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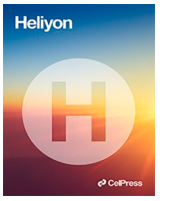

Research article

Detection of prolong excretion of *Escherichia albertii* in stool specimens of a 7-year-old child by a newly developed *Eacdt* gene-based quantitative real-time PCR method and molecular characterization of the isolates

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A B S T R A C T

Escherichia albertii is an emerging zoonotic foodborne pathogen. The clinical significance of this bacterium has increasingly been recognized worldwide. However, diagnostic method has not yet been established and its clinical manifestations are not fully understood. Here, we show that an *Eacdt* gene-based quantitative real-time PCR (qRT-PCR) developed in this study is 100% specific and sensitive when tested with 39 *E. albertii* and 36 non-*E. albertii* strains, respectively. Detection limit of the real-time PCR was 10 colony forming unit (CFU) and 1 pg of genomic DNA per PCR tube. When *E. albertii* was spiked with 4×10^0–10^6 CFU per mL to stool of healthy person, detection limit was 4.0×10^3 and 4.0 CFU per mL before and after enrichment culture, respectively. Moreover, the qRT-PCR was able to detect *E. albertii* in five children out of 246 (2%) but none from 142 adults suffering from gastroenteritis. All five *E. albertii* strains isolated carried *eae* and *paa* genes, however, only one strain harbored *stx2f* genes. Long-term shedding of *stx2f* gene-positive *E. albertii* in a child stool could be detected because of the qRT-PCR developed in this study which might have been missed if only conventional PCR and culture methods were employed. Furthermore, *E. albertii* isolated from siblings with diarrhea showed clonality by PFGE analysis. Taken together, these data suggest that the *Eacdt* gene-based qRT-PCR developed for the detection of *E. albertii* is useful and will assist in determining the real burden and clinical manifestation of *E. albertii* infections.

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1. Introduction

Escherichia albertii was first isolated from a child with diarrhea in Bangladesh and identified as *Halfnia alvei* [1]. Subsequently, this bacterium was reclassified as *E. albertii* on the basis of DNA-DNA hybridization and biochemical properties [2]. However, *E. albertii* was often misidentified as enteropathogenic *Escherichia coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and *Shigella boydii* serotype 13 because these bacteria have similar biochemical properties and carry same virulence genes as *E. albertii*. Like EPEC and EHEC, *E. albertii* strains commonly carry the *eae* gene, the genetic determinant associated with attaching and effacing lesion on intestinal mucosa. Certain strains of *E. albertii* also carry *stx2a/stx2f* genes encoding the Shiga toxin 2a/2f, a major virulence factor of EHEC [3–5]. Therefore, prevalence of *E. albertii* could be underestimated, and real burden of *E. albertii* infections remains unclear. Furthermore, *E. albertii* might cause symptoms as severe as those caused by EHEC. *E. albertii* also chromosomally harbours the *cdt* genes (called *Eacdt*), which encodes the cytolethal distending toxin (CDT). CDT is comprised of CdtA, CdtB and CdtC subunits [6]. In addition, the *paa* gene, which encodes the porcine attaching and effacing associated protein, is an important virulence factor of *E. coli* causing diarrhea in piglets and it is also highly conserved in *E. albertii* [7,8].

In addition to *E. albertii*, CDT has been identified in several Gram-negative bacteria including *E. coli, Shigella* spp., *Providencia* spp. *Campylobacter* spp. etc. Although, clinical significance of CDT is obscure, it is considered that CDT might be associated with diarrhea, severity of diseases and colonization of the pathogens [9–11]. Among *E. coli* CDTs, five subtypes have been reported, namely, EcCDT-I to EcCDT-V [12]. However, our previous studies have confirmed that *Eccdt-II* gene-positive *E. coli* are actually *E. albertii* and *Eccdt-II* genes are highly homologous or nearly identical to *Eacdt* genes present in *E. albertii* [6,13]. Till date, *Eacdt* genes have been reported in all *E. albertii* strains examined with only few exception [14–16]. Infact, *cdt* genes have been also targeted for species identification in certain bacteria, which ubiquitously carry those genes. For example, *C. jejuni, C. coli* and *C. fetus* carry *cdt* genes in a species-specific manner and those *cdt* genes have been used for the identification of respective *Campylobacter* species [17,18].

Since *E. albertii* could not be distinguished by conventional tests from *Escherichia/Shigella* spp., genetic analysis is required for their identification. Until recently, multilocus sequence analysis was used to identify *E. albertii* which is expensive and time-consuming [13, 15]. More recently, Hatanaka et al., 2020 [19] reported improved accuracy of *E. albertii* identification by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS), however, this assay also requires expensive equipment such as MALDI biotyper system. On the other hand, over the years various PCR based assays have been developed to detect and identify *E. albertii* [6,16,20]. We have also recently reported a *cdt* gene-based PCR assay for the detection and identification of *E. albertii* [21], which showed better result in comparison to the other target genes by *in-silico* analysis [22]. Although these PCR assays could be used for accurate identification of *E. albertii* but they are conventional end-point PCR assays and could not be used for quantification of *E. albertii* in crude samples such as clinical and environmental specimens or enrichment culture, thus a quantitative real-time PCR (qRT-PCR) assay is required. Therefore, the objectives of this study were to develop and validate a qRT-PCR assay employing fluorescence based intercalating dye for quantification of *E. albertii,* to evaluate the performance of the assay using clinical stool specimens, and lastly, to characterize the isolated *E. albertii* strains by molecular and biological assays.

