Microbiological Research 262 (2022) 127109

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Longitudinal surveillance and comparative characterization of *Escherichia albertii* in wild raccoons in the United States

Atsushi Hinenoya [a,b,c,d], Huiwen Wang [a], Erin M. Patrick [e], Ximin Zeng [a], Liu Cao [a], Xing-Ping Li [a,f], Rebecca L. Lindsey [g], Barbara Gillespie [a], Qiang He [h], Shinji Yamasaki [b,c,d,*], Jun Lin [a,*]

[a] Department of Animal Science, University of Tennessee, Knoxville, TN, USA
[b] Graduate School of Veterinary Science, Osaka Metropolitan University, Osaka, Japan
[c] Asian Health Science Research Institute, Osaka Metropolitan University, Osaka, Japan
[d] Osaka International Research Center for Infectious Diseases, Osaka Metropolitan University, Osaka, Japan
[e] United State Department of Agriculture, Animal Plant Health Inspection Service, Wildlife Services, Knoxville, TN, USA
[f] College of Animal Science and Technology, Henan University of Science and Technology, Luoyang, China
[g] Centers for Disease Control and Prevention, Atlanta, GA, USA
[h] Department of Civil and Environmental Engineering, University of Tennessee, Knoxville, TN, USA

ARTICLE INFO

Keywords:
Escherichia albertii
Reservoir
Raccoon
Chicken
Environment

ABSTRACT

Escherichia albertii is an emerging enteric bacterial pathogen causing watery diarrhea, abdominal distension, vomiting and fever in humans. *E. albertii* has caused many foodborne outbreaks in Japan and was also reported in other countries worldwide. However, the important animal reservoirs of this pathogen are still largely unknown, impeding us to combat this emerging pathogen. Recently, we reported that wild raccoons (*Procyon lotor*) and broiler chickens are significant reservoirs of *E. albertii* in Japan and the U.S., respectively. Here, we performed a longitudinal surveillance to monitor prevalence of *E. albertii* in wild raccoons in the U.S. and conducted comprehensive comparative analyses of the *E. albertii* of different origins. A total of 289 fecal swab samples were collected from wild raccoons in Tennessee and Kentucky in the U.S. (2018–2020). Approximately 26% (74/289) of the raccoons examined were PCR-positive for *E. albertii* and eventually 22 *E. albertii* isolates were obtained. PFGE analysis showed the U.S. raccoon *E. albertii* were phylogenetically distant even though the corresponding raccoons were captured from a small area. Unlike the high prevalence of multidrug resistance (83%) observed in previous chicken *E. albertii* survey, antibiotic resistance was rarely observed in all the U.S. raccoon and 22 Japan raccoon strains with only one Japan strain displaying multidrug resistance (2%). Whole genome sequencing of 54 diverse *E. albertii* strains and subsequent comparative genomics analysis revealed unique clusters that displayed close evolutionary relationships and similar virulence gene profiles among the strains of different origins in terms of geographical locations (*e.g.*, U.S. and Japan) and hosts (raccoon, chicken, swine, and human). Challenge experiment demonstrated raccoon *E. albertii* strains could successfully colonize in the chicken intestine at 3 and 8 days postinfection. A pilot environmental survey further showed all the four tested water samples from Tennessee river were *E. albertii*-positive; two different *E. albertii* strains, isolated from a single water sample, showed close relationships to those of human origin. Together, the findings from this study provide new insights into the ecology, evolution, and pathobiology of *E. albertii,* and underscore the need to control the emerging *E. albertii* in a complex ecosystem using One Health approach.

1. Introduction

Escherichia albertii is an emerging zoonotic foodborne pathogen causing watery diarrhea, abdominal distention, vomiting and fever in humans (Bhatt et al., 2019; Huys et al., 2003; Oaks et al., 2010). This bacterium has been frequently misidentified as other bacterial species

* Corresponding author at: Graduate School of Veterinary Science, Osaka Metropolitan University, Osaka, Japan.
* Correspondence to: Department of Animal Science, The University of Tennessee, 2506 River Drive, Knoxville, TN 37996-4574, USA.
E-mail addresses: shinji@vet.osakafu-u.ac.jp (S. Yamasaki), jlin6@utk.edu (J. Lin).

https://doi.org/10.1016/j.micres.2022.127109
Received 6 April 2022; Received in revised form 27 June 2022; Accepted 28 June 2022
Available online 2 July 2022

Table 1
Major *E. ablbertii* strains used in this study.

Name	Origin	Country	Collection site (year) or modification[a]	Source or Reference
TN18229	Raccoon	US	Hawkins, TN (2018)	This study
TN18236	Raccoon	US	Hawkins, TN (2018)	This study
TN18249	Raccoon	US	Sullivan, TN (2018)	This study
TN19003	Raccoon	US	Sullivan, TN (2019)	This study
TN19007	Raccoon	US	Washington, TN (2019)	This study
TN19201	Raccoon	US	Sullivan, TN (2019)	This study
TN19305	Raccoon	US	Marion, TN (2019)	This study
KY19009	Raccoon	US	Harlan, KY (2019)	This study
TN19001	Raccoon	US	Sullivan, TN (2019)	This study
TN19004	Raccoon	US	Sullivan, TN (2019)	This study
TN19006	Raccoon	US	Sullivan, TN (2019)	This study
TN19116	Raccoon	US	Hamilton, TN (2019)	This study
TN19123	Raccoon	US	Hamilton, TN (2019)	This study
TN19202	Raccoon	US	Sullivan, TN (2019)	This study
TN19207	Raccoon	US	Sullivan, TN (2019)	This study
TN19226	Raccoon	US	Hawkins, TN (2019)	This study
TN19357	Raccoon	US	Marion, TN (2019)	This study
TN19310	Raccoon	US	Marion, TN (2020)	This study
TN19336	Raccoon	US	Marion, TN (2020)	This study
TN20127	Raccoon	US	Hamilton, TN (2020)	This study
TN20348	Raccoon	US	Marion, TN (2020)	This study
TN20403	Raccoon	US	Marion, TN (2020)	This study
RAC-7A	Raccoon	Japan	Osaka	Hinenoya et al. (2020a)
RAC-34A	Raccoon	Japan	Osaka	Hinenoya et al. (2020a)
RAC-44A	Raccoon	Japan	Osaka	Hinenoya et al. (2020a)
RAC-58	Raccoon	Japan	Osaka	Hinenoya et al. (2020a)
RAC-199	Raccoon	Japan	Osaka	Hinenoya et al. (2020a)
RAC-247	Raccoon	Japan	Osaka	Hinenoya et al. (2020a)
RAC-258	Raccoon	Japan	Osaka	Hinenoya et al. (2020a)
RAC-263	Raccoon	Japan	Osaka	Hinenoya et al. (2020a)
RAC-266	Raccoon	Japan	Osaka	Hinenoya et al. (2020a)
RAC-281	Raccoon	Japan	Osaka	Hinenoya et al. (2020a)
RAC-300	Raccoon	Japan	Osaka	Hinenoya et al. (2020a)
RAC-305A	Raccoon	Japan	Osaka	Hinenoya et al. (2020a)
RAC-310A	Raccoon	Japan	Osaka	Hinenoya et al. (2020a)
RAC-324	Raccoon	Japan	Osaka	Hinenoya et al. (2020a)
RAC-342	Raccoon	Japan	Osaka	Hinenoya et al. (2020a)
RAC-357A	Raccoon	Japan	Osaka	Hinenoya et al. (2020a)
RAC-409A	Raccoon	Japan	Osaka	Hinenoya et al. (2020a)
RAC-410A	Raccoon	Japan	Osaka	Hinenoya et al. (2020a)
RAC-414	Raccoon	Japan	Osaka	Hinenoya et al. (2020a)
RAC-431A	Raccoon	Japan	Osaka	Hinenoya et al. (2020a)
P2855	Human	Japan	Okayama	Hinenoya et al. (2017)
P5093	Human	Japan	Okayama	Hinenoya et al. (2017)
P6796	Human	Japan	Okayama	Hinenoya et al. (2017)
P3502	Human	Japan	Okayama	Hinenoya et al. (2017)
P6648	Human	Japan	Okayama	Hinenoya et al. (2017)
AKT130	Human	Japan	Akita	Hinenoya et al. (2019a)
JCM17328^T	Human	Bangladesh	Dhaka, original ID:19982	Albert et al. (1991)
Sw-9	Swine	Japan	Nara	Hinenoya et al. (2014)
WB7-2	Wild bird	Japan	Kochi	Hinenoya et al. (2021)b
WB7-4	Wild bird	Japan	Kochi	Hinenoya et al. (2021)b
TN-C1	Chicken	US	TN broiler farm (2019)	Hinenoya et al. (2021)a
PS211	Chicken	US	MS broiler farm (2020)	Wang et al. (2022)
PS107	Chicken	US	MS broiler farm (2020)	Wang et al. (2022)
PT102	Chicken	US	AL broiler farm (2020)	Wang et al. (2022)
JL1694	River	US	TN (2020)	This study
JL1695	River	US	TN (2020)	This study
JL1513	Raccoon	US	TN18229/pZE21, Kan^r	This study
JL1514	Raccoon	US	TN18236/pZE21, Kan^r	This study
JL1515	Raccoon	US	TN18249/pZE21, Kan^r	This study

[a] The name of specific county where the raccoon was captured is indicated for each US raccoon sample. The number in parenthese indicates sampling year of the corresponding US strain. Abbreviation of specific state in the US: TN, Tennessee; KY, Kentucky; MS, Mississippi; AL, Alabama. Kan^r, kanamycin resistant

due to phenotypic and genotypic resemblance with other members belonging to the family *Enterobacteriaceae*, such as enteropathogenic or enterohemorrhagic *E. coli* (Gomes et al., 2020). This bacterium can express type III secretion system and *eae*-encoded adhesin called intimin, resulting in the formation of attaching-and-effacing (A/E) lesions on host intestinal epithelium (Bhatt et al., 2019). *E. albertii* also produces cytolethal distending toxin (CDT) and occasionally Shiga toxin (Stx) that may lead to intoxication and death of epithelium (Gomes et al., 2020). The *cdt* gene in *E. albertii* (*Eacdt*) has been targeted for PCR-based rapid and accurate identification of *E. albertii* (Hinenoya et al., 2019b).

