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Development of a simple paper-based colorimetric diagnostic platform for sensitive detection of *Salmonella typhimurium*

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Abstract

Salmonella has long been recognised as the most common and primary cause of food poisoning in many countries. Its detection is still primarily based on conventional microbiological culture methods, which are considered as time-consuming and labour-intensive. Accordingly, the development of a rapid, simple, and reliable detection method to detect *Salmonella* is crucial to maintain public health safety and security. We report herein the development of a simple and rapid paper-based platform to detect *S. typhimurium*. *Salmonella* polyclonal antibody was utilised to capture the target cells, and a direct sandwich enzyme-linked immunosorbent assays (ELISA) format was then developed using an immunoglobulin G (IgG) conjugated with alkaline phosphatase (ALP) as the enzyme label. Nitro blue tetrazolium-(5-bromo-4-chloro-3-indolyl phosphate) was used as enzyme mediator system, which produced a bluish-purple colour on the surface of the paper platform. The developed colour signal was then analysed using a scanner for quantitative analysis. This method was highly sensitive with a limit of detection of 6 CFU mL^{-1} without requiring expensive or advanced equipment. Bare eyes observation can be used for qualitative analysis, thus showing the ability of this method to be used for on-site detection and in resources-limited environment.

Introduction

Salmonella is a ubiquitous enteric pathogen, and one of the leading causes of intestinal illness around the world. This pathogen is transmitted to humans mainly through the consumption of contaminated food or drinking water (Levantesi *et al.,* 2012; Duan *et al.,* 2016). *Salmonella* infection substantially contributes to global morbidity and mortality. The World Health Organization reported that salmonellosis is the most frequently reported food-borne disease worldwide. Salmonellosis causes an estimated 1.2 million infections and 450 deaths annually in the United States (Scallan *et al.,* 2011). Therefore, rapid detection and identification of this pathogen is extremely important to maintain public health safety and security.

The conventional methods to detect *Salmonella* based on microbiological techniques such as swabbing, pre-enrichment, and selective plating, are accurate and reliable. However, these methods are time-consuming with complicated preparation procedures and labour intensiveness (Lazcka *et al.,* 2007; Widyastuti *et al.,* 2018). Nowadays, PCR-based methods has become common analytical techniques to detect and identify pathogens (Postollec *et al.,* 2011). However, PCR-based analytical methods are still far from being reliable to on-site analysis, since they need well-equipped laboratories (Bagheryan *et al.,* 2016). Therefore, it is still challenging to develop a simple, rapid, sensitive, and easy to use analytical methods to detect *Salmonella*.

Recently, paper devices including paper-based assays, paper strip test, and paper-based microfluidics offer new opportunities for pathogen detection. These devices are attractive for portable point-of-measurement and on-site detection due to several advantages such as rapid, economical, portable, and ease of use (Sia and Kricka, 2008). Most of the commercial paper devices are using colorimetric method, and have been applied for analyte detection qualitatively and/or semi-quantitatively (Ratnarathorn *et al.,* 2012). A colorimetric paper-based microspot assay has been

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developed for the detection of *S. typhimurium*, which is capable to detect *Salmonella* at a concentration of 10 CFU mL^{-1}. Unfortunately, the sensitivity of this method is only obtained by the enrichment step of *Salmonella*, and within 10 h (Jokerst *et al.,* 2012). Another report documented the use of colorimetric paper-based device coupled with immunomagnetic separation. The limit of detection of *S. typhimurium* using this method was 10^2 CFU mL^{-1} within 90 min assay in culturing solution without any pre-enrichment step (Srisa-Art *et al.,* 2018).

The focus of the present work was therefore to develop paper-based colorimetric sensor for *S. typhimurium* detection as a rapid, simple, and highly sensitive method; feasible for on-site diagnosis. Paper-based assays are gaining popularity as a simple and rapid method of disease screening in resources limited environment (Martinez *et al.,* 2010). Promising colorimetric assay for pathogen detection have been demonstrated using camera and scanner (Zaytseva *et al.,* 2005; Abe *et al.,* 2010).