2. Material and methods

2.1. Bacterial strains

E. albertii type strain 19982^T [1] and *E. coli* strain C600 were used as a positive and negative control for the PCR assay, respectively. *E. albertii* strains ($n = 39$) previously isolated in our laboratory from diarrheal stool swabs or collected from culture collections were used for the sensitivity assay (Table S1). Non-*E. albertii* strains ($n = 36$) belonging to 18 different species under 9 genera were used for the specificity assay (Table S2). The non-*E. albertii* strains include *cdt* gene-positive strains such as *E. coli* strains NT3363 (*Eccdt-I*), AH-6 (*Eccdt-III*), AH-9 (*Eccdt-IV*), AH-26 (*Eccdt-V*); *Providencia alcalificiens* strain AH-31 (*Pacdt*) and *P. rustigianii* strain JH-1 (*Prcdt*) as reported previously [23–25].

2.2. Primer designing

Reference *cdt* gene sequences of *E. albertii* [*Eacdt,* ($n = 25$)], *E. coli* [*Eccdt-I* ($n = 3$)], *Eccdt-III* ($n = 1$), *Eccdt-IV* ($n = 1$), *Eccdt-V* ($n = 2$), *P. alcalifaciens* [*Pacdt,* ($n = 3$)], and *P. rustigianii* [*Prcdt,* ($n = 1$)] were either extracted from GenBank or previously sequenced in our laboratory [23–25]. *Eacdt* gene-specific primers were designed manually by targeting the conserved sequence regions of the gene. Accession numbers of the *cdt* gene sequences used and primer binding sites are shown in Fig. S1. The primers were evaluated *in-silico* at NCBI to confirm their specificity.

2.3. DNA template preparation

DNA template was prepared by boiling method as described previously [21]. Bacterial culture was grown in tryptic soy broth (TSB; Becton, Dickinson and Co., Franklin Lakes, NJ, USA) at 37 °C for 18 h with shaking followed by 10 times dilution with 1× TE buffer (10 mM Tris-HCl, 1.0 mM EDTA, [pH 8.0]), boiling for 10 min and snap cooled. The boiling suspension was centrifuged at 9800 *g* at 4 °C for 10 min, supernatant was collected and used as DNA templates. In some cases, DNA templates were prepared by alkaline lysis method. In brief, 100 μL of a bacterial culture was centrifuged at 9800 *g* at 4 °C for 10 min, cell pellet was collected and re-suspended in 85 μL of 50 mM NaOH followed by boiling of the suspension for 10 min. Boiled sample was snap cooled, neutralized with 15 μL of 1.0 M

Tris-HCl (pH 7.2), centrifuged at 9800 *g* at 4 °C for 5 min and the supernatant was used as DNA templates for qRT-PCR.

2.4. Quantitative real-time PCR

qRT-PCR was carried out in 20 μL reaction mix, including 10 μL of GoTaq qPCR master mix (Promega, Corporation, Madison, MD), Eacdt-CF1 (5′-GAGAGACTATTGGATGGGAAA-3′)/Eacdt-CR1 (5′-TGGTCTGTGTTTGGCGTTCG-3′) primers at a final concentration of 0.5 mM each, and 1.0 μL of the DNA template by Thermal Cycler Dice Real Time System II (TaKaRa Bio Inc., Shiga, Japan) using FAM filter with optimized PCR conditions as follows: initial denaturation at 95 °C for 2 min followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 56 °C for 15 s and extension at 60 °C for 30 s. The dissociation conditions were followed as per default settings from 60 °C–95 °C. Ct value of 32 was set as cut-off.

2.5. Detection limit

E. albertii 19982^{T} was grown in 3 mL of TSB at 37 °C, 180 rpm for 14 h. An overnight culture was diluted 1000 times with 3 mL of fresh TSB and again incubated under the same condition for 3 h. This early log phase culture was used for the preparation of DNA template and CFU enumeration. In brief, the bacterial cells were collected by centrifugation at 3500 *g* at 4 °C for 5 min and washed with 10 mM phosphate-buffered saline (PBS; pH 7.4). The bacterial pellet was suspended in PBS and CFU was enumerated by serial dilution with sterilized PBS and plating on tryptic soy agar (TSA). Simultaneously, DNA templates were prepared by boiling cells in 1xTE buffer as described above. Genomic DNA was isolated from *E. albertii* 19982^{T} using conventional method and DNA concentration was determined by using a nanodrop lite spectrophotometer (Thermo Scientific, Waltham, MA). Prepared boiled and genomic DNA samples were used as templates for determination of the detection limit of *E. albertii* using the qRT-PCR developed in this study.

2.6. Spiking of E. albertii in stool

A stool sample was collected from a healthy individual and it was confirmed *E. albertii* negative by the newly developed qRT-PCR method described in this study as well as using an *Eacdt* gene-based conventional PCR assay reported earlier from our group [21]. Briefly, the collected stool sample (ca 150 mg) was suspended in 3 mL of TSB and spiked with early log phase cells of *E. albertii* 19982^{T} at a concentration of 1–10^{6} CFU per mL. The spiked stool suspension was incubated at 37 °C with shaking at 180 rpm for 14 h followed by preparation of template DNA from the TSB enriched stool sample by alkaline lysis method as described above. For comparison, template DNA from non-enriched sample was also prepared by alkaline lysis method as described above.

