti-Stx2fA rabbit serum (8), which was preincubated with crude toxin samples at 37°C for 30 min. The mixture was applied to the cytotoxicity assay.

Nucleotide Sequence Accession Numbers

All nucleotide sequences obtained in this study were registered into the DNA Data Bank of Japan database. The accession numbers are LC504574–LC504632 (for *eae* genes) and LC504633–LC504634 (for *stx2f* genes).

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			PCR conditions (30 cycles)			Amplicon	
Target gene	Primer name	Sequence (5'-3')	Denaturing	Annealing	Extension	size (bp)	Reference
Eacdt	EaCDTsp-F2	GCTTAACTGGATGATTCTTG	94°C, 30 sec	50°C, 30 sec	72°C, 60 sec	449	(9)
	EaCDTsp-R2	CTATTTCCCATCCAATAGTCT					
Eccdt-IA	EcCDT1A-F	GAT CGG TGA TTC ACC TTC	94°C, 30 sec	50°C, 30 sec	72°C, 60 sec	499	This study
	EcCDT1A-R	TTT CTC AAG GGT GAT TGT AA					
Eccdt-IB	EcCDT1B-F	GAT TTT GCC GGG TAT TTC T	94°C, 30 sec	50°C, 30 sec	72°C, 60 sec	640	This study
	EcCDT1B-R	TCA AGA ACA CCA CCA CTG					
Eccdt-IC	EcCDT1C-F	TAC TGC TGA CAG GTT GTG	94°C, 30 sec	50°C, 30 sec	72°C, 60 sec	540	This study
	EcCDT1C-R	CAG CTC GTT AAT GGA GAC					(10)
Eccdt-IVA	EcCDT4A-F	TCT CCA ACA TTT GGG AG	94°C, 30 sec	50°C, 30 sec	72°C, 60 sec	427	(10)
	EcCDT4A-R	CTT TTG CAC CAG GAC AC					This study
Eccdt-IVB	EcCDT4B-F	ACC ATC TTC AGC TAC ACT A	94°C, 30 sec	50°C, 30 sec	72°C, 60 sec	286	This study
	EcCDT4B-R	GCT CCA GAA TCT ATA CCT					-
Eccdt-IVC	EcCDT4C-F	TCA GAA ACC CTG TAG GTC	94°C, 30 sec	50°C, 30 sec	72°C, 60 sec	202	This study
	EcCDT4C-R	GTA AAT AAT GCA TTG CGA TTG	,	,	,		

Appendix Table 1. PCR primers for detection of *E. albertii*, and differentiating *Eccdt-I* and *Eccdt-IV* genes; PCR conditions (30 cycles).

