Rapid colorimetric lactoferrin-based sandwich immunoassay on cotton swabs for the detection of foodborne pathogenic bacteria

Saleh Alamer\textsuperscript{a,b}, Shimaa Eissa\textsuperscript{b}, Raja Chinnappan\textsuperscript{b}, Paul Herron\textsuperscript{a}, Mohammed Zourob\textsuperscript{b,c,⁎}

\textsuperscript{a} Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, 161 Cathedral Street, Glasgow G4 0RE, United Kingdom
\textsuperscript{b} Department of Chemistry, Alfaisal University, Al Zahrawi Street, Al Maather, Al Takhasusi Rd, Riyadh 11533, Saudi Arabia
\textsuperscript{c} King Faisal Specialist Hospital and Research Center, Zahrawi Street, Al Maather, Riyadh 12713, Saudi Arabia

\textbf{A R T I C L E  A B S T R A C T}

Cotton swab is the conventional swabbing tool that is usually applied for collecting pathogens from contaminated surfaces, followed by cells lysis and DNA extraction before subjecting to genetic analysis. However, such an approach is time consuming as it involves several steps and requires highly trained personnel to perform the experiment. In this study, we developed a new cotton swab-based detection system that involved integrating bacterial collection, preconcentration and detection on Q-tips. The platform is based on a sandwich assay that can detect different pathogens visually by color changes. Lactoferrin-immobilized cotton is used as a general capturing tool to collect various pathogens from surfaces. The presence of particular bacteria is then detected by immersing the cotton in antibodies attached to different colored nanobeads. The target cell is captured between the lactoferrin and specific antibody-conjugated beads which results in a certain color development. The effectiveness of this simply fabricated sensor was demonstrated using Salmonella typhimurium, Salmonella enteritidis, Staphylococcus aureus and Campylobacter jejuni. The intensity of color on the cotton surfaces increased with increasing the concentration of the pathogenic bacteria. The detection limit was as low as 10 cfu/ml for Salmonella typhimurium and Campylobacter jejuni, 100 cfu/ml for Salmonella enteritidis and 100 cfu/ml for Staphylococcus aureus on chicken meat surface. Moreover, this method showed high selectivity and was further confirmed by loop-mediated isothermal amplification (LAMP). The simplicity and the low cost of this colorimetric sensor renders it applicable to a wide range of other pathogens on different surfaces.

\textbf{1. Introduction}

Microorganisms, such as bacteria, viruses and fungi are found everywhere in the environment. These pathogenic microorganisms can cause human foodborne illness due to the ingestion of contaminated food or water [1]. Foodborne diseases have been considered among the most important public health problems in both developed and developing countries [2–5] and there have been significant outbreaks of foodborne pathogenic bacteria in recent years [1,2,4,6].

\textit{Salmonella} can be transmitted to humans through poultry products such as chicken meat and eggs [7,8]. In tropical countries, salmonellosis is one of the major pathogens that cause gastroenteritis due to the consumption of contaminated eggs and poultry products. \textit{Staphylococcus aureus} (Sa) is a potentially pathogenic bacteria also responsible for food poisoning; it is frequently found in contaminated foods such as raw or semi cooked meats, dairy products and ready-to-eat foods [9,10]. Sa is one of the top five pathogens causing the most foodborne illnesses in the USA. It produces a variety of heat stable pathogenic staphylococcal enterotoxins (SEs) that are transported to the blood stream and T cells by super antigen activity. \textit{Campylobacter jejuni} (Cj) is a zoonotic organism which is found mostly in the intestinal mucosa of warm blooded animals. It plays a major role in bacterial diarrhoeal disease worldwide [11,12]. Due to its pathogenicity, even a low dose causes major harm to human health.