2.7. Collection and analysis of clinical samples

Three hundred and eighty-eight diarrheal stool swabs (SWEEDSWAB γ1, Eiken chemical Co., Tokyo, Japan) including 142 and 246 samples from adults and children, respectively, were collected at Mizushima Central Hospital, Okayama, Japan, between November 2020 and August 2021. At the hospital, the diarrheal stools were initially tested for enteric pathogens including adenovirus, norovirus, rotavirus, *Salmonella enterica, Shigella* spp., Shiga toxin-producing *E. coli* (STEC), *Clostridium difficile,* and *Campylobacter* spp. These viral and bacterial pathogens were screened by immunochromatography and culture methods, respectively. To detect and quantitate *E. albertii*, these stool samples were transported from hospital to our laboratory in Cary-Blair transport medium (Becton Dickinson, Sparks, MD, USA), a standard recommended method for transportation of stool/swab samples [26], at ambient temperature and analyzed within a week of collection. Briefly, each swab sample was vigorously stirred in 0.5 mL of sterile PBS (pH 7.4) and the suspended solution was used to prepare template DNA by alkaline lysis method as described above. For enrichment, 100 μL of the prepared PBS suspension was added to 3 mL of TSB and incubated at 37 °C with shaking at 180 rpm for 14 ± 2 h. Glycerol stock as well as DNA templates were prepared using the enriched culture and stored at −80 °C for future use. Informed consent was always taken from patients for using their fecal specimens for research purpose. In case of children, consent was obtained from their respective parents or legal guardians. Clinical analysis conducted in this study was approved by the ethical committee of Osaka Prefecture University (OPUGSLES-19-E12 and OPUGSLES-20-E18).

2.8. Detection, isolation, molecular characterization, and antibiotic sensitivity of E. albertii isolates from stool specimens

Presence of *E. albertii* in the collected stool specimens ($n = 388$) was examined by the newly developed qRT-PCR method described in this study as well as by *E. albertii* specific *Eacdt*-gene PCR assay [21]. Next, *E. albertii* detected by PCR methods in TSB enriched diarrheal stool samples was further checked by the culture method using an *E. albertii* selective medium, called XRM-MacConkey agar developed in our laboratory [27]. *E. albertii* strains obtained by culture method were also used for preparation of agarose blocks containing intact genomic DNA for pulsed-field gel electrophoresis (PFGE) and molecular characterization of *E. albertii* isolates. For PFGE profile analysis, intact *E. albertii* genomic DNA containing agarose slice was digested by the restriction enzyme *Xba*I followed by electrophoresis as recommended in the CDC PulseNet protocol for the *E. coli* genome (https://www.cdc.gov/pulsenet/pathogens/pfge.html).

Presence of *eae, paa, stx1, stx2, stx2f, Eccdt-I* and *Eccdt-IV* virulence genes in the isolated *E. albertii* genomes was examined by the colony hybridization method [28] using ^{32}P-labelled specific gene probes as described previously [23,29]. Further, Stx2f toxin production by *stx2f* gene-positive *E. albertii* was examined by the Vero cell cytotoxicity assay using cell free culture supernatant [30]. In

brief, an aliquot of the overnight grown *E. albertii* strain was inoculated in 3.0 mL of TSB and incubated at 37 °C for 3 h at 180 rpm followed by addition of mitomycin C (Kyowa Hakko Kirin Co., Ltd, Tokyo, Japan) at 0.5 μg/mL concentration in the culture and continuation of incubation of the culture for another 6 h. As a control, in another tube containing bacterial culture without mitomycin C incubation was continued for 9 h at 37 °C with shaking. Next, bacterial culture was subjected to centrifugation at 4 °C, supernatant was collected, filter sterilized using a 0.2 μm pore size filter (Asahi glass Co., Ltd., Tokyo, Japan), and used for the Vero cell cytotoxicity assay. For the assay, Vero cells were seeded at density of 1×10^4 cells in a 96-well plate (Asahi glass Co., Ltd., Tokyo, Japan) and 10 μL of 2-fold serially diluted *E. albertii* cell free culture supernatant was added to wells. The cytotoxic effect was examined microscopically after 72 h of incubation.

Antimicrobial susceptibility tests were carried out by disk diffusion method essentially as recommended earlier (CLSI, 2019) using 15 different antimicrobials disks (BD BBL Sensi-Disc, Becton, Dickinson and Co.) including ampicillin (10 μg), cefotaxime (30 μg), ceftazidime (30 μg), meropenem (10 μg), imipenem (10 μg), cefoxitin (30 μg), fosfomycin (30 μg), gentamicin (10 μg), kanamycin (30 μg), streptomycin (10 μg), tetracycline (30 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg) and sulfamethoxazole/trimethoprim (23.75 μg/1.25 μg) (https://clsi.org/media/3062/clsi-update-2019_21819_final). *E. coli* strain ATCC25922 was used as a quality control.

