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Prevalence of *eae*-positive, lactose non-fermenting *Escherichia albertii* from retail raw meat in China

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SUMMARY

Escherichia albertii is a newly emerging enteric pathogen that has been associated with gastroenteritis in humans. Recently, *E. albertii* has also been detected in healthy and sick birds, animals, chicken meat and water. In the present study, the prevalence and characteristics of the *eae*-positive, lactose non-fermenting *E. albertii* strains in retail raw meat in China were evaluated. Thirty isolates of such strains of *E. albertii* were identified from 446 (6·73%) samples, including duck intestines (21·43%, 6/28), duck meat (9·52%, 2/21), chicken intestines (8·99%, 17/189), chicken meat (5·66%, 3/53), mutton meat (4·55%, 1/22) and pork meat (2·44%, 1/41). None was isolated from 92 samples of raw beef meat. Strains were identified as *E. albertii* by phenotypic properties, diagnostic PCR, sequence analysis of the 16S rRNA gene, and housekeeping genes. Five intimin subtypes were harboured by these strains. All strains possessed the II/III/V subtype group of the *cdtB* gene, with two strains carrying another copy of the I/IV subtype group. Pulsed-field gel electrophoresis showed high genetic diversity of *E. albertii* in raw meats. Our findings indicate that *E. albertii* can contaminate various raw meats, posing a potential threat to public health.

Key words: *Escherichia albertii*, cytolethal distending toxin (CDT), intimin, raw meat.

INTRODUCTION

Escherichia albertii is a newly described and emerging diarrhoeagenic pathogen, which is associated with sporadic infections and outbreaks in humans and birds [1–7]. *E. albertii* was originally recovered from the faeces of Bangladeshi children with accompanying symptoms of diarrhoea, vomiting, fever, mild dehydration and abdominal distention, and was initially identified as *Hafnia alvei* [1]. Nevertheless, subsequent analysis based on phenotypic characterization, 16S rDNA sequencing and DNA–DNA hybridization, indicated that these *H. alvei*-like strains belong to a new *Escherichia* species, named *E. albertii* [8].

E. albertii belongs to the attaching and effacing (A/E) group of pathogens, which form A/E lesions on intestinal epithelial cell surfaces by the combined action of an outer membrane protein, intimin,

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encoded by *eae* gene, and other type III secretion system effectors of the locus of enterocyte effacement pathogenicity island [9]. Currently, more than 30 distinct intimin subtypes have been reported and *E. albertii* possess intimin subtypes that are rarely described or have not been described in *E. coli* [3]. Donato *et al*. found that *E. albertii* can express enterohaemorrhagic *E. coli* O157:H7 type III secretion system effectors EspE and EspF and cause a redistribution of the tight junction protein zona occludens-1 of the polarized epithelial MDCK-I and T84 cells [10]. In addition to intimin, cytolethal distending toxin (CDT) has also been reported as a putative virulence factor in *E. albertii* [11], which is a conserved bacterial genotoxin that blocks cell cycle progression, leading to apoptosis of a broad range of mammalian cell lineages [12]. CDT consists of a heterotrimeric complex of three subunits designated CdtA, CdtB and CdtC [12]. CdtB has DNase I activity, and is responsible for CDT-induced cell cycle arrest [13, 14]. Subunits CdtA and CdtC associate with CdtB to translocate it into the host cell [15]. CdtB has distinct subtypes, and most *E. albertii* strains possess a *cdtB* belonging to the II/III/V subtype group [3]. Shiga toxins (Stx) are the most significant virulence factors of Stx-producing *E. coli* in human infections and Stx2 are classified into seven subtypes (Stx2a to Stx2g) [16]. Stx2f, originally identified in *E. coli* strains isolated from the faeces of feral pigeons [17], has also been detected in some *E. albertii* strains [3, 5]; however, the clinical significance of Stx2f-producing *E. albertii* is unknown. Recently, a stx_{2a}-positive *E. albertii* strain was identified from a patient with bloody diarrhoea [18].

