Evaluating the Occurrence of *Escherichia albertii* in Chicken Carcass Rinses by PCR, Vitek Analysis, and Sequencing of the *rpoB* Gene

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Escherichia albertii is a recently described species that has been associated with gastroenteritis in humans and with healthy and ill birds. Most recently, it has been identified as the causative agent in a food-borne outbreak in Japan. The distribution and clinical importance of *E. albertii* are not well studied because its importance is unclear. Culture methods for clinical isolation frequently miss *E. albertii* or incorrectly identify it as *Shigella* spp., *Escherichia coli*, or *Hafnia alvei*. This study was designed to determine if *E. albertii* could be recovered from chicken carcass rinses collected at slaughter during a 1-year period from November 2009 until October 2010. Colonies were isolated from chicken carcass rinses and tested by PCR for the presence or absence of *clpX*, *lysP*, *mdh*, intimin (*eae*), Shiga toxins 1 and 2 (*stx*₁, *stx*₂, and *stx*_{2c}), heat-stable enterotoxin A (*stxA*), and cytotoxin distending toxins 1 and 2 (*cdtB*) genes. Sixty-five isolates were analyzed by sequencing a section of the *rpoB* gene. Analysis of the *rpoB* gene sequences revealed 14 fixed differences between *E. albertii* and other, closely related organisms. The fixed differences found in the *rpoB* gene could aid in future discrimination of *E. albertii* from closely related bacteria.

Escherichia albertii is an emerging enteric pathogen first described by Albert and associates, Huys et al., and Hyma et al. (1–4). Isolates initially recovered from stool specimens from sick Bangladeshi children (1, 2) were preliminarily identified as atypical *eae*-positive *Hafnia alvei* and later were determined to be a new species, named *E. albertii*. From 1992 until now, *E. albertii* has been recovered on multiple continents from healthy and diseased birds, humans, and a cat (5–9). Most recently, *E. albertii* has been detected in sick humans in Japan, where Ooka et al. and Konno et al. found it to be the major causative agent in a restaurant-associated outbreak (9, 10). Additional studies have shown that *E. albertii* can be transmitted via food-borne routes, including lettuce, ground beef, and turkey (11–13).

The true clinical importance and prevalence of *E. albertii* are unknown, which is in part attributable to the inability to identify isolates using commercially available biochemical identification systems, such as Vitek (bioMérieux, Inc., Durham, NC) and API biochemical test strips (bioMérieux). *E. albertii* is not in the Vitek or API database, so currently, isolates may be incorrectly identified as *Shigella* spp., *Escherichia coli*, or *H. alvei* (3, 14–17). Molecular genetic approaches, such as PCR, for identification and sequencing for phylogenetic studies of specific genes have been used to distinguish *E. albertii* (4, 6, 8, 16, 18). Sequence analysis of the 16S rRNA gene has been used for *E. albertii* identification (3, 4, 6), but it is not sufficiently discriminative (19). Multilocus sequence typing (MLST), diagnostic multiplex PCR, and sequencing of the 16S rRNA, *eae*, and *cdtB* genes was used to show that atypical *Shigella* sp. strain B13 isolates were in fact *E. albertii* (6). Ooka et al. (8) added intimin subtyping and PCR analysis of Shiga toxin genes to detect *E. albertii* from a population previously described as intimin-positive *E. coli*. Isolates of *E. albertii* are clearly distinguished from *E. coli* by MLST (6, 8, 20, 21).

New methods for identification of *E. albertii* are needed to determine its prevalence and pathogenicity in people and animals. With the availability of sequencing, a definitive sequence-based approach for identification of *E. albertii* is possible. Another useful target for species identification has been the gene for RNA poly-

merase (RNAP). RNAP is responsible for synthesizing mRNA, rRNA, and tRNA in bacteria (22). The β subunit of RNAP is encoded by the *rpoB* gene and is highly conserved. The *rpoB* gene is an established tool for bacterial species and subspecies identification and is more robust than 16S rRNA gene sequencing (23). Analysis of the *rpoB* gene for species identification has been shown to accurately reflect DNA-DNA hybridization (DDH) studies (24–27). Mollet et al. were unable to distinguish *E. coli* from *Escherichia fergusonii* using 16S rRNA gene analysis; however, the partial *rpoB* sequences diverged by 2.3% (28). *rpoB* gene sequence alignments have been used for resolution at the species and subspecies levels for *Aeromonas* and the family *Pasteurellaceae* (26–29, 42). The hypervariable region of the *rpoB* gene (located between bp 2300 and 3300) has been reported to be the most suitable place to sequence for phylogenetic discrimination and identification at the species and subspecies levels (23, 25, 27, 29–34).

This study was designed to determine if *E. albertii* was present

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in chicken carcass rinse samples from federally inspected poultry slaughter and processing facilities during a 1-year period from November 2009 until October 2010 and to characterize recovered isolates. Presumptive positive isolates were tested using the standard multiplex PCR for the presence of *clpX*, *lysP*, and *mdh* genes (4). The isolates were tested for the presence or absence of seven additional genes. Isolates positive for the *cdtB* and *eae* genes were analyzed further to determine genetic relationships. A 521-bp segment of the *rpoB* gene was sequenced to determine an additional sequence-based method for positive identification of *E. albertii* and was analyzed for the presence or absence of fixed differences, i.e., character states that are the same in all members of one population but different in members of other populations (species, etc.), between *E. albertii* and other, closely related organisms.