In the present work, we developed a simple and highly sensitive colorimetric paper-based immunoassay for *S. typhimurium* detection, based on a direct sandwich ELISA format with alkaline phosphatase (ALP) used as the enzyme label and nitro blue tetrazolium-(5-bromo-4-chloro-3-indolylphosphate) (NBT-BCIP) as the substrate. The developed method combines the specificity of antibodies to the target analyte with the effective catalytic properties of enzymes for signal amplification to provide highly sensitive detection (Mubarok *et al.,* 2016). The catalytic reaction of the enzyme produces a coloured signal, which can be analysed using a scanner. Additionally, the qualitative results of the developed assay can be identified by naked eyes, showing the feasibility to use for on-site detection and in resources-limited environment. The developed paper-based sensor showed potential application for monitoring *Salmonella* in environmental and drinking water to ensure public health.

Materials and methods

Bacterial cultures, chemicals, and instrumentation

All pathogenic cultures (*S. typhimurium*, *Escherichia coli*, and *Listeria monocytogenes*) used in the present work were acquired from Laboratory of Food Microbiology, Brawijaya University. *Salmonella typhimurium* polyclonal antibody was purchased from Bioss Antibodies (USA). Goat anti-rabbit IgG conjugated with alkaline phosphatase (ALP) was purchased from Chemicon (USA). Phosphate buffered saline (PBS, pH 7.4) was obtained from Merck (Germany). Nitro blue tetrazolium-(5-bromo-4-chloro-3-indolyl phosphate) (NBT-BCIP) was purchased from Thermo Fisher Scientific (USA). Bovine serum albumin was purchased from Nacalai Tesque, Inc. (Japan). Glutaraldehyde (25%, v/v) was obtained from Sigma-Aldrich (USA). Tween-20, acetic acid (0.25 M), and Whatman No.1 filter paper were bought from a local chemical store in Malang, Indonesia.

All scanner images of the paper devices were obtained using CanoScan LiDE 220 (Canon Inc., Japan). Adobe Photoshop CS6 program was used to obtain the RGB colour readings.

Glutaraldehyde and antibody solution

Glutaraldehyde solution was prepared by diluting glutaraldehyde stock solution (25% in H_2O) in PBS to obtain 2, 3, 4, 5, and 6% concentration. Primary antibody solution was prepared by diluting 2 µL of *Salmonella* spp. polyclonal antibody stock solution (1 µg mL^{-1}) in 198 µL of PBS. Secondary antibody solution was prepared by diluting 1 µL of goat anti-rabbit IgG-ALP conjugate stock solution (0.1 mg mL^{-1}) in 499 µL of PBS.

Detection of S. typhimurium on the paper devices

Filter paper (Whatman No.1) was cut into small pieces (1 cm × 1 cm), and used as a paper-based platform. Next, 4 µL glutaraldehyde solution was dropped onto the paper platform, and the platform was then dried for 10 min at room temperature. Then, 4 µL of the primary antibody solution was dropped on the paper platform and left for 10 min at room temperature for the immobilisation process. The paper platforms were then washed with 100 µL of PBS containing 1% Tween 20 (PBS-T). Then, 4 µL of BSA was added to the platform as a blocking agent. Next, 10 µL of sample suspension containing various concentration of *S. typhimurium* was spotted to the paper platform, and dried for 10 min at room temperature. Subsequently, 4 µL of secondary antibody was added to the platform, after which the platform was allowed to dry for another 10 min at room temperature. The platform was then rinsed with 100 µL of PBS-T to remove unbounded secondary antibody. Finally, 4 µL of NBT-BCIP was dropped onto the platform. The enzymatic reaction was then conducted at room temperature, and the optimum reaction time was investigated. After the reaction, the paper platform was scanned to obtain the RGB readings. The development of bluish-purple colour indicated the presence of *S. typhimurium*. The resulting blue (B) colour component was taken as analytical response.

The use of various concentrations of

glutaraldehyde solution (2 - 6%) was investigated to obtain the optimum condition for primary antibody immobilisation on the surface of the paper platform. The experiments with various dilution of *S. typhimurium* (10^1 - 10^8 CFU mL^{-1}) were conducted to obtain the limit of detection. In the case of selectivity study, we investigated the presence of other pathogens (*E. coli* and *L. monocytogenes*).