The specific biochemical characteristics of *E. albertii* are poorly defined; therefore, no commercial system currently includes this species in its database. Thus, it remains difficult to discriminate *E. albertii* from *E. coli* and other members of the Enterobacteriaceae using routine bacterial identification systems, which often misidentify *E. albertii* strains as *Hafnia, Salmonella, Yersinia ruckeri*, and, in particular, *E. coli*, with high probability [19]. The lack of motility and the inability to ferment D-xylose and lactose have been reported to be the common biochemical properties of confirmed *E. albertii* strains, which can help to distinguish *E. albertii* from other *Escherichia* species [3, 6, 8, 19–21]. Hyma *et al*. devised a diagnostic multiplex polymerase chain reaction (PCR) to detect conserved sequences that distinguish members of the *E. albertii* lineage from *E. coli* and *Shigella* spp. strains. This method was based on nucleotide polymorphisms of the housekeeping genes *lysP* and *mdh* in the *E. albertii* lineage [11]. Multiplex PCR is independent of biochemical and antigenic phenotypes and, therefore, provides a useful method to screen *E. albertii* strains [2, 4–6, 21, 22].

Although *E. albertii* is found in humans, animals, birds and water, the prevalence, epidemiology and clinical relevance of the species are still poorly defined, in part because it is likely to either remain unidentified or be misidentified. *E. albertii* may be one of the unknown aetiological agents that contribute to the estimated 62 million cases of foodborne illnesses and 3200 deaths in the United States [23]. An *eae*-positive isolate of *H. alvei* was isolated from minced meat in Sweden, but it was doubtful whether this *H. alvei* strain was actually *E. albertii* [24]. The lack of identification of this pathogen has resulted in limited investigations into its incidence in foodstuffs. Current *E. albertii* studies are mainly based on the type strains and reference strains, which show the presence of the *eae* gene, lack of motility, and an inability to ferment D-xylose and lactose. Yet, the phenotypic and genetic descriptions tend to vary as a greater number of *E. albertii* isolates are identified from different sources. In this study, we investigated the prevalence and characteristics of *eae*-positive, lactose non-fermenting phenotype *E. albertii* strains which represent a substantial proportion of *E. albertii* in retail raw meats collected from Zigong city, Sichuan province, China.

MATERIALS AND METHODS

Sample collection

A total of 446 raw meat and intestine samples were collected from supermarkets and farmers' markets in Zigong city, Sichuan province, China, from March 2013 to July 2014. The samples included beef meat ($n = 92$), pork meat ($n = 41$), mutton meat ($n = 22$), chicken meat ($n = 53$), chicken intestines ($n = 189$), duck meat ($n = 21$), and duck intestines ($n = 28$). The samples were transported in ice-bags to the laboratory of Zigong Centre for Disease Control and Prevention within 4 h and tested immediately.

Screening *eae*-positive samples

Twenty-five grams of each minced raw meat or sliced intestine sample was enriched with 225 ml EC broth (Oxoid, UK) and incubated at 20 °C for 24–36 h on a shaking platform (220 rpm). One microlitre of

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each enrichment sample was centrifuged at 1500 ***g*** for 1 min to pellet large meat debris. The supernatant was centrifuged at 13000 ***g*** for 2 min and the pellet was suspended in 100 μl lysis buffer [100 mM NaCl, 10 mM Tris-HCl (pH 8·3), 1 mM EDTA (pH 9·0), and 1% Triton X-100], boiled for 10 min, and centrifuged again. The resulting supernatant was used as a template to test the presence of *eae* by PCR, using previously published primers (Supplementary Table S1) [2].

Isolation of lactose non-fermenting strains

One loopful of each *eae*-positive enrichment culture was directly streaked on MacConkey agar (Oxoid, UK) and CHROMagar ECC agar (CHROMagar, France). After overnight incubation at 36 °C, colourless, round and moist, presumptive *E. albertii* colonies on both agars were chosen to test for the presence of *eae*. The *eae*-positive colonies were plated on Luria–Bertani (LB) agar (Oxoid) and incubated overnight for further identification. If more than one *eae*-positive lactose non-fermenting isolate was identified, only one from each sample was retained for further investigation.

