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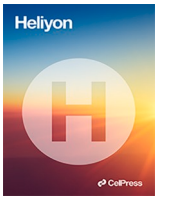

Research article

Preparation of polyclonal antibody against a universal bacterial antigen OmpA deduced by bioinformatic analysis and preliminary evaluation of concentration effects on foodborne pathogens

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ABSTRACT

Rapid and ultrasensitive microbial detection in actual samples have challenges because of target pathogen diversity and low abundance. In this study, we attempted to capture and concentrate multiple pathogens by combining magnetic beads with polyclonal antibodies against a universal antigen of *ompA*, LAMOA-1, before further detection. A protein sequence consisting of 241 amino acids with spatial conformation similar to *E. coli ompA* was identified and expressed as a recombinant protein in prokaryotes according to the results of sequence alignment among 432 sequences of *ompA* belonging to intestinal bacteria from gram-negative bacteria. Purified from immunized rabbits, the anti-LAMOA-1 antibody was shown to effectively recognize 12 foodborne bacterial species. Antibody-conjugated beads were used to concentrate the bacteria when the bacterial concentration in artificially contaminated samples is between 10 and 100 CFU/mL, which shortens detection duration by 8–24 h. The enrichment strategy is potentially beneficial for detection of foodborne pathogens.

1. Introduction

Foodborne diseases remain a global economic burden, particularly for the food industry [1,2]. The majority of conventional testing for bacterial detection is culture-based methods. Conventional culturing methods are still the gold standard for confirmation of obtained results in emergency cases [3]. Alternative methods have emerged, including PCR detection [4], 16S rRNA phylogenetic tree [5], and homogeneous laser chemiluminescence immunoassay technologies among others. Sample preparation in direct food sample analysis is the most challenging problem. This is due to food matrices heterogeneity, the nonuniform pathogen distribution, and low target pathogen abundance in the food. Culture-based growth on a selective agar medium is generally required for traditional methods for detecting foodborne bacteria. When target bacteria are few in the growth medium, the following procedure would fail to detect them. It is therefore essential to concentrate low-abundance pathogens prior to detection. The immunomagnetic separation method has been applied for target bacterial pathogens selective concentration to eliminate PCR inhibitors and reduce food sample heterogeneity [6]. Immunomagnetic beads (IMBs) application to enrich single strains such as *Escherichia coli* [7], *Staphylococcus aureus* [8],

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Salmonella [9], and Listeria monocytogenes [10] has been reported by many researchers, but preparing single antibody magnetic beads application to capture more than one bacteria has not been reported.

Outer membrane proteins are β barrel structures with surface-exposed epitopes, and almost all share a common evolutionary ancestor [10]. The outer membrane porin A OmpA is surface exposed in gram-negative bacteria and can be abundantly expressed. OmpA plays important roles in structural integrity, cellular morphology, and porin activity. It shares the conserved domain at the carboxyl terminal with signal sequence at the N-terminal and characteristic β-barrel structure of recognized porins [11].

OmpA protein might be conserved in many gram-negative bacteria considering high abundance and conservation. In this study, we analyzed the OmpA protein sequence conservation in gram-negative bacteria and to predict the spatial conformational sequences exposed to the outer cell membrane using a bioinformatics approach. Designated as LAMOA-1, a deduced OmpA analog was expressed and purified in vitro, which is conserved in many bacterial pathogens. The goal is to have antibodies that recognize as many bacterial species as possible. The anti-LAMOA-1 antibody was then obtained by subcutaneously immunizing the rabbit with recombinant protein. Magnetic beads were activated and directly conjugated with anti-LAMOA-1 antibody. The performance of concentrating the pathogen captured magnetic beads was then evaluated by bacterial plate counting. Our results demonstrated that the strategy of combining the universal antigen OmpA with immunomagnetic separation was effective in increasing the low level pathogenic bacteria amount and enrichment time reduction during the pathogenic identification process from practical samples.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A total of 17 American Type Culture Collection (ATCC) or Center for Medical Culture Collections (CMCC) references strains were used in this study according to the National Food Safety Standard of the People's Republic of China for food microbiological examination. ATCC and China National CMCC were used to collect these strains as follows: *Escherichia coli* (ATCC 11775, pathogenic & ATCC 25922, nonpathogenic), *Salmonella enterica* (ATCC 43971), *Salmonella typhimurium* (CMCC 50115), Shigella flexneri (ATCC 29903), *Shigella dysenteriae* (ATCC 13313), Shigella boydii (CMCC 51180), Shigella sonnei (CMCC 51182), Yersinia enterocolitica (ATCC 9610), *Enterobacter cloacae* (ATCC 13047), Enterobacter aerogenes (ATCC 13048), *Enterococcus faecalis* (ATCC 19433), Cronobacter sakazakii (ATCC 29544), Vibrio parahaemolyticus (ATCC 17802), *Staphylococcus aureus* (ATCC 25923), Bacillus cereus (ATCC 14579) and Listeria monocytogenes (ATCC 15313). Bacterial strains were routinely grown on appropriate culture media at 37 °C. Overnight cultures were diluted and continuously maintained under the same conditions ranging from 18 to 48 h, which allows the cells to grown to the late-exponential phase for the following procedures. All experiments involving live bacterial strains were conducted in a biosafety laboratory (BSL-2) facility.