Results and discussion

Colorimetric immunoassay for sensing S. typhimurium

The simple and rapid paper-based immunoassay developed for monitoring *S. typhimurium* was successfully performed. Cellulose-based materials, such as paper, are a good medium for colorimetric tests, since they provide white background. Moreover, cellulose-based paper is compatible with biological samples (Costa *et al.,* 2014). In the present work, we used Whatman No.1 paper as a platform for colorimetric assay, since they have high absorbency and good wicking rate (Chan and Lim, 2016). This paper is manufactured from high quality cotton linters with high alpha cellulose content (> 98%) to guarantee quality, reproducibility, and uniformity (Costa *et al.,* 2014). Glutaraldehyde solution was used to activate the surface of the paper, which can form a linkage with the primary antibody.

The assay developed in the present work for *S. typhimurium* detection was based on a direct sandwich ELISA format with ALP used as the enzyme label, and NBT-BCIP as the substrate. The mechanism of the colorimetric immunoassay applied to detect *S. typhimurium* is illustrated in Figure 1. The primary antibody (*Salmonella* polyclonal antibody) functioned as the capture molecule. Meanwhile, secondary antibody (goat anti-rabbit IgG conjugated with ALP) functioned as reporter antibody, which bound to the captured *Salmonella* cells to complete the sandwich. NBT-BCIP was used as a substrate for colorimetric detection. BCIP is the substrate for enzymatic hydrolysis, which produces indolyl intermediate when it is reacted with ALP. In the presence of oxygen, the indolyl intermediate reacts with NBT, which develops a bluish-purple product in the paper platform (Chen *et al.,* 2016). The colour development in the paper platform indicated the presence of *Salmonella* cells. The blue (B) colour component from RGB reading was taken as analytical response for *Salmonella* cells quantification (Birch and Stickle, 2003).

Optimisation of glutaraldehyde concentration

Glutaraldehyde is one of the most widely used cross-linking agents due to its high reactivity. It is also commercially available and inexpensive (Migneault *et al.,* 2004). Glutaraldehyde is used to immobilise primary antibody, which can form an imine linkage with primary amine on the protein (Williams and Blanch, 1994). Glutaraldehyde concentration affects the immobilisation efficiency of primary antibody on the surface of the paper platform (Albarghouthi *et al.,* 2000). The colour signal intensity increased as glutaraldehyde concentration changed from 2 to 5%, and reached the peak value at 5% concentration. Then, the colour signal intensity began to decrease at 6% concentration (Figure 2A). Low concentration of glutaraldehyde solution produced lower primary antibody densities on the paper surface, which made it difficult for the antigen to bind thus producing low colour signal. Therefore, increasing concentration of glutaraldehyde can raise primary antibody density attached on the paper surface, thus increasing the colour signal. However, a very high primary antibody density can create steric hindrance, which attenuates antigen binding and reduces the colour signal (Pivetal *et al.,* 2017). Thus, 5% concentration of glutaraldehyde solution was selected as the optimal value, and used in subsequent experiments to detect *Salmonella* cells.

Optimisation of enzymatic reaction time

Enzymatic reaction time is an important factor

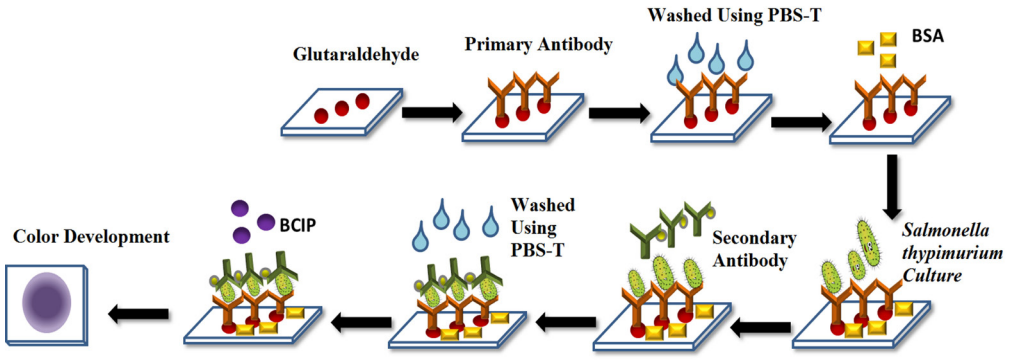

Figure 1. Fabrication of paper-based colorimetric immunosensor to detect *Salmonella*.

