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Nonspecificity of Primers for *Escherichia albertii* Detection

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It is important for public health to identify foods that are potential sources of bacterial infections such as food poisoning. Polymerase chain reaction (PCR) is often used to detect pathogens that are poorly defined by biochemical characteristics (1). *Escherichia albertii* is a newly-described enteropathogen of humans and birds (2,3). This bacterium occasionally causes outbreaks of gastroenteritis in humans following ingestion of contaminated foods (4). In this study, we used PCR to identify foods that may be sources of *E. albertii* infection. However, we found that the species-specific primers for *E. albertii* detection (5) also detected genes from bacterial species other than *E. albertii*.

A total of 188 seed sprouts (105 bean sprouts, 25 Japanese radish sprouts, 17 broccoli sprouts, 8 red cabbage sprouts, 8 pea sprouts, 7 mustard sprouts, 6 green perilla sprouts, 5 watercress sprouts, 3 buckwheat sprouts, 2 peanut sprouts, 1 arugula sprout, and 1 kale sprout) were collected from 11 supermarkets in Fukuoka Prefecture, Japan, from March 2013 to March 2014. Samples were incubated in 9 volumes of buffered peptone water (BPW) (Oxoid, Hampshire, UK) at 35°C for 18 h. Each BPW culture was centrifuged at 13,800 × *g* for 5 min, and the supernatant was discarded. The pellet was resuspended in normal saline (1 mL) and centrifuged at 13,800 × *g* for 5 min. The supernatant was discarded, and the pellet was resuspended in 1 mL of 0.1 × TE buffer with 5% Chelex 100 (Bio-Rad Laboratories, Hercules, CA, USA) before boiling for 10 min and then centrifuging at 13,800 × *g* for 10 min. The supernatant was used as a template for primary PCR to detect *E. albertii* using primer pairs lysP107F/lysP358R and mdh50F/mdh164R, as described previously (3,5). GoTaq Green Master Mix (Promega, Madison, WI, USA) was used in the PCR reaction mixture. Reaction conditions involved denaturation at 95°C for 2 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Reactions were performed using a Veriti Thermal Cycler (Foster City, CA, USA). The primers used in the present study have been previously used in many studies to differentiate between *E. albertii* and *E. coli* strains (6,7).

PCR results showed that 72 (38%) samples, including of 63 bean sprouts, 3 broccoli sprouts, 2 Japanese radish sprouts, 2 red cabbage sprouts, and 2 peanut sprouts, were positive for *E. albertii* genes. Subsequently, 68 of the 72 samples were analyzed using a second set of primers, EA0134-283F (5'-TTGCGTACTAACGCGGATG) and EA0134-446R (5'-TGTGACTGTTGGGCTATTGG); these primers were designed in the current study to target unique sequences in *E. albertii* TW07627 (ABKX000000000.1). PCR reaction conditions involved initial denaturation at 95°C for 2 min, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. However, no samples tested positive using this set of primers. Subsequently, we attempted to culture suspected *E. albertii* from samples that were positive by PCR with primers lysP107F/lysP358R and mdh50F/mdh164R. However, no *E. albertii* was isolated from PCR-positive samples using the culture methods. Consequently, the PCR products generated from each of 3 bean sprout samples using lysP107F/lysP358R were sequenced. The results showed that the sequences of the amplified genes differed from those of a known *E. albertii* sequence (GenBank accession: GU967959; 88% identity over 130 bp). Therefore, the PCR products from a different bean sprout sample were cloned and sequenced. Cloning was performed using the pGEM-T Easy Vector System (Promega, Fitchburg, WI, USA), and colonies were incubated overnight in Luria-Bertani broth (Becton, Dickinson and Company, Sparks, MD, USA) supplemented with ampicillin (100 mg/L). Recombinant plasmid DNA was purified using a QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). The purified DNA was sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit on a 3500 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA), and the resulting sequences were compared with those in sequence databases using the Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/BLAST/).

The sequences of the cloned PCR products differed from a previously deposited *E. albertii* sequence (GenBank accession: GU967959 and GU969742; 87% identity over 203 bp for *lysP* and 86% identity over 59 bp for *mdh*). The cloned sequences were most similar to those of *Klebsiella variicola* (GenBank accession CP001891: 91% identity over 203 bp for *lysP* [in CP001891; the region is described as an amino acid permease-associated region] and 100% identity over 59 bp for *mdh*) (Fig. 1). This indicated that the PCR products could have probably been derived from organisms other than

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lysP

mdh

Fig. 1. Phylogenetic tree showing *lysP* (upper, comparing 203 bp) and *mdh* (lower, comparing 59 bp) nucleotide sequence clusters from the cloned DNA from bean sprout and reference enterobacterial sequences. GenBank accession numbers for reference sequences are in parentheses. The scale bar indicates the number of nucleotide substitutions per site.