2.2. Amino acid sequence alignment of bacterial OmpA protein

Bacterial OmpA protein amino acid sequences with more than 150 amino acid length were downloaded using Batch Entrez from the Gene Bank database of National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/sites/batchentrez). The resulting sequences were subject to local Blast alignment. Those sequences with less than 50% similarity with *E. coli* K12 OmpA sequence was excluded from the analysis. The alignment of amino acid sequences among the OmpA homologues was made using software CLUSTALW 2.1. A consensus of OmpA amino acid sequences, LAMOA-1, was obtained based on the results of multiple sequence alignment. Using the Mega tool (version 7.0.26), the novel protein sequence was then added to the phylogenetic tree to observe its group affiliation. Homologues were divided into clades based on the consensus among the trees.

2.3. Preparation of the recombinant LAMOA-1 protein

The identified LAMOA-1 protein duplicate wells were reversely translated and generated a DNA sequence based on the codon usage table in prokaryotes. Using the GenSmart™ Codon Optimization tool (21′https://www.genscript.com.cn/gensmart-free-gene-codon-optimization.html), the corresponding nucleotide sequence was further codon optimized. The optimized sequences were artificially synthesized and PCR-amplified using specific primers (Forward primers: 5'-GACACCATATGAAGAAAACCGCGGCGGCGGTTGCGGCGGGTGCGACCGTGGCGGCGGC-3', Reverse primers: 5'-GTGTCCTCGAGCGCGGTCACAACGTCTTTACCCTTCACAA-3'. Using the ArrayDesigner tool 2.0). The PCR product was digested with NdeI and XhoI and cloned into plasmid pET21a (Novagen, His-tagged). The recombinant plasmid was transformed into *E. coli* BL21 (DE3). The addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG) induced protein secretion. The recombinant LAMOA-1 protein was purified by Ni-NTA agarose gel and verified by SDS-PAGE and Western blot.

2.4. Rabbit immunization with the recombinant LAMOA-1

The Institutional Animal Care and Use Committee of Laboratory Animal Center under approval number IACUC-DWZX-2017-006 approved the animal experiment. Upon arrival, two Japanese white rabbit (female, 3 months old, weight about 2.5 kg) were subject to quarantine observation for 1 week. The rabbits were immunized with the purified LAMOA-1 protein by subcutaneous multipoint injection in the axillary and inguinal regions three, or four times, if necessary. Freund's complete adjuvant was used in the first injection and incomplete adjuvant in other injections. The injection dose of each rabbit was 250, 500, 1,000, and 1,000 μg on days 0, 14,

28, and 42, respectively. Small aliquots of sera samples were collected and analyzed for antibody response by enzyme-linked immunosorbent assay (ELISA) at regular intervals up to day 52 in order to monitor the immunogenic responses of LAMOA-1 in rabbits. The serum sample on day 0 was used as a negative control. The immunization manipulation was terminated and the rabbit serum was harvested for the antibody purification once the titer of antiserum against LAMOA-1 reached more than 1:51,200 on day 38. Otherwise, the fourth injection of immunization was carried out with the dose of 1,000 μg on day 42.

2.5. Purification of anti-LAMOA-1 IgG polyclonal antibody

The octanoic acid/saturated ammonium sulfate precipitation and membrane filtration method was used to purify the anti-LAMOA-1 polyclonal antibody from the antiserum. Briefly, the collected rabbit sera were centrifuged at 5,000 r/min with three times the sodium acetate volume. Octanoic acid was slowly added to the final concentration of 7.5% with thorough mixing at room temperature for 30 min and kept at 4 °C for 1 h. The supernatant was collected by centrifugation at 10 °C for 30 min at 12,000 r/min. Then, 50% of saturated ammonium sulfate was added to the supernatant while stirring for 30 min. Pellets collected by centrifugation was suspended with sterile normal saline and restored to the volume of the original serum. Saturated ammonium sulfate was added dropwise to 30% of serum volume, and kept at 4 °C for 3 h. The supernatant was discarded, and the resuspended pellet was transferred with 0.005 mol/L PBS into dialysis bag at 4 °C overnight. The precipitate was discarded after centrifugation of 9,000 rpm/min at 10 °C for 10 min. The IgG concentration was measured by NanoDrop2000 spectrophotometer. The titers were determined by ELISA.

2.6. Determination of antibody titers by using ELISA

The purified LAMOA-1 protein or different foodborne bacteria were used as coated antigens by indirect ELISA method as follows: The coating antigens with a concentration of 1 μg/mL (LAMOA-1) or 10^7–10^8 CFU/mL (bacteria) were coated on the detection plate with the coating solution at 4 °C overnight. The plates were washed twice and incubated with casein blocking buffer in PBS at 37 °C for 2 h. A 100 μL of 2-fold serial diluted rabbit serum or purified IgG antibody were added to each well at 37 °C for 1 h after washing. Washing steps were repeated and 100 μL of goat antirabbit IgG horseradish peroxidase conjugate at a 1:5000 dilution in PBS was added to each well and incubated at 37 °C for 30 min. The plates were then washed five times and 100 μL of TMB solution was added to each well. Plates were incubated at 37 °C for 20 min. Reaction was stopped by adding a stopping solution. All the tests were done in duplicate wells and the absorbance values at 450 nm from duplicate wells of more than 0.2 were considered positive.