3. Results

3.1. Evaluation of the Eacdt-gene based quantitative real-time PCR assay

The primers designed in this study when evaluated at NCBI, the hits were only for *E. albertii.* However, when *E. albertii* was excluded from the BLAST analysis, 3 hits each for *E. coli* and *S. boydii* were observed with 100% query coverage and identity. This discrepancy in results could be attributed to the misidentification of *E. albertii* in those studies [6,23]. qRT-PCR assay was standardized using *E. albertii* 19982^T and *E. coli* C600 strains as positive and negative controls, respectively, to see the amplification and dissociation profile of the PCR amplicon. Specific amplification was observed with *E. albertii* 19982^T and the melting temperature (Tm) was determined to be 80.5 °C. As expected, no amplification was obtained from the control strain *E. coli* C600. Sensitivity of the qRT-PCR developed in this study was further evaluated using 39 different clinical *E. albertii* strains (Tables 1 and S1) and desired amplification was obtained from each of these *E. albertii* strains including the type strain 19982^T, which was used as a positive control (Fig. S2). The average Tm value was 80.3 ± 0.16 °C, which is close to the expected Tm and there was no other Tm peak, suggesting that the amplification in each case is specific (Fig. S2). Therefore, the results indicated 100 % sensitivity of the qRT-PCR method developed in this study. Furthermore, specificity assay was also performed with non-*E. albertii* strains ($n = 36$) belonging to 9 different genera with 18 species (Tables 1 and

Table 1
Sensitivity and specificity of *Eacdt* gene-based quantitative real-time PCR assay.

Genus	Species/serovars	*cdt*-gene	Strains tested (*n*)	*Eacdt* qRT-PCR
Escherichia	*albertii*	*Eacdt*[a]	**39**	39[e]
Non-*E. albertii*			**36**	NA
Escherichia	*coli*	*Eccdt*[b]	4	0
	coli	None	5	0
Providencia	*alcalifaciens*	*Pacdt*[c]	3	0
	alcalifaciens	None	4	0
	rustigianii	*Prcdt*[c]	1	0
	rustigianii	None	1	0
	rettgeri	None	1	0
	heimbachae	None	1	0
	stuartii	None	1	0
Shigella	*dysenteriae*	None	2	0
	flexnerii	None	1	0
	sonnei	None	1	0
	boydii	None	1	0
Salmonella	Braenderup	None	1	0
	Typhimurium	None	1	0
	Enteritidis	None	1	0
Campylobacter	*jejuni*	*Cjcdt*[d]	1	0
	coli	*Cccdt*[d]	1	0
	fetus	*Cfcdt*[d]	1	0
Acinetobacter	*baumannii*	None	1	0
Vibrio	*cholerae*	None	1	0
Lactobacillus	*casei*	None	1	0
Clostridium	*difficile*	None	1	0

[a] *E. albertii cdt.*
[b] *E. coli cdt.*
[c] *Providencia cdt.*
[d] *Campylobacter cdt.*
[e] Apart from *E. albertii* type strain 19982^T used as a positive control.

S2). As expected, no amplication was obtained from any of these non-*E. albertii* strains including *cdt* gene-positive bacteria. Thus, the results further support 100 % specificity of the qRT-PCR method developed in this study for *E. albertii*.

3.2. Detection limit of E. albertii and its detection in spiked stool specimens

Detection limit was determined using boiled template DNA as well as purified genomic DNA from *E. albertii* 19982^T strain. The detection limit of the qRT-PCR for boiled template DNA and purified genomic DNA was 10 CFU and 1 pg per PCR tube, respectively (Table 2). *E. albertii* 19982^T was spiked in *E. albertii* free stool sample obtained from an apparently healthy individual. The results of *E. albertii* detection from non-enriched and TSB-enriched spiked stools are summarized in Table 3. In non-enriched spiked stool, PCR amplification was observed up to 4.0×10^3 CFU per ml. On the other hand, in TSB-enriched stool, the limit of detection was 4.0 CFU per mL (after enrichment *E. albertii* cells increased from 4 to 4.3×10^3 CFU/mL).

3.3. Evaluation of Eacdt quantitative real-time PCR with patient stool specimens and its clinical course

Out of 246 stool specimens from children, five gave positive result for *Eacdt* gene by the qRT-PCR method described in this study (Table 4). It is to be noted that these *Eacdt* gene-positive stool samples were negative for other enteric etiological agents including *Salmonella enterica, Shigella* spp., STEC, *Clostridium difficile, Campylobacter* spp., rotavirus, adenovirus, and norovirus (data not shown). The results were also confirmed by using *E. albertii* specific *Eacdt*-gene PCR assay described earlier from this laboratory [21]. None of the stool specimens from adults were positive for *E. albertii* (Table 4). Clinical symptoms of the five *E. albertii* positive patients are chronologically summarized in Table 5.

It is interesting to note that one patient, OKY361, shed *E. albertii* in stool for almost four weeks (Fig. 1). Clinical symptoms such as abdominal pain, nausea and diarrhea began on June 21st and the patient admitted to the hospital on June 23rd. No enteropathogenic bacteria and viruses were detected by hospital diagnosis. However, *E. albertii* was detected in the stool sample collected on June 23rd by both conventional *Eacdt*-gene PCR and qRT-PCR assays (Ct value was 22.5) conducted at Osaka Prefecture University. Lebenin, which contains powder of antimicrobial resistant lactic acid-producing enterococcus was prescribed to the patient for 5 days. Patient continued to experience mild abdominal pain and watery stool till July 3rd, and *E. albertii* was detected in the stool sample only by the qRT-PCR (Ct value was 31.9) but not by conventional PCR assay or by culture method. Subsequently, on July 10th and 17th, although the patient had no symptom, *E. albertii* was detectable by the conventional *Eacdt*-gene PCR, qRT-PCR (Ct values were 27.1 and 31.8, respectively) and by culture method. Fosfomycin was prescribed for 7 days, as a result the stool sample collected on July 28th was negative for *E. albertii* by both conventional PCR and qRT-PCR methods. When tested with DNA templates from non-enriched stool specimens of the patient, *E. albertii* load varied from 9.8×10^3 to 7.1×10^6 CFU/mL between June 23rd and July 17th as determined by the qRT-PCR assay (Table 6).