Phenotypic tests

Carbohydrate-fermenting abilities were determined after 3 days of incubation at 36 °C in Andrade peptone water containing lactose or D-xylose (Land Bridge, China). *In vitro* motility was determined on motility agar plates (0·3% LB agar) incubated at 36 °C for up to 48 h. Additional biochemical characterization was performed using the API 20E system (bioMérieux, France).

Diagnostic multiplex PCR and 16S rDNA sequencing

All *eae*-positive, lactose non-fermenting strains were screened by diagnostic multiplex PCR for the *E. albertii* lineage-specific housekeeping genes *lysP* and *mdh*. The *clpX* gene, which is conserved in all *E. coli, Shigella* spp. and the *E. albertii* lineages, was used as a positive control. The PCR primers and conditions used were as described previously (Supplementary Table S1) [6, 11].

16S rRNA genes were amplified and sequenced according to published protocols [25] and nucleotide sequences were aligned using ClustalW. Phylogenetic trees were constructed using the neighbour-joining algorithm with 1000 bootstrap resamplings to assess the relationships between individual pathogens using MEGA 6 (www.megasoftware.net) [26].

Multi-locus sequence typing (MLST)

All *eae*-positive, lactose non-fermenting strains were analysed by MLST to determine their phylogenetic relationships according to the *E. coli* MLST website (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). A neighbour-joining dendrogram was constructed, based on the concatenated nucleotide sequences of the seven genes and the maximum composite likelihood model using MEGA 6. The genome-sequenced *E. coli, E. fergusonii, E. albertii, Shigella* spp., and *Salmonella enterica* serovar Typhi strains were included in the phylogenetic representation, as described by Ooka *et al.* [3].

Detection of virulence genes and sequence analysis

In addition to *eae*, eight diarrhoeagenic *E. coli* marker genes, i.e. *stx$_1$, stx$_2$, ipaH, aggR, elt, estIa, estIb* and *daaD*, were screened with published primers [27] and the *stx$_{2f}$* subtype was determined as described by Schmidt *et al.* [17]. The entire *eae* gene was amplified by PCR using the cesT-F9/eae-R3 primer pair for the 5′ half of the gene, and the eae-F1/escD-R1 primer pair for the 3′ half of the gene [28]. PCR amplicons were sequenced using the above-mentioned primers and an additional sequence primer (1669–1688) was used to fully sequence the 3′ half of the *eae* gene, as described by Ooka *et al.* [3].

All sequencing results were checked and assembled using SeqMan II software (DNASTAR Inc., USA). Using the ClustalW program in MEGA 6, the predicted amino acid sequences obtained from this study were aligned with those of the reference intimin subtypes downloaded from GenBank. A phylogenetic tree was constructed as detailed above.

cdtB type I/IV was detected by the PCR primer pair CDT-s2 and CDT-as2 and type II/ III/ V by PCR primer pair CDT-s1 and CDT-as1, as described previously [29]. The partial amino acid sequences of the CdtB subunit were used to construct a neighbour-joining tree.

Pulsed-field gel electrophoresis (PFGE)

DNA extracts of *E. albertii* strains were digested with *Xba*I and fragments separated on a 1% agarose gel using a CHEF-DR III PFGE apparatus (Bio-Rad, USA). The pulse times were ramped from 2·2 to

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Table 1. *Prevalence of* Escherichia albertii *in raw meat and intestinal samples*

Samples	No. of samples	No. of *E. albertii* strains (%)
Beef meat	92	0 (0)
Pork meat	41	1 (2·44)
Mutton meat	22	1 (4·55)
Chicken meat	53	3 (5·66)
Duck meat	21	2 (9·52)
Chicken intestines	189	17 (8·99)
Duck intestines	28	6 (21·43)
Total	446	30 (6·73)

54·2 s over 18 h, according to the protocol for *E. coli* O157:H7 from PulseNet, USA (http://www.cdc.gov/pulsenet/pathogens/index.html). Gel images were captured with a Gel Doc™ XR+ system (Bio-Rad, USA), converted to TIFF files, and analysed by BioNumerics v. 4·0 (Applied Maths, Belgium). An unweighted pair-group method with arithmetic mean dendrogram was constructed using BioNumerics software.