2.7. Preparation of meat samples with artificially bacterial contaminations

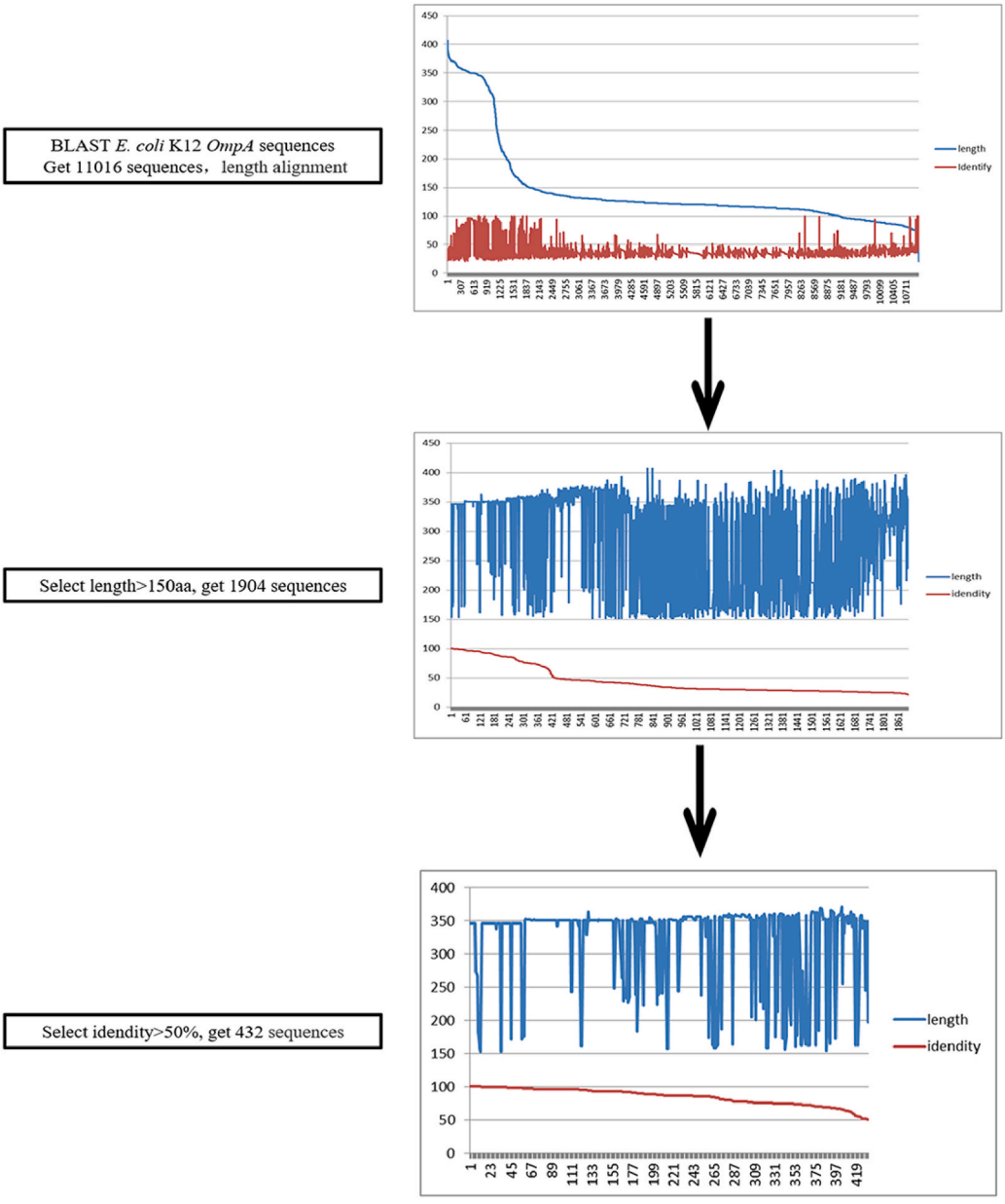
Fresh pork was purchased from a retail market and verified to be negative for three tested species (*S. dysenteriae*, *S. typhimurium* and *S. aureus*). The pork was cut into 5 g pieces with scissors and mixed with 45 mL of PBS solution to make a pork-bacteria mixture suspension. Specific bacterial suspensions containing 10-10^1, 10^1-10^2, 10^2-10^3, 10^3–10^4 CFUs at 1 μL were mixed thoroughly with 1 mL of meat suspension, respectively. The final mixtures were used in the following bead-based detection.

2.8. Preparation of immunomagnetic beads

The carboxylic magnetic bead coupling kit (Sangon Bioengineering Shanghai Co., Ltd.) and highly qualified IgG antibodies selected above were used to prepare the magnetic bead. The magnetic beads carboxyl groups were activated with 50 mg/mL EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) and 50 mg/mL SULFO-NHS (Sulfosuccinimidobiotin). Each immunomagnetic beads microgram were conjugated with 100 μg of antibodies in the coupling buffer (50 mM MES, pH 6.0, 0.01% Triton X-100) at 37 °C

Table 1
National Food Safety Standard for microbiological examination complied by this study.

Standard ID	Culture medium & growth conditions	Colony features
GB 4789.3–2016 (*E. coli*)	VRBA (Violet Red Bile Agar); 36 ± 1 °C; 18–24h	Purplish red, surrounded by red precipitation rings; 0.5 mm or larger in diameter
GB 4789.4–2016 (*Salmonella*)	BS (Bismuth Sulfite Agar); 36 ± 1 °C; 40–48 h	Black with metallic luster, brown or gray, the surrounding medium may be black or brown.
GB 4789.5–2012 (*Shigella*)	XLD (XyloseLysineDesoxycholate Agar) 36 ± 1 °C; 18–24 h;	Pink with or without a black center;
GB 4789.6–2016 (*E. coli* O157: H7)	MAC (MacConkey Agar) 36 ± 1 °C 18–24 h;	Brick red to peach red
GB 4789.8–2016 (*Y. enterocolitica*)	CIN-1 (Cepulodin Irgasan Novobiocin Agar) 26 ± 1 °C 48 ± 2 h;	Dark red, surrounded by a colorless transparent circle;
GB 4789.10–2016 (*S. aureus*)	BP (Baird-Parker Agar) 36 ± 1 °C 24–48h	Round, smooth, raised, moist, grayish-black to black, shiny, often light (not white) edges