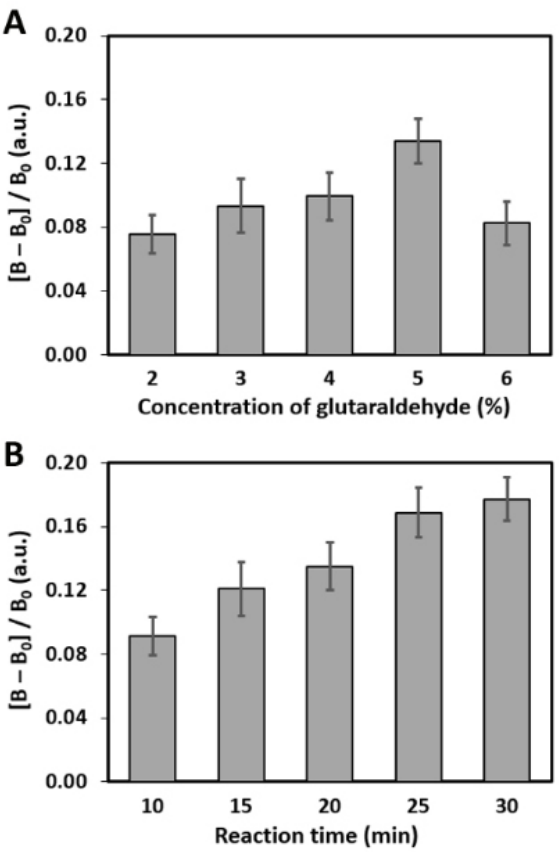

Figure 2. (A) Colour signal enhancement with the presence of *S. typhimurium* (10^8 CFU mL^{-1}) with different concentrations of glutaraldehyde for primary antibody immobilisation. (B) Influence of enzymatic reaction time on colour signal enhancement with the presence of *S. typhimurium* (10^8 CFU mL^{-1}).

influencing the colour development, thus affecting the sensitivity of the developed platform. In order to develop rapid detection method, we investigated different enzymatic reaction time between ALP and NBT-BCIP from 10 to 30 min. The colour signal intensity greatly increased when the enzymatic reaction time increased from 10 to 25 min, and slightly increased when the enzymatic reaction time increased from 25 to 30 min (Figure 2B). 25 min reaction time was chosen as optimum since increasing the reaction time beyond 25 min did not significantly increase the colour signal.

Sensitivity of S. typhimurium detection

The sensitivity of the developed paper-based sensor was examined by using *S. typhimurium* at different concentrations. A linear calibration curve of colour signal enhancement *versus Salmonella* cells concentration was established (Figure 3). The colour signal increased as the concentration of *Salmonella* cells increased, and showed a linear trend over a wide *Salmonella* cell concentration range of 10 - 10^8 CFU mL^{-1}. The limit of detection of *S. typhimurium* was calculated to be 6 CFU mL^{-1} using the equation LOD = 3sb/S, where sb is the standard deviation of blank signal, and S is the slope of linear calibration curve (Mubarok *et al.,* 2016). The sensitivity of our developed method to detect *Salmonella* was found to be superior as compared to the previous report with enrichment-ELISA method (with LOD of 10^2 CFU mL^{-1} in 10 h assay) (Kumar *et al.,* 2008), colorimetric nanosensor (with LOD of 23 CFU mL^{-1} in 24 h assay) (Ma *et al.,* 2017), electrochemical immunosensor (with LOD of 21 CFU mL^{-1} in 2 h assay) (Salam and Tothill, 2009), and surface-enhanced Raman scattering-based aptasensor (with LOD of 15 CFU mL^{-1} in 95 min assay) (Duan *et al.,* 2016). When compared with reported colorimetric paper-based assay (with LOD of 102 CFU mL^{-1} in 90 min assay) (Srisa-Art *et al.,* 2018), our developed paper-based method was found to be more sensitive and less time-consuming. Moreover, we could easily differentiate the colour signal between blank sample and 10^2 CFU mL^{-1} *Salmonella* cells by naked eye observation (Figure 3). This result showed the ability of the developed method for on-site *Salmonella* detection.