Although the clinical importance of *E. albertii* has been increasingly recognized worldwide, epidemiological studies of this emerging pathogen are still severely lacking, particularly in terms of its major animal reservoirs and environmental prevalence. This significant knowledge gap has impeded us to develop effective strategies for prevention and control of the emerging *E. albertii* infections in humans (Bhatt et al., 2019). Recently, examination of the prevalence of *E. albertii* in the broiler chickens from various farms in multiple U.S. states provided direct evidence showing chickens as an important reservoir for human *E. albertii* pathogen (Hinenoya et al., 2021; Wang et al., 2022). Intriguingly, our recent study indicated raccoons also could be a significant reservoir of *E. albertii* in Japan and the isolates from raccoon had the potential to cause human diseases (Hinenoya et al., 2020a). Approximately 58% (248/430) of raccoons examined were *E. albertii*-positive (Hinenoya et al., 2020a). Given that wild raccoons in Japan were initially introduced from the U.S. and currently raccoons are deemed as invasive animals with increasing numbers worldwide (Hinenoya et al., 2020a), the raccoons may serve as a significant natural wildlife host to carry and disseminate *E. albertii* worldwide. Notably, raccoons reside in a wide range of habitats with preference to live in areas near a source of water and prey on various animals, such as small mammals, fish, birds, amphibians, reptiles, and insects. Raccoons also forage for vegetables, crops, and fruits in the fields as well as feeds in food animal farms (Larivière, 2004). Therefore, raccoons can potentially serve as an active carrier for *E. albertii* in complex agroecosystem and

may play a critical role in the dynamic interactions among the enteric *E. albertii* pathogen, animals, humans, and their shared environment, which is considered within the One Health umbrella for disease control.

In this study, we performed a longitudinal surveillance to monitor prevalence of *E. albertii* in wild raccoons in the U.S. Using the *E. albertii* of different origins from geographically diverse areas, comprehensive microbiological, molecular, comparative genomics, and animal studies were performed as well. The novel findings from this study provide insights into the ecology, evolution, and pathobiology of *E. albertii,* and underscore the need to control the emerging *E. albertii* in a complex ecosystem using One Health approach.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Major *E. albertii* strains and their sources are listed in Table 1. The *E. albertii* strains were routinely grown in Luria-Bertani broth (LB; Becton, Dickinson and Company, Franklin Lakes, NJ) or tryptic soy broth (TSB; Becton, Dickinson and Company) with shaking (250 rpm) at 37 °C overnight. When necessary, culture media were supplemented with kanamycin (50 μg/mL).

2.2. PCR for detection and validation of E. albertii

PCR was performed for diagnosis and characterization of *E. albertii*. Each PCR was performed with a 20 μL mixture using GoTaq® Green Master Mix (Promega, Madison, WI). The DNA template was subjected to PCR using the primer pair (forward, GCTTAACTGGATGATTCTTG; reverse, CTATTTCCCATCCAATAGTCT) targeting *E. albertii* cytolethal distending toxin (*Eacdt*) gene (469 bp) that is highly specific for *E. albertii* (Hinenoya et al., 2019b). The temperature-cycling parameters are as follows: initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation (95 °C for 0.5 min), annealing (50 °C for 0.5 min), and extension (72 °C for 1 min), with a final extension at 72 °C for 2 min

2.3. Detection and isolation of E. albertii from raccoons in the U.S

To sample raccoon feces, rectal swabs were collected from cage-trapped wild raccoons in November-December over three successive years (2018–2020) in Tennessee and Kentucky. The raccoon fecal samples collected in 2018 were placed in individual plastic bags and kept at 4 °C while the Culture Swab™ Cary-Blair Agar Transport System (BD BBL™) was used for those collected in 2019 and 2020.

The swabs were subjected to *E. albertii* detection and isolation procedure according to previous publications (Hinenoya et al., 2019b, 2021, 2020b; Wang et al., 2022). Briefly, each raccoon fecal swab was suspended in l mL sterile saline. An aliquot (200 μL) of the suspension was inoculated into 4 mL of TSB and grown for enrichment at 37 °C for 14–16 h with vigorous shaking. Subsequently, a total of 500 μL enrichment culture was used for preparation of DNA template for PCR by using the boiling method as described previously (Hinenoya et al., 2019b). DNA templates were subjected to PCR as described above. The DNA from *E. albertii* JCM17328^T (Table 1) was used as a positive control. Upon identification of a PCR-positive sample, both the corresponding original fecal suspension and the enriched culture in TSB were serially diluted with sterile PBS and spread on XRM-MacConkey agar plates, a selective medium we recently developed for isolation of *E. albertii* from clinical samples (Hinenoya et al., 2020b). Following 24 h of incubation at 37 °C, the suspected *E. albertii* colonies (white color) on agar plates were selected (up to 10 colonies per swab sample) and individually inoculated into 200 μL TSB. After the incubation at 37 °C for 3 h, 1 μL of bacterial culture was directly used as template for the *Eacdt*-targeting PCR as described above. The PCR-positive culture was subsequently streaked on XRM-MacConkey agar plate again and incubated for 18 h at 37 °C; a single colony was picked, inoculated into 3 mL of TSB, and grown at 37 °C with vigorous shaking for 4 h. After confirmation of the culture using another round of *Eacdt*-targeting PCR and Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI--TOF MS) analysis as described previously (Hinenoya et al., 2021), the *E. albertii* culture was stored with 25% glycerol at -80 °C. If multiple isolates were obtained from single swab sample, one representative isolate was randomly selected for further analyses described below.

2.4. Detection and isolation of E. albertii from river samples

The water samples were collected from two creeks (*i.e.*, Fourth Creek and Ten Mile Creek, tributary of Tennessee river) in Knox County of Tennessee in both June and August, 2020. Briefly, the stream water was collected by submerging a sterile 1 L polypropylene bottle at least two inches below the stream surface for representation of typical base flow per standard methods (Wilde, 2010). All samples were stored on ice-packs during transportation and were analyzed within 6 h. The biomass in 1 L of water sample was captured on 0.45-μm nitrocellulose membrane filter (Fisher brand, Cat. No. 09–719–2E) using MilliporeSigma filtration system. Subsequently, membrane filter was placed in 5 mL of TSB and grown for enrichment at 37 °C for 14–16 h with vigorous shaking. The procedure of *E. albertii* detection and isolation was the same as described above for raccoon fecal samples except that up to 96 putative colonies per water sample were randomly picked from XRM-MacConkey agar plates for screening *E. albertii*.

2.5. Pulsed-field gel electrophoresis (PFGE)

PFGE of the macrorestriction fragment patterns of genomic DNA using *Xba*I enzyme was performed by following the Centers for Disease Control and Prevention (CDC) standardized protocol for *E. coli* (Gautom, 1997; Ribot et al., 2006) with slight modifications on electrophoresis conditions. A *Salmonella* serotype Braenderup strain (H9812) was used as the universal size standard (Hunter et al., 2005). The electrophoresis was performed using a CHEF Mapper XA system (Bio-Rad, Hercules, CA) and the electrophoretic conditions were as follows: initial switch time, 2.2 s; final switch time, 54.2 s; run time, 17 h; angle, 120°; gradient, 6.0 V/cm; temperature, 14 °C; ramping factor, linear. The PFGE image was analyzed in the software GelCompar II (Applied Maths, Belgium) by following its standard protocol.

2.6. Antimicrobial susceptibility testing

Antimicrobial susceptibility assay was performed using the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (M02-A12, 2015). The *E. albertii* strains were tested against 20 antimicrobial agents belonging to 14 classes, which include ampicillin (10 μg), piperacillin (100 μg), cephalothin (30 μg), cefuroxime (30 μg), ceftriaxone (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), cefoxitin (30 μg), cefepime (30 μg), azthreonam (30 μg), meropenem (10 μg), imipenem (10 μg), streptomycin (10 μg), kanamycin (30 μg), gentamicin (10 μg), chloramphenicol (30 μg), tetracycline (30 μg), ciprofloxacin (5 μg), nalidixic acid (30 μg), and trimethoprim-sulfamethoxazole (1.25 μg/23.75 μg). The antimicrobial disks were purchased from Becton, Dickinson and Company. The *E. coli* strain ATCC 25922 served as quality control. The sizes of growth inhibition zones were interpreted based on the criteria of CLSI M100 (ED30:2020) for the order Enterobacterales.

2.7. Whole genome sequencing

A total of 54 diverse *E. albertii* strains were subjected to whole genome sequence (WGS) analysis, including those isolated in this study (22 U.S. raccoon and 2 U.S. water strains) and those isolated in Japan (20 raccoon, 7 human, 1 swine, and 2 bird strains) (Table 1). Genomic DNA of each strain was sequenced using Illumina Miseq Desktop

Sequencer at Iowa State University Veterinary Diagnostic Laboratory (Ames, IA). The procedures, including genomic DNA extraction and purification, library preparation, sequencing, and *de novo* assembly of draft genome, were described in previous publications (Hinenoya et al., 2021; Wang et al., 2022).

2.8. Comparative genomics analysis

Before conducting genomics analysis, completeness and contamination of each assembled draft genome were assessed *via* CheckM (v1.0.18) (Parks et al., 2015) in KBase server (https://www.kbase.us/) (Arkin et al., 2018). In addition, to further confirm that the isolates were indeed *E. albertii*, complete genome sequence of *E. albertii* strain KF1 (Fiedoruk et al., 2014) was used as a reference to calculate its average nucleotide identity (ANI) to the draft genome of each strain *via* FastANI (v0.1.3) (Jain et al., 2018) in KBase server (Arkin et al., 2018). If the ANI value exceeded 95%, the isolate could be confirmed as *E. albertii* species (Jain et al., 2018).

To identify antimicrobial resistance (AR) genes and plasmid replicon types of the *E. albertii* strains, draft genome of each strain was uploaded to ResFinder (v4.1) (Bortolaia et al., 2020) and PlasmidFinder (v2.1) (Carattoli et al., 2014), respectively, in Center for Genomic Epidemiology (http://www.genomicepidemiology.org/). For the querying, minimum sequence identity and coverage were set as 90% and 80%, respectively.

Whole genomes of a total of 68 diverse *E. albertii* strains were subjected to phylogenetic analysis, including draft genomes of the 54 *E. albertii* strains obtained from this study and the 4 strains from chicken from our recent studies (Hinenoya et al., 2021; Wang et al., 2022), as well as complete genomes of other 10 geographically diverse *E. albertii* from public database (9 human isolates and 1 bird isolate) (Gomes et al., 2020). The phylogenetic analysis was based on whole-genome single nucleotide polymorphism (wgSNP) using kSNP (v3.0) software (Gardner et al., 2015) without reference genome. The resulting phylogenetic tree was visualized using Interactive Tree of Life (https://itol.embl.de/) (Letunic and Bork, 2019). Whole genomes of the 4 chicken strains from our recent studies (Hinenoya et al., 2021; Wang et al., 2022) and the 10 strains from public database (Gomes et al., 2020) were retrieved from NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) with accession numbers as follows: KF1, CP007025; 1551-2, CP025317; 2014C-4356, CP024282; NIAH_Bird_3, AP014855; EC06-170, AP014857; CB9786, AP014856; 2012EL-1823B, CP030783; 2014C-4015, CP034166; NCTC 9362, CP034213; 06-3542, CP034162; TN-C1, SAMN14781548; PT102, SAMN20716681; PS211, SAMN20716680; PS107, SAMN20716676.