MATERIALS AND METHODS

Bacterial isolation, identification, and growth conditions. Chicken carcass rinsates ($n = 1,644$) were collected at federally inspected slaughter plants in regions 1 through 4 in the United States as defined for the National Antimicrobial Resistance Monitoring System (NARMS) program (35). The 5 NARMS regions are as follows: Northeast (region 1), Southeast (region 2), Midwest (region 3), Southwest (region 4), and West (region 5). The chicken rinses were processed using two different methods based on studies of growth conditions of isolates originally described by Albert et al. (1). For the first method, 1 ml of chicken carcass rinsate was diluted with 9 ml of buffered peptone, and a sterile cotton swab was used to streak it directly onto Chromagar EEC (Hardy Diagnostics, Santa Maria, CA) plates. The plates were incubated for 18 to 24 h at 42°C, after which white colonies matching the phenotype of *E. albertii* control strains were selected. When available, multiple colonies per rinse were selected. Separate rinse samples were identified with the letter codes A through AA (Table 1). Selected colonies were reisolated by subsequent plating on Luria-Bertani (LB) medium (Hardy Diagnostics, Santa Maria, CA) and incubated at 37°C overnight.

For the second method, chicken carcass rinses were diluted 1:10 in EE Mossel Broth (Becton Dickinson, Sparks, MD), and replicate broths were incubated at either 37°C or 42°C for 4 h. One hundred microliters of diluent was then plated on xylose lysine deoxycholate agar (XLD) (Hardy Diagnostics, Santa Maria, CA) and Hektoen enteric agar (HEA) (Hardy Diagnostics, Santa Maria, CA), and replicate plates were incubated overnight at 37°C or 42°C. Suspect *E. albertii* isolates were pink, with a slightly yellow or cream-colored center on XLD and/or green on HEA. If present, multiple colonies per plate were selected. The isolates were tested further on each of five biochemical agar slants (sulfur-indole motility [SIM] [Oxoid, Hampshire, England], Simmons citrate [Sigma, St. Louis, MO], urea [Sigma, St. Louis, MO], triple-sugar iron [TSI] [Becton Dickinson, Sparks, MD], and lysine iron [Becton Dickinson, Sparks, MD]). Suspected isolates and *E. albertii* control isolates were negative for SIM, Simmons's citrate, and urea, alkaline over acid with no hydrogen sulfide production for TSI, and alkaline over neutral or alkaline over alkaline with no hydrogen sulfide production for lysine iron agar. Selected isolates were grown in LB medium, liquid or agar, at 37°C and frozen at −80°C in LB medium with 10% (vol/vol) glycerol.

Multiplex PCR for detection of putative positive *E. albertii*. Template DNA for PCR from suspect *E. albertii* isolates was prepared by suspending cells from a single colony in 200 µl sterile water and treating in a boiling-water bath for 10 min. All primers (Table 2) were obtained from Eurofins MWG Operon (Huntsville, AL). Positive controls, as well as a negative control without DNA, were included in each PCR discussed below.

The initial screen consisted of a multiplex PCR to confirm putative positive *E. albertii* isolates (http://www.shigatox.net/stec/protocols/PCR_EB.pdf) with published primers (Table 1) for the *clpX*, *lysP*, and *mdh* genes (4). The PCRs were performed according to the manufactur-

TABLE 1 Study isolates with unique rinse, growth conditions, PCR results, or *rpoB* type

Isolate identifier	Rinse ^a	Medium/ temp (°C) ^b	PCR result ^c			<i>rpoB</i> type ^d	Isolate multiple ^e
			Screening	<i>eae</i>	<i>cdtB</i>		
EA-1	A	XLD/42	Neg	Neg	Neg	5	1
EA-2	B	HEA/37	Neg	Neg	Neg	5	1
EA-3	C	HEA/42	Pos	Pos	Pos	5	4
EA-7	C	XLD/37	Pos	Pos	Pos	5	1
EA-8	C	CHR/37	Pos	Pos	Pos	5	2
EA-10	C	CHR/37	Neg	Neg	Neg	3	1
EA-14	D	CHR/37	Neg	Neg	Neg	3	1
EA-16	E	CHR/37	Pos	Pos	Pos	3	2
EA-18	F	HEA/37	Pos	Pos	Pos	5	1
EA-19	G	HEA/37	Pos	Pos	Pos	5	1
EA-21	H	CHR/37	Pos	Pos	Pos	5	1
EA-22	I	CHR/37	Pos	Pos	Pos	3	2
EA-26	J	XLD/37	Pos	Pos	Pos	3	1
EA-27	K	HEA/37	Pos	Neg	Pos	4	1
EA-28	K	HEA/37	Pos	Pos	Pos	4	1
EA-29	L	CHR/37	Pos	Pos	Pos	4	1
EA-30	L	CHR/37	Pos	Neg	Pos	4	1
EA-32	M	HEA/37	Pos	Pos	Pos	5	1
EA-33	N	HEA/42	Pos	Pos	Pos	4	1
EA-34	N	HEA/42	Pos	Neg	Pos	4	1
EA-35	O	XLD/42	Pos	Pos	Pos	3	4
EA-39	O	HEA/42	Pos	Pos	Pos	3	4
EA-43	P	CHR/37	Pos	Pos	Pos	3	2
EA-45	Q	XLD/37	Pos	Pos	Pos	3	2
EA-47	R	CHR/37	Pos	Pos	Pos	3	2
EA-49	S	HEA/42	Pos	Pos	Pos	4	1
EA-50	S	XLD/42	Pos	Pos	Pos	4	1
EA-51	T	HEA/37	Pos	Pos	Pos	5	1
EA-52	U	HEA/42	Pos	Pos	Pos	3	2
EA-54	V	CHR/37	Pos	Pos	Pos	4	1
EA-55	W	HEA/37	Pos	Pos	Pos	3	1
EA-56	W	HEA/42	Pos	Pos	Pos	3	2
EA-58	X	HEA/42	Pos	Pos	Pos	3	1
EA-59	Y	HEA/42	Pos	Pos	Pos	3	4
EA-63	Z	HEA/37	Pos	Pos	Pos	6	4
EA-67	Z	HEA/42	Pos	Pos	Pos	6	1
EA-68	Z	XLD/37	Pos	Pos	Pos	6	3
EA-75	AA	HEA/37	Pos	Pos	Pos	3	3