Street food, contaminated drinking water and preparation of ready-to-eat foods without proper safety have become important public health issues, and thus detection of foodborne pathogens is demanded for consumer protection. Therefore, several conventional and sensitive methods have been developed for the detection of foodborne pathogens. However, these methods are time consuming, require experienced technicians and are expensive. In order to protect public health, the spread of these diseases must be controlled; therefore, there is a demand for rapid and sensitive alternative methods. Several advanced methods have been developed based on various principles [1,13,14]. Enzyme-linked immunosorbent assay (ELISA) is one such detection method. However, ELISA suffers from the high-cost of the antibody...
production, pre-processing and long analysis time [15]. Polymerase chain reaction (PCR) is widely used for the detection of pathogens using specific primers. Real-time PCR (RT-PCR) is used for the quantitative detection of pathogens using DNA intercalating fluorescent dyes [1,12] and the multiplexed-PCR (mPCR) approach can be used for the detection of more than one pathogen simultaneously [12,15,16]. For example, Chen et al. detected five pathogens simultaneously using mPCR [17]. In addition to these methods, more sophisticated analytical methods such as liquid/gas chromatography coupled with mass spectrometry have been used for the analysis of pathogens. Despite this, these methods are relatively sensitive and accurate; they cannot be used for point-of-care pathogen detection and are also very expensive.

Cotton swabs are widely applied for recovering pathogens from contaminated surfaces. This is usually followed by vortexing to release the pathogens in extraction buffer. After extraction, the samples are then subjected to culturing or any other analysis technique. In this work, we aimed to exploit cotton swab for both sample collection and as a supporting matrix for the sensor. This would minimize the number of analysis steps and reduce the cost of the assay.

Lactoferrin (LF) is a globular glycoprotein. It binds to iron, DNA, RNA, polysaccharides, heparin, bacteria, proteins and viruses and is mainly produced from saliva, milk and exocrine secretions [18]. A high concentration of LF can be found in human colostrum. A number of studies have demonstrated the ability of LF to bind most bacterial cells [19–21]. Some of these studies suggested that the binding between LF and bacteria is due to electrostatic interactions between LF and bacterial cells themselves [22]. Other studies have identified a LF- binding protein on the surface of bacteria such as Streptococcus pneumoniae [23] and Escherichia. coli [24]. For instance, based on solid-phase binding studies, strong binding was demonstrated between LF and purified E. coli OmpC trimer showing a dissociation constant of 39 ± 18 nM [25].

Taking advantage of the binding of LF to bacterial cells, we used LF as a general capturing agent for collecting various pathogens from food samples. Compared with antibodies, the use of LF in immunoassays can offer several advantages. Particularly, it can be easily extracted at a low-cost compared with the long and expensive procedure of antibodies production.

A simple, versatile, portable, rapid and highly sensitive colorimetric immunoassay for bacteria detection is reported. The proposed sensor consists of a cotton swab-based sandwich LF-immunoassay. LF-immobilized cotton swab is used to preconcentrate the bacteria cells from the chicken. Specific antibody coupled with colored nanoparticle beads were then used for color development. Different colours were used as indicator of the presence of various bacteria strains.

2. Experimental section

2.1. Materials and reagents

Sodium periodate (NaIO₄), phosphate buffer saline (PBS), Bovine serum albumin (BSA), 1-ethyl-(3-dimethylaminopropy) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich. *Salmonella Typhimurium*, St (ATCC6538), *Listeria monocytogenes*, Lm (ATCC14028), *Streptococcus pneumoniae* (ATCC10498), *Listeria monocytogenes*, Lm (ATCC7644), Escherichia coli, Ec (ATCC8739) and *Campylobacter jejuni*, Cj (ATCC 29248) were purchased from American Type Culture Collection (Manassas, VA). Stock cultures of all strains except Cj were stored at −80 °C in 20% glycerol solution. Prior to use, the frozen culture was activated in trypticase soy broth (TSB, Oxoid, Hampshire, UK) at 37 °C with two consecutive transfers after 18 ± 2 h incubation periods. The culture was centrifuged at 10000 × g for 10 min at 4 °C and washed twice with trypti-case soy broth. Cell suspensions were prepared and adjusted to an OD of 0.5 at 600 nm, equivalent to 10⁶ cfu/ml. The cells were then serially diluted in trypticase soy broth. Stock cultures of Cj were grown for 4 h at 37 °C and then for 24–48 h at 42 °C under microaerophilic conditions in Bolton broth media (Oxoid LTD, UK) in an anaerobic jar with an active catalyst and a microaerophilic gas generator pack. 10-fold serial dilutions were made in maximum recovery diluent (Oxoid LTD, UK) and the viable cell numbers of Cj were determined by surface plating on Columbia blood agars (Oxoid LTD, UK). Anti- monoclonal antibodies of St and Se and Murine anti-Cj and rabbit polyconal antibody of Sa were purchased from Biospecific (Emeryville, CA, USA).