To examine virulence factors present in the above 68 *E. albertii* strains, draft or complete genome of each strain was uploaded to Virulence Factor Database (http://www.mgc.ac.cn/VFs/) for analysis with a collection of different pathotypes of *E. coli* as the reference (Liu et al., 2019). The major virulence factors were further selected based on previous publications (Bhatt et al., 2019; Gomes et al., 2020). Virulence factor profile of each *E. albertii* strain was incorporated with the above phylogenetic tree and visualized using Interactive Tree of Life (Letunic and Bork, 2019).

2.9. Chicken colonization experiment

The chicken experiment was approved by Institutional Animal Care and Use Committee at The University of Tennessee (IACUC No. 2597) prior to start of the experiment. One-day-old broiler chicks were obtained from Pilgrim's Pride Corporation (Cohutta, GA). The chickens were negative for *E. albertii* as determined by culturing cloacal swabs prior to use in this study. All chickens were managed in a sanitized wire-floor cage and provided with water and antibiotic-free feed *ad libitum* throughout the trial.

Newly hatched broiler chicks were assigned into four groups (15 chicks per group). Following four days of acclimatization, the chicks were challenged with PBS (control), or one of the three raccoon *E. albertii* strain derivatives (JL1513, JL1514, and JL1515; Table 1). Inoculation was performed *via* oral gavage using the dose of approximately 10^8 CFU bacteria per bird. Notably, given the consistent lack of kanamycin-resistant bacterial populations in newly hatched chicks, to increase ease and accuracy to differentiate the inoculated *E. albertii* from other organisms on selective agar plates, we introduced a small plasmid pZE21 (Lutz and Bujard, 1997) into parent raccoon *E. albertii* strains (TN18229, TN18236, and TN18249; Table 1) to confer kanamycin resistance in their derivatives. For each group, five birds were euthanized and cecal samples were collected at 3, 9 and 15 days post-infection (DPI). The cecal contents from each bird were weighed and subsequently suspended and serially diluted in PBS. The diluted samples were plated on the selective XRM-MacConkey agar plates supplemented with 50 μg/mL of kanamycin. Following 24 h of incubation at 37 °C, the suspected white colonies of potential *E. albertii* isolates were enumerated. Three colonies from each plate were randomly selected and subjected to *E. albertii* confirmation by using the *Eacdt* gene-based PCR assay (Hinenoya et al., 2019b). The CFU of *E. albertii* per g of cecal contents was calculated for each chicken and used as an indicator of the colonization level. The detection limit of the plating methods was 100 CFU/g of cecal contents.

Table 2
Longitudinal surveillance and isolation of *E. albertii* in wild raccoons in Tennessee (TN) and Kentucky (KY) in the U.S.

Sampling duration	Sample No.	County No.	No. of PCR Positive (%)	No. of Isolates
11/27/2018 – 12/07/2018	32	3 in TN	6 (18.8%)	3
11/06/2019 – 12/11/2019	105	10 in TN, 1 in KY	36 (34.3%)	14
11/17/2020 – 12/11/2020	152	6 in TN	32 (21.1%)	5
TOTAL	289	14	74	22

2.10. In vitro stability of pZE21 in E. albertii strains

Since introduction of the Kan^r pZE21 plasmid in the inoculated *E. albertii* may lead to an underestimate of *E. albertii* colonization in the chicken intestine due to potential plasmid stability issues, an *in vitro* experiment for assessing the stability of pZE21 in *E. albertii* was performed in parallel to the chicken colonization experiment. Briefly, each of the pZE21-bearing strains (JL1513, JL1514, and JL1515; Table 1) was inoculated in 5 mL of antibiotic free LB broth for overnight growth at 37 °C with vigorous shaking (250 rpm). Approximately 5 μL of the overnight culture was transferred into 5 mL of sterile LB broth for subculture every day for up to 21 days. At different passage days (*i.e.*, 1, 3, 7, 14, and 21), the culture was serially diluted in sterile PBS and the diluted samples were plated on LB agar plates without any antibiotics for enumeration of total *E. albertii* population, and LB agar plates supplemented with the Kan (50 μg/mL) for enumeration of the pZE21-carrying *E. albertii*.

2.11. Data availability

Genome assemblies of the 54 *E. albertii* strains obtained from this study were deposited in NCBI Sequence Read Archive under BioProject PRJNA814751.

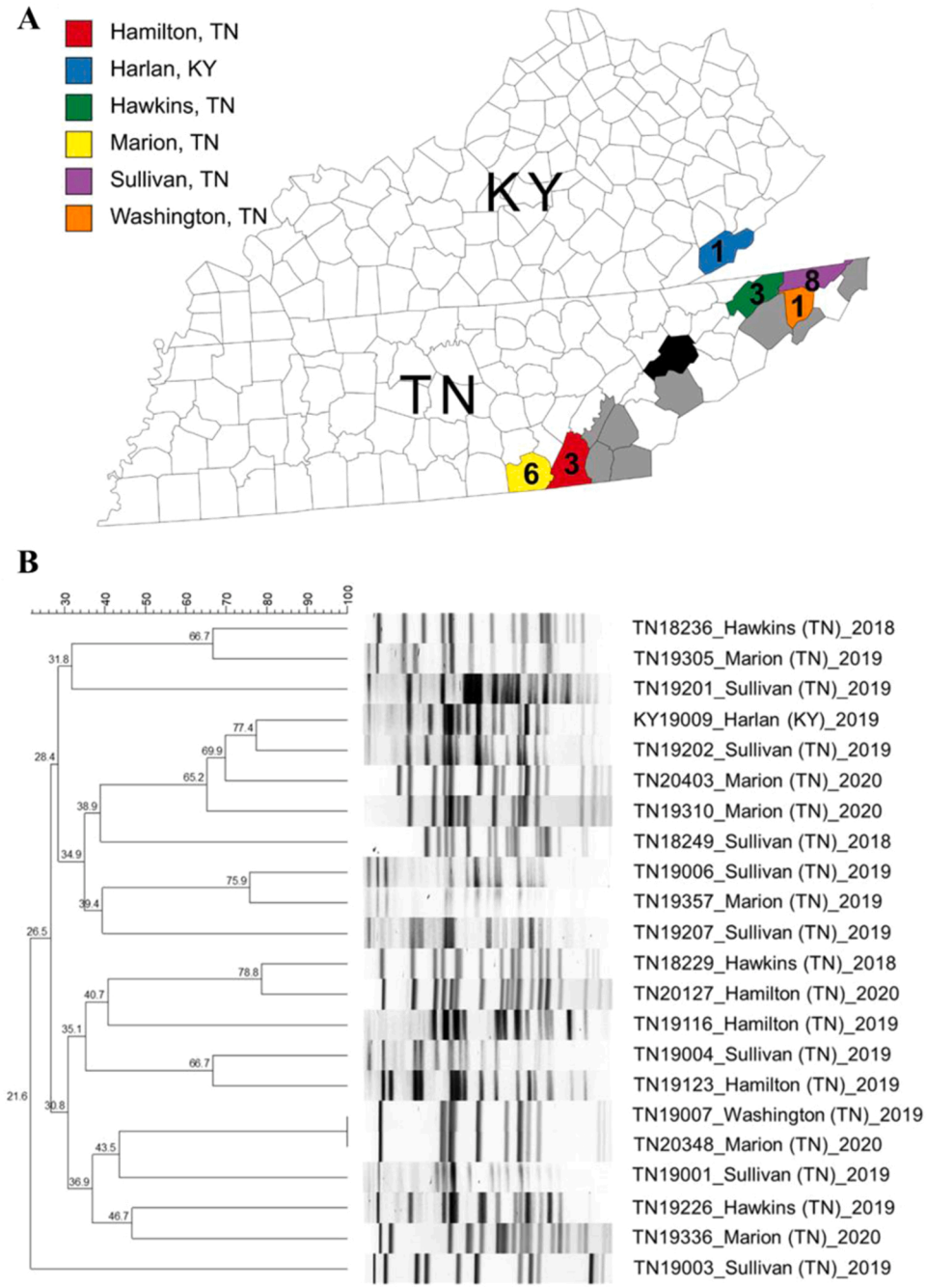


Fig. 1. Phylogenetic relationship of the *Escherichia albertii* strains isolated from wild raccoons in the U.S. (A) Geographical locations of the wild raccoons captured in Tennessee (TN) and Kentucky (KY) over three-year period (2018 – 2020). *E. albertii* was successfully isolated in the fecal samples from the raccoons trapped in the counties highlighted in bright color; the total number of isolated *E. albertii* in corresponding county was indicated. The counties highlighted in dark gray indicated the areas where raccoons were sampled but *E. albertii* was failed to be isolated from fecal samples. The water samples were collected in Knox county highlighted in solid black. (B) Pulsed-field gel electrophoresis (PFGE) analysis and phylogenetic tree of the U.S. raccoon *E. albertii* strains. The Genomic DNA of each *E. albertii* strain was digested with *Xba*I and separated by PFGE. Each US strain is labeled using format of "strain name_county (state) _year".

3. Results

3.1. *E. albertii* was isolated from wild raccoons in Tennessee and Kentucky

Our recent study has shown high prevalence of *E. albertii* in raccoons in Japan (Hinenoya et al., 2020a), which prompted us to determine if the raccoons in the U.S. could also serve as a significant reservoir for *E. albertii*. As shown in Table 2, a total of 32, 105, and 152 raccoon fecal swab samples were collected in fourteen counties in 2018, 2019, and 2020, respectively, for the survey of *E. albertii*.

In the initial survey in 2018, the 32 rectal swab samples were collected from the raccoons captured in three counties in East Tennessee. Six of 32 fecal samples (18.8%) from wild raccoons were PCR-positive for *E. albertii*. Given the high relative abundance of other enteric bacterial populations on selective plates (pink and red colonies) in some raccoon fecal samples, we were only able to isolate *E. albertii* from three PCR-positive raccoon samples (one to three isolates obtained per sample). One representative isolate was selected from each sample and further confirmed as *E. albertii* using both PCR and MALDI-TOF MS analyses. The 3 raccoon *E. albertii* strains isolated from the fecal samples in 2018 are described in Table 1.

In the second large-scale survey in 2019, the fecal samples were collected from 105 raccoons captured in a broad area in Tennessee (ten counties) and Kentucky (one county). Of the 105 fecal samples, 36 (34.3%) were PCR-positive for *E. albertii*. We finally isolated *E. albertii* from 14 PCR-positive raccoon samples with only one isolate successfully identified from each raccoon sample. Each isolate was further confirmed as *E. albertii* using PCR. The 14 raccoon *E. albertii* strains are described in Table 1.

Table 3
Antimicrobial resistance data of the tested *E. albertii* strains that displayed resistance to at least one antibiotic.