^a All rinses were identified by a letter from A to AA. Rinses that yielded *E. albertii* by multiple methods are highlighted in boldface.

^b Agar used and temperature of incubation. CHR, Chromagar ECC.

^c Screening PCR shows results for *clpX*, *lysP*, and *mdh* genes (11). Pos, positive; Neg, negative.

^d *rpoB* types 3 to 6 were found in this study.

^e The number of isolates that were isolated separately but have the same profile.

er's instructions with 10 µl of 2× Multiplex master mix (Qiagen Multiplex PCR kit), 10 µM each forward and reverse primer, 3 µl boiled colony lysate, and sterile water in a final volume of 20 µl. The PCR cycle conditions were as follows: 95°C for 15 min, followed by 35 cycles of 94°C for 60 s, 65°C for 60 s, and 72°C for 60 s, with a final extension at 72°C for 10 min. Amplicons were visualized on 1× Tris-borate-EDTA, and 1% agarose gels were run for 2 h at 80 V alongside a TrackIt 1-kb Plus DNA ladder (Invitrogen Corporation). An isolate was considered putative positive *E. albertii* if amplicons of the expected size for all three genes were observed.

Multiplex PCR for *staA*, *eae*, *stx*₁, and *stx*₂. All putative positive *E. albertii* isolates were tested with a multiplex PCR modified from that of Franck et al. (36) to amplify heat-stable enterotoxin A (*staA*), intimin (*eae*), and Shiga toxin 1 and 2 (*stx*₁ and *stx*₂) genes. PCRs were performed

TABLE 2 Primers used in this study

Gene	Primer name	Primer sequence (5'–3')	Amplicon size (bp)	Reference or source
<i>clpX</i>	clpX_28F	TGG CGT CGA GTT GGG CA	384	4
	clpX_411R	TCC TGC TFC GGA TGT TTA CG		
<i>lysP</i>	lysP_107F	GGG CGC TGC TTT CAT ATA TTC TT	252	4
	lysP_358R	TCC AGA TCC AAC CGG GAG TAT CAG GA		
<i>mdh</i>	mdh_50F	CTG GAA GGC GCA GAT GTG GTA CTG ATT	115	4
	mdh_164R	CTT GCT GAA CCA GAT TCT TCA CAA TAC CG		
<i>sta</i>	sta F	GCT AAT GTT GGC AAT TTT TAT TTC TGT A	190	34
	sta R	AGG ATT ACA ACA AAG TTCA CAG CAG TAA		
<i>eae</i>	eae1 F	ATA TCC GTT TTA ATG GCT ATC T	425	34
	eae1 R	AAT CTT CTG CGT ACT GTG TTC A		
<i>stx₁</i>	stx1 F	TTC GCT CTG CAA TAG GTA	555	34
	stx1 R	TTC CCC AGT TCA ATG TAA GAT		
<i>stx₂</i>	stx2F	GTG CCT GTT ACT GGG TTT TTC TTC	118	34
	stx2R	AGG GGT CGA TAT CTC TGT CC		
<i>stx_{2f}</i>	stx2f-F1	TGG GCG TCA TTC ACT GGT TG	424	35
	stx2f-R1	TAA TGG CCG CCC TGT CTC C		
<i>cdtB</i>	CDT-s1-F	GAA AGT AAA TGG AAT ATA AAT GTC CG	466	18
	CDT-as1-R	AAA TCA CCA AGA ATC ATC CAG TTA		
<i>rpoB</i>	Ea_rpoB1	TCA GGG ACG CAC CGA CGG AT	610	This study
	Ea_rpoB2	CGC GTT GGC CTG GTA CGT GT		

according to the manufacturer's instructions with 10 µl of 2× Multiplex master mix (Qiagen Multiplex PCR kit), 10 µM each forward and reverse primer, 3 µl boiled colony lysate, and sterile water in a final volume of 20 µl. The PCR cycle conditions were as follows: 95°C for 15 min, followed by 30 cycles of 94°C for 30 s, 50°C for 45 s, and 70°C for 90 s, with a final extension at 70°C for 10 min. Amplicons were visualized as described above. An isolate was considered positive for a particular gene if an amplicon of the expected size was observed.