3.4. Isolation of E. albertii from stool samples

E. albertii was successfully isolated from all five *Eacdt* gene-positive stools using XRM-MacConkey agar (Table 5). One to three colonies were collected from each sample for further analysis. In the case of patient OKY361, who exhibited long-term shedding, *E. albertii* was isolated from three out of four *E. albertii* PCR-positive samples (Fig. 1). Three isolates were collected from the first and third sample, but only one isolate could be obtained from the fourth sample. No *E. albertii* was isolated from second and fifth (PCR negative) samples. It should be emphasized that when PBS suspension of stool specimen obtained on June 23rd was inoculated onto XRM-MacConkey agar plate from 10^{-4} dilution tube, 421 colonies obtained were colorless and most probably *E. albertii* while 17 bacterial colonies were red. On the other hand, when the stool sample of July 3rd was inoculated onto XRM-MacConkey agar, all 361

Table 2
Limit of detection of *E. albertii* by quantitative real-time PCR.

S. No.	Strain	CFU or DNA per PCR tube	qRT-PCR (Ct)
1	*E. albertii* 19,982	1.21×10^4	**22.45**
2		1.21×10^3	**25.87**
3		1.21×10^2	**29.27**
4		1.21×10^1	**31.93**
5		1.21×10^0	–
6	*E. albertii* 19,982	100 ng	**16.06**
7		10 ng	**19.86**
8		1 ng	**22.81**
9		100 pg	**26.1**
10		10 pg	**28.79**
11		1 pg	**31.73**
12		100 fg	–
13	*E. coli* Sakai	1.00E+05	–
14	DW	NA	–

Colony forming unit (CFU), Cycle threshold (Ct).
Ct value of 32 was set as cutoff.

Table 3
Co-relation between Ct value of quantitative real-time PCR and the number of *E. albertii* by spiking experiment with healthy stool specimen.

Spiked *E. albertii* (CFU/mL)	Non-enriched		TSB-enriched[a]	
	qRT-PCR (Ct)	(CFU/mL)	qRT-PCR (Ct)	(Log CFU/mL)
4.0×10^6	22.6	3.31×10^6	15.7	8.04×10^8
4.0×10^5	25.9	2.41×10^5	18.1	1.19×10^8
4.0×10^4	28.7	2.71×10^4	20.9	1.29×10^7
4.0×10^3	31.5	2.96×10^3	23.1	2.19×10^6
4.0×10^2	–	–	26.4	1.58×10^5
4.0×10^1	–	–	28.9	2.24×10^4
4.0×10^0	–	–	30.9	4.37×10^3
0	–	–	–	–

Trypticase soy broth (TSB), Colony forming unit (CFU), Cycle threshold (Ct).
[a] Incubated at 37 °C, with shaking at 180 rpm for 14 h.

Table 4
Detection of *E. albertii* in stool specimens of diarrheal patients by quantitative real-time PCR.

Group	No. of samples	*Eacdt* gene-based qRT-PCR positive (%)
Children[a]	246	5 (2.0)
Adult[b]	142	0 (0)
Total	**388**	**5 (1.3)**

[a] 1–18 years old.
[b] Above 18 years old.

Table 5
Detail information about *E. albertii* positive patients, the results of quantitative real-time PCR, the number of *E. albertii* load and its isolation.

Patient ID	Age/Sex	Date	Symptom	qRT-PCR (Ct)	CFU/mL	*E. albertii* isolation (*n*)
OKY98	13/M	01/03/2021	AP, LS	23.1	4.7×10^6	Yes [3]
OKY203	14/F	21/04/2021	AP	23.8	2.8×10^6	Yes [3]
OKY361	7/F	23/06/2021	AP, N, D	22.5	7.1×10^6	Yes [3]
		03/07/2021	AP, WS	31.9	9.8×10^3	No
		10/07/2021	NS	27.1	2.8×10^5	Yes [3]
		17/07/2021	NS	31.8	1.0×10^4	Yes [1]
		28/07/2021	NS	–	NA	NA
OKY374	2/M	21/07/2021	WS	23.5	3.5×10^6	Yes [3]
		30/07/2021	NS	–	NA	NA
OKY375	3/F	21/07/2021	MS	29.1	6.9×10^4	Yes [3]
		30/07/2021	NS	–	NA	NA

AP, abdominal pain; LS, loose stool; N, nausea; D, diarrhea; WS, watery stool; NS, no symptom; MS, muddy stool; NA, not applicable.

colonies obtained were red. When the same experiment was done with stool samples of July 10th and 17th, 15 and 1 colorless and 499 and 330 red colonies were obtained, respectively (Table 6).

3.5. Characterization of isolated E. albertii

To see the DNA fingerprint of each *E. albertii* strain isolated from five patients, PFGE analysis was performed. As shown in Fig. 2, the isolates obtained from 5 patients showed different PFGE patterns except for strains OKY374 and OKY375 which were isolated from siblings. It should be noted that the isolates obtained from the patient (OKY361) who exhibited prolong shedding, showed almost identical PFGE patterns indicating that *E. albertii* could colonize at least for four weeks in the intestine of the patient. Based on the PFGE results, one isolate was selected from each sample for further analysis.