Lactoferrin from camel milk was purchased from Monojo (Amman, Jordan). Blue, orange and green dye coated polymer nano-beads with less than 50 nm containing carboxylic acid functional groups were purchased from Bangs Laboratories Inc. (Warrington, USA). Carboxylic acid functionalized cobalt based magnetic nano-particles with 50 nm diameter were purchased from Turbo beads (Zurich, Switzerland).

2.2. Procedures

2.2.1. Activation of cotton swabs

2.4 g of sodium periodate (NaIO₄) and 1 ml of concentrated sulfuric acid (H₂SO₄) in 100 ml of water were mixed for 10 min. The cotton swabs were then immersed in the solution overnight to oxidize the hydroxyl groups. The oxidized cotton was then washed with cold distilled water. FTIR was used to confirm the conversion of the hydroxyl groups to aldehyde. The appearance of characteristic peak at 1730 cm⁻¹ confirmed the formation of active aldehyde group on the cotton surface.

2.2.2. Immobilization of lactoferrin on cotton

The activated cotton was immersed in a solution of LF (40 µl, 50 ng/ml) and 2 ml of PBS buffer overnight at 4 °C. After that, the LF-conjugated cotton swabs were washed extensively with PBS to remove the unbound lactoferrin. In order to block the unreacted active aldehyde groups, the cotton was incubated in 1 ml of bovine serum albumin (BSA) for 30 min. The cotton was then washed with PBS and stored at 4 °C until further use. The control samples were prepared by using the same protocol except that the 1 mg/ml BSA was used instead of LF.

2.2.3. Immobilization of the antibodies on the nanobeads

1 ml of 50% polymer nanobeads slurry (blue, orange and green) or a suspension of black magnetic nanobeads was washed with PBS buffer and collected by centrifugation at 16000–18000 rpm for 10 min. The supernatants were removed and 1.5 ml of buffer was added for each beads. The washing step was repeated three times. 500 µl of the mixture of 1-ethyl-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) solution were added to 500 µl of beads and mixed for 20 min at room temperature. The activated beads were washed with PBS three times and suspended in 0.5 ml of PBS buffer. The beads were then incubated with 20 µl of specific antibodies for different bacteria overnight. Each set of colored beads corresponded to a particular bacterial strain; St, Se, Sa and Cj specific antibodies were immobilized on black, blue, orange and green beads, respectively. After washing with PBS, 1 mg/ml of BSA was incubated with the antibody-immobilized beads to block the unreacted active sites on the beads.

2.2.4. Screening procedure

The screening procedure consisted of two steps, the first step was the bacterial capturing step and the second step was the sandwich formation with the secondary antibody for the color development as shown in Scheme 1. In the first step, the cotton-immobilized LF was swabbed over the contaminated chicken surfaces to capture the bacteria. The chicken meat was initially contaminated with St, Se, Sa and Cj cells and the number of the cells was determined by cell counting. After 10 min, the cotton-immobilized LF-bacteria complex was washed with PBS buffer to remove the free cells from the cotton. In the second step (detection step), the bacteria were sandwiched between the LF and