R7 A 2016 Jun RC-7B R8 B 2016 Jun RAC-8 R8 B 2016 Jun RAC-30 R30 C 2016 Jun RAC-30 R30 C 2016 Jun RAC-33A R33 B 2016 Jun RAC-33B RAC-33C 2016 Jun RAC-33C R33 B 2016 Jun RAC-33C R34 B 2016 Jun RAC-34A R34 B 2016 Jun RAC-34B RAC-34B RAC-34E RAC-34E RAC-34E RAC-34E RAC-44D RAC-44D RAC-44D RAC-44D RAC-118 R118 B <	Appendix Table 2 Strain	Raccoon ID	City captured*	a albertii strains were isolated. Month of sampling
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AAC-30 R30 C 2016 Jun AAC-333 R33 B 2016 Jun AAC-336 R33 B 2016 Jun AAC-340 R34 B 2016 Jun AAC-340 R4 B 2016 Jun AAC-340 R4 A 2016 Jun AAC-340 R4 A 2016 Jun AAC-340 R4 A 2016 Jun AAC-341 R1 A 2016 Jun AAC-440 A 2016 Jun A AAC-410 A 2016 Jun A AAC-410 A 2016 Jun A AAC-116 R118 B 2017 Jun AAC-118 R118 A 2017 Jun AAC-118 R243 C 2017 Jun AAC-244 R243 C 2017 Jun	RAC-7B			
AAC-33A R33 B 2016 Jun AAC-33G R34 B 2016 Jun AAC-33G R34 B 2016 Jun AAC-34B R42 B 2016 Jun AAC-34B R44 B 2016 Jun AAC-34D R44 B 2016 Jun AAC-34B R41 A 2016 Jun AAC-34B R18 B 2016 Jun AAC-112 R112 E 2016 Jun AAC-112 R118 B 2016 Jun AAC-118B R118 B 2017 Jun AAC-118D R18 A 2017 Jun AAC-118D R243 C 2017 Jun AAC-218 R245 C 2017 Jun AAC-2261 R245 C 2017 Jun AAC-2261 R262 J 2017 Jun AAC-2263 R263 D 2017 Jun AAC-264 H 2017 Jun AAC-264 R 2017 Jun	RAC-8	R8	В	2016 Jun
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RAC-264E RAC-264G RAC-264G RAC-2666 R266 RAC-2674B R274 RAC-274B R274 RAC-274B R274 RAC-274B R274 RAC-274D R274 RAC-274D R274 RAC-274D R274 RAC-274E R278 RAC-278B R278 RAC-278C R278 RAC-278D R278 RAC-278C R2017 Jul RAC-278C R2017 Aug RAC-278G R2017 Aug RAC-278G R2017 Aug RAC-302B R303 E RAC-303B R305 K 2017 Aug RAC-305A R305 K 2017 Aug RAC-306B R306 K 2017	RAC-264C			
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RAC-274A R274 F 2017 Jul RAC-274B RAC-274C RAC-274C RAC-274C RAC-274C RAC-274C RAC-274C RAC-274C RAC-274E RAC-278A R278 G 2017 Jul RAC-278B RAC-278C RAC-278C RAC-278C RAC-278C RAC-278F RAC-278F RAC-278F RAC-278F RAC-278F RAC-278F RAC-278G R300 K 2017 Jul RAC-278F RAC-300 R300 K 2017 Aug RAC-302A R302 L 2017 Aug RAC-302B R303 E 2017 Aug RAC-303B R303 E 2017 Aug RAC-305A R305 K 2017 Aug RAC-305A R305 K 2017 Aug RAC-306B R306 K 2017 Aug RAC-306C R306 K 2017 Aug RAC-306F RAC-306F RAC-306F RAC-310A RAC-310B R310 G 2017 Aug	RAC-264H			
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RAC-278D RAC-278E RAC-278F RAC-278G RAC-278G RAC-278G RAC-278G RAC-281 R281 RAC-278G RAC-278G RAC-278G RAC-278G RAC-278G RAC-278G RAC-278G RAC-300 R300 KAC-302A R302 L 2017 Aug RAC-302B R303 RAC-303A R303 RAC-303B R305 RAC-305B R305 RAC-306B R306 RAC-306B R306 RAC-306F R310 RAC-310A R310 GAC-310B Satisfies				
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RAC-278G RAC-281 R281 C 2017 Jul RAC-300 R300 K 2017 Aug RAC-302A R302 L 2017 Aug RAC-302B R303 E 2017 Aug RAC-303A R303 E 2017 Aug RAC-303B R305 K 2017 Aug RAC-305B R305 K 2017 Aug RAC-306A R306 K 2017 Aug RAC-306B R306 K 2017 Aug RAC-306D RAC-306E R306 K 2017 Aug RAC-306F R306 K 2017 Aug R306 RAC-310A R310 G 2017 Aug				
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RAC-302B R303 E 2017 Aug RAC-303B R305 K 2017 Aug RAC-305A R305 K 2017 Aug RAC-305B R306 K 2017 Aug RAC-306B R306 K 2017 Aug RAC-306B R306 K 2017 Aug RAC-306C RAC-306E RAC-306E RAC-306E RAC-306F R310 G 2017 Aug				0
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RAC-305A R305 K 2017 Aug RAC-305B R306 K 2017 Aug RAC-306A R306 K 2017 Aug RAC-306B K 2017 Aug RAC-306C K 2017 Aug RAC-306C K 2017 Aug RAC-306E K 2017 Aug RAC-306F K 2017 Aug RAC-310A R310 G 2017 Aug		1,000	L	2017 Aug
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RAC-306B RAC-306C RAC-306D RAC-306E RAC-306F RAC-310A R310 G 2017 Aug RAC-310B		R306	ĸ	2017 400
RAC-306C RAC-306D RAC-306E RAC-306F RAC-310A R310 G 2017 Aug RAC-310B		1,000	IX.	2017 Aug
RAC-306D RAC-306E RAC-306F RAC-310A R310 G 2017 Aug RAC-310B				
RAC-306E RAC-306F RAC-310A R310 G 2017 Aug RAC-310B				
RAC-306F RAC-310A R310 G 2017 Aug RAC-310B				
RAC-310A R310 G 2017 Aug RAC-310B				
RAC-310B		R310	G	2017 Aug
		1.010	0	2017 Adg
		R313	G	2017 Aug
		1.010	5	2017 Aug