(a)

Amino acid sequence of LAMOA-1:

MKKTAAAVAAGATVAAAKDNTWYTGAKGWSYHDTGNNGTHNGAGAGGYVNYGMGYDWGRMYK
GDVNGAKAGVTAKGYTDDDYTRGGMVWRADSKNVGGSKDHDTGVSVAGGVYATATRYWTNNGD
ATGTRDNGSGVSYRGAAVVAAAAVTKHTKSDVNNKATKGADYSSNDKDGSVVVGTDRGSDAYNGS
KRASVVDYSKGADKSARGMGSNVTGNTCDNVKRAADCADRRVVKGKDVVTA

(b)

(caption on next page)

Fig. 1. Bioinformatics strategy of identifying homologous amino acid sequences of the bacterial OmpA protein (a) and the deduced LAMOA-1 sequence (b).

for 90 min. The coupled beads were stored at 4 °C until further use after treatment with a blocking solution (TBS (25 mM Tris-Cl, 130 mM NaCl, 2.7 mM KCl) pH 8, 0.01% Triton X-100) and a preservation solution (TBS or PBS containing 0.01% Triton X-100 or 0.01% Tween 20, 0.05%NaN_3).

2.9. Beads-based detection of pure bacterial cultures and meat samples

Bacteria with better recognition effect of LAMOA-1 protein IgG antibody were selected to evaluate whether the detection efficiency could be improved after enrichment of antibody magnetic beads. The pure bacterial cultures were serially diluted 10-fold. Different tubes with bacterial suspensions or meat samples containing final concentration of 10°-10^{1}, 10^{1}-10^{2}, 10^{2}-10^{3}, 10^{3}–10^{4} CFUs per mL were used in the following procedure. Various concentrations of 1 μL of pure bacterial or meat-bacteria mixture suspensions were separately added into 1 mg of immunomagnetic beads conjugated with LAMOA-1 IgG antibody, and placed on a high-frequency thermostatic oscillator incubated at 37 °C for 45 min. A magnetic rack was used for magnetic separation for 30–60 s. A 100 μL PBS was used to resuspend the magnetic beads after the supernatant was discarded. Appropriate media and culture conditions listed in Table 1 were used for various bacteria, respectively. Colonies were counted on solid media after 48 h incubation at 37 °C. Data are presented as average ± standard deviations of three independent experiments. Meanwhile, other aliquots of pure bacterial or meat-bacteria mixture suspensions were processed exactly the same except for omitting magnetic beads absorption as described above.

3. Result

3.1. Determination of amino acid sequence of LAMOA-1 protein

Fig. 1a shows the steps to obtain OmpA protein homologous amino acid sequences from a variety of bacteria. *E. coli* K12 OmpA sequence was compared with NCBI Microbial Genomes database using the BLAST program. A total of 11,016 homologous sequences

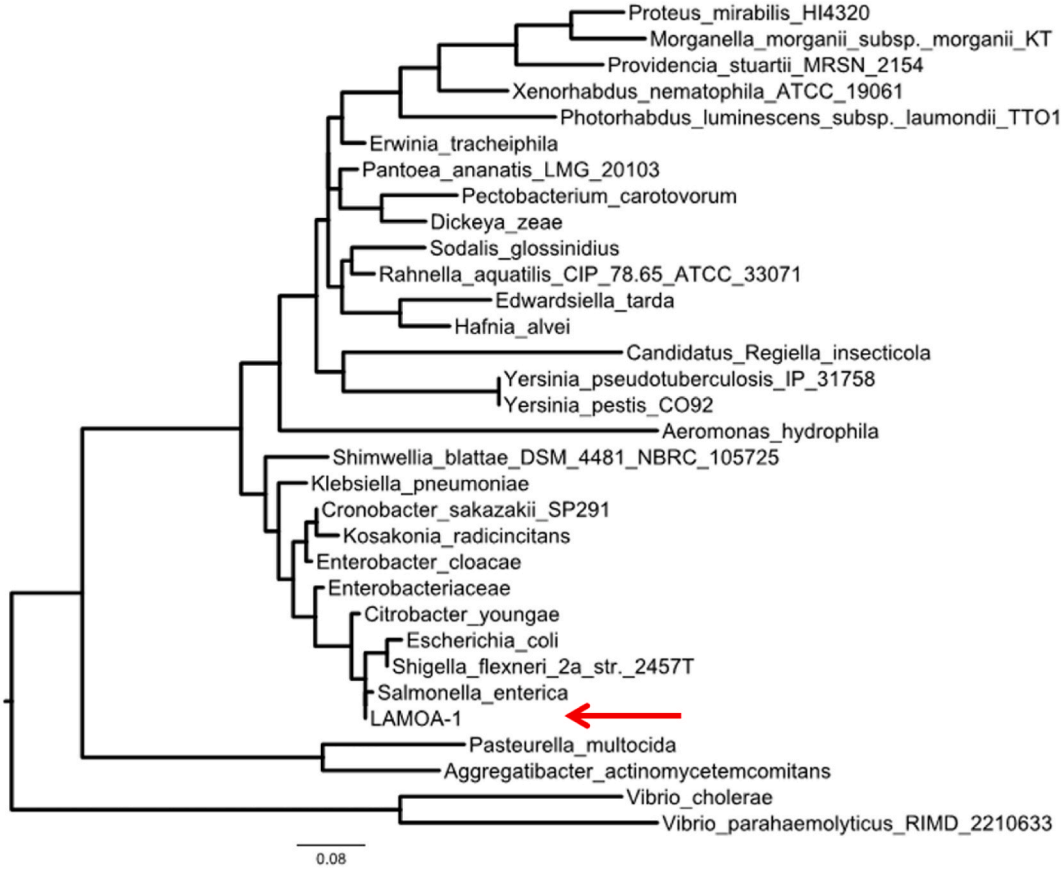


Fig. 2. Phylogenetic tree of the OmpA proteins and deduced LAMOA-1 in bacteria.

were found in the NCBI database of which 1,904 sequences of at least 150 amino acids in length were extracted for the following analysis. The sequences with similarity of greater than 50% were chosen. A total of 432 ompA sequences were finally obtained from the available bacterial genome of which more than 70% belong to 10 genera in Enterobacteriaceae. Homologous amino acid sequences were merged into a 241-amino-acid consensus sequence, LAMOA-1 (Fig. 1b).