Antimicrobial susceptibility testing showed that all *E. albertii* isolates were susceptible to 15 different antimicrobials examined in this study. Virulence gene profiling indicated that *E. albertii* strains isolated from five patients carry *eae* and *paa* genes (Table 7). Furthermore, except for *E. albertii* strain OKY203, all the isolates carried *Eccdt*-I genes in addition to *Eacdt.* It should be noted that the *E. albertii* isolates from OKY361 patient were positive for *stx2fA* gene throughout the shedding period (Table 7). The Vero cell cytotoxicity assay revealed the toxicity titer of 2^6 or 2^{10} in the absence or presence of mitomycin C, respectively (data not shown).

4. Discussion

E. albertii has been increasingly recognized as an emerging zoonotic enteropathogen worldwide [22,31]. Although a number of food-poisoning outbreaks and sporadic cases of gastroenteritis have been reported, infection source and route of *E. albertii* remain

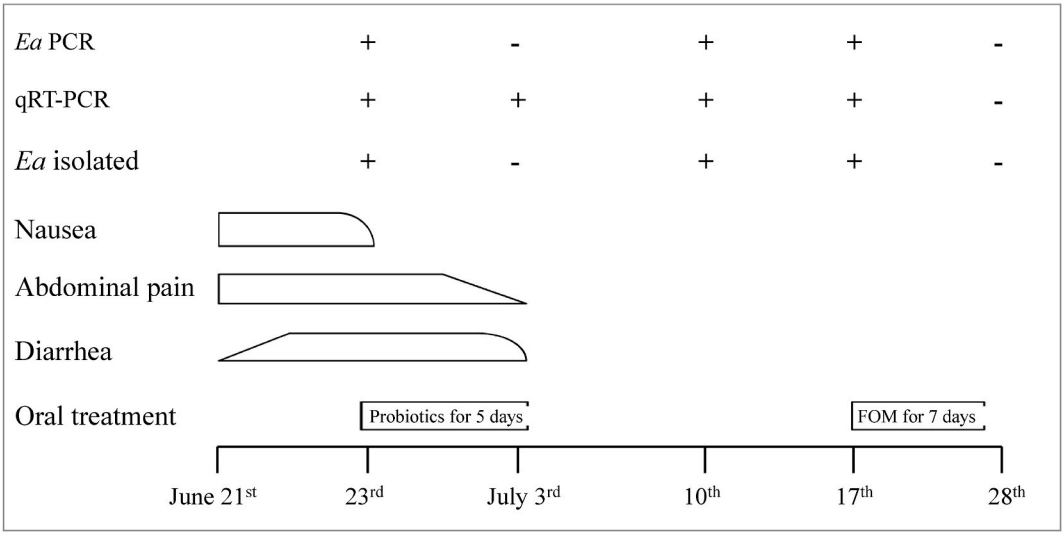

Fig. 1. Clinical course of the patient (OKY361) with prolong shedding of *E. albertii* A 7-year-old girl showed nausea, abdominal pain and diarrhea two days before visiting the hospital. On June 23rd, intermittent abdominal pain and watery stool were observed. No pathogen was detected at the hospital but *E. albertii* (*Ea*) was detected by the conventional (*Ea* PCR), quantitative real-time PCR (qRT-PCR) and by culture method. Lebenin, an antibiotic-resistant lactic acid bacterial formulation as probiotics (3 g/day) was prescribed for 5 days. On July 3rd, patient had no clinical symptom, however, *Ea* was detectable by qRT-PCR assay but not by *Ea* PCR or bacteriological method. No medicine was further prescribed. On July 10th and 17th, patient had no symptom but *Ea* was detectable by *Ea* PCR, qRT-PCR, and bacteriological methods. Fosfomycin (1.6 g/day) was prescribed for 7 days. On July 28th, patient had no symptom and *Ea* was not detectable by *Ea* PCR, qRT-PCR and bacteriological methods.

Table 6
The number of red and colorless colonies on XRM-MacConkey agar (10^{-4} dilution) and Ct value of quantitative real-time PCR of the patient (OKY361).

Date	Red colony (*n*)	Colorless colony (*n*)	qRT-PCR (Ct)	*E. albertii* (CFU/mL)
23 June 2021	17	421	22.5	7.1×10^6
03 July 2021	361	0	31.9	9.8×10^3
10 July 2021	499	15	27.1	2.8×10^5
17 July 2021	330	1	31.8	1.0×10^4

XRM (Xylose-Rhamnose-Melibiose) MacConkey agar.
Bacterial culture plates were incubated at 37 °C for 16 h.

unclear [5,15,16,32,33]. Furthermore, incidence of *E. albertii* infections is still underestimated because reliable test methods have not yet been established [13,22,34]. Some PCR assays targeting different *E. albertii*-specific genes for the detection of *E. albertii* have been reported [6,16,20,21]. However, these PCR assays are all endpoint assays. To address the limitation of endpoint assays in quantifying the *E. albertii*, we have developed an intercalating dye (BRYT™) based qRT-PCR that targets the *Eacdt* genes for the quantification of *E. albertii*. Being an intercalating dye-based qRT-PCR, the assay is economically feasible.