Class	Antimicrobial (μg)	Breakpoints (R, I, S)	TN19001 (raccoon, US)	TN19116 (raccoon US)	TN19226 (raccoon, US)	JL1694 (river, US)	RAC-342 (raccoon, Japan)	P3502 (human, Japan)	P6648 (human, Japan)	AKT130 (human, Japan)	JCM17328 (human, Bangladesh)	SW-9 (pig, Japan)	WB7-2 (bird, Japan)	WB7-4 (bird, Japan)
1st generation cephalosporin	cephalothin (30)	14, 15–17, 18	14 (R)	14 (R)	14 (R)	13 (R)	14 (R)	12 (R)	12 (R)	14 (R)	16 (I)	13 (R)	14 (R)	14 (R)
2nd generation cephalosporin	cefuroxime (30)	14, 15–17, 18	23	24	23	22	20	20	20	22	21	22	21	21
3rd generation cephalosporin	cefotaxime (30)	22, 23–25, 26	30	32	30	30	26	28	29	27	29	28	29	28
	ceftazidime (30)	17, 18–20, 21	26	29	26	28	23	25	25	23	27	25	26	25
	ceftriaxone (30)	19, 20–22, 23	20	32	29	28	24	25	28	27	30	27	28	27
4th generation cephalosporin	cefepime (30)	18, 19–24, 25	32	31	32	30	26	29	30	29	31	31	31	31
Carbapenem	imipenem (10)	19, 20–22, 23	27	28	25	27	26	25	28	23	26	25	26	26
	meropenem (10)	19, 20–22, 23	32	31	28	30	31	30	31	27	30	28	29	29
Penicillin	ampicillin (10)	13, 14–16, 17	20	20	20	20	7 (R)	17	7 (R)	7 (R)	19	19	18	19
	piperacillin (100)	17, 18–20, 21	25	28	25	26	11 (R)	24	12 (R)	15 (R)	24	26	26	26
Monobactam	aztreonam (30)	17, 18–20, 21	31	31	29	30	25	26	29	28	27	28	27	27
Cephamycin	cefoxitin (30)	14, 15–17, 18	24	25	24	23	21	21	22	22	25	23	24	24
Aminoglycoside	kanamycin (30)	13, 14–17, 18	21	21	20	20	6 (R)	25	6 (R)	18	20	19	19	19
	streptomycin (10)	11, 12–14, 15	17	16	17	17	19	8 (R)	6 (R)	15	17	15	16	16
	gentamicin (10)	12, 13–14, 15	24	22	23	22	23	32	25	19	21	20	20	20
Tetracycline	tetracycline (30)	11, 12–14, 15	23	23	19	22	22	7 (R)	7 (R)	23	7 (R)	21	23	22
New quinolone	ciprofloxacin (5)	21, 22–25, 26	32	39	33	35	22	34	28	32	30	30	30	30
Quinolone	nalidixic acid (30)	13, 14–18, 19	24	24	22	24	24	7 (R)	25	23	23	23	21	22
Metabolic inhibition	trimethoprim-sulfamethoxazole (1.25/23.75)	10, 11–15, 16	26	27	28	28	30	30	6 (R)	12 (I)	23	26	27	26
Chloramphenicol	chloramphenicol (30)	12, 13–17, 18	25	27	27	28	6 (R)	29	6 (R)	19	27	23	28	28

The value indicates diameter size (mm) of growth inhibition zone.

In the large-scale survey performed in 2020, the rectal swab samples were collected from 152 raccoons captured in a broad area in Tennessee (six counties). Of the 152 fecal samples, 32 (21.1%) were PCR-positive for *E. albertii*. Due to high false positive rate of the randomly selected *E. albertii*-like colonies on the XRM-MacConkey selective agar plates, we finally only isolated *E. albertii* from 5 PCR-positive raccoon samples with one to four isolates obtained from each sample. One representative isolate was selected from each sample and further confirmed as *E. albertii* using PCR. The 5 raccoon *E. albertii* strains are described in Table 1.

Together, a total of 22 U.S. raccoon *E. albertii* strains were successfully isolated during the 3-year surveillance, which is briefly summarized in Table 2. Geographical locations of the wild raccoons captured in different counties over the three year (2018–2020), particularly those counties with success for isolation of *E. albertii* from trapped raccoons, are highlighted in the map as shown in Fig. 1A.

3.2. *E. albertii was detected and isolated in river samples*

All the four examined water samples were PCR-positive for *E. albertii*. However, despite our intensive isolation effort by screening up to 96 randomly selected *E. albertii*-like colonies on the XRM-MacConkey selective agar plates for each individual sample, we only obtained *E. albertii* isolates from one sample, which was collected in Fourth Creek in June, 2020. Specifically, we obtained four *E. albertii* isolates from this water sample. PFGE analysis revealed two clusters of the isolates, with identical PFGE patterns within each cluster (Data not shown). Thus, two different water *E. albertii* strains were selected and designated as JL1694 and JL1695 (Table 1).

3.3. *Genome diversity of the U.S. raccoon E. albertii strains*

To understand the phylogenetic relationships among the isolated U. S. raccoon *E. albertii* strains, the DNA fingerprints of these isolates were examined by PFGE (Fig. 1B). In general, the 22 U.S. raccoon *E. albertii* displayed significant genomic diversity even for those isolated from the raccoons trapped in same area within the same year. For example, with respect to the three strains (TN18229, TN18236, and TN18249) isolated from the samples in 2018 (Table 1), the corresponding three raccoon hosts were captured from a small area with distance as short as 0.78 km (TN18229 and TN18236) and no more than 38.8 km apart (TN18236 and TN18249). However, clearly, the three raccoon *E. albertii* strains were phylogenetically distant as shown by PFGE analysis (Fig. 1B). Similar to what we observed in the raccoon surveillance in 2018, the 14 and 5 *E. albertii* strains obtained from the large-scale surveys performed in 2019 and 2020, respectively, also showed significant diversity with no clear clonal relationship observed (Fig. 1B).

Interestingly, the PFGE analysis also revealed that some of the strains displayed clonal relationship but were isolated from distant areas in different years. For example, TN19007 and TN20348 displayed the same DNA fingerprint pattern and showed clear clonal relationship (Fig. 1B). However, TN19007 was isolated from a raccoon captured in 2019 in Washington county (northeast of TN, orange color, Fig. 1A) while TN20348 was isolated from a raccoon captured in 2020 in Marion county (southeast of TN, yellow color, Fig. 1A). TN18229 and TN20127 displayed similar PFGE pattern and showed close phylogenetic relationship (Fig. 1B). However, TN18229 was isolated from a raccoon captured in 2018 in Hawkins county (northeast of TN, green color, Fig. 1A) while TN20127 was isolated from a raccoon captured in 2020 in Hamilton county (southeast of TN, red color, Fig. 1A).

3.4. *Antimicrobial resistance (AR) profile of E. albertii strains*

A panel of *E. albertii* strains of different origins were examined for AR profile against 20 antibiotics belonging to 14 classes in this study. These strains include those from raccoons (n = 22) and river (n = 2) in the U. S., those from raccoons (n = 20), humans (n = 6), swine (n = 1), and

Table 4
Antibiotic resistance (AR) profile and genomics analysis of all the examined *E. albertii* strains.

Strain ID	Origin	AR phenotype[a]	AR genotype[b]	Plasmid replicon[c]
P6648	human_Japan	CEF, AMP, PIP, KAN, STR, TET, CHL, SXT	bla_{TEM-1B}, *aph* (3')-Ia, *aph* (3'')-Ib, *aph* (6)-Id, *tetA*, *sul2*, *dfrA12*, *aadA2*, *qacE*, *catA1*	IncFIB, IncFII (29), IncFII (pSE11), IncHI2, IncHI2A
P3502	human_Japan	CEF, STR, TET, NAL	*tetA*, *aph*(3'')-Ib, *aph*(6)-Id	IncFIB, IncFII (29), IncFII (pHN7A8), IncFII(pSE11), IncI2(Delta), Col(pHAD28)
RAC-342	raccoon_Japan	AMP, CEF, PIP, KAN, CHL	*floR*, *aph*(3')-Ia, *qnrS1*, $bla_{TEM-176}$	IncFIB, IncFII (pHN7A8), IncI1-I(Alpha), IncX1
AKT130	human_Japan	CEF, AMP, PIP	bla_{TEM-1B}, *dfrA1*	IncFIB, IncFII, IncI1-I(Alpha)
JCM17328^T	human_Japan	TET	*tetB*	–
SW-9	pig_Japan	CEF	–	–
WB7-2	bird_Japan	CEF	–	IncFIB, IncFII
WB7-4	bird_Japan	CEF	–	IncFIB, IncFII
TN19001	raccoon_US	CEF	–	–
TN19116	raccoon_US	CEF	–	–
TN19226	raccoon_US	CEF	–	IncFIB, IncFII
JL1694	river_US	CEF	–	IncFIA, IncFIB, IncFII, p0111
TN18229	raccoon_US	–	–	IncFIA(HI1), IncFIB, IncFIC (FII)
TN18236	raccoon_US	–	–	IncFIA(HI1), IncFIB
TN18249	raccoon_US	–	–	IncFIB, IncFII
TN19003	raccoon_US	–	–	IncFIA(HI1), IncFIB, IncFIC (FII), Col (pHAD28)
TN19007	raccoon_US	–	–	–
TN19201	raccoon_US	–	–	–
TN19305	raccoon_US	–	–	–
KY19009	raccoon_US	–	–	IncFIA(HI1), IncFIB, IncFII
TN19004	raccoon_US	–	–	–
TN19006	raccoon_US	–	–	IncFIA(HI1), IncFIB, IncFIC (FII), Col (pHAD28)
TN19123	raccoon_US	–	–	IncFIA(HI1), IncFIB
TN19202	raccoon_US	–	–	IncFIA(HI1), IncFIB, IncFII
TN19207	raccoon_US	–	–	IncFIA(HI1), IncFIB, IncFII
TN19357	raccoon_US	–	–	IncFIA(HI1), IncFIB, IncFIC (FII)
TN19310	raccoon_US	–	–	IncFIA(HI1), IncFIB, IncFII (pSE11)
TN19336	raccoon_US	–	–	IncFIB, IncFII (pCoo), Col440I
TN20127	raccoon_US	–	–	IncFIA(HI1), IncFIB, IncFIC (FII)
TN20348	raccoon_US	–	–	–
TN20403	raccoon_US	–	–	IncFIA(HI1), IncFIB, IncFII
RAC-7A	raccoon_Japan	–	–	–
RAC-34A	raccoon_Japan	–	–	–
RAC-44A	raccoon_Japan	–	–	–

(*continued on next page*)

Table 4 (*continued*)

Strain ID	Origin	AR phenotype[a]	AR genotype[b]	Plasmid replicon[c]
RAC-58	raccoon_Japan	–	–	IncFIB, IncFII, IncFII (pHN7A8), IncX4
RAC-199	raccoon_Japan	–	–	IncFIB, IncFII (pSE11)
RAC-247	raccoon_Japan	–	–	IncFII
RAC-258	raccoon_Japan	–	–	IncFIA(HI1), IncFIB, IncFIC (FII)
RAC-263	raccoon_Japan	–	–	IncFIB, IncFII
RAC-266	raccoon_Japan	–	–	–
RAC-281	raccoon_Japan	–	–	IncFIA, IncFIB, IncFII
RAC-300	raccoon_Japan	–	–	–
RAC-305A	raccoon_Japan	–	–	–
RAC-310A	raccoon_Japan	–	–	IncFIA(HI1), IncFIB, IncY
RAC-324	raccoon_Japan	–	–	IncFIA(HI1), IncFIB, IncFIC (FII), IncFII (pCoo)
RAC-357A	raccoon_Japan	–	–	–
RAC-409A	raccoon_Japan	–	–	IncFIA(HI1), IncFIB, IncFIC (FII)
RAC-410A	raccoon_Japan	–	–	–
RAC-414	raccoon_Japan	–	–	IncFIB, IncFII
RAC-431A	raccoon_Japan	–	–	IncFIA(HI1), IncFIB
P2855	human_Japan	–	–	IncFIB, IncFII (pHN7A8), IncI1-I(Alpha), pSL483
P5093	human_Japan	–	–	–
P6796	human_Japan	–	–	IncFIB, IncFII (pHN7A8)
JL1695	river_US	–	–	–

Abbreviations: AMP, ampicillin; CEF, cephalothin; PIP, piperacillin; CHL, chloramphenicol; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline.