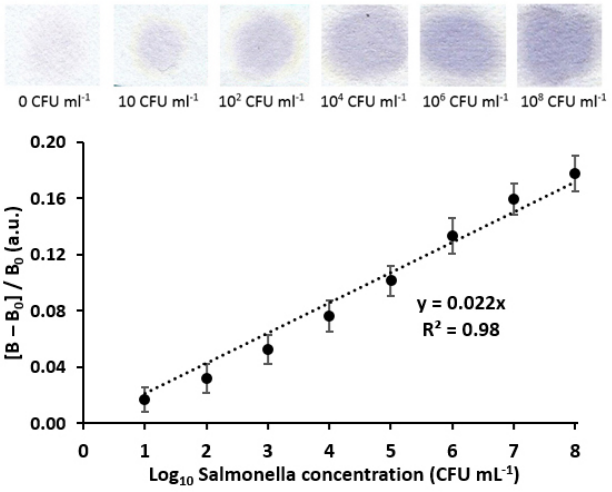

Figure 3. Colour development on the surface of paper platform, for the sensitivity analysis and linear calibration curve obtained with the presence of different concentrations of *S. typhimurium*. B = blue colour component obtained from RGB reading at the indicated concentration of *S. typhimurium*, and B_0 = blue colour component of blank sample.

Specificity study

In order to evaluate the effectiveness of the developed paper-based immunosensor, the specificity study was performed with the presence of other pathogens namely *E. coli* and *L. monocytogenes*. Both are known to be harmful and can cause serious

foodborne illnesses (Välimaa *et al.,* 2015; Wu *et al.,* 2015). The developed paper-based immunosensor based on a direct sandwich ELISA format showed a high sensitivity for *Salmonella* cell detection (Figure 4). The colour signal obtained for *E. coli* and *L. monocytogenes* was 84 and 94%, respectively, lower than the one corresponding to *S. typhimurium*. The high sensitivity of the sensor to detect Salmonella cells using antigen-antibody interaction is similar to the previous report (Salam and Tothill, 2009). Moreover, the result obtained for a mixture of both pathogens (*E. coli* and *S. typhimurium*) is similar to that obtained for the sample spiked only with *Salmonella* cells. This result showed that the presence of other bacteria would not interfere with the result obtained from the developed paper-based sensor.

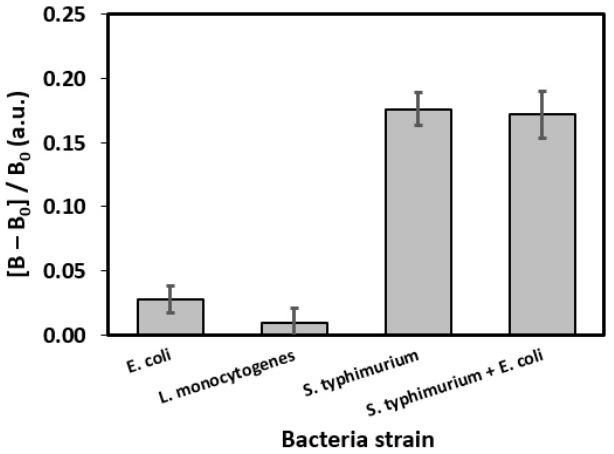

Figure 4. Specificity study performed in the presence of *E. coli* (10^8 CFU mL^{-1}), *L. monocytogenes* (10^8 CFU mL^{-1}), *S. Typhimurium* (10^8 CFU mL-1), and a mixture of *E. coli* and *S. typhimurium* (10^8 CFU mL^{-1} of each bacteria).

Conclusion

A simple paper-based immunoassay for the colorimetric detection of *S. typhimurium* has been developed. Concentration of glutaraldehyde, as a cross-linking agent, and enzymatic reaction time have been optimised for a rapid and sensitive method for future deployment for on-site diagnosis. The developed sensor showed high sensitivity with a limit of detection of 6 CFU mL^{-1}. The different colour signal on the surface of the paper platform can be used for qualitative detection of *Salmonella* cells. This simple method is potentially applicable to detect *Salmonella* for on-site detection and in limited-resources environment.

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