The nucleotide sequences determined in this study have been submitted to GenBank. Their accession numbers are KP197062–KP197093 for 16S rRNA genes; KP015856–KP016011 and KP064411–KP064472 for the seven housekeeping genes; KP197094–KP197126 for the *eae* genes; and KP197127-KP197158 for the *cdtB* genes.

RESULTS

Prevalence of *eae*-positive, lactose non-fermenting strains in raw meat

Thirty isolates from 446 (6·73%) samples were identified as *eae*-positive, lactose non-fermenting strains. Seventeen (56·7%) were recovered from chicken intestines and six (20·0%) from duck intestines. The sources of the remaining isolates are given in Table 1; none was isolated from the 92 raw beef meat samples.

All isolates were oxidase-negative, fermented glucose but not lactose or D-xylose, and were non-motile. Variation was observed in utilization of *β*-galactosidase, production of indole or acetoin, and fermentation of D-manitol, D-sorbitol or D-melibiose. All strains were identified phenotypically as *E. coli* with different probabilities.

None of the strains tested positive for stx_1, stx_2, *ipaH, aggR, elt, estIa, estIb* or *daaD* genes and all were positive for the *E. albertii*-specific alleles of *lysP* and *mdh*, and for the *clpX* gene conserved in *E. coli, Shigella* spp., and *E. albertii* lineages, which tentatively identified these strains as *E. albertii*.

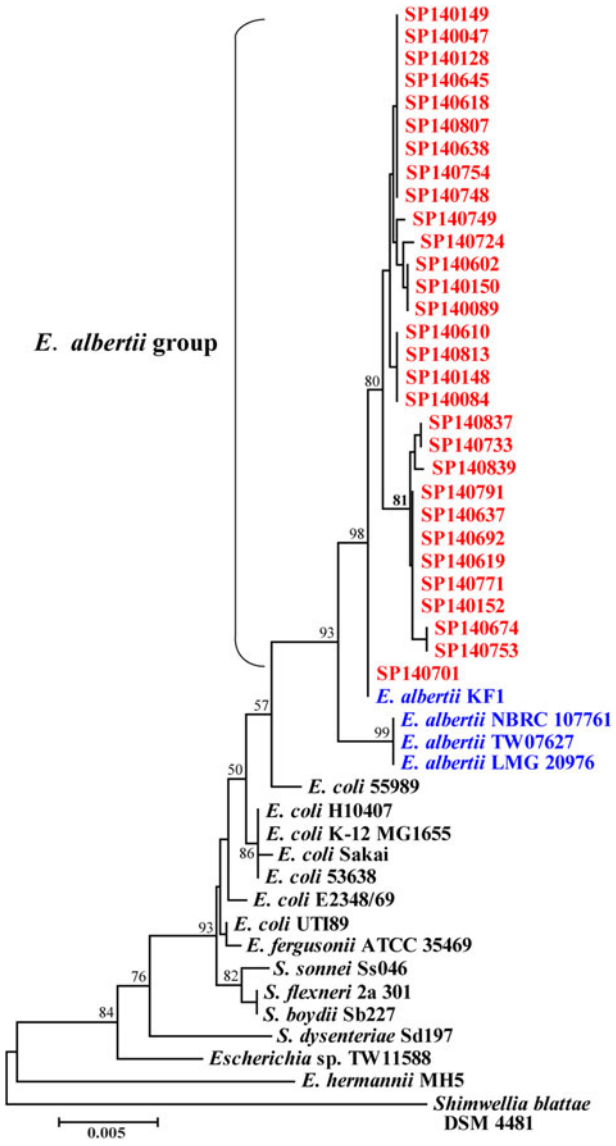

Fig. 1. Phylogenetic tree based on a 1404 bp portion of the 16S rRNA gene of *Escherichia albertii* strains isolated in this study, the *E. albertii* type strain LMG 20976 and other related genome-sequenced bacterial species.

Sequencing of a 1506 bp amplicon of the 16S rRNA genes revealed ten distinct sequence types in the 30 *E. albertii* strains with a percentage similarity ranging from 99·7% to 100%, with 99·6% identity to the *E. albertii* type strain LMG 20976 (Fig. 1). One strain (SP140701) from chicken meat was identical to *E. albertii* KF1, a clinical strain. These results support the identification of the strains as *E. albertii*.