Primer pair *lysP*107F/*lysP*358R

5' -GGGCGCTGCTTTCATATATCTT/TCCTGATACTCCCGGTGGATCTGGA-3'

.....

·C·····C·····G·C·G·····G·····

.....G·T·····G·C·····C·G·C·····

·C··T··T·C··T·····G·C·G·C·G·G·C·····

···AT·····C·G·····G·G·C·G·····A·G·C·····

Primer pair *mdh*50F/*mdh*164R

5' -CTGGAAGGCGCAGATGTGGTACTGATT/CGGTATTGTGAAGAATCTGGTTCAGCAAG-3'

.....

.....T··T·····C·····C·····C·····C·····

.....T·····C··T··T·C·····C··C·····A·C·····A·····

.....T·····G·····C·····C··C··T·····C·····G·····GA

·C·····T··T·····G·····C··T··C·····A·····C·····A

Fig. 2. Phylogenetic tree showing the nucleotide sequence clusters for sequences of the primers *lysP*107F and *lysP*358R (upper, comparing 49 bp) and *mdh*50F and *mdh*164R (lower, comparing 56 bp) from the designed primers and corresponding sequences from reference enterobacteria. GenBank accession numbers for reference sequences are in parentheses. The scale bar indicates the number of nucleotide substitutions per site. On the right of the tree is an alignment of the primer pair DNA sequences and corresponding sequences from the reference enterobacteria. Dots indicate the nucleotide is identical to that of the primer. The reverse primers and the corresponding sequences have been converted to the reverse complement.

E. albertii; however, the 2 PCR products were not necessarily derived from the same organism. In the *mdh* region, the sequences in CP001891 (*K. variicola*) corresponding to the *mdh*50F and *mdh*164R primer sites showed 88.9% (24/27) and 75.9% (22/29) identity to the primer sequences, respectively (Fig. 2). The novel cloned *lysP* and *mdh* sequences were deposited in the DDBJ under accession numbers AB935988 and AB935989, respectively. Data suggests that nonspecific amplification of *K. variicola* sequences might have occurred despite the low-temperature handling of samples prior to PCR analysis. Nevertheless, in the *lysP* region, it was unclear as to which organism was detected with the *lysP* primer pair. In this region, the amplified product showed 91% identity to CP001891 (*K. variicola*), and only low level identity was observed in the primer binding sites (Fig. 2). This suggests that the PCR products may have been obtained from non-*K. variicola* bacteria whose sequences are not yet available in the database.

To determine the ability of the primers to distinguish *E. albertii*, PCR analysis using primer pairs EA0134-283F/EA0134-446R, *lysP*107F/*lysP*358R, and *mdh*50F/*mdh*164R was conducted to test 69 *E. albertii* strains/isolates, including *E. albertii* LMG 20976^T, *E. albertii* LMG 20973, *Shigella boydii* ATCC 12032 (previously identified as *E. albertii* [5]), and 66 laboratory *E. albertii* isolates, as well as 16 non-*E. albertii* strains, including *Morganella morganii* ATCC 25830, *Citrobacter freundii* ATCC 8090, *K. oxytoca* ATCC 8724, *Salmonella enterica* subsp. *enterica* serovar Braenderup, *S. Typhimurium* ATCC 13311, *K. pneumoniae* ATCC 4352, *K. pneumoniae* ATCC 700603, *E. coli* ATCC 11775^T, *E. coli* O157 ATCC 43894, *Proteus mirabilis* ATCC 7002, *Yersinia enterocolitica* O3, and 5 other *E. coli* strains. The results showed that all the 3 pairs of primers amplified products from the 69 *E. albertii* strains. However, *lysP*107F/*lysP*358R also amplified a product from *M. morganii* ATCC 25830, while *mdh*50F/*mdh*164R amplified products from *C. freundii* ATCC 8090, *K. oxytoca* ATCC 8724, *S. Braenderup*,

and *S. Typhimurium* ATCC 13311. EA0134-283F/EA0134-446R was the only primer pair that did not amplify a product from non-*E. albertii* strains.

In conclusion, the present study revealed that other microorganisms often found in bean sprouts could be nonspecifically amplified using the primer pairs *lysP*107F/*lysP*358R and *mdh*50F/*mdh*164R (3,5). This finding indicated that these primers may be not specific enough for accurate detection of *E. albertii* in food samples when used under the PCR conditions used in this study. Therefore, additional methods, such as the use of the primers EA0134-283F/EA0134-446R (product size: 209 bp) designed in this study, may be needed for the detection of *E. albertii* in food samples.

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Conflict of interest None to declare.

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