PCR for *stx_{2f}*. All putative positive *E. albertii* isolates were tested by PCR to amplify the Shiga toxin 2f (*stx_{2f}*) gene (37). PCRs were performed according to the manufacturer's instructions with 10 µl of 2× HotstarTaq master mix (Qiagen HotstarTaq PCR kit), 5 µM each forward and reverse primer, 5 µl boiled colony lysate, and sterile water in a final volume of 20 µl. The PCR cycle conditions were as follows: 95°C for 15 min, followed by 35 cycles of 94°C for 50 s, 56°C for 40 s, and 72°C for 60 s, with a final extension at 72°C for 10 min. Amplicons were visualized as described above. An isolate was considered positive for a particular gene if an amplicon of the expected size was observed.

PCR for detection of *cdtB*. All putative positive *E. albertii* isolates were tested separately with two PCR primer pairs designed from the consensus region of all cytolethal distending toxin (*cdtB*) genes (18). The PCRs were performed according to the manufacturer's instructions with 10 µl of 2× Multiplex master mix (Qiagen Multiplex PCR kit), 10 µM each forward and reverse primer (Table 2), 3 µl boiled colony lysate, and sterile water in a final volume of 20 µl. The PCR cycle conditions were as follows: 95°C for 15 min, followed by 30 cycles of 94°C for 60 s, 55°C for 60 s, and 72°C for 60 s, with a final extension at 72°C for 10 min. Amplicons were visualized as described above. An isolate was considered positive for the *cdtB* gene if an amplicon of the expected size was observed.

PCR and sequencing of a section of the *E. albertii* *rpoB* gene. The Basic Local Alignment Search Tool (BLAST) (38) search algorithm was used to search the database at the National Center for Biotechnology

Information (NCBI), National Institutes of Health, Bethesda, MD, for the *rpoB* sequences of 68 *Enterobacteriaceae* species closely related to *E. albertii*, including various *E. coli*, *Shigella*, *Citrobacter*, *Salmonella*, and *E. fergusonii* strains (data not shown). Fixed differences in the *rpoB* gene between *E. albertii* and these closely related strains from NCBI were visually detected using sequence alignment and comparison. Primers were designed to amplify a 610-bp section of the *rpoB* gene using NCBI/Primer-BLAST (39). The primers were designed in a hypervariable region of the *rpoB* gene but optimized specifically for *E. albertii* fixed differences deduced from the nucleotide sequences recovered from NCBI (26). Total DNA from putative positive *E. albertii* isolates was extracted from 2 ml of overnight cultures using the QIAprep Spin miniprep kit (Qiagen Corp., Valencia, CA) following the manufacturer's instructions. The DNA concentration was determined by measuring the optical density at 260 nm with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). PCRs for sequencing were performed according to the polymerase manufacturer's instructions (Advantage-HF 2 PCR Kit; Clontech Laboratories) and contained 1× HF 2 PCR buffer, 400 ng of template DNA, 10 µM each forward and reverse primer, 1× deoxynucleoside triphosphate (dNTP) mixture, 1× polymerase mixture, and sterile water in a final volume of 50 µl. The PCR cycle conditions were as follows: 94°C for 15 s, followed by 25 cycles of 94°C for 15 s, 61°C for 15 s, and 68°C for 60 s, with a final extension of 68°C for 3 min. Amplicons were visualized as described above. All isolates had amplified products at the expected 610-bp size. The PCR products were sent to Retrogen, Inc. (San Diego, CA), for primer extension sequencing from the forward and reverse primers.

Sequences were analyzed using Geneious Pro version 5.1.7, created by Biomatters. After sequencing and trimming, the shortest DNA sequence was 521 bp. For analysis of polymorphic bases, an alignment was made, including the corresponding 521-bp regions of the *rpoB* gene for five different *E. coli* strains—APEC O1 (NCBI accession no. NC_008563), ATCC 8739 (NCBI accession no. CP000946), O111:H- strain 11128

position	11111	1122222233	3333333444	4444444455
position	3356725678	9922458822	4455678133	455667780
position	1721047921	0369436758	3928492201	747092845
Consensus ^a	TCATGACTCT	CGCCTGTCCG	ACCGCCGTCC	GTACTTTTC
<i>E. coli</i> APEC O1 2	.TGC.GGA.C	TA..ACC.T.	...TTT.C..	AA...CCC.
<i>E. coli</i> ATCC 8739	.TGC.G.A.C	TA..AC..T.	...TT..C..	AA...CCCT
<i>E. coli</i> O111:H- str. 11128	.TGC.G.A.C	TA..AC..T.	...TT..C..	AA...CCC.
<i>E. coli</i> O157:H7 str. EDL933	.TGC.G.A.C	TA..AC..T.	.T.TT..C..	AA...CCCT
<i>E. coli</i> K12	ATGC.G.A.C	TA..AC..T.	...TT..C.T	AA...CCC.
<i>S. boydii</i> CDC 3083-94	.TGC.G.A.C	TA..AC..T.	...TT..C..	AA.T.CCC.
<i>Shigella</i> sp. 1-5 ^b	.TGC.G.A.C	TA..AC..T.	...TT..C..	AA...CCC.
<i>S. sonnei</i> Ss046	.GC.G.GTC	TAT.AC.TT.	G..TT.TC..	AA...CCCT
<i>Shigella</i> C13 773557 ^c	.TGC.G.A.C	TA..AC..TA	.T.TT..C..	AA...CCCC.
<i>E. albertii</i> subpopulation 1 ^d			.T.....T	.G..CCC.
<i>E. albertii</i> subpopulation 2 ^e	T....		.T.....T	
<i>E. albertii</i> subpopulation 3 ^f		T.....		
<i>E. albertii</i> subpopulation 4 ^g	T....			.G..CCC.
<i>E. albertii</i> subpopulation 5 ^h	.G.....		.T.....T	
<i>E. albertii</i> subpopulation 6 ⁱ	T....		.T.....	
<i>E. albertii</i> fixed difference^j	* * * *	* * *	* * *	* *