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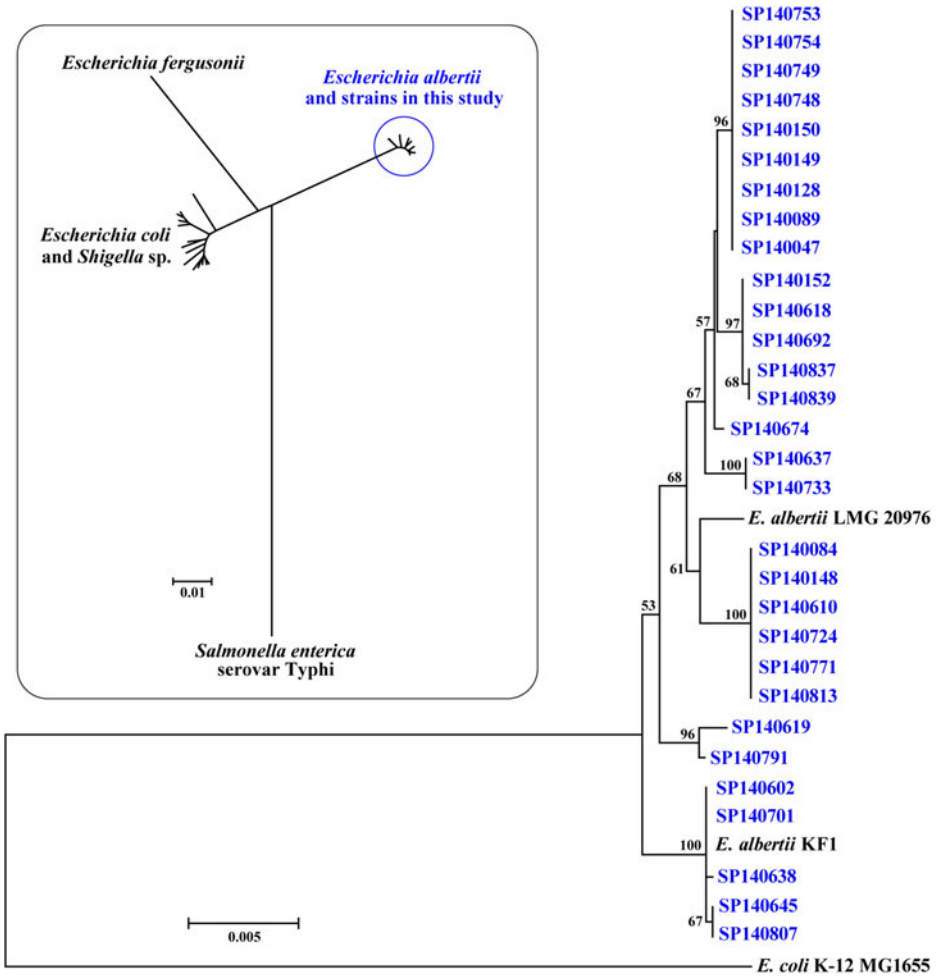

Fig. 2. Neighbour-joining dendrogram of *Escherichia albertii* strains and reference strains based on the 3423 bp concatenated partial nucleotide sequences of seven housekeeping genes. Inset is an image of the relationship of the strains isolated in this study and reference strains of *E. albertii, E. coli*/*Shigella* spp., *E. fergusonii* and *Salmonella enterica* serovar Typhi.

MLST analysis and virulence gene sequence

By MLST all strains from meat and intestine samples were highly divergent from *E. coli*/*Shigella* spp., *E. fergusonii* and *Salmonella enterica* serotype Typhi stains, and formed a separate lineage with *E. albertii* reference strains (Fig. 2, inset). Eleven different sequence types, with pairwise differences of 0·0003–0·0079, were identified in the test *E. albertii* strains, each type comprising 1–9 strains. All sequence types were closely related to the *E. albertii* type strain LMG 20976, but were less related to *E. coli* K-12 MG1655 (Fig. 2). Two strains (SP140602 and SP140701) were of identical sequence type to *E. albertii* KF1.