[a] A total of 20 diverse antibiotics were used for susceptibility test using disk diffusion method on Mueller Hinton agar. –: no any antibiotic resistance was detected

[b] The AR genes were identified from draft genome of each strain using ResFinder (v4.1) with nucleotide identity above 90% and length coverage above 80%. –: no AR gene was detected on genome.

[c] The plasmid replicon types were identified from draft genome of each strain using PlasmidFinder (v2.1) with nucleotide identity above 90% and length coverage above 80%. –: no plasmid replicon was detected on genome.

wild bird (n = 2) in Japan, and the first human clinical strain JCM17328^T (Table 1). For the 42 raccoon strains, regardless geographical origin, most of them (19 of 22 U.S. strains, 19 of 20 Japan strains) were susceptible to all tested antimicrobials. Three U.S. strains (TN19001, TN19116, and TN19226) were resistant to cephalothin, the first generation of cephalosporin (Table 3). Only one Japanese raccoon *E. albertii*, RAC-342, displayed multidrug resistance (MDR) phenotype with resistance to cephalothin, penicillin, piperacillin, kanamycin, and chloramphenicol (Table 3).

With respect to the 6 human clinical strains isolated in Japan, three were susceptible to all antimicrobials while the other three (P3502, P6648, AKT130) displayed MDR phenotype with resistance to up to 8 antimicrobials (Table 3). The JCM17328^T, the strain isolated from an infant in 1991 (Albert et al., 1991), was resistant to tetracycline (Table 3). The tested Japanese swine strain (Sw-9) and bird strains (WB7-2 and WB7-4) were only resistant to cephalothin (Table 3). Original AR data are shown in Table 3 for the tested *E. albertii* strains that displayed resistance to at least one antimicrobial.

3.5. Whole genome sequencing and genomic analysis

A total of 54 *E. albertii* strains were subjected to whole genome sequencing using MiSeq platform with sequencing coverage over 80X for each strain. For each assembled draft genome, completeness was above 97.6% while contamination was below 1.26%, indicating high quality of draft genome. In addition, ANIs of the 54 draft genomes to reference genome of *E. albertii* KF1 were all above 98%, further confirming the 54 isolates were indeed *E. albertii* species.

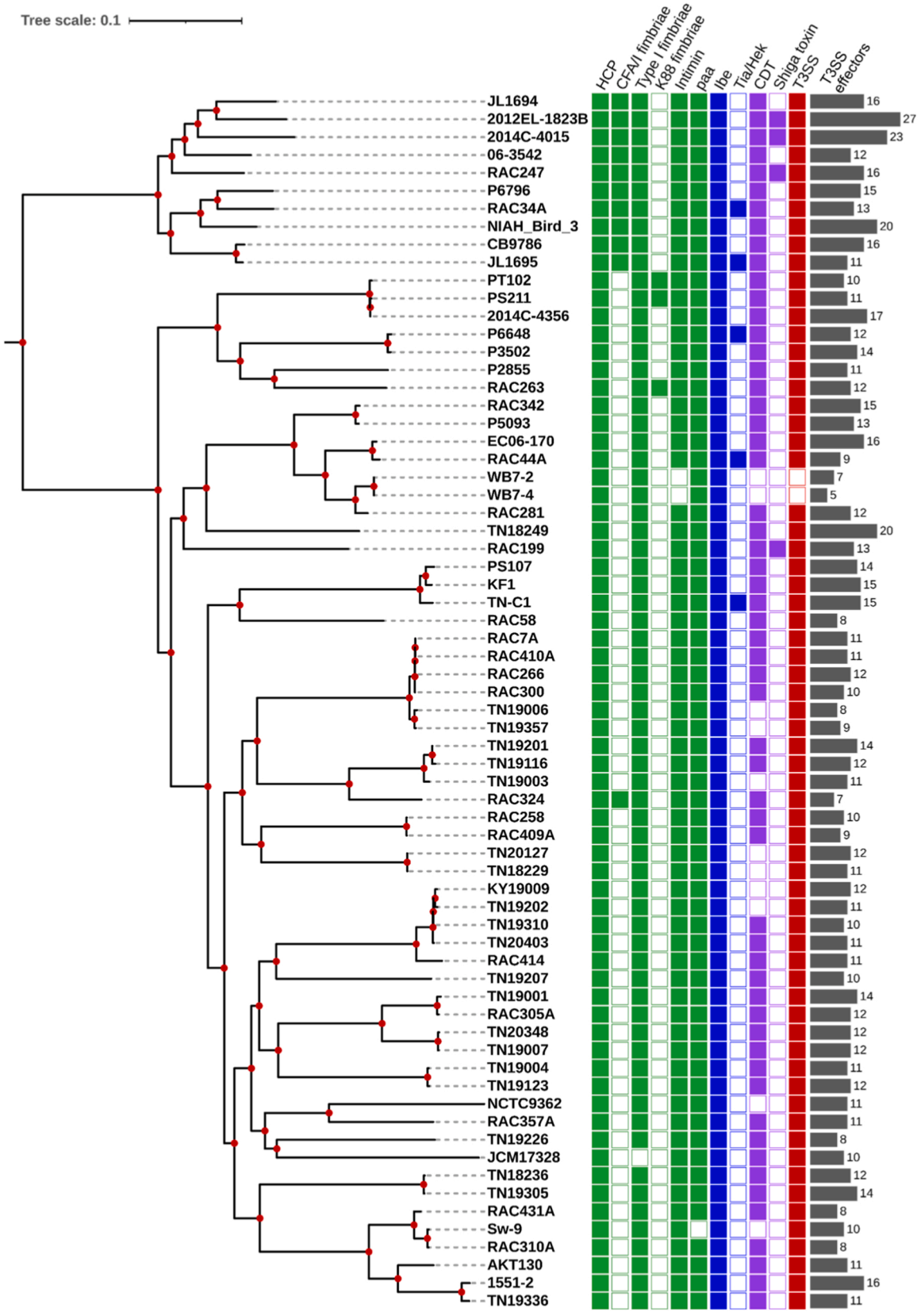
The assembled draft genomes of the 54 *E. albertii* strains were then subjected to comprehensive gene mining with emphasis on AR gene and plasmid replicon, which are summarized in Table 4. As expected, gene mining using ResFinder did not identify any AR genes in all the antimicrobial susceptible strains as well as those only displaying resistance to cephalothin (CEF) (Table 4). However, a panel of AR genes were discovered in three human MDR strains (P6648, P3502, and AKT130) and one raccoon MDR strain RAC-342 of Japan origin (Table 4). Briefly, the $bla_{\text{TEM-1B}}$ and $bla_{\text{TEM-176}}$ were identified as beta-lactamase genes. The AR genes responsible for resistance to aminoglycoside (*aph(3')-Ia, aph(3'')-Ib*, and *aph(6)-Id*), chloramphenicol (*floR, catA1*), and tetracycline (*tet*(A) and *tet*(B)) were identified in the genomes. Additionally, *sul2* and *dfrA12* genes related to sulfonamide and trimethoprim resistance, respectively, were discovered in P6648 MDR strain, consistent with its AR phenotype (Table 4). Nevertheless, there were still discrepancies between AR phenotype and genotype in the strains. Specifically, P3502 displayed quinolone resistance but lacked corresponding resistant gene; RAC-342 was susceptible to quinolone but contained the resistance gene *qnrS1*.

Plasmids play a critical role in horizontal gene transfer, a process involved in the exchange of AR genes as well as virulence genes among bacteria in different ecological niches. Thus, we subsequently performed bioinformatics analysis of plasmid origins for the draft genomes of all 54 strains using PlasmidFinder. The four MDR strains carried 3–6 plasmid replicon types (Table 4). IncFIB was the only common plasmid replicon type in the 4 strains. The rest of the plasmid replicon types were either unique to a specific strain or only shared by 2 of the 4 strains (Table 4). Notably, plasmid replicons were also frequently identified in most *E. albertii* strains that lacked AR genes and were susceptible to all tested antimicrobials (Table 4). Plasmid replicon types IncFIB and IncFIA(HI1) were prevalent in these antimicrobial-susceptible strains and coexisted in many of the strains. IncFII and IncFIC(FII) were less prevalent and never coexisted with each other. The rest of the plasmid replicon types were relatively unique and only shared by no more than 3 of the susceptible strains (Table 4). In addition, for MDR strains P3502 and RAC-342, the AR genes were found to be on the same contigs where the plasmid replicons were (data not shown). Specifically, a contig of the strain P3502 harbored both AR genes *tet*(A), *aph(3'')-Ib*, and *aph(6)-Id*, and plasmid replicons IncFII(29) and IncFII(pHN7A8); AR genes $bla_{\text{TEM-176}}$ and *aph(3')-Ia* in RAC-342 shared the contig with plasmid replicon IncX1.

3.6. Comparative genomics analysis of diverse E. albertii strains

To better understand evolution and virulence of the *E. albertii* strains isolated in this study and previous studies (Albert et al., 1991; Hinenoya et al., 2022, 2019a, 2021, 2020a, 2014, 2017; Wang et al., 2022), the 54 *E. albertii* strains (Table 1), together with 9 human and 1 bird *E. albertii* strains obtained from public database, were subjected to in-depth comparative genomics analysis.