FIG 1 *rpoB* gene polymorphic sites for positive identification of *E. albertii*. The sequence descriptions are identified by superscript letters as follows: a, consensus sequence generated by Geneious based on *E. albertii* isolates in this study; b, five *Shigella* sequences from NCBI that had identical sequences in this region (*S. flexneri* 5 strain 8401, *S. flexneri* 2a strain 2457T, *S. flexneri* 2a strain 301, *S. dysenteriae* Sd197, and *S. boydii* Sb227); c, atypical *Shigella* C13 3557-77; d, subpopulation 1, *E. albertii* strains originally described by Albert (the Albert numbers are identical to the International Centre for Diarrhoeal Disease Research, Bangladesh [ICDDR] numbers, 12502, 10790, 10457, 9194, and 19982 (2) (the same *rpoB* sequence as in GenBank accession number [BBMY01000050](#), *E. albertii* NBRC 107761); e, subpopulation 2, *E. albertii*, initially identified as *S. boydii* C13 616 and *S. boydii* c-425 (ATCC 12032) (GenBank accession number [KP100164](#)); f, subpopulation 3, 35 isolates from 12 rinses in this study (GenBank accession number [KP090209](#)); g, subpopulation 4, 14 isolates from 8 rinses in this study (GenBank accession number [KP090210](#)); h, subpopulation 5, 10 isolates from 7 rinses in this study (GenBank accession number [KP090211](#)); i, subpopulation 6, 6 isolates from 1 rinse in this study (GenBank accession number [KP090212](#)); j, the asterisks indicate the positions of fixed differences, i.e., sites that distinguish *E. albertii* from the other populations.

(NCBI accession no. [NC_013364](#)), O157:H7 strain EDL933 (NCBI accession no. [AE005174.2](#)), and K-12 MG1655 (NCBI accession no. [NC_000913.2](#))—and seven *Shigella* strains—*Shigella boydii* CDC3083-94 (NCBI accession no. [NC_010658.1](#)), *Shigella flexneri* 5 strain 8401 (NCBI accession no. [NC_008258.1](#), *S. flexneri* 2a strain 2457T (NCBI accession no. [NC_004741.1](#)), *S. flexneri* 2a strain 301 (NCBI accession no. [NC_004337.1](#)), *Shigella dysenteriae* Sd197 (NCBI accession no. [NC_007606.1](#)), *S. boydii* Sb227 (NCBI accession no. [NC_007613.1](#)), and *Shigella sonnei* (NCBI accession no. [NC_016822.1](#)). A table of polymorphic sites was generated (Fig. 1) using the software SITES (40).

Phenotypic characterization of *E. albertii* isolates using Vitek-2. Further analysis of the *E. albertii* isolates was conducted by analyzing the isolates with the Vitek-2 Compact system (bioMérieux, Marcy l'Etoile, France). Isolates were grown on tryptic soy agar (TSA) with 5% sheep blood (Becton Dickinson, Sparks, MD) before testing. The inoculum suspension was prepared in 0.45% saline, giving the equivalent of 0.5-McFarland standard turbidity. A Vitek test card (Gram negative for identification) for biochemical analysis was filled with cell suspension, and testing was completed according to the manufacturer's instructions.

Nucleotide sequence accession numbers. Newly determined sequence data were submitted to GenBank under accession numbers [KP100164](#), [KP090209](#), [KP090210](#), [KP090211](#), and [KP090212](#).

RESULTS

Bacteria isolated. *E. albertii* isolates were recovered from a total of 27 of the original 1,644 chicken carcass rinse samples taken at slaughter from November 2009 until October 2010 (Table 1). *E. albertii* isolates were detected from rinses in each medium category: Chromagar at 37°C (8 rinses), HEA at 37°C (9 rinses), HEA at 42°C (9 rinses), XLD at 37°C (3 rinses), and XLD at 42°C (3 rinses). None of the rinse samples yielded *E. albertii* by all four methods, two samples (C and Z) were positive by three methods, three samples were positive by two methods, and 22 samples were positive by only one method. Four isolates that presented as white or purple and were initially rejected as *E. albertii* were labeled as putative negative isolates but were kept for comparison because their biochemical phenotypes (see below) were atypical for *E. coli*

or *H. alvei*. They were later found to have *rpoB* sequences characteristic of *E. albertii*. Both motile and nonmotile *E. albertii* isolates were detected.