The phylogenetic tree of the amino acid sequences of the intimin protein encoded by the *eae* gene in all meat and intestine sample strains showed identical or close relation to five known intimin subtypes, σ, ρ, $\iota 2$, $\epsilon 3$, ν (Supplementary Fig. S1A). Seventeen strains contained the σ subtype and were isolated from all meat samples excepting those of beef meat and pork meat. Five and six strains from chicken meat or chicken intestines samples were subtyped as ρ and $\epsilon 3$, respectively; one strain from chicken intestines was of the $\iota 2$ subtype and the single pork meat isolate was of the ν subtype (Fig. 3).

All test strains were PCR-positive for *cdtB* using the CDT-s1/CDT-as1 primer pair and two strains, SP140637 from chicken intestines and SP140733 from duck intestines were also positive for *cdtB* using the CDT-s2/CDT-as2 primer pair. Sequencing and alignment of the CDT-s1/CDT-as1 PCR products showed 94–100% nucleotide and 95–100% amino acid identities and they were closely related to types II, III

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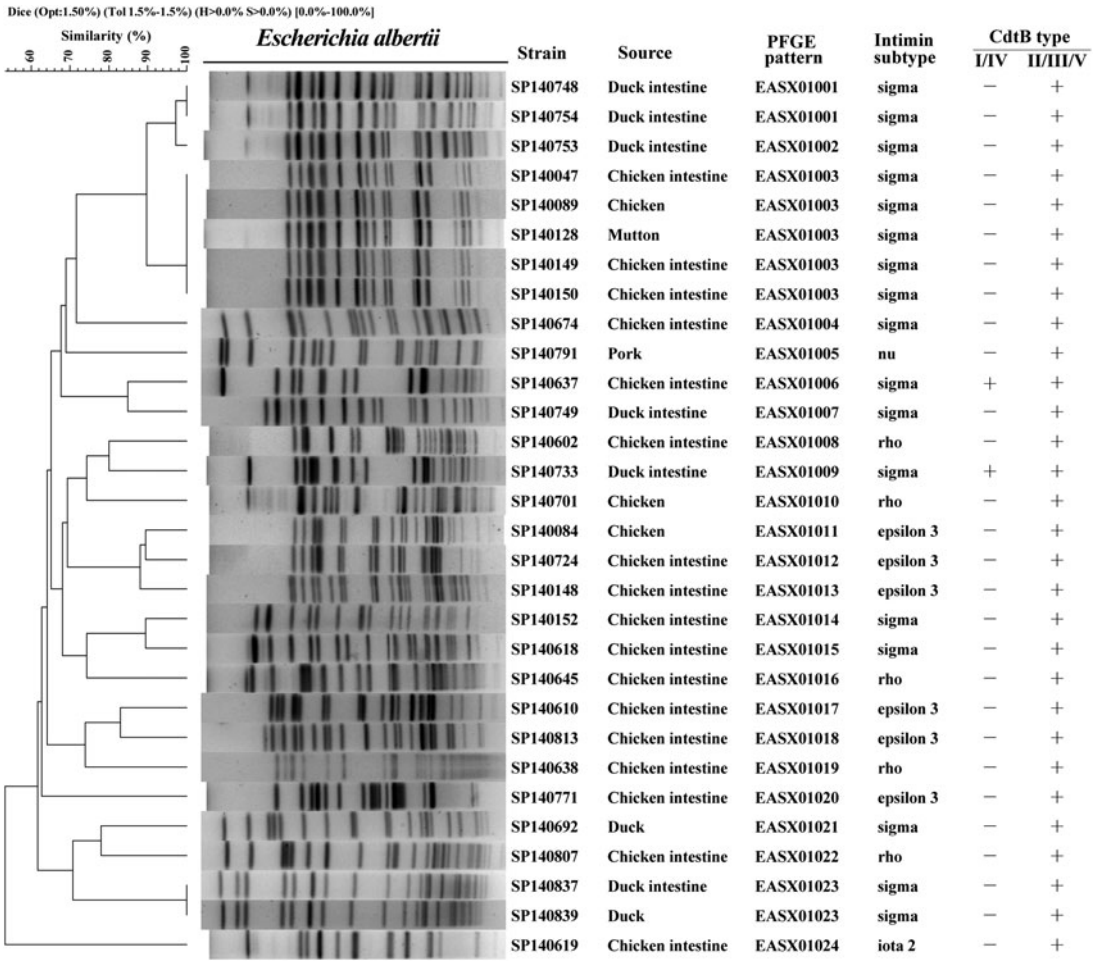