When tested with 39 *E. albertii* and 36 non-*E. albertii* strains belonging to 9 different genera and 18 species, specificity and sensitivity of the qRT-PCR developed in this study were both 100 % (Table 1). Furthermore, the detection limit of the qRT-PCR was 10 CFU and 1 pg of genomic DNA per PCR tube, respectively, when pure culture and purified genomic DNA were employed (Table 2), and 4.0×10^3 and 4.0 CFU per mL before and after enrichment of culture when *E. albertii* was spiked to stool of a healthy person (Table 3). Analysis of 388 stool swabs including 246 children and 142 adults with gastroenteritis revealed that five stool specimens (2.0%) from children were positive for *Eacdt* by the qRT-PCR assay developed in this study (Table 4). Ct values were varied from 29.1 to 22.5 in these five stool samples indicating that 6.9×10^4 to 7.1×10^6 CFU/mL of *E. albertii*, respectively, might be present in the samples suspended in PBS (Table 5). Indeed, *E. albertii* was isolated from each of these 5 stool specimens by using XRM-MacConkey agar, a selective agar for *E. albertii* [27]. It should be noted that none of the other enteric pathogens such as STEC, *Campylobacter, Salmonella,* norovirus, rotavirus and adenovirus were detected in *E. albertii* positive stool specimens (data not shown). These data indicated that *E. albertii* was most likely the causative agent of gastroenteritis in these children. However, as *Eacdt* gene-negative *E. albertii* had been reported earlier [16], we could not rule out the possibility of missing some of those *E. albertii* in the specimens. No non-specific amplification was detected by the qRT-PCR when tested with 388 diarrheal stool swab samples. Furthermore, when we utilized this qRT-PCR to determine the abundance of *E. albertii* in 1606 raccoon rectal swabs, no non-specific amplification was observed [35]. Taken together, these data suggest that the qRT-PCR assay developed in this study is highly specific and may be useful for the detection and quantification of *E. albertii* in clinical samples.

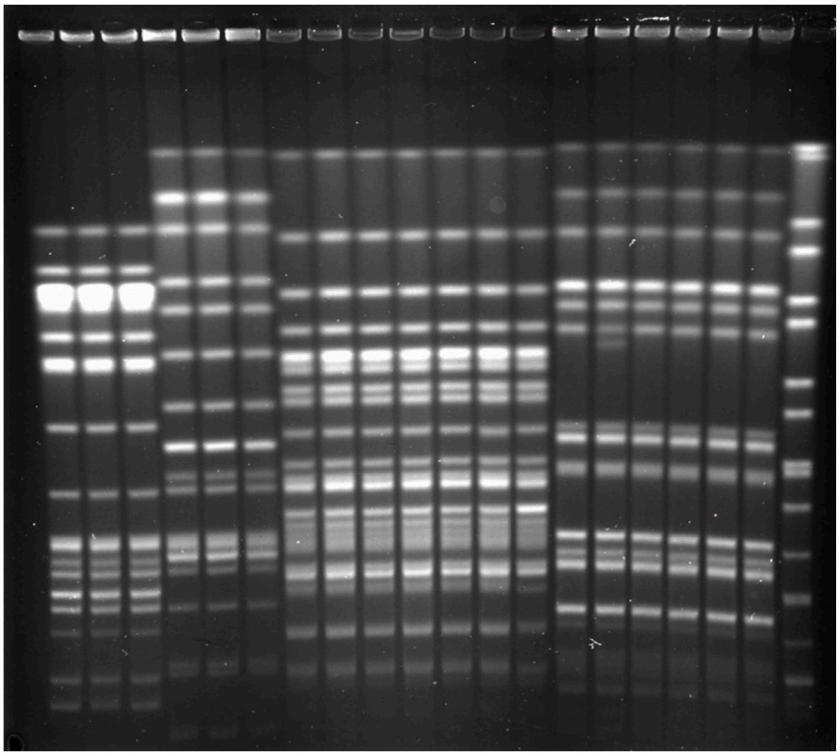

Fig. 2. PFGE profiles of *Xba*I-digested *E. albertii* isolates from diarrheal stool samples of infected children. Lanes: 1–3, *E. albertii* OKY98; 4–6, *E. albertii* OKY203; 7–13, *E. albertii* OKY361; 14–16, *E. albertii* OKY374; 17–19, *E. albertii* OKY375; M, molecular size marker (*Xba*I-digested genomic DNA of *Salmonella* Braenderup strain H9812). In the case of *E. albertii* isolates from patient OKY361, lanes 7–9 are isolates from first sampling, lanes 10–12 from third sampling and lane 13 from fourth sampling.

Table 7
Virulence gene profile of *E. albertii* isolates from diarrheal stool samples.

Strain	*eae*	*paa*	*stx1*	*stx2*	*stx2f*	*Eccdt-I*	*Eccdt-IV*
OKY98	+	+	−	−	−	+	−
OKY203	+	+	−	−	−	−	−
OKY361_1st	+	+	−	−	+	+	−
OKY361_3rd	+	+	−	−	+	+	−
OKY361_4th	+	+	−	−	+	+	−
OKY374-1	+	+	−	−	−	+	−
OKY374-2	+	+	−	−	−	+	−
OKY375	+	+	−	−	−	+	−

eae: gene encoding intimin associated with attaching and effacing lesion.
paa: gene encoding porcine attaching and effacing-associated.
stx: Shiga toxin genes.
cdt: cytolethal distending toxin genes.