For the raccoon *E. albertii* strains isolated in the U.S., WGS-based analysis (Fig. 2) confirmed the great genomic diversity as shown in PFGE analysis (Fig. 1B). In addition, the strains with clonal relationship revealed by PFGE analysis (*i.e.*, TN19007 *vs.* TN20348; TN18229 *vs.* TN20127, Fig. 1B) was further confirmed by the wgSNP-based phylogenetic analysis (Fig. 2). The examined Japanese raccoon *E. albertii* strains also exhibited significant genomic diversity (Fig. 2). Remarkably,



(caption on next page)

Fig. 2. Comparative genomics analysis of the *E. albertii* strains isolated from geographically diverse areas and different origins. Majority of the *E. albertii* strains listed on Table 1, which include those from the wild raccoons (22), chickens (4) and river (2) in the U.S., the wild raccoons (20), humans (6), swine (1), and wild birds (2) in Japan, and the human (1) in Bangladesh, were compared with human (9) and bird (1) *E. albertii* strains obtained from public database for genomics analysis. Information of the *E. albertii* strains retrieved from public database is described in *Materials & Methods*. The phylogenetic tree was constructed using CSI Phylogeny (v1.4) and visualized using iTOL (v6). The profiles of major virulence factors (displayed on the right of each strain) were identified and generated by using Virulence Factor Database (http://www.mgc.ac.cn/VFs/) in which different pathotypes of *E. coli* were used as the reference. Solid and open squares denote presence and absence of a virulence factor, respectively. Notably, further in-depth annotation and PCR analysis indicated that all the examined strains were indeed positive for *Eacdt*, *paa*, and the intimin gene *eae* although some strains were shown negative by using the Virulence Factor Database. The square colors green, blue, violet, and red represent virulence categories adherence, invasion, toxin, and type III secretion system, respectively, while the gray bar denotes the number of type III secretion system effectors.

some of the raccoon strains displayed very close evolutionary relationship with the Japanese human clinical strains. Specifically, the raccoon strain RAC-342 and RAC-44A showed clonal relationship to human strains P5093 and EC06–170, respectively (Fig. 2). Interestingly, some of Japanese raccoon strains were phylogenetically close to those of the U.S. origin. For example, the Japanese RAC-305A strain was highly similar to the U.S. strain TN19001 (Fig. 2). In addition, the clade containing four Japanese strains (RAC-300, RAC-266, RAC-410A, and RAC-7A) was phylogenetically close to the clade comprised of two U.S. strains (TN19357 and TN19006). Finally, we also observed that the Japanese swine strain Sw-9 showed clonal relationship to the raccoon strain RAC-310A that was isolated in Japan (Fig. 2).

Using a recently developed virulence gene analysis software, the Virulence Factor Database, we also examined and compared major virulence factors that potentially contribute to *E. albertii* pathogenicity, such as those required for adherence and invasion of host cells. For adherence-associated factors, the genes encoding hemorrhagic *E. coli* pilus (HCP), type I fimbriae, intimin, and porcine attaching-effacing associated protein (Paa) were highly prevalent in all the *E. albertii* strains while colonization factor antigen I (CFA/I) fimbriae and K88 fimbriae genes were rarely identified (Fig. 2). With regard to invasion-related factors, the *ibe* was common for all the *E. albertii* strains while *tia/hek* was only present in 5 of the strains (Fig. 2). Type III secretion system (T3SS), which is responsible for the ability of *E. albertii* to form A/E lesions (Gomes et al., 2020), was highly prevalent in the *E. albertii* strains, and the numbers of T3SS effectors encoded by the locus of enterocyte effacement (LEE) or non-LEE genes had substantial variations across all the *E. albertii* strains (Fig. 2). The *stx* genes were rare and only present in 4 of the strains.

Interestingly, of all the 68 *E. albertii* strains examined, the CDT gene was not detected in 11 strains when using the Virulence Factor Database although all these strains were *Eacdt* positive during isolation process when using the PCR primers highly specific for *E. abertii* (Hinenoya et al., 2019b). Due to the limitation of the virulence analysis software by using *E. coli* genomes as the references (Liu et al., 2019), this bioinformatics tool may lead to false negative virulence profile when analyzing

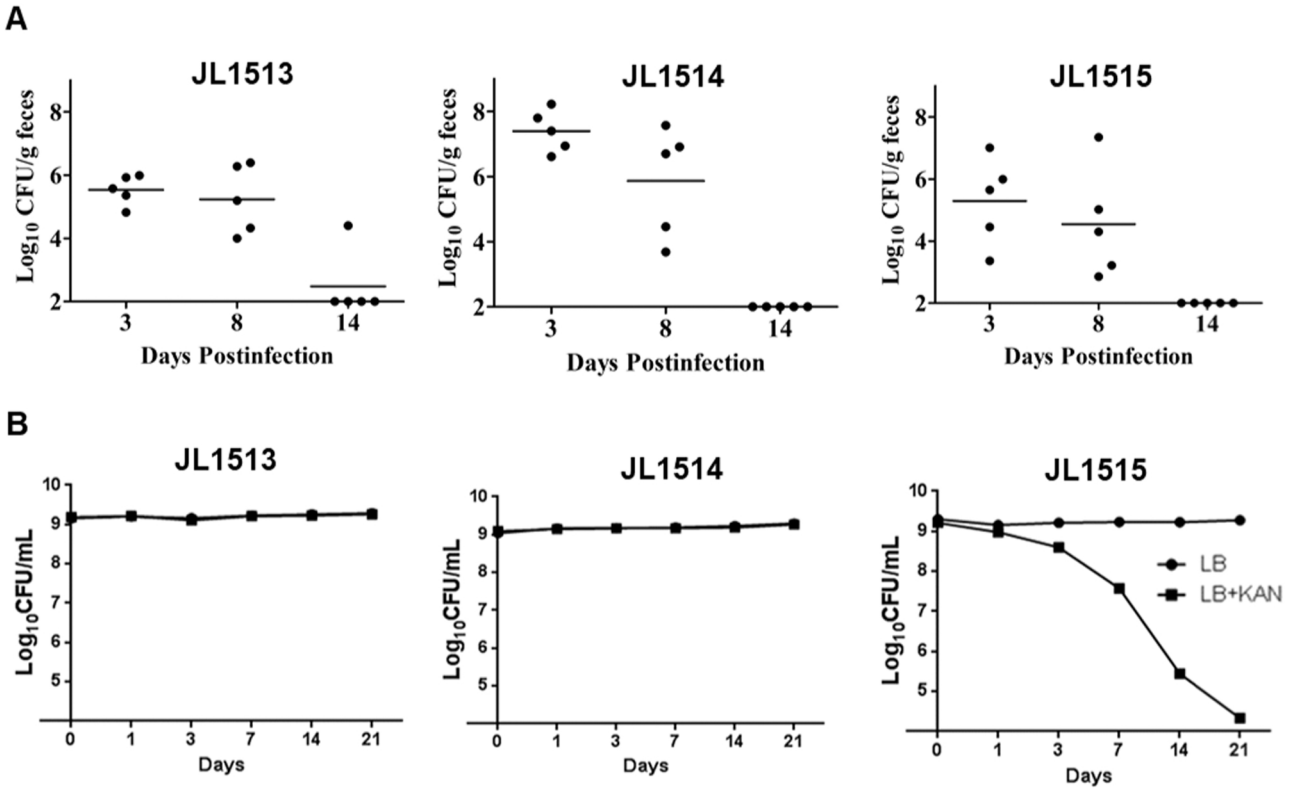


Fig. 3. Colonization of the U.S. raccoon *E. albertii* strains in the chick intestine. (A) chicken challenge trial. The chicks at age of 4-day old (15 birds per group) were orally challenged with raccoon *E. albertii* derivative JL1513 (TN18229/pZE21), JL1514 (TN18236/pZE21), and JL1515 (TN18249/pZE21), respectively. Cecal contents were collected from 5 sacrificed chicks in each group at 3, 8 and 14 days postinfection for *E. albertii* CFU enumeration using the selective agar plates. The detection limit of the plating methods was 100 CFU/g of cecal content. (B) *In vitro* stability of pZE21 in raccoon *E. albertii* derivatives. Each of the three pZE21-bearing raccoon *E. albertii* strains was grown and daily passaged in antibiotic-free LB broth at 37°C for 21 days. At different passage days, the culture was serially diluted and plated onto LB agar plates for total *E. albertii* enumeration (solid circle) and onto LB agar plates supplemented with the kanamycin (KAN) for enumeration of the pZE21-carrying *E. albertii* (solid square).

the genomes of different species, particularly when concerning the well-recognized heterogeneity of CDT gene sequence between *E. coli* and *E. albertii* (Hinenoya et al., 2019b). Therefore, we subsequently performed BLAST search, genome annotation analysis as well as PCR analysis for the three genes that have been reported prevalent in *E. albertii*, including *Eacdt*, *paa*, and the intimin gene *eae*. As expected, further in-depth analysis of the assembled *E. albertii* genomes together with PCR analysis using specific primers (Hinenoya et al., 2022) not only demonstrated that all the examined *E. albertii* strains were positive for *Eacdt* but also the two bird strains (WB7-2 and WB7-4, Fig. 2) and one swine strain (SW-9, Fig. 2) were indeed positive for *eae* and paa, respectively (Data not shown).

3.7. Colonization of raccoon E. albertii in the chicken intestine

We have successfully isolated *E. albertii* from broiler cloacal samples in recent studies (Hinenoya et al., 2021; Wang et al., 2022), indicating that chickens could serve as a reservoir for *E. albertii*. However, the ability of *E. albertii* originating from other animal hosts, such as raccoon, to colonize in the chicken intestine is still unknown. Addressing this issue is critically important for us to understand transmission of this emerging pathogen in complex ecosystems. Thus, in this study, we performed a challenge experiment to assess the ability of three different raccoon *E. albertii* strains to colonize chicken intestine.

As expected, no chick in the control group showed clinical signs and none had detectable *E. albertii* in the cecal contents (data not shown). Similar to the control groups, no clinical signs were observed in the chicks from the *E. albertii*-challenged groups throughout the chicken trial. As shown in Fig. 3A, all the three raccoon *E. albertii* strains efficiently colonized the intestines of all chicks by 3 DPI, with JL1514 displaying the highest colonization (approximately 7.5 $\log_{10}$ CFU/g cecal contents). Colonization of the *E. albertii* in the chicken intestine could persist to 8 DPI with slightly lower colonization levels (up to 2 log unit reduction) in the intestine (Fig. 3A). However, by 14 DPI, the raccoon strains were not isolated from almost all the chicks (Fig. 3A).

To improve efficiency and accuracy of recovering the *E. albertii* inoculum strains, we introduced a Kan^r marker in the three wild-type raccoon strains *via* transformation of a small plasmid pZE21 (Table 1). As expected, the inoculated Kan^r derivatives in chicken cecal samples could be easily distinguished on selective plates. In addition, all randomly selected putative *E. albertii* (3 colonies per strain per time point) have been confirmed as *E. albertii* using the *Eacdt* gene-based PCR assay, demonstrating reliability of this *in vivo* assessment. It is possible that the plasmid can be unstable during infection, leading to underestimated CFU counts obtained on plates supplemented with Kan. To address this issue, *in vitro* stability of pZE21 in the three strains was assessed. As shown in Fig. 3B, two strains (JL1513 and JL1514) displayed exceptional stability by 21 daily passages in antibiotic-free broth medium. The plasmid in JL1515 was not stable and gradually disappeared from the host strain during growth. For example, by day 3, the Kan^r population only accounted for approximately 2% of the total *E. albertii* population enumerated (Fig. 3B).

