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Development of a novel multiplex lateral flow assay using an antimicrobial peptide for the detection of Shiga toxin-producing *Escherichia coli*

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ABSTRACT

The binding capacity of peptides with broad antimicrobial activity, or antimicrobial peptides (AMPs), to microbes has recently been applied to the specific detection of bacteria and viruses. We established a novel lateral flow assay (LFA) that combines AMPs labeled with colloidal gold and a target-specific antibody immobilized on a nitrocellulose membrane. α-Helical AMPs, especially cecropin P1 (CP1), magainin 2 (MG2), and ceratotoxin A (CtxA), were shown to have optimal properties as probes in LFA. We also established a multiplex LFA for the simultaneous detection and identification of three serogroups of Shiga toxin-producing *Escherichia coli* (STEC) using the CP1 probe with polyclonal antibodies anti-O157, anti-O26, and anti-O111. Each serogroup of *E. coli* could easily and rapidly be detected by multiplex LFA using CP1 and each was clearly visualized in a different position on the LFA strip. The multiplex LFA could detect all tested *E. coli* strains from serogroups O157 (22/22), O26 (17/17), and O111 (7/7), and the detection limit was 10^4 CFU/mL. No other serogroups of *E. coli*, including STEC O45, O91, O103, O121, and O145, or non-*E. coli* strains, reacted. The multiplex LFA could detect *E. coli* O157, O26, and O111 in food samples at very low levels (6.3, 2.9, and 5.6 CFU per 25 g of ground beef, respectively) after 18-h enrichment, and these results were in accordance with the results of the culture method, immunochromatography (IC) strip, and PCR. Given the broad binding capacity, AMP probes in combination with specific antibodies in the novel multiplex LFA may have the potential to detect various microbes simultaneously with identification on a single strip.

1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are important foodborne pathogens that cause severe diseases such as hemorrhagic colitis (HC) and hemolytic–uremic syndrome (HUS; Gyles, 2007). Although the major serogroup of STEC is O157, non-O157 STEC, including O26 and O111, have been reported worldwide (Bettelheim, 2007; Brooks et al., 2005; Gerber et al., 2002). The gold standard for detecting STEC is a culture method (Safarikova and Safarik, 2001), but it is time-consuming, laborious, and inefficient. Because STEC testing is important for the diagnosis of illness and for preventing the spread of foodborne illness, many rapid detection methods have been developed, including, real-time PCR (Sharma, 2002), loop-mediated isothermal amplification (LAMP; Hara-Kudo et al., 2008), and a lateral flow assay (LFA; Jung et al., 2005).

A LFA utilizes the antigen-antibody interaction as the detection method in a simple and rapid format that does not require expensive equipment or complicated procedures. Various LFAs have been developed for routine on-site clinical or food testing and for detecting pathogens such as *E. coli* O157 (Jung et al., 2005), *Listeria monocytogenes* (Shim et al., 2007), and *Campylobacter jejuni* and *C. coli* (Kawatsu et al., 2008). However, the use of antibodies in a LFA has some drawbacks due to production, stability, and modifications. Various applications have been developed by replacing antibodies with nucleic acid probes, which hybridize to specific DNA/RNA sequences (Posthuma-Trumpie et al., 2009), or with aptamers, which bind specific targets (Huang et al., 2007).

Here, we consider, for the first time, the use of antimicrobial peptides (AMPs) as recognition molecules for bacteria in a LFA. AMPs are widely distributed in species of bacteria, protozoa, fungi, plants, and multicellular animals, and over 1200 different, naturally occurring peptides have been discovered to date (Wang et al., 2009). AMPs are generally small, comprised of between 15 and 45 amino acids, positively charged, and amphipathic. Most AMPs display broad-spectrum activity,

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and the broad binding capacity may be exploited for the specific detection of bacteria.

AMPs are categorized into two major structural classes: linear α-helical peptides or β-sheet peptides stabilized by intramolecular disulfide bridges. There are three major models explaining the attachment and insertion of AMPs into lipid bilayers and eventual membrane disruption (Brogden, 2005). In the carpet model, the first interaction of AMPs with target cells is mediated by charge; AMPs are electrostatically attracted to the anionic phospholipid head groups at numerous sites and cover the surface of the membrane in a carpet-like manner. At high peptide concentrations, surface-oriented peptides are thought to disrupt the bilayer in a detergent-like manner (Brogden, 2005). The mode of action of cecropin P1 (CP1), a major AMP in the cecropin family, is described as the carpet model (Gazit et al., 1995).

Recently, other bacteria detection methods utilizing AMPs as recognition molecules have been reported. In those studies, the target bacteria were captured by AMPs immobilized on glass slides and could be detected by a labeled fluorescent dye (direct assay) or by an anti-target antibody (sandwich assay; Kulagina et al., 2005, 2006). Cy5-labeled AMPs were used to detect the capture of *E. coli* O157 by anti-*E. coli* O157 immuno-magnetic beads (Arcidiacono et al., 2008). These methods have high sensitivity but have limited application because fluorescence detection equipment is required. In another method, bacteria immobilized by AMPs were detected electrically (Mannoor et al., 2010), but a spectrum analyzer is required for this analysis. For a more practical method, colorimetric detection using AMP was developed. *E. coli* captured by microplate-immobilized-CP1 is detected colorimetrically with the horseradish peroxide conjugate of anti-*E. coli* antibody (Gregory and Mello, 2005). Utilizing AMPs as antigen capture agents, a polymyxin-based enzyme-linked immunosorbent assay (polymyxin-ELISA) was established for the detection for *E. coli* serogroups O157 (Blais et al., 2004), O26, and O111 (Blais et al., 2006). We recently developed a sandwich colorimetric detection assay that combines the use of an immobilized antibody for antigen capture with biotin-labeled AMP as the detection molecule (manuscript in preparation). However, these methods require multiple steps to detect the target and also require a microplate reader, making it imperative to develop new detection methods that are easy, rapid, and portable in field conditions.

