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Sensitive and rapid detection of *staphylococcus aureus* in milk via cell binding domain of lysin

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ABSTRACT

Staphylococcus aureus (*S. aureus*) is an important food-borne pathogen in dairy products contaminated through raw ingredients or improper food handling. Rapid detection of *S. aureus* with high sensitivity is of significance for food quality and safety. In this study, a new method was developed for detecting *S. aureus* in milk by coupling immunomagnetic separation with enzyme linked cell wall binding domain (CBD) of lysin plyV12, which can bind to *S. aureus* with high affinity. There are millions of binding sites present on the cell surface of *S. aureus* for the CBD attachment, which greatly improves the detection sensitivity. The method has the overall testing time of only 1.5 h with the detection limit of 4×10^3 CFU/mL in spiked milk. Because it is simple, rapid and sensitive, this method could be used for the detection of *S. aureus* in various food samples.

1. Introduction

Staphylococcus aureus (*S. aureus*) is one of the most common pathogenic bacteria associated with food poisoning worldwide (Hennekinne et al., 2012). A wide spectrum of infections, from superficial skin infections to severe, and potentially fatal, invasive diseases can be caused by *S. aureus* (Chaibenjawong and Foster, 2011). Annually there are about 241,000 food-borne illnesses due to *S. aureus* in the United States (Kadariya et al., 2014). The number of *S. aureus* in raw milk or other dairy products needs to be less than 10^4 CFU/g according to the regulation of FDA in USA (http://www.fda.gov/food/foodscienceresearch/safepracticesforfoodprocesses/ucm094156.htm). Similar limit is suggested for other countries, such as Australia (Walcher et al., 2014) and New Zealand (Hill et al., 2012). Therefore, sensitive and rapid detection of *S. aureus* is important for food quality and safety.

During recent decades, some methodologies have been developed to detect *S. aureus* in milk. Traditional cultivation methods are standard methods, but they are time consuming (requiring several days) and laborious. (Zanardi et al., 2014). Some molecular methodologies such as the polymerase chain reaction (PCR) (Jin et al., 2009; Riyaz-Ul-Hassan et al., 2008; Zanardi et al., 2014) and in situ hybridization (ISH) (Gey et al., 2013) (both having detection limits around 10^3 CFU/mL) are rapid and sensitive to identify *S. aureus* in food, but they need DNA extraction and require skills and facilities to avoid cross-contamination. Immunoassays, such as enzyme-linked immunosorbent assay (ELISA), commonly used to detect *S. aureus* related proteins or toxins (Chang and Huang, 1994; Yazdankhah et al., 1999), are fast, cheap and requiring fewer skills than PCR based methods. However, there were no ELISA methods published to detect *S. aureus* itself, probably because of Staphylococcal protein A (SPA), which exists on the surface of 99% *S. aureus* isolates (Goding, 1978; Sjoquist et al., 1972). SPA has been a marker for detecting *S. aureus* in agglutination tests (Wilkerson et al., 1997), which are only suitable for detecting high concentration of bacteria. For detecting *S. aureus* in food by ELISA, the main problems would be to find a suitable antibody pair and to meet the sensitivity requirement to detect the small number of bacteria in food. It is possible to use an IgG antibody to capture *S. aureus* bacteria through its binding with SPA, but the second antibody for detection could compete with the IgG capture antibody to bind with SPA also, which would release the captured bacteria and decrease the sensitivity. Therefore, it is pivotal to discover a candidate to substitute one of the two antibodies to improve the sensitivity of ELISA.

Bacteriophage endolysins or lysins are a class of bacteria cell wall peptidoglycan hydrolase synthesized at the late stage of the bacteriophage lytic cycle, mediating lysis of the host cell and the release of progeny phages (Fischetti, 2008; Yang et al., 2012, 2014b). Lysins generally contain two modular domains: N-terminal catalytic

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domain and C-terminal cell wall binding domain (CBD) (Yang et al., 2012, 2014b). The CBDs of the majority of lysins are responsible for attaching the hydrolases to their specific substrates in the bacterial cell walls via non-covalent binding of carbohydrate ligands (Loessner et al., 2002). It has been reported that the number of CBD binding sites on one bacterial cell can be 10^7 or more (Yang et al., 2012). Moreover, the size of CBD (usually 10 to 20 kilodaltons) is much smaller than that of antibodies (usually 150 kilodaltons). Therefore, CBD might be a good candidate to substitute one of the two antibodies in ELISA. Lysin CBDs have been used in biosensors to detect some pathogenic bacteria (Kretzer et al., 2007; Schmelcher et al., 2010; Tolba et al., 2012). In our recently published study (Dong et al., 2015), we found that plyV12C, the CBD from a lysin plyV12 (Yoong et al., 2004), can bind to *S. aureus* with high affinity.

In addition, immunomagnetic particles (IMPs) based separation techniques, which utilize the antibody-coated magnetic beads, have been introduced to separate and enrich the pathogens in complex media such as milk (Ochoa and Harrington, 2005; Sung et al., 2013; Wang et al., 2013). The magnetic separation could remove or decrease the matrix interference during signal detection. The magnetic particles also have highly activated surface area, allowing them to disperse rapidly in samples and accelerate the interactions between magnetic particles and the target bacteria, which would save the assay time and simplify the sample processing procedure.

Herein, with an intention to develop a fast immunoassay with sensitivity for detecting the limit of *S. aureus* bacteria in the milk set by many countries, we used enzyme linked CBD plyV12C (ELCBD) to detect the *S. aureus* bacteria captured by IMPs. This novel ELCBD based method was found able to detect 400 CFU of *S. aureus* in 100 μL milk within 1.5 h.