Strain	Raccoon ID	City captured*	Month of sampling
RAC-318A	R318	M	2017 Aug
RAC-318B			
RAC-318C			
RAC-318D			
RAC-318E			
RAC-318F			
RAC-324	R324	С	2017 Aug
RAC-333A	R333	Ĵ	2017 Aug
RAC-333B			
RAC-334A	R334	F	2017 Aug
RAC-334B			
RAC-335	R335	F	2017 Aug
RAC-336	R336	D	2017 Aug
RAC-337	R337	D	2017 Aug
RAC-342	R342	Ğ	2017 Aug
RAC-349	R349	Ň	2017 Aug
RAC-351A	R351	A	2017 Aug
RAC-351B	1(351	7	2017 Aug
RAC-351C			
RAC-351D			
RAC-351D	R355	А	2017 Aug
	R355 R357	M	
RAC-357A	1667	IVI	2017 Aug
RAC-357B RAC-359A	Daro	С	2017
	R359	C	2017 Aug
RAC-359B	DOGE	н	0047
RAC-365	R365		2017 Aug
RAC-376	R376	D	2017 Aug
RAC-381A	R381	K	2017 Sep
RAC-381B			
RAC-381C	Daga	0	0017.0
RAC-382	R382	0	2017 Sep
RAC-386	R386	P	2017 Sep
RAC-393A	R393	В	2017 Sep
RAC-393B			
RAC-393C			
RAC-393D			
RAC-393E			
RAC-393F			
RAC-396	R396	Ν	2017 Sep
RAC-400	R400	E	2017 Sep
RAC-404	R404	L	2017 Sep
RAC-406	R406	K	2017 Sep
RAC-409A	R409	A	2017 Sep
RAC-409B			
RAC-410A	R410	N	2017 Sep
RAC-410B			
RAC-410C			
RAC-410D			
RAC-413A	R413	С	2017 Sep
RAC-413B			·
RAC-413C			
RAC-414	R414	I	2017 Sep
RAC-419A	R419	Â	2017 Sep
RAC-419B			- 1
RAC-419C			
RAC-419D			
RAC-419E			
RAC-423A	R423	В	2017 Sep
RAC-423B		2	2011 000
RAC-431A	R431	Н	2017 Sep
RAC-431B			2011 000
RAC-431C			
RAC-431D			
RAC-431D			
RAC-431F	D420	Н	2017 Sep
RAC-439A	R439	11	2017 Sep
RAC-439B			
RAC-439C		P).	

*City names are coded by alphabetical letters (A–P).

Appendix Table 3. Detailed information and characteristics of E. albertii raccoon strains.

	Virulence genes				
Strain*	Eacdt	eae	Eccdt-l	stx2f	Intimin subtypes
RAC-7A	+	+	-	-	rho
RAC-30A	+	+	-	-	xi
RAC-33A	+	+	-	-	omicron
RAC-34A	+	+	-	-	beta3
RAC-44A	+	+	-	-	xi

	Virulence genes				
Strain*	Eacdt	eae	Eccdt-I	stx2f	Intimin subtypes
RAC-44D	+	+	+	-	gamma5
RAC-58	+	+	-	-	N4
RAC-81A	+	+	-	-	sigma
RAC-112	+	+	-	-	N5
RAC-116A	+	+	-	-	N1.3
RAC-118A	+	+	-	-	alpha8
RAC-199	+	+	-	+	Ń1.2
RAC-243	+	+	_	_	N2
RAC-244	+	+	-	-	N5
RAC-245	+	+	_	_	Unknown (2013C-4143)
RAC-247	+	+	+	+	gamma5
RAC-258	+	+	· _	-	iota2
RAC-263	+	+	_	_	N2
RAC-264A	+	+	—	_	Unknown (1261-6/89)
RAC-266			—		rho
	+	+	-	_	
RAC-274A	+	+	+	-	N3
RAC-281	+	+	-	-	sigma
RAC-300	+	+	-	-	rho
RAC-302A	+	+	-	-	sigma
RAC-303A	+	+	+	-	N3
RAC-305A	+	+	+	-	unknown (1261-6/89)
RAC-305B	+	+	-	-	N3
RAC-306A	+	+	-	-	Unknown (4281-7/89)
RAC-310A	+	+	-	-	omicron
RAC-313	+	+	-	-	UT1
RAC-318A	+	+	-	-	UT2
RAC-318F	+	+	-	-	UT2
RAC-324	+	+	_	_	N3
RAC-333A	+	+	_	_	UT1
RAC-336	+	+	_	_	N3
RAC-337	+	+	_	_	N5
RAC-342		+	_	_	sigma
RAC-349	т ,	+	_	_	Unknown (4281-7/89)
	+		-		
RAC-355	+	+	-	-	rho
RAC-357A	+	+	-	-	UT1
RAC-359A	+	+	-	—	UT1
RAC-365	+	+	-	-	rho
RAC-376	+	+	-	-	iota2
RAC-383	+	+	-	-	N4
RAC-386	+	+	-	-	UT2
RAC-393A	+	+	-	-	N5
RAC-396	+	+	-	-	Unknown (2013C-4143)
RAC-404	+	+	-	-	UT1
RAC-406	+	+	-	-	rho
RAC-409A	+	+	-	-	iota2
RAC-410A	+	+	_	-	rho
RAC-413A	+	+	_	_	Unknown (2013C-4143)
RAC-414	+	+	_	_	iota2
RAC-419A	+	+	_	_	iota2
RAC-419D	+	, +	_	_	UT1
RAC-419E	' -	+	_	-	rho
	+		-	-	N4
RAC-423A	+	+	-	-	
RAC-431A	+	+	_	-	omicron
RAC-439A	+	+	-	-	omicron

*RAC, number, letter (A–F) indicate raccoon, raccoon ID, and *E. albertii* colony ID, respectively. Strains with identical numbers were isolated from the same raccoons. +, present; –, not present.