PCR for expected *E. albertii* genes. A total of 1,040 colonies from the original rinses were screened by the multiplex PCR for the presence of the *clpX*, *lysP*, and *mdh* genes (4). *clpX* was used as a positive control for the PCR, as all *E. coli*, *E. albertii*, and *Shigella* strains were positive for this gene. *lysP* and *mdh* are housekeeping genes specific to *E. albertii*. Sixty-one isolates yielded PCR products of the expected size for the *clpX*, *lysP*, and *mdh* genes and were labeled as putative positive *E. albertii* isolates; four isolates were negative for the *lysP* and *mdh* genes and were labeled as putative negative and kept for further analysis.

Table 1 shows 38 conditions yielding isolates unique in rinse, growth conditions, PCR results (*lysP*, *mdh*, *eae*, and *cdtB*), and *rpoB* type. However, if only isolates with differing subtypes from a single sample are considered independent, there were a minimum of 31 independent isolates. Further analysis of all 65 isolates included a multiplex PCR to detect heat-stable enterotoxin A (*stxA*), intimin (*eae*), and Shiga toxin 1 and 2 (*stx₁* and *stx₂*) genes (9). *E. albertii* isolates are expected to be positive for the intimin (*eae*) and a *cdtB* gene and negative for heat-stable enterotoxin A (*stxA*) and Shiga toxin 1 and 2 (*stx₁* and *stx₂*) genes (2, 18). *E. albertii* isolates can be positive for *stx_{2f}* (37). Fifty-eight isolates followed the expected PCR profile for *E. albertii*: positive for *cdtB1* and *eae* but negative for *stxA*, *stx₁*, and *stx₂* genes. Seven isolates were negative for *eae*. All of our isolates were negative for *stx_{2f}*. Isolates from four rinses (C, K, L, and N) showed variation in the PCR results in the same rinse, suggesting that multiple *E. albertii* strains were isolated.

Polymorphic sites detected in the *E. albertii* *rpoB* gene. A section of the *rpoB* gene was sequenced for 65 isolates, and an alignment was prepared with known sequences from *E. albertii* and other, closely related organisms. Five different *E. coli* and

seven *Shigella* strains were obtained from NCBI and included in the polymorphic analysis. After sequencing and trimming to the shortest DNA sequence, 521 bp of all isolates was analyzed. A table of polymorphic sites was generated using the software SITES (40). The alignment revealed 39 base positions with polymorphisms (Fig. 1); 14 of these polymorphisms were fixed differences that discriminated the *E. albertii* population from the *E. coli* and *Shigella* populations. Within the *E. albertii* populations, there were 10 single-nucleotide polymorphisms (SNPs) relative to the species consensus at bp 52, 70, 229, 352, 430, 431, 457, 472, 478, and 484 of the alignment (Fig. 1). None of the SNPs introduced a base that differed from the genus consensus and thus are likely to be ancestral in origin. Based on the pattern of SNPs, four *rpoB* subpopulations were detected among the 65 chicken isolates analyzed here. Two additional subpopulations were determined in the *E. albertii* reference isolates used in this study. Two different *rpoB* subpopulations were detected in isolates from rinse C (Table 1), in which 8 isolates were tested; 1 isolate was *rpoB* type 3, and the remaining 7 isolates were *rpoB* type 5. Interestingly, 7 isolates that were PCR negative for at least one expected gene had sequences characteristic of *E. albertii*. *rpoB* type 3 was the most common, with 34 isolates from 14 rinse samples, and was found in all five different medium categories tested. *rpoB* type 4 was found in 9 isolates from 5 rinses and in four of the five medium categories tested. *rpoB* type 5 was found in 14 isolates from 8 rinses and was found in all five medium categories tested. *rpoB* type 6 was the least common and was found in 8 isolates from one rinse and in three of the five medium categories tested.

Biochemical analysis of *E. albertii* isolates. Further biochemical analysis of the 65 isolates was conducted using the Vitek-2 Compact system (bioMérieux, Marcy l'Etoile, France) Gram-negative card. This system is not programmed to detect *E. albertii*, and there were no consistent phenotypic differences found between *E. albertii* (isolates and controls) and *H. alvei*, *E. coli*, and *Shigella* controls. When isolates were analyzed by grouping them as *rpoB* subpopulations, consistent phenotypic patterns emerged in the Vitek-2 results (Table 3). All *E. albertii* isolates were negative for the following Vitek-2 tests: Ala-Phe-Pro-acrylamidase (APPA), L-arabidose (IARL), H₂S production (H₂S), beta-N-acetyl-glucosaminidase (BNAG), glutamyl arylamidase pNA (AGLTp), beta-glucosidase (BGLU), beta-xylosidase (BXYL), beta-alanine arylamidase pNA (BAIap), D-tagatose (dTAG), lipase (LIP), palatinose (PLE), urease (URE), saccharose/sucrose (SAC), citrate sodium (CIT), malonate (MNT), 5-keto-D-gluconate (5KG), alpha-glucosidase (AGLU), beta-N-acetyl-galactosaminidase (NAGA), L-histidine assimilation (IHISa), and Glu-Gly-Arg-arylamidase (GGAA).

DISCUSSION

Albert et al. (1, 2) first described the species of bacteria that later became known as *E. albertii* (3). Current *E. albertii* studies are based on the type strains and reference strains they described. The distinguishing phenotypic, biochemical, and genetic properties of *E. albertii* include the following: nonmotile; unable to ferment sucrose, xylose, lactose, and ducitol; and unable to produce β-D-glucuronidase (1, 2, 20, 21). The phenotypic and biochemical descriptions tend to vary, which is understandable due to the limited number of *E. albertii* isolates studied to date.