It has been reported that long-term shedding of EHEC can occur in some patients and lasts for several weeks to months [36]. A few studies estimated the duration of the EHEC infectious (shedding) period to be < 3 weeks, while others have reported longer durations [36,37]. However, no such information is available regarding *E. albertii* infection. Interestingly, one patient of this study, a 7-year-old girl, excreted *stx2f* gene-positive *E. albertii* for almost four weeks (Fig. 1 and Table 5). If qRT-PCR developed in this study was not employed, prolonged shedding of *E. albertii* in this patient (OKY361) remain undetected. As shown in Fig. 1, although *E. albertii* was detected by PCR as well as bacteriological methods in the first sample collected on June 23rd but it was detectable only by the qRT-PCR assay in the second sample on July 3rd. To the best of our knowledge, this is the first report about prolong excretion of *E. albertii* (at least for 4 weeks) in stool by a recovered patient (Fig. 1 and Table 6). Furthermore, the study also records that patients may excrete *E. albertii* in stool for a long period of time without showing any symptom of the disease.

It is important to note that although the first stool sample of June 23rd gave 421 colorless (*E. albertii* candidates) and 17 red colonies (non-*E. albertii* candidates) on XRM-MacConkey agar, the second sample of July 3rd failed to give colorless colonies and only 361 red colonies were observed. Furthermore, as shown in Table 6, the Ct value of the qRT-PCR was 22.5 corresponding to 7.1×10^6 CFU/mL

of *E. albertii* on June 23rd, but the value declined to 31.9 (9.8×10^3 CFU/mL of *E. albertii*) on July 3rd. Together, these observations suggest that most probably Lebenin prescribed to the patient inhibited the growth of *E. albertii* and helped in restoring the intestinal flora. Despite showing consistent number of red colonies in the selective agar from July 3rd, 15 and 1 colorless colonies were obtained from the stool samples collected on July 10th and 17th, respectively. However, the patient showed no symptoms of the disease during this period. It should be noted that during various sampling instances, randomly selected 46 and 8 colorless colonies out of 421 and 15, as well as one colorless colony, were confirmed to be *E. albertii* by the qRT-PCR (data not shown).

Another interesting observation in this study was *E. albertii* infection in siblings. As shown in Fig. 2, strains OKY374 and OKY375 isolated from siblings showed identical PFGE patterns, indicating that same source of infection or person to person infection between siblings might be involved. It should be noted that no *E. albertii* was detected and isolated from their parents (data not shown). Additionally, PFGE analysis revealed *XbaI* digested different genomic profiles of the *E. albertii* strains isolated from other four patients indicating that diverse genotypes of *E. albertii* are circulating near the hospital area. Recently, it has been reported that natural reservoir of *E. albertii* might be wild animals such as raccoon and wild birds but not ruminants such as cattle for EHEC [29,30,38,39]. Although *E. albertii* has been detected in chicken and bivalves [40] but the positivity rate was not high. These wild animals might contaminate environmental water or agricultural products and become the source of infection. Although there is no evidence showing the infection source to these patients, but these children might be infected by contaminated water or food through wild animals who carried *E. albertii*. Climate changes might affect the area of activity of wild animals. Further studies are required to understand the infection source and route and the qRT-PCR assay developed in this study may help in future epidemiological studies of *E. albertii*.

Some of the *E. albertii* strains isolated from patients with gastroenteritis may possess the *stx2f* genes and produced the Stx2f toxin [3,5]. It is to be noted that Stx2f-producing *E. albertii* has been isolated from a patient with HUS [41]. *E. albertii* has recently been also reported from urinary tract infections and their isolation from patients [42,43]. Furthermore, febrile infection associated with *E. albertii* bacteremia in a 76-year-old woman with gastric dysplasia has also been reported [44]. These observations indicate that *E. albertii* is not only the causative agent of intestinal infections but also has the ability to cause extraintestinal infections. Nevertheless, real burden of *E. albertii* infections still remain unclear due to the lack of a rapid, simple and reliable test method and a specific selective medium for *E. albertii*. The qRT-PCR method developed in this study will hopefully open the door in revealing the true picture of *E. albertii* infection in clinical settings.

In conclusion, a qRT-PCR method targeting *Eacdt* genes, which seem to be ubiquitously present in *E. albertii*, has been developed in this study. The specificity as well as sensitivity of the qRT-PCR is 100%. Utility of the developed qRT-PCR has successfully been demonstrated by clinical investigation by which *E. albertii* was detected from five (2%) stool samples of children with gastroenteritis out of 246 clinical samples examined. This is the first report showing prolong excretion of *E. albertii,* at least for 4 weeks, from a patient with gastroenteritis even during the recovery phase when there was practically no symptom of the disease. Further studies are definitely needed to clarify the importance of *E. albertii* in children with gastroenteritis not only in Japan but also in other countries.

Data availability statement

All data regarding the presented work was included in the main manuscript.

CRediT authorship contribution statement

Sharda Prasad Awasthi: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Akira Nagita:** Resources, Formal analysis, Data curation. **Noritoshi Hatanaka:** Supervision, Data curation. **Jayedul Hassan:** Investigation. **Bingting Xu:** Validation, Investigation. **Atsushi Hinenoya:** Writing – review & editing, Supervision, Project administration, Formal analysis. **Shinji Yamasaki:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

Shinji Yamasaki reports financial support was provided by Japan Society for the Promotion of Science. Shinji Yamasaki reports a relationship with Sakura Cooperation that includes: consulting or advisory. Shinji Yamasaki has patent pending to Detection of Escherichia albertii. None If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e30042.

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