Strain	Source	PFGE pattern	Intimin subtype	CdtB type I/IV	CdtB type II/III/V
SP140748	Duck intestine	EASX01001	sigma	−	+
SP140754	Duck intestine	EASX01001	sigma	−	+
SP140753	Duck intestine	EASX01002	sigma	−	+
SP140047	Chicken intestine	EASX01003	sigma	−	+
SP140089	Chicken	EASX01003	sigma	−	+
SP140128	Mutton	EASX01003	sigma	−	+
SP140149	Chicken intestine	EASX01003	sigma	−	+
SP140150	Chicken intestine	EASX01003	sigma	−	+
SP140674	Chicken intestine	EASX01004	sigma	−	+
SP140791	Pork	EASX01005	nu	−	+
SP140637	Chicken intestine	EASX01006	sigma	+	+
SP140749	Duck intestine	EASX01007	sigma	−	+
SP140602	Chicken intestine	EASX01008	rho	−	+
SP140733	Duck intestine	EASX01009	sigma	+	+
SP140701	Chicken	EASX01010	rho	−	+
SP140084	Chicken	EASX01011	epsilon 3	−	+
SP140724	Chicken intestine	EASX01012	epsilon 3	−	+
SP140148	Chicken intestine	EASX01013	epsilon 3	−	+
SP140152	Chicken intestine	EASX01014	sigma	−	+
SP140618	Chicken intestine	EASX01015	sigma	−	+
SP140645	Chicken intestine	EASX01016	rho	−	+
SP140610	Chicken intestine	EASX01017	epsilon 3	−	+
SP140813	Chicken intestine	EASX01018	epsilon 3	−	+
SP140638	Chicken intestine	EASX01019	rho	−	+
SP140771	Chicken intestine	EASX01020	epsilon 3	−	+
SP140692	Duck	EASX01021	sigma	−	+
SP140807	Chicken intestine	EASX01022	rho	−	+
SP140837	Duck intestine	EASX01023	sigma	−	+
SP140839	Duck	EASX01023	sigma	−	+
SP140619	Chicken intestine	EASX01024	iota 2	−	+

Fig. 3. Dendrogram of pulsed-field gel electrophoresis (PFGE) profiles of 30 *Escherichia albertii* strains from meat and intestinal samples; strain designations, animal source, PFGE patterns, intimin subtypes and CdtB types.

and V *cdtB* reference alleles. Sequencing and alignment of the CDT-s2/CDT-as2 PCR product showed that the sequences from the two strains were identical to each other and the type I *cdtB* reference allele (Fig. S1B).

Twenty-four different PFGE patterns were distinguishable (<97% similarity) in the strains indicating a high degree of genetic diversity in this collection of *E. albertii* (Fig. 3).

Strains did not appear to cluster on the basis of their source, intimin subtype or CdtB type. Three PFGE patterns (EASX01001, EASX01003, EASX01023) contained two, five and two strains, respectively; the remaining patterns were each represented by single strains. The five strains of pattern EASX01003 from different sources (chicken intestines, chicken meat, mutton meat) had the same intimin subtype and CdtB type. Strains SP140748 and SP140754 from duck intestines were identical by PFGE,16S rRNA gene sequence, MLST, *eae* gene and *cdtB* gene sequences, indicating that the strains may belong to the same *E. albertii* clone (EASX01001, Fig. 3). Strain SP140753 from duck intestine had a similar pattern to EASX01001, identical MLST, *eae* gene and *cdtB* gene sequences, but different 16S rRNA gene sequence.