OmpA proteins phylogenetic tree generated showed that the identified LAMOA-1 protein classifies in the branch of Enterobacteriaceae, but more closely related to *S. enterica*, S. flexneri and *E. coli* than to other members of Enterobacteriaceae (Fig. 2).

3.2. Protein expression and purification of artificially synthesized LAMOA-1

The consensus sequences after codon optimization were artificially synthesized and produced good yields when and expressed in *E. coli*. SDS-PAGE (Fig. 3a) and Western blot (Fig. 3b) using anti-His antibody showed an obvious band corresponding to the expected ~27 kDa molecular weight indicating that the protein was successfully expressed and purified. Approximately 12.0 mg (0.58 mg/mL 10 mL, 0.55 mg/mL 4 mL, 2 mg/mL 2 mL) of purified proteins were finally obtained and was sufficient for the immunization steps. The protein was subpacked into a microtube and stored at −80 °C.

3.3. Preparation of rabbit polyclonal antibody against LAMOA-1 protein

Subcutaneous rabbit immunization with the recombinant protein generated rabbit polyclonal antibody. LAMOA-1 antiserum collected from the two rabbits was examined using ELISA for LAMOA-1 antigen specific antibodies. The high titer (1:102,400) was detected in sera from rabbits suggesting that the rabbits were effectively immunized with LAMOA-1. The anti-LAMOA-1 IgG antibodies were then purified with the lowest detectable concentrations of 31.25 ng/mL upon detection of LAMOA-1.

3.4. Determining ranges of anti-LAMOA-1 antibody on recognizing various foodborne bacteria

A total of 17 bacterial species belonging to 11 genera were chosen to examine the recognition ranges of the purified polyclonal antibodies specific for the theoretically universal antigen, LAMOA-1, using ELISA methods. These bacterial species were considered efficiently detected if the lowest detectable concentrations of anti-LAMOA-1 antibody in recognizing some bacterial species are less than or equal to 25 μg/mL; otherwise, they would be excluded from the following beads-based assays. As shown in Table 2, 12 out of 17 strains were observed positive, including pathogenic and nonpathogenic *E. coli*, four species of Shigella genus (S. flexneri, *S. dysenteriae*, S. boydii, S. sonnei), two species of Enterobacter (E. aerogenes, *E. cloacae*), *S. typhimurium*, Y. enterocolitica, *E. faecalis* and *S. aureus*. The anti-LAMOA-1 antibody had a high degree of similarity in identifying different species of the same genera. For example, four species of Shigella genus (S. flexneri, *S. dysenteriae*, S. boydii, S. sonnei), two species of Enterobacter (E. aerogenes, *E. cloacae*). The results showed that the OmpA of bacteria from the same genera but different species had more similar structures. On the other hand, this indicated that the anti-LAMOA-1 antibody had a broad spectrum.

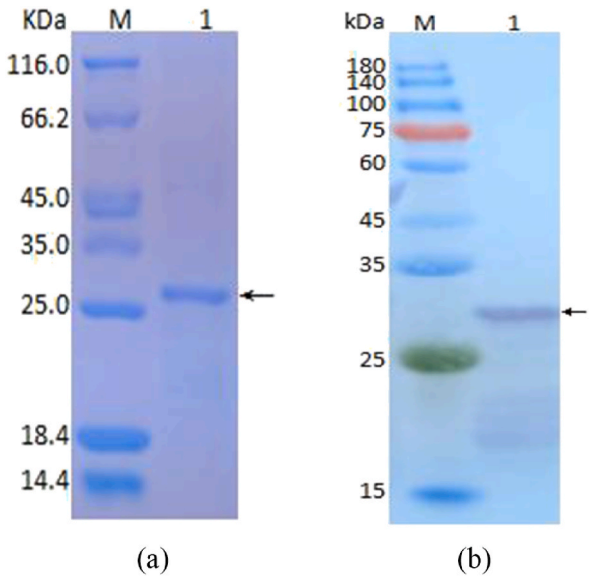


Fig. 3. SDS-PAGE (a) and Western Blot (b) of His-tagged LAMOA-1 protein
Lane M, Protein marker; Lane 1, Fusion of target protein.

Table 2
Lowest detectable concentrations of anti-LAMOA-1 antibody for 17 bacterial species.

Bacterial genus	Detectable species with lowest concentrations of anti-LAMOA-1 IgG antibody	
	≤25 μg/mL	>25 μg/mL
Escherichia	*E. coli* O157:H7 (25); *E. coli* (25)	
Salmonella	*S. typhimurium* (12.5)	*S. enterica* (200)
Shigella	S. flexneri (12.5), S. boydii (1.5625), *S. dysenteriae* (6.25), S. sonnei (12.5)	
Yersinia	Y. enterocolitica (12.5)	
Enterococcus	*E. faecalis* (12.5)	
Enterobacter	*E. cloacae* (25), E. aerogenes (25)	
Cronobacter		C. sakazakii (50)
Vibrio		V. parahaemolyticus (>200)
Staphylococcus	*S. aureus* (3.125)	
Bacillus		B. cereus (50)
Listeria		L. monocytogenes (200)

Note: The exact values of IgG concentrations for different strains are indicated in parentheses.