Given the finding of *E. albertii* in birds (6, 7), we expected to find the organism in poultry products. Indeed, *E. albertii* was de-

TABLE 3 Vitek-2 analysis of *E. albertii* controls and isolates

Vitek well ^a	% <i>rpoB</i> positive ^d		Subpopulation					
	<i>E. coli</i> ^b	Atypical <i>Shigella</i> ^c						
			1	2	3	4	5	6
ADO	0	0	0	0	0	8	0	0
PyrA	0	0	0	0	0	8	0	0
dCEL	0	0	0	0	0	8	0	0
BGAL	0	0	100	0	9	50	100	100
dGLU	100	100	100	100	100	100	100	100
GGT	0	0	40	0	94	0	85	0
OFF	100	100	100	50	100	100	100	100
BGLU	0	0	0	0	0	0	0	0
dMAL	100	100	20	0	97	20	15	0
dMAN	100	100	100	100	100	100	100	100
dMNE	100	100	100	100	100	100	100	100
ProA	0	100	80	50	71	85	20	100
TyrA	100	0	100	100	77	100	100	100
dSOR	100	0	0	100	100	0	11	100
dTAG	0	0	0	0	0	0	0	0
dTRE	100	0	20	100	100	80	100	0
ILATk	100	100	100	100	100	100	100	100
SUCT	100	100	100	100	100	40	100	100
AGAL	100	100	0	0	6	0	70	0
PHOS	0	0	20	0	23	0	46	0
GlyA	0	100	60	0	71	20	85	100
ODC	100	0	100	100	94	80	100	100
LDC	100	0	100	0	94	100	100	100
CMT	100	100	100	100	100	100	100	100
BGUR	100	0	0	0	3	0	8	0
O129R	0	0	100	50	14	60	70	0
IMLTa	0	0	60	50	80	0	85	100
ELLM	100	100	0	50	97	70	100	100
ILATa	0	0	0	0	26	0	54	62
Total no. ^e	1	1	5	2	35 (12)	14 (8)	10 (7)	6 (1)

^a Vitek wells: adonitol (ADO), L-pyrrolydonyl-arylamidase (PyrA), D-cellobiose (dCEL), beta-galactosidase (BGAL), D-glucose (dGLU), gamma-glutamyl-transferase (GGT), fermentation/glucose (OFF), D-maltose (dMAL), D-mannitol (dMAN), D-mannose (dMNE), L-proline arylamidase (ProA), tyrosine arylamidase (TyrR), D-sorbitol (dSOR), D-trehalose (dTRE), L-lactate alkalization (ILATk), Succinate alkalization (SUCT), alpha-galactosidase (AGAL), phosphatase (PHOS), glycine arylamidase (GlyA), ornithine decarboxylase (ODC), lysine decarboxylase (LDC), courmate (CMT), beta-glucuronidase (BGUR), O/129 resistance (O129R), L-malate assimilation (IMLTa), Ellman (ELLM), and L-lactate assimilation (ILATa).

^b A single *E. coli* 25922-1 isolate used as a control.

^c Atypical *Shigella* B13 3557-77.

^d *rpoB* subpopulation 1 comprised five *E. albertii* strains originally described by Albert: (i) 97-3259, (ii) 97-3260, (iii) 97-3261, (iv) 97-3262, and (v) 97-3263 (2). In *rpoB* subpopulation 2, *S. boydii* C13 616 and ATCC 12032 are *E. albertii*. *rpoB* subpopulations 3 to 6 were found in this study.

^e For each *rpoB* subpopulation, the total number of isolates (rinses) in this study is shown.

tected in 1.6% (27/1,644) of the carcass rinses analyzed from broiler chickens from federally inspected poultry slaughter and processing plants. *E. albertii* was detected in NARMS regions 1 through 4 (Northeast, Southeast, Midwest, and Southwest) from November 2009 until October 2010 (35). In this study, no *E. albertii* isolates were detected in chicken rinses from the West region (region 5, comprising Arizona, California, Colorado, Idaho, Montana, New Mexico, Nevada, Oregon, Utah, Washington, and Wyoming). Previously, *E. albertii* had only been reported to be detected in birds found in Alaska, Washington, and Idaho (6). With the current study, *E. albertii* has been detected in birds from

all regions of the United States. *E. albertii* in region 5 may not have been detected because of the low number of federally inspected poultry slaughter and processing facilities; less than 10% (23/241) of the establishments are located in region 5 (U.S. Department of Agriculture Food Safety and Inspection Service [FSIS], personal communication).

Culture medium tests were conducted with the five *E. albertii* reference isolates. These reference isolates presented as pink colonies with a slightly yellow or cream-colored center on XLD, which detects rapid fermentation of lactose and an acid pH; as green colonies on HEA, showing lack of hydrogen sulfide production; or as blue colonies on Chromagar EEC. Additionally the *E. albertii* reference isolates were nonmotile at 37°C.