DISCUSSION

E. albertii has been recently recognized as a potential enteric human pathogen. These organisms isolated from diarrhoeal patients were originally identified as enteropathogenic *E. coli* or enterohaemorrhagic *E. coli* by routine diagnostic protocols [3, 5]. *E. albertii* strains were isolated from outbreaks of gastroenteritis in Japan [2, 4, 7] and were implicated as the probable cause of death of wild birds in Alaska, USA [6], although also found in healthy wild birds from Korea [22]. *E. albertii* has been detected in other bacterial

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opportunistic pathogens in the drinking water distribution system of a hospital in Budapest, Hungary [30], and in different environmental waters from Québec, Canada [31], suggesting that water could be a potential source of this pathogen.

To date, relatively few studies have investigated the prevalence of *E. albertii* in foodstuffs of animal origin. We isolated the *eae*-positive, lactose non-fermenting strains from a variety of raw meats and intestines, but none was recovered from beef meat samples. Phenotypic and biochemical analyses, diagnostic PCR, 16S rDNA sequencing, and MLST analyses classified the isolates as *E. albertii*, indicating that a wide range of raw meats could be a source of the species. Lindberg *et al*. reported an *eae*-positive strain of *H. alvei* isolated from minced meat in Sweden, which might have been *E. albertii* [24], and recently, *E. albertii* isolates were also detected in chicken rinses from three out of five regions of the United States, and in chicken giblets (liver) collected in Japan [32, 33]. The contaminant sources of raw meats are unclear; however, our limited investigation showed a higher prevalence of this pathogen in chicken intestines and duck intestines, suggesting that *E. albertii* could colonize the intestines of these animals. Ooka *et al*. analysed 278 *eae*-positive strains isolated from humans, animals, and the environment in Japan, and found a strain of *E. albertii* from a domestic cat [3]. The shedding of *E. albertii* from animal faeces requires further investigation.

We found that more colourless (lactose non-fermenting) colonies appeared on MacConkey agar after the sample was enriched at 20 °C for 24–36 h compared to 37 °C (data not shown) and this may reflect the low tolerance of *E. albertii* to heat, acid, and pressure which is reported to be significantly lower than for wild-type *E. coli* O157:H7 [34]. The growth of *E. albertii* is uncertain when it competes with other bacteria, especialy *E. coli*. In the present study, diagnostic PCR proved useful for rapid identification of the *E. albertii* strains. However, the specificity of the primers has recently been questioned and may not be optimal for the detection of *E. albertii* in food samples [35].

The protein intimin is a critical virulence factor of *E. albertii*, which is characterized by intimin subtypes that are rare or have not been previously described in *E. coli* [3]. Ooka *et al*. found that 26 *E. albertii* strains from humans, birds and a cat were grouped into nine known intimin subtypes and five new subtypes [3] and other investigations have shown that *E. albertii* strains from birds possess a variety of intimin subtypes, some novel and some similar to those previously reported [6, 22]. Five intimin subtypes, σ, ρ, $\iota 2$, $\epsilon 3$, and ν, were identified in this study and with the exception of $\iota 2$ were reported previously in *E. albertii* strains in several other studies [3, 6, 22]. The $\iota 2$ subtype was identified in *Shigella boydii* serotype 13, which is regarded as within the *E. albertii* lineage [11]. Additionally, all 30 *E. albertii* strains possessed the II/III/V subtype group *cdtB* gene, and two strains possessed another copy of the I/IV subtype group *cdtB* gene, which is in agreement with previous findings [3, 6].

In conclusion, heterogeneous *eae*-positive, lactose non-fermenting *E. albertii* strains were recovered from a variety of retail animal meat samples and intestines collected in Zigong city, Sichuan province, China. To the best of our knowledge, this is the first investigation of this newly recognized pathogen in China, and also the largest-scale survey into the prevalence of *E. albertii* in raw meats worldwide. The transmission of *E. albertii* from foodstuffs of animal origin to humans is possible, which may pose a potential threat to public health. Our study mainly focused on the type strains; other various phenotypic/genotypic strains might be missed, which may lead to an underestimation of its prevalence in retail raw meats. Further investigations are needed to assess the occurrence, pathogenicity and clonal diversity of all phenotypic/genotypic variants, and their public health significance in causing human disease in China.

SUPPLEMENTARY MATERIAL

For supplementary material accompanying this paper visit http://dx.doi.org/10.1017/S0950268815001120.

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DECLARATION OF INTEREST

None.

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