3.5. Impact of adding antibody-coupled beads capture step on detection efficiency of pure bacterial culture

About 70% (12/17) of tested bacterial species belonging to multiple genera have been identified by anti-LAMOA-1 antibody as described above. The results encouraged us to explore the possibility of anti-LAMOA-1 antibody application in bacterial concentration by magnetic beads capture. The activated magnetic beads were directly conjugated with anti-LAMOA-1 antibody. Pure bacterial cultures with concentrations ranging from 10^0 to 10^4 CFUs per mL were subject to being or not being captured by antibody immobilized magnetic beads. The number of recovered cells was recorded as indicators of the detection efficiency as shown in Table 3. Bacterial cells recovered from IMBs-treated group at the concentration of 10^1–10^2 CFUs per mL were increased to varying degrees than those from IMBs-untreated group in *S. typhimurium* and *E. coli* O157:H7. This indicates that the antibody has a broad spectrum, and the enrichment based on antibody magnetic beads is conducive to improving the detection sensitivity, but our current magnetic bead experiment conditions still need to be improved. However, IMBs seemed to have no concentrated effects on pure culture of all tested bacteria at 10^2–10^4 CFUs per mL. Overnight or longer incubation with IMBs or without IMBs was required when bacterial concentration is lower than 10 CFU/mL. IMBs failed to recover more bacteria from the pure culture of Y. enterocolitica and *S. dysenteriae*, compared with the IMBs-untreated group. The interactive strength between antibody and these two antigens might not be high enough so that antigen-antibody complex was partly dissociated during beads washing steps even though they could be specifically recognized by anti-LAMOA-1 antibody.

Table 3
Colony detection of bacterial pure cultures based on standard methods combining or not combining LAMOA-1 IgG-coupled IMBs methods.

Bacteria	Initial concentration (CFU/mL)	Detected concentration (CFU/mL) upon treatment		
		Without IMBs	With IMBs	Overnight incubation without IMBs
E. coli	10°–10^1	–	–	nd
	10^1–10^2	$(0.83 \pm 1.18) \times 10^1$	$(0.67 \pm 0.23) \times 10^1$	nd
	10^2–10^3	$(1.50 \pm 0.20) \times 10^2$	$(0.25 \pm 0.08) \times 10^2$	nd
	10^3–10^4	$(1.43 \pm 0.14) \times 10^3$	$(0.20 \pm 0.01) \times 10^3$	nd
S. typhimurium	10°–10^1	–	–	+
	10^1–10^2	$(0.83 \pm 1.18) \times 10^1$	$(5.00 \pm 0.00) \times 10^1$	+
	10^2–10^3	$(1.33 \pm 0.31) \times 10^2$	$(2.33 \pm 0.24) \times 10^2$	+
	10^3–10^4	$(1.09 \pm 0.07) \times 10^3$	$(0.17 \pm 0.02) \times 10^3$	+
S. dysenteriae	10°–10^1	–	–	+
	10^1–10^2	$(3.33 \pm 2.36) \times 10^1$	-	+
	10^2–10^3	$(2.41 \pm 0.59) \times 10^2$	$(0.17 \pm 0.24) \times 10^2$	+
	10^3–10^4	$(1.49 \pm 0.06) \times 10^3$	$(0.05 \pm 0.02) \times 10^3$	+
E. coli O157:H7	10°–10^1	–	–	+
	10^1–10^2	–	$(0.17 \pm 0.24) \times 10^1$	+
	10^2–10^3	$(1.17 \pm 0.24) \times 10^2$	$(0.02 \pm 0.01) \times 10^2$	+
	10^3–10^4	$(1.50 \pm 0.09) \times 10^3$	$(0.07 \pm 0.00) \times 10^3$	+
Y. enterocolitica	10°–10^1	–	–	+
	10^1–10^2	$(0.83 \pm 1.18) \times 10^1$	$(0.17 \pm 0.24) \times 10^1$	+
	10^2–10^3	$(0.83 \pm 0.24) \times 10^2$	$(0.10 \pm 0.14) \times 10^2$	+
	10^3–10^4	$(1.45 \pm 0.13) \times 10^3$	$(0.03 \pm 0.01) \times 10^3$	+
S. aureus	10°–10^1	-	-	+
	10^1–10^2	$(0.83 \pm 1.18) \times 10^1$	$(0.50 \pm 0.41) \times 10^1$	+
	10^2–10^3	$(1.50 \pm 0.35) \times 10^2$	$(0.21 \pm 0.02) \times 10^2$	+
	10^3–10^4	$(0.95 \pm 0.17) \times 10^3$	$(0.25 \pm 0.02) \times 10^3$	+

Note: "-" represents no bacteria was detected, "+" represents too much colonies to be counted, “nd" means “not done".

3.6. Impact of adding antibody-coupled beads capture step on detection efficiency of artificially contaminated samples

S. dysenteriae, *S. typhimurium* and *S. aureus* are pathogenic bacteria not allowed to be detected in food by the China National Food Safety Standard and were chosen to observe whether magnetic beads could effectively play a role in enrichment and further improve detection efficiency without medium enrichment. The results shown in Table 4 were in agreement with those in Table 3. Reduced counts were consistently observed in all the bacteria-pork suspension compared to the pure culture, likely due to pork bacterial adsorption. The actual sample is much more complicated than this, which makes detection more challenging. The detection efficiency of *S. dysenteriae* using IMBs was even lower than that without IMBs at the concentration of 10^1–10^2 CFUs per mL, while effects of IMBs on *S. typhimurium* and *S. aureus* was visible in the case of omitting the incubation steps. Immune magnetic beads have potential role in reducing impurities interference in the process of microbial detection in food. However, the specificity of the OmpA polyclonal antibody is limited due to its broad-spectrum nature, and there is a need to further improve the antibody magnetic beads capture effect by sequence optimization.