2. Materials and experiments

2.1. Materials

The gene of lysin PlyV12 (Genbank accession No. AAT01859.1) on plasmid pUC was synthesized by Songon Biotech Co. (Shanghai, China). The gene of tdTomato was amplified from plasmid pLVX-IRES-tdTomato (Clontech, CA, US). *Pyrobest* polymerase and the Competent Cell Preparation Kit were purchased from Takara (Dalian, China). T4 DNA ligase was purchased from NEB (New England Biolabs Inc., UK). The restriction endonucleases were obtained from Thermo Scientific Fermentas (USA). Oligonucleotides were synthesized by Invitrogen (Shanghai, China). Skim milk, BSA and (+)-Biotin N-hydroxysuccinimide ester were purchased from Oxiod (UK), Roche (USA) and Sigma (USA), respectively. Isothiocyanic acid labeled magnetic particles (ITC-MPs, 3 μm in diameter) and the magnetic separation rack were purchased from Goldmag Biotech Co. (Xi'an, China). Streptavidin labeled HRP (strep-HRP) and TMB (3,3′,5,5′-Tetramethylbenzidine) were purchased from Kangwei Century Biotech Co. (Beijing, China) and Bofeng Biotech Co. (Beijing, China), respectively. 96 Serological microplates were bought from Jet Bio-Filtration Co. (Guangzhou, China). Sterile fresh milk was bought in a local supermarket. Mouse IgG was a generous gift from Prof. Bing Yan (Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China). All other chemicals used in the experiments were of analytical grade unless stated otherwise. Deionized water (18.2 MΩ, 0.22-μm filter, Merck Millipore, Germany) was used in all experiments.

2.2. Construction of plasmids

The fragment encoding the putative CBD gene (amino acid residues 146 to 314, plyV12C) was derived from the plyV12 gene by PCR as described previously (Dong et al., 2015). The plasmids of pET28a-$(G_4S)_2$-plyV12CBD (pET28a-CBD) and pET28a-tdTomato-$(G_4S)_3$-CBD (pET28a-T-CBD) were constructed by cloning the corresponding genes into the plasmid pET28a (Novagen, USA) for expressing the respective recombinant proteins. The corresponding primers are listed here: pET28a-T-CBD forward: TTTAC ATATG GTGAG CAAGG GCGAG GAGG TCAT; pET28a-CBD forward: TATAG AATTC GGCGG AGGTG GCAGC GGCGG TGGCG GATCG TTAAA CGGTG GAAGC ACT; the reverse primer of pET28a-CBD and pET28a-T-CBD: TCGTC TCGAG TTATT ACTTAA ATGTA CCCCA TGCT. After confirmation by sequencing, the correct plasmids were transformed into *Escherichia coli* BL21 (DE3) for expression of the recombinant proteins.

2.3. Protein expression and purification

The constructed plasmids were transformed in *E. coli* BL21 (DE3) bacteria to express the proteins. When the $OD_{600\ nm}$ of the bacteria medium arrived around 0.5, Isopropyl β-D-1-thiogalactopyranoside (IPTG, with a final concentration of 0.5 mM) was added to induce the expression of the proteins, tdTomato-CBD (T-CBD) and CBD, overnight at 18 °C, respectively. Then the bacteria were suspended in the binding buffer (20 mM Tris–HCl pH 8.0, 20 mM imidazole, and 0.5 M NaCl) and lysed on an ultra-sonication system (Sonics vibra-cell, Ultrasonic processor VCX 750 W; Sonics & Materials, Newtown, CT, USA). After centrifuging to remove bacteria fragments, the lysate was subsequently filtrated through a 0.4 μm syringe filter before purification. The proteins were purified by nickel affinity chromatography using a HisTrap FF column (GE Healthcare, USA) on the AKTA purifier (GE Healthcare, USA). The purified proteins were dialyzed in PBS buffer to remove imidazole and concentrated using an Amicon Ultra-15 centrifugal filter device (10,000 kDa, Merck KGaA, Darmstadt, Germany). The protein sizes were analyzed using SDS-PAGE and the protein concentrations were assessed by the bicinchoninic acid (BCA) assay (Pierce Rockford, IL, USA). T-CBD or CBD and biotin N-hydroxysuccinimide ester were mixed by the molar ratio of 1:20 (T-CBD or CBD: biotin) to get biotinylated T-CBD or CBD. The biotinylated T-CBD or CBD was changed into PBS buffer by filtering through the centrifugal filter device. The final concentration of biotinylated T-CBD or CBD was determined by the BCA assay.

2.4. Preparation of bacteria

S. aureus strain N315, *E. coli* O157:H7 strain (EDL933), *Bacillus subtilis* (ATCC9372), *Salmonella* (CICC21497), *Streptococcus dysgalactiae* (ATCC35666), *Enterococcus faecalis* (isolated from raw milk), *Enterococcus faecium* (isolated from raw milk) were used in this study. Briefly, 200 μL of the bacterial strains were inoculated in 20 mL Mueller-Hinton broth (MH, Huankai, Guangzhou, China) first, respectively. After incubation for 12 h at 37 °C, the bacteria were centrifuged at 3000 rpm and 4 °C for 10 min. After rinsing by sterile PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) with 15% glycerol, the bacteria precipitates were re-suspended in 20 mL sterile PBS with 15% glycerol. The bacterial concentrations were then determined by serial dilution and plating on agar plates containing the culture medium. The final concentrations of bacteria were adjusted to 10^8 CFU/mL. Finally, the bacteria were stored in 100 μL aliquots at −80 °C until use.

2.5. Preparation of IgG modified magnetic beads

The superparamagnetic ITC-MPs were conjugated with the IgG antibody as described previously (Zhang et al., 2014). Briefly, 0.8 mg ITC-MPs was washed with 200 μL of PBS (pH 7.4) for three times to balance the salt concentration. After mixing 250 μL of