There was not an ideal medium for isolation of *E. albertii* from chicken carcasses (Table 3). HEA recovered the most *E. albertii* isolates, with nine positive rinses at each temperature (37°C and 42°C), and XLD recovered the least, with three positive rinses at each temperature (37°C and 42°C). Any colony color selected from Chromagar EEC could be *E. albertii*. Thus, the percentage of *E. albertii* isolates detected was probably inaccurately low due to the lack of a phenotypic method to select suspect isolates. The possibility that multiple strains of *E. albertii* may be found in a single chicken was suggested by finding *E. albertii* isolates with two different *rpoB* SNP types in rinse C and isolates with different PCR genotypes in rinses C, K, L, and N.

The currently accepted protocol for identifying *E. albertii* is to initially screen isolates by PCR for *lysP* and *mdh* genes and find those that are positive (3, 4). *E. albertii* is usually positive for the *eae* and *cdtB* genes but negative for *stx*₁, *stx*₂, and *sta* (1, 4, 18, 36). However, variations have been found; Ooka et al. described two *stx*_{2F}-positive *E. albertii* isolates (9). In this study, 65 isolates from 27 poultry carcass rinses obtained from healthy birds at slaughter were tested by PCR. Fifty-eight isolates were positive for *clpX*, *lysP*, *mdh*, *eae*, and *cdtB* and negative for *staA*, *stx*₁, *stx*₂, and *stx*_{2F}. An additional seven isolates did not carry all of the expected genes. Four isolates were negative for *lysP*, *mdh*, *eae*, and *cdtB*, and an additional three isolates were negative for the *eae* gene alone (Table 1). All the isolates were analyzed further using the *rpoB* sequencing and analysis method described here. An *E. albertii* consensus sequence was generated, and 14 fixed differences distinguished *E. albertii* from closely related species (Fig. 1). *rpoB* sequence analysis revealed that the *E. albertii* isolates, including the six isolates that did not have all of the expected genes, fell into six different subpopulations based on SNPs relative to the *E. albertii* consensus sequence (Fig. 1). Subpopulation 1 had identical SNPs at nucleotides 352T, 431T, 457G, 472C, 478C, and 484C and included the five reference *E. albertii* isolates originally described by Albert (2). Subpopulation 1, all human isolates from sick pediatric patients in Bangladesh, had a distinct Vitek-2 profile (Table 3). Subpopulation 2 had identical SNPs at nucleotides 70T, 352T, and 431T and consisted of two isolates (C13 616 and C-425) that were originally thought to be *S. boydii* but are in fact *E. albertii*. Isolates from chicken rinses tested in this study fell into *rpoB* subpopulations 3 to 6 (Fig. 1). Subpopulation 3 had one SNP at nucleotide 229T. It comprised the largest subpopulation in our isolates and was found in 48% of the original rinses (14 original rinses; 32 isolates). Subpopulation 4 had identical SNPs at nucleotides 70T, 457G, 472C, 478C, and 484C and was found in 26% of the original rinses (13 original rinses; 32 isolates). Subpopulation 5 had identical SNPs at nucleotides 52G, 352T, and 430T and was

found in 18.5% of the original rinses (5 original rinses; 10 isolates). It was noted that the recently published genome sequence for *E. albertii* KF1 (41) has an *rpoB* sequence fragment identical to that in our subpopulation 5. Subpopulation 6 had identical SNPs at nucleotides 70T and 352T. It consists of 7.5% of the original rinses (2 original rinses; 9 isolates).

We detected seven positive *E. albertii* isolates by *rpoB* testing that did not carry some of the expected genes (*lysP*, *mdh*, *eae*, and *cdtB*). It is possible that rare sequences of these genes exist in the *E. albertii* populations that are not detected with the PCR primers used here. Recently, new *eae* variants of *E. albertii* isolates have been described by Oh et al. (7) and Maheux et al. (20). Also, Toth et al. found new variants of *cdt* (18). However, the patterns for *rpoB*-based subpopulations remained intact.

All the isolates were analyzed by Vitek-2 using the Gram-negative AST card. We identified different profiles based on *rpoB* types, but no phenotype was 100% consistent for all isolates. These results agree with those of Maheux et al. (20) and were not surprising because the Vitek-2 system has not been optimized to detect *E. albertii*. *E. albertii* was initially described as nonmotile, but we identified motile isolates using SIM medium. As can be seen in Table 1, most of the samples yielded identifiable *E. albertii* with only one of the media and temperatures that were tested, showing that media bias recovery and that further research on isolation methods is necessary. At this time, there are two genomic sequences for *E. albertii* in a public database (GenBank accession numbers ABKX01000019.1 and CP007025.1), and both isolates were from the five originally described by Albert (1). An *in silico* genome analysis of *E. albertii* did not identify a potentially unique method for medium-based discrimination of *E. albertii* from closely related species.

Further studies are warranted, due to the ability of *E. albertii* to cause sickness in humans and to be transmitted through food-borne routes. Here, we report on a definitive *rpoB*-based sequence analysis approach for identification of *E. albertii*. *rpoB*-based analysis identified six subpopulations in the isolates studied here. Additionally, *E. albertii* was detected in chicken carcass rinses at slaughter, indicating that *E. albertii* is present in birds across the United States, which may represent a route for human exposure. Better detection methods will increase the likelihood of finding additional subpopulations from various sources (human, environmental, and food animals), which is needed to accurately assess the prevalence of *E. albertii* in food animals and other sources.

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