4. Discussion

Outer membrane proteins are common components in gram-negative bacteria. OmpA has been well-studied and characterized in model species, such as *E. coli* and *S. enterica* [12]. It is a highly abundant porin, evolutionarily conserved, and structurally stable across bacterial species, even among gram-negative bacteria. OmpA family plays key roles in adhesion, biofilm formation, and host immune response. Therefore, it has been regarded as a potential vaccine candidate or a potential therapeutic target [13,14]. OmpA was additionally used as a useful antigen to rapidly detect *E. coli* [15] or to induce OmpA-against antibody for bacterial enrichment step before molecular detection experiment [16]. We noticed the features of high conserved sequence of OmpA and its expression and high immunogenicity on the cell membrane surface. The antibody produced by the universal OmpA antigen could be used in gram-negative bacteria broad-spectrum detection containing OmpA protein. The release of massive information on bacterial genome makes it possible to obtain the sequence OmpA universal antigen. Similarly, antibody against OmpA protein could be used in the enrichment steps during rapid detection of bacteria, especially for those with low abundance. Guan et al. showed that the homology of *E. coli* OmpA protein with Shigella, Salmonella, and Pseudomonas was 96%–100%, 90%–94%, and 45%, respectively, by bioinformatics analysis. This also tentatively verified our conjecture [17].

The concentration step addition would be beneficial for detection efficiency and even shorten the detection period once used as an alternative to overnight culture in the established method, although different countries may have standardized pathogen detection procedures in food samples. Immunomagnetic separation (IMS) method could utilize antibody-coupled beads to efficiently capture and isolate target pathogens from complex samples based on the principle of antigen-antibody specific binding. Food composition detection is complicated due to the interference from a variety of microorganisms and the difficulty of isolating a single bacterium. IMS is a potentially effective method for selective target bacterial pathogen concentration and lowering the heterogeneity of food samples. It could shorten detection time and improve the efficiency of bacterial detection combined with the existing detection methods [18,19]. Just recently, the polyclonal antibody against OmpA was used in IMS to enrich the *E. coli* in milk samples. The detection sensitivity of PCR was improved more than 100 times than those not treated with IMS [16].

We tried to use a universal antibody against an artificially synthesized OmpA in the enrichment detection step in this study instead of using the commonly chosen antibody against a single bacterial pathogen. Numerous OmpA homologues are easily accessible in the sequenced genomes by using bioinformatics methods. A consensus sequence of 241-amino-acid LAMOA-1 was identified by blasting various OmpA homologues in more than 40 bacterial genera. The artificially designed LAMOA-1 protein was successfully expressed after codon optimization. Phylogenetic analysis of OmpA proteins indicated that LAMOA-1 was found to be closely related to other common members of Enterobacteriaceae. LAMOA-1 was also closely related to other Gram-negative bacteria due to the highly conserved nature of OmpA protein in Gram-negative bacteria. The codon-optimized OmpA gene was efficiently expressed in *E. coli*. We used the expressed LAMOA-1 as the universal antigen to immunize rabbits. The anti-LAMOA-1 polyclonal antibody detection spectrum was verified using indirect ELISA. Universal antibodies could be preferentially applied to the situation where pathogen species is

Table 4
Colony detection of bacterial contaminated pork based on standard methods combining or not combining LAMOA-1 IgG-coupled IMBs methods.

Bacteria	Initial Concentration (CFU/mL)	Detected concentration (CFU/mL)		
		Bacterial suspension before mixing with pork	Bacteria-pork suspension	Bacteria-pork suspension with IMBs
S. typhimurium	10^1–10^2	$(0.83 \pm 1.18) \times 10^1$	–	$(0.50 \pm 0.00) \times 10^1$
	10^2–10^3	$(1.50 \pm 0.20) \times 10^2$	$(0.50 \pm 0.00) \times 10^2$	$(0.23 \pm 0.02) \times 10^2$
	10^3–10^4	$(1.08 \pm 0.08) \times 10^3$	$(0.42 \pm 0.02) \times 10^3$	$(0.18 \pm 0.02) \times 10^3$
S. dysenteriae	10^1–10^2	$(1.67 \pm 1.18) \times 10^1$	$(0.17 \pm 0.24) \times 10^1$	–
	10^2–10^3	$(1.33 \pm 0.31) \times 10^2$	$(0.23 \pm 0.02) \times 10^2$	$(0.77 \pm 0.59) \times 10^2$
	10^3–10^4	$(1.45 \pm 0.10) \times 10^3$	$(0.21 \pm 0.12) \times 10^3$	$(0.97 \pm 0.03) \times 10^3$
S. aureus	10^1–10^2	$(0.08 \pm 0.12) \times 10^1$	$(0.83 \pm 1.17) \times 10^1$	$(1.67 \pm 1.17) \times 10^1$
	10^2–10^3	$(1.08 \pm 0.47) \times 10^2$	$(0.92 \pm 0.24) \times 10^2$	$(0.50 \pm 0.20) \times 10^2$
	10^3–10^4	$(1.15 \pm 0.02) \times 10^3$	$(0.50 \pm 0.07) \times 10^3$	$(0.22 \pm 0.04) \times 10^3$

Note: "-" represents no bacteria was detected.

uncertain in actual samples despite the problem of not exactly matching all epitopes [20].