In this study, we developed a novel LFA that combines a colloidal gold-labeled AMP and target-specific antibodies immobilized on a nitrocellulose membrane. Moreover, we developed a multiplex LFA using an AMP for the simultaneous detection and identification of STEC O157, O26, and O111.

2. Materials and methods

2.1. Bacterial strains

Bacterial strains used in this study (listed in Table 4) were obtained from the following sources: American Type Culture Collection (ATCC), Manassas, VA, USA; Research Institute for Microbial Diseases (RIMD), Osaka University, Osaka, Japan; International Research Center for Infectious Diseases (IID), Institute of Medical Science, The University of Tokyo, Tokyo, Japan; and Japan Collection of Microorganisms (JCM), RIKEN BioResource Center, Tsukuba, Japan.

2.2. Preparation of polyclonal antibody against E. coli O157, O26, and O111

Preparation of antigen was basically according to the protocol described previously (Ahlstedt and Holmgren, 1975). *E. coli* strains O157, O26, and O111 were cultured on nutrient agar (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) plates at 37 °C for 18 h. Formalin-killed cells were prepared by suspending harvested cells in 10% formalin solution and incubating at room temperature for 18 h. The formalin-killed cells were then washed three times and suspended in phosphate buffered saline (PBS). The suspension of each strain was mixed in a 1:1 ratio with Freund's complete adjuvant and injected intraperitoneally into Japanese white female rabbits as the first injection of immunogen. Subsequent injections of immunogen were administered at 2-week intervals for 10 weeks, giving a total of 6 injections. Ten days after the final injection, whole blood was collected from all rabbits under anesthesia and centrifuged to obtain serum. Affinity purification of the serum was performed with a protein G column (GE Healthcare UK, Ltd., Buckinghamshire, UK) to obtain rabbit IgG. The obtained rabbit IgG was used as the anti-O157, anti-O26, and anti-O111 polyclonal antibody. The protocol was carried out according to the guidelines for animal experiments of R&D Center, Nippon Meat Packers, Inc.

2.3. Preparation of colloidal gold conjugate solution

AMPs used in this study are shown in Table 1. AMPs containing a C-terminal biotin were chemically synthesized by Sigma-Aldrich Japan G.K. (Hokkaido, Japan). Synthesized peptides were purified (to over 70% purity) by HPLC.

To prepare colloidal gold conjugate, 300 μL of the biotinyl AMP solution (30 μg/mL) was added to 300 μL of a streptavidin–colloidal gold solution (BioAssay Works, LLC, Ijamsville, MD). After a 30-min incubation at room temperature, 10% bovine serum albumin (BSA) solution was added to the mixture. After another 30-min incubation at room temperature, the mixture was centrifuged at 10,000 ×*g* for 20 min, and the precipitate was suspended in 2 mM borate buffer (pH 8.0) containing 1% BSA and 1% trehalose. The prepared colloidal gold conjugate solutions were stored at 4 °C until use.

2.4. Development of the LFA

Schematic diagram of the LFA strip is shown in Fig. 1. The platform on which the LFA was carried out was composed of a sample pad (Whatman International Ltd., Maidstone, UK), and a conjugate pad, a nitrocellulose membrane, and an absorbent pad (EMD Millipore Corporation, Billerica, MA, USA). Anti-O157 and/or anti-O26 and/or anti-O111 antibodies in 10 mM phosphate buffer were immobilized on the nitrocellulose membrane by jet dispenser (CyberJet 2; Musashi Engineering, Inc., Tokyo, Japan). Anti-streptavidin antibody (GenScript USA Inc., Piscataway, NJ, USA) was also immobilized as a control line. Each membrane was placed on a plastic film with each membrane partially overlapping end-to-end. The prepared membranes were cut into 4-mm-wide strips and stored at 4 °C until use. Colloidal gold conjugate solutions of AMP were used for testing with LFA strips.

Table 1
Antimicrobial peptides used in this study.

Antimicrobial peptide	Sequences	Residues	Structure
BLP-7	GIGGALLSAGKSALKGLAKGLAEHFAN	27	Putative α-helix*
CP1	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR	31	α-Helix
CtxA	SIGSALKKALPVAKKIGKIALPIAKAALP	29	α-Helix
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	37	α-Helix
MG2	GIGKFLHSAKKFGKAFVGEIMNS	23	α-Helix
pBD-2	DHYICAKKGGTCNFSPCPLFNRIEGTCYSGKAKCCIR	37	β-Sheet
Tachyplesin I	KWCFRVCYRGICYRRCR	17	β-Sheet
TEWP	EKKCPGRCTLKCGKHERPTLPYNCGKYICCVPVKVK	36	β-Sheet

BLP-7, bombinin-like peptide 7; CP1, cecropin P1; CtxA, ceratotoxin A; LL-37, leucine leucine-37; MG2, magainin 2; pBD-2, porcine beta-defensin 2; and TEWP, turtle egg-white protein.

* It was thought to be a modified α-helix although the structure of BLP-7 was not cleared because other BLPs showed α-helical structure.