4. Discussion

The findings from this longitudinal surveillance provide new and strong evidence to support our recent Japanese raccoon study showing that the raccoon is likely a significant reservoir of *E. albertii* (Hinenoya et al., 2020a). In the 2-year survey in Japan, a total of 430 rectal swabs were collected from wild raccoons captured in Osaka Prefectural area between 2016 and 2017, and approximately 58% of the raccoons examined in Japan were *E. albertii*-positive (Hinenoya et al., 2020a). In this study, the examined raccoons were from a much broader area in the U.S. (Fig. 1A) over a 3-year surveillance. Although overall *E. albertii*-positive rate (26%) in U.S. raccoons is lower than that reported in Japanese raccoon study, the real *E. albertii*-positive rate in the U.S. is likely higher due to sample quality issue in this study. In the survey performed in Japan, the collected fecal samples were immediately transported to the laboratory for processing within 6 h of collection (Hinenoya et al., 2020a). However, in this study, two factors greatly affected the quality of the examined U.S. raccoon fecal samples. The first factor is storage time. The interval time between field sampling and laboratory processing is 50 days in average with some up to 100 days for U.S. raccoon fecal samples. Such long waiting time was caused by multiple challenging issues associated with logistic management of sampling by field workers, periodical transportation of samples from field to central USDA office, transportation of samples from USDA office to the laboratory for processing, lengthy process of *E. albertii* isolation and validation due to high false positive rate, and the uncertainties of laboratory operation due to COVID-19 pandemic for the third batch of samples collected in 2020. The second factor is storage method. The raccoon fecal samples tested in the first survey had been unexpectedly desiccated before culture due to long-term storage of swab in a plastic bag, leading to a significant reduction in viable bacterial organisms that could be recovered. To ensure appropriate preservation of specimens for *E. albertii* examination, the method for fecal sample storage was modified in the second and third raccoon survey. As expected, the prevalence of *E. albertii* in the second survey is higher than the first survey in this study (Table 2).

In this study, overall isolation rate of *E. albertii* was still lower than the PCR positive rate in this raccoon study, which has been observed in our recent chicken study (Wang et al., 2022). This is due to the limitation of the current methods for enrichment and isolation of *E. albertii*. In this study, we used the protocol for the detection and isolation of *E. albertii* in human clinical fecal specimens, in which *E. albertii* generally could be isolated with high successful rate from PCR-positive samples (Hinenoya et al., 2020b). However, given significant differences in the composition and relative abundance of gut microbiota in raccoons and humans, we had high false positive rate of the randomly selected *E. albertii*-like colonies on the XRM-MacConkey agar, the selective medium used in this study. To increase efficiency for isolation of *E. albertii* from enriched culture, more efforts should be placed on optimization of existing selective medium. Recently, a novel selective enrichment broth was reported for isolation of *E. albertii* from poultry samples (Wakabayashi et al., 2021). In future studies, 16S rRNA-based examination of the identities of false positive colonies in various samples, such as water and feces of different animal origins, would facilitate us to develop better selective enrichment broth as well as selective agar for *E. albertii* isolation from samples of different sources, which is critically important for large scale of epidemiological study on this emerging zoonotic pathogen.

Both phenotypic and genomics examination revealed distinct patterns of the *E. albertii* isolated from raccoons and chickens with respect to AR profile and its diversity. In recent chicken studies at pre-harvest level (Hinenoya et al., 2021; Wang et al., 2022), most of isolated *E. albertii* (15 of 18) displayed resistance to multiple antimicrobials (up to 12 antimicrobials). One strain was even resistant to imipenem, a clinically important carbapenem antibiotic (Wang et al., 2022). In contrast, besides one raccoon strain of Japan origin (RAC-342) that displayed multidrug resistance (Table 3), all the rest 41 strains are either susceptible to all tested antimicrobials (38 of 41) or only slightly resistant to cephalothin (CEF) (3 of 41, Tables 3 and 4). Notably, CEF has been observed to be a poor predictor of susceptibility to certain oral cephalosporins because of high rates of overcalling resistance. Thus, CLSI removed the cephalothin surrogacy claim for oral cephalosporins from the M100 document in 2016 (https://clsi.org/media/2270/clsi_astnewsupdate_june2018_final.pdf). Consequently, raccoon *E. albertii*, regardless of specific country origin, are basically susceptible to wide range of antimicrobials, which was further supported by the lack of AR genes in the genome of these raccoon isolates (Table 4). In terms of strain diversity, the *E. albertii* of chicken origin consistently displayed clonal relationship for those isolated from same

farm (Hinenoya et al., 2021; Wang et al., 2022). This finding together with absence of *E. albertii* in some chicken farms (Wang et al., 2022) suggest that chicken is not the original natural animal host for *E. albertii*, but can be a significant reservoir, or an intermediate host, if exposed to *E. albertii* through various channels, such as contaminated food and water, interaction with farmers, or the feces of other animals. In contrast, the raccoon *E. albertii* showed strikingly high diversity. Taking the three U.S. raccoon *E. albertii* strains isolated in 2018 as an example (Table 1), the three raccoon strains are phylogenetically distant even though the corresponding three *E. albertii* raccoon hosts were captured from a small area, suggesting wild raccoons harbor diverse *E. albertii* strains. Given the lack of AR phenotype/genotype and the high genome diversity observed among the examined raccoon *E. albertii* strains, we propose that the raccoon is likely the original natural animal host for *E. albertii*. If *E. albertii* carried by raccoon enters an agroecosystem, the *E. albertii* would have many opportunities to interact with food-producing animals, and the environment, consequently posing a threat to human health. This hypothesis needs to be tested in future comprehensive molecular epidemiological studies within a well-managed and coordinated system.

Using an established chicken model, in this study, we have also demonstrated that three different raccoon *E. albertii* strains could colonize in the chicken intestine, which partly supported above hypothesis about transmission of the raccoon-carried *E. albertii* into animal production systems, such as poultry. In addition, examination of limited *E. albertii* of Japan origin in this study also revealed clonal relationship of a swine strain (Sw-9) to raccoon strains, suggesting active *E. albertii* transmission between swine and raccoons in Japan. Thus, food-producing animals could potentially serve as an intermediate host for *E. albertii*, posing a threat to public health. Notably, some *E. albertii* strains of chicken origin identified in previous chicken surveys (Hinenoya et al., 2021; Wang et al., 2022) not only displayed multidrug resistance phenotype but also were phylogenetically close to human clinical strains. For example, the PT102 and PS211 strains, which were isolated from different farms in the U.S., showed clear clonal relationship to the U.S. human strain 2014C-4356 (Fig. 2). Hinenoya et al. (2021a) also demonstrated that the *E. albertii* strains isolated from human could successfully colonize in the chicken intestine, further supporting the importance of chickens as a reservoir for human *E. albertii* pathogen. Strikingly, the pilot environmental survey performed in this study suggested that *E. albertii* is prevalent in river; more importantly, comparative genomics analysis showed the water *E. albertii* strains were phylogenetically close to those of human origins. Together, the findings from this study and our previous *E. albertii* epidemiological research (Hinenoya et al., 2021; Wang et al., 2022) provide new insights into the evolution, ecology, and transmission of *E. albertii*.

The comparative genomics analysis in this study also provided additional information helpful for future epidemiological research on *E. albertii*. First, despite limited *E. albertii* strains of Japan origin were examined in this study, some Japanese raccoon *E. albertii* strains have shown clear clonal relationship to the human and swine strains isolated in Japan. This is likely attributed to the limited or small sampling area where these strains were isolated. In this U.S. raccoon *E. albertii* study, geographically diverse areas were targeted, which is needed for the purpose of prevalence surveillance. However, given lack of parallel examination of the samples collected from other sources (*e.g.* food animals and river) in the same area, at this stage, there is no evidence showing potential *E. albertii* transmission among livestock, wildlife, and humans in the U.S. To address this knowledge gap, a larger scale of longitudinal surveillance should be performed by collecting samples of different origins in a limited region with participation and cooperation of multiple units for sampling and diagnosis. Another important finding from comparative genomics analysis is discovery of some U.S. raccoon strains showing clonal relationship to those of Japan origin, which may result from international trade. Raccoons are native to North American but have been introduced as pet or game animals into other countries including Japan. This observation highlights a new factor that should be taken into consideration for *E. albertii* epidemiology in the future. Finally, it is important to mention that the insightful findings from this study and previous studies (Hinenoya et al., 2021; Wang et al., 2022) were greatly benefited from active international collaboration and information sharing. A recent comparative genomics study (Luo et al., 2021) provided baseline information on the population structure, virulence variation, and antimicrobial resistance of *E. albertii* from 15 countries, further stressing on the importance of collaboration of different units at local, regional, and global levels for elucidation of the ecology, evolution and pathobiology of *E. albertii* in the future.

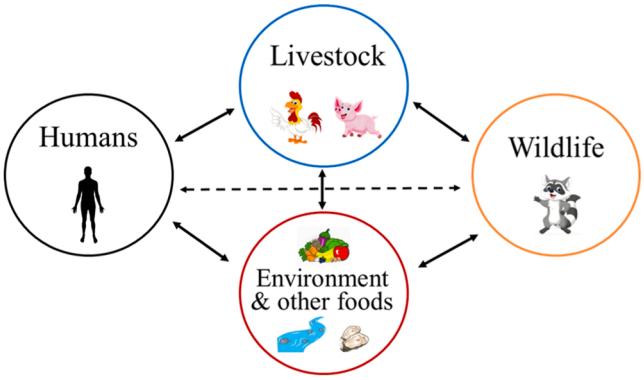


Fig. 4. Transmission of the emerging *E. albertii* pathogen in complex ecosystem. The unique dynamic interactions among the enteric *E. albertii* pathogen, livestock, wildlife, humans, and their shared environment with other food products (*e.g.* crops and seafood), is considered within the "*One Health*" approach. The direct interaction between human and raccoon (*e.g.* petting) is feasible but rare, and thus is shown as dotted line.

In conclusion, this study demonstrated that raccoons could be an important reservoir of *E. albertii* and a potential source of human infections in the U.S., and raccoons and chickens may serve as unique and significant players in the dynamic interactions among the enteric *E. albertii* pathogen, animals and humans, and their shared environment with other food products (*e.g.* crops and seafood) (Fig. 4). Notably, in addition to raccoons, other wildlife may also serve as natural reservoir of *E. albetii* and should be surveyed in future studies. In the future, the systemic and integrated One Health approach is critically needed to control the emerging *E. albertii* in a complex ecosystem. In particular, well-controlled comprehensive epidemiological studies are highly warranted to improve our understanding of *E. albertii* transmission and to identify critical control points for reducing *E. albertii* infections in humans.