Our results confirmed that the LAMOA-1 protein sequence had good immunogenicity and produced the polyclonal antibody with the standard titer indicating that the predicted sequence had good natural structure and antigenic determinants. We found that the anti-LAMOA-1 polyclonal antibody could recognize 12 out of the 17 foodborne bacterial pathogenic species specified in the National Food Safety Standard with a sensitivity threshold of less than 25 μg/mL. The polyclonal antibody is supposed to have lower specificity but greater adaptation to antigen recognition than monoclonal antibody. Wang et al. and Mwale et al. used OmpA monoclonal antibodies to recognize Acinetobacter baumannii and *Escherichia coli* [21,22]. In contrast, the anti-LAMOA-1 IgG antibody prepared by our lab has obvious advantages in broad spectrum.

Since outer membrane protein is unique to Gram-negative bacteria, the Gram-positive bacterium *Staphylococcus* were speculated not to be detected. Therefore, we initially design it as control group. Unexpectedly, *Staphylococcus aureus* was detected in the pure culture. Unexpectedly, the anti-LAMOA-1 IgG antibody was found to have a good binding effect on the gram-positive bacteria, *S. aureus*. We speculated that the high expression level of *Staphylococcus* A (SPA), a membrane protein on the cell surface of *S. aureus*, might nonspecifically bind to the Fc terminal of IgG immunoglobulin. The specific mechanism and principle need to be further studied and confirmed.

According to the experimental results of Table 3, the current magnetic bead enrichment conditions are only suitable for some bacteria. Therefore, all the bacteria in Table 3 were not included in the experimental design of the artificially contaminated samples, and only three of them were selected. In the future, after the enrichment conditions of magnetic beads are optimized, artificially contaminated samples experiments can be carried out for more bacteria".

Further attempts were made to combine anti-LAMOA-1 antibody-coupled beads capturing the standard method, which provides evidence of the role of antibody in concentrating several bacterial species, especially *S. typhimurium* and *S. aureus*, from artificially contaminated samples. The enrichment step addition makes it possible to skip the incubation process and go directly to the subsequent procedure when the concentration of bacterial pure culture is between 10 and 100 CFU per mL. This would supposedly save 8–24 h due to omitting the step of liquid cultivation, compared with national standard culture method.

However, this study has many shortcomings compared with the detection of a single bacterial antibody IMB, especially in verifying the enrichment effect. For example, combining IMS with molecular detection or flow cytometry technology can eliminate the interference of impurities, improve detection limit, and detection efficiency. Kubo et al. combined IMS with PCR to detect *Salmonella enteritis* in egg yolks. Magnetic beads were first used to remove the yolk components that would interfere with PCR, and then PCR was used to detect salmonella within 6 h [23]. Combining IMS with PCR technology can eliminate the interference of impurities on the substrate. Li et al. combined IMS with chemiluminescent particle immunochromatography to detect trace salmonella in milk and chicken, and could complete the detection of trace *Salmonella* in five different serotypes in milk and chicken within 8 h without false negative or false positive results [24]. The combination of IMS technology and flow cytometry can also better complete the detection of foodborne pathogens. Pan et al. combined IMS technology with immunosensor fluorescence and flow cytometry to effectively detect octadic acid (a typical diarrheal shellfish poisoning toxin), the detection time is less than 50 min, and the minimum detection limit is 0.05 g/L [25]. Jong-Hui Kim et al. used different concentrations of magnetic beads and incubation times specifically in order to determine the optimal amount of IMB and immune response time in capturing bacteria [26]. There is still room for improvement, especially in sensitivity and detection limits during the enrichment steps, which could be achieved by further optimizing conditions.

The LAMOA-1 protein was obtained by homologous alignment of a large number of gram-negative bacteria OmpA protein sequences using the bioinformatics method. In theory, antibodies for LAMOA-1 protein could recognize a wider variety of gram-negative bacteria. However, we only focused on the bacterial species closely related to food safety in this study due to the limitation of experimental conditions. We also additionally found encouraging effects of this universal antibody in other bacterial species, such as *Yersinia pestis* and *Burkholderia pseudomallei* (data not shown). Further work is needed to improve and optimize our methodology to allow anti-LAMOA-1, the antibody of the universal antigen, to play better roles in bacterial detection.

5. Conclusion

In this study, 241-amino-acid LAMOA-1 consensus sequence was deduced by comparing various OmpA homologues in more than 40 bacterial genera. The anti-LAMOA-1 antibody was obtained by subcutaneous rabbit immunization with the recombinant protein. Magnetic beads were activated and directly conjugated with the anti-LAMOA-1 antibody. Their performances of concentrating the pathogen captured magnetic beads were then evaluated by bacterial plate counting. The number of recovered cells from IMBs-treated group tends to increase in *S. typhimurium* and *E. coli* O157:H7 at low concentration of pure bacterial culture. The strategy of combining the universal antigen OmpA with immunomagnetic separation was potentially promising in increasing the amount of low level of pathogenic bacteria and shortening the enrichment time during the process of pathogenic identification from practical samples after enrichment method optimization.

Production notes

Author contribution statement

Yanping Han: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Lei Wang: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis

tools or data; Wrote the paper.

Yuehua Ke; Yajun Song: Conceived and designed the experiments; Analyzed and interpreted the data.

Ye Li; Yixuan Li; Yanfeng Yan; Bo Gao: Conceived and designed the experiments; Performed the experiments.

Ruifu Yang: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Data availability statement

Data included in article/supplementary material/referenced in article.

Additional information

No additional information is available for this paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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