Acknowledgement

The authors thank Abigail M. Sallee for her technical assistance. This project was supported by AgResearch and the One Health Initiative Seed Program at The University of Tennessee. Atsushi Hinenoya was awarded the travel grant of the Overseas Research Scholar Program in Osaka Prefecture University. Xing-Ping Li was supported by the Graduate Student Overseas Study Program of South China Agricultural University (2018LHPY007). This work was supported in part by JSPS KAKENHI Grant Numbers 20K06396 to A.H.

References

Albert, M.J., Alam, K., Islam, M., Montanaro, J., Rahaman, A., Haider, K., Hossain, M.A., Kibriya, A., Tzipori, S., 1991. *Hafnia alvei*, a probable cause of diarrhea in humans. Infect. Immun. 59, 1507–1513. https://doi.org/10.1128/iai.59.4.1507-1513.1991.

Arkin, A.P., Cottingham, R.W., Henry, C.S., Harris, N.L., Stevens, R.L., Maslov, S., Dehal, P., Ware, D., Perez, F., Canon, S., et al., 2018. KBase: the united states department of energy systems biology knowledgebase. Nat. Biotechnol. 36, 566–569. https://doi.org/10.1038/nbt.4163.

Bhatt, S., Egan, M., Critelli, B., Kouse, A., Kalman, D., Upreti, C., 2019. The evasive enemy: insights into the virulence and epidemiology of the emerging attaching and effacing pathogen *Escherichia albertii*. Infect. Immun. 87, e00254–18. https://doi.org/10.1128/IAI.00254-18.

Bortolaia, V., Kaas, R.S., Ruppe, E., Roberts, M.C., Schwarz, S., Cattoir, V., Philippon, A., Allesoe, R.L., Rebelo, A.R., Florensa, A.F., et al., 2020. ResFinder 4.0 for predictions of phenotypes from genotypes. J. Antimicrob. Chemother. 75, 3491–3500. https://doi.org/10.1093/jac/dkaa345.

Carattoli, A., Zankari, E., García-Fernández, A., Voldby Larsen, M., Lund, O., Villa, L., Møller Aarestrup, F., Hasman, H., 2014. *In silico* detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob. Agents Chemother. 58, 3895–3903. https://doi.org/10.1128/AAC.02412-14.

Fiedoruk, K., Daniluk, T., Swiecicka, I., Murawska, E., Sciepuk, M., Leszczynska, K., 2014. First complete genome sequence of *Escherichia albertii* strain KF1, a new potential human enteric pathogen. Genome Announc 2, e00004–e00014. https://doi.org/10.1128/genomeA.00004-14.

Gardner, S.N., Slezak, T., Hall, B.G., 2015. kSNP3.0: SNP detection and phylogenetic analysis of genomes without genome alignment or reference genome. Bioinformatics 31, 2877–2878. https://doi.org/10.1093/bioinformatics/btv271.

Gautom, R.K., 1997. Rapid pulsed-field gel electrophoresis protocol for typing of *Escherichia coli* O157: H7 and other Gram-negative organisms in 1 day. J. Clin. Microbiol. 35, 2977–2980. https://doi.org/10.1128/jcm.35.11.2977-2980.1997.

Gomes, T.A., Ooka, T., Hernandes, R.T., Yamamoto, D., Hayashi, T., 2020. *Escherichia albertii* Pathogenesis. EcoSal 9. https://doi.org/10.1128/ecosalplus.ESP-0015-2019.

Hinenoya, A., Shima, K., Asakura, M., Nishimura, K., Tsukamoto, T., Ooka, T., Hayashi, T., Ramamurthy, T., Faruque, S.M., Yamasaki, S., 2014. Molecular characterization of cytolethal distending toxin gene-positive *Escherichia coli* from healthy cattle and swine in Nara, Japan. BMC Microbiol. 14, 97. https://doi.org/10.1186/1471-2180-14-97.

Hinenoya, A., Yasuda, N., Mukaizawa, N., Sheikh, S., Niwa, Y., Awasthi, S.P., Asakura, M., Tsukamoto, T., Nagita, A., Albert, M.J., Yamasaki, S., 2017. Association of cytolethal distending toxin-II gene-positive *Escherichia coli* with *Escherichia albertii*, an emerging enteropathogen. Int. J. Med. Microbiol. 307, 564–571. https://doi.org/10.1016/j.ijmm.2017.08.008.

Hinenoya, A., Ichimura, H., Awasthi, S.P., Yasuda, N., Yatsuyanagi, J., Yamasaki, S., 2019a. Phenotypic and molecular characterization of *Escherichia albertii*: further surrogates to avoid potential laboratory misidentification. Int. J. Med. Microbiol. 309, 108–115. https://doi.org/10.1016/j.ijmm.2018.12.003.

Hinenoya, A., Ichimura, H., Yasuda, N., Harada, S., Yamada, K., Suzuki, M., Iijima, Y., Nagita, A., Albert, M.J., Hatanaka, N., 2019b. Development of a specific cytolethal distending toxin (*cdt*) gene (*Eacdt*)-based PCR assay for the detection of *Escherichia albertii*. Diagn. Microbiol. Infect. Dis. 95, 119–124. https://doi.org/10.1016/j.diagmicrobio.2019.04.018.

Hinenoya, A., Nagano, K., Awasthi, S.P., Hatanaka, N., Yamasaki, S., 2020a. Prevalence of *Escherichia albertii* in Raccoons (*Procyon lotor*), Japan. Emerg. Infect. Dis. 26, 1304–1307. https://doi.org/10.3201/eid2606.191436.

Hinenoya, A., Nagano, K., Okuno, K., Nagita, A., Hatanaka, N., Awasthi, S.P., Yamasaki, S., 2020b. Development of XRM-MacConkey agar selective medium for the isolation of *Escherichia albertii*. Diagn. Microbiol. Infect. Dis. 97, 115006 https://doi.org/10.1016/j.diagmicrobio.2020.115006.

Hinenoya, A., Li, X.P., Zeng, X., Sahin, O., Moxley, R.A., Logue, C.M., Gillespie, B., Yamasaki, S., Lin, J., 2021. Isolation and characterization of *Escherichia albertii* in poultry at the pre-harvest level. Zoonoses Public Health 68, 213–225. https://doi.org/10.1111/zph.12812.

Hinenoya, A., Awasthi, S.P., Yasuda, N., Nagano, K., Hassan, J., Takehira, K., Hatanaka, N., Saito, S., Watabe, T., Yoshizawa, M., Inoue, H., Yamasaki, S., 2022. Detection, isolation, and molecular characterization of *Escherichia albertii* from wild birds in West Japan. Jpn. J. Infect. Dis. 5, 156–163. https://doi.org/10.7883/yoken.JJID.2021.355.

Hunter, S.B., Vauterin, P., Lambert-Fair, M.A., Van Duyne, M.S., Kubota, K., Graves, L., Wrigley, D., Barrett, T., Ribot, E., 2005. Establishment of a universal size standard strain for use with the PulseNet standardized pulsed-field gel electrophoresis protocols: converting the national databases to the new size standard. J. Clin. Microbiol. 43, 1045–1050. https://doi.org/10.1128/JCM.43.3.1045-1050.2005.

Huys, G., Cnockaert, M., Janda, J.M., Swings, J., 2003. *Escherichia albertii* sp. nov., a diarrhoeagenic species isolated from stool specimens of Bangladeshi children. Int. J. Syst. Evol. Microbiol. 53, 807–810. https://doi.org/10.1099/ijs.0.02475-0.

Jain, C., Rodriguez-R, L.M., Phillippy, A.M., Konstantinidis, K.T., Aluru, S., 2018. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. Nat. Commun. 9, 5114. https://doi.org/10.1038/s41467-018-07641-9.

Larivière, S., 2004. Range expansion of raccoons in the Canadian prairies: review of hypotheses. Wildl. Soc. Bull. 32, 955–963.

Letunic, I., Bork, P., 2019. Interactive Tree Of Life (iTOL) v4: recent updates and new developments. Nucleic Acids Res. 47, W256–W259. https://doi.org/10.1093/nar/gkz239.

Liu, B., Zheng, D., Jin, Q., Chen, L., Yang, J., 2019. VFDB 2019: a comparative pathogenomic platform with an interactive web interface. Nucleic Acids Res. 47, D687–D692. https://doi.org/10.1093/nar/gky1080.

Luo, L., Wang, H., Payne, M.J., Liang, C., Bai, L., Zheng, H., Zhang, Z., Zhang, L., Zhang, X., Yan, G., et al., 2021. Comparative genomics of Chinese and international isolates of *Escherichia albertii*: population structure and evolution of virulence and antimicrobial resistance. Microb. Genom. 7, 000710 https://doi.org/10.1099/mgen.0.000710.

Lutz, R., Bujard, H., 1997. Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/I_1-I_2 regulatory elements. Nucleic Acids Res. 25, 1203–1210. https://doi.org/10.1093/nar/25.6.1203.

Oaks, J.L., Besser, T.E., Walk, S.T., Gordon, D.M., Beckmen, K.B., Burek, K.A., Haldorson, G.J., Bradway, D.S., Ouellette, L., Rurangirwa, F.R., 2010. *Escherichia albertii* in wild and domestic birds. Emerg. Infect. Dis. 16, 638–646. https://doi.org/10.3201/eid1604.090695.

Parks, D.H., Imelfort, M., Skennerton, C.T., Hugenholtz, P., Tyson, G.W., 2015. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res. 25, 1043–1055. https://doi.org/10.1101/gr.186072.114.

Ribot, E.M., Fair, M., Gautom, R., Cameron, D., Hunter, S., Swaminathan, B., Barrett, T. J., 2006. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157: H7, *Salmonella*, and *Shigella* for PulseNet. Foodbourne. Pathog. Dis. 3, 59–67. https://doi.org/10.1089/fpd.2006.3.59.

Wakabayashi, Y., Seto, K., Kanki, M., Harada, T., Kawatsu, K., 2021. Proposal of a novel selective enrichment broth, NCT-mTSB, for isolation of *Escherichia albertii* from poultry samples. J. Appl. Microbiol. 132, 2121–2130. https://doi.org/10.1111/jam.15353.

Wang, H., Zhang, L., Cao, L., Zeng, X., Gillespie, B., Lin, J., 2022. Isolation and characterization of *Escherichia albertii* originated from the broiler farms in Mississippi and Alabama. Vet. Microbiol. 267, 109379 https://doi.org/10.1016/j.vetmic.2022.109379.

Wilde, F.D., 2010. Water-quality sampling by the U.S. Geological Survey: standard protocols and procedures. U. S. Geol. Surv. Fact. Sheet 3121. https://doi.org/10.3133/fs20103121.