Development of a rapid diagnostic kit for detection of Salmonella Enteritidis in food using indirect coagglutination technique

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ABSTRACT

The objective of this study was to develop a rapid, simple, cheap, sensitive, and specific assay for detection of Salmonella Enteritidis in food. The kit-prototype was developed by using indirect coagglutination technique with three main components, namely Staphylococcus aureus Cowan I, rabbit IgG anti-chicken Fc IgY and chicken IgY anti-S. Enteritidis. Isa Brown layer chickens were used to produce specific antibodies against S. Enteritidis. Monospecific antisera were prepared by absorption method. Staphylococcus aureus Cowan I was coupled with rabbit IgG anti-chicken Fc IgY and monospecific antisera anti-S. Enteritidis. Kit-prototype was compared with multiplex polymerase chain reaction to determine sensitivity and specificity of kit-prototype. Artificially inoculated food sample was used to determine the limit of detection of kit-prototype in a food sample. Indirect coagglutination kit-prototype was able to differentiate positive control from negative control without self-agglutination reaction. This assay has a high specificity to S. Enteritidis without significant cross-reactivity towards other bacteria. Kit-prototype was able to detect \(10^8\) CFU/mL of S. Enteritidis in the buffer and 1 CFU/mL of S. Enteritidis in a food sample after selective enrichment procedure. The application of this kit was able to give a fast result (reaction can be observed in 10 sec), to be applied in a sample without extraction in the preparation of antigen and to reduce detection time of S. Enteritidis in food until 4 days.

Keywords Salmonella Enteritidis – indirect coagglutination – food - diagnostic kit

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Introduction

Salmonellosis is one of the most widespread infectious diseases in the world, and causes high incidences of gastrointestinal food poisoning (34). Salmonella enterica serotype Enteritidis (S. Enteritidis) is the most frequent cause of food contamination leading to salmonellosis in humans (14). Salmonella Enteritidis outbreaks in humans have typically been associated with consumption of raw or undercooked chicken and eggs (5).

The surveillance for the presence of Salmonella in food has therefore become routine all over the world in order to ensure a safe food supply, to minimize the occurrence of foodborne diseases and subsequently ensure public health (4). The culture-based techniques were used as the gold standard for the detection of Salmonella, but the conventional procedures required multiple subculture steps, biochemical and serological confirmation, and were time-consuming, labor-intensive and require considerable technical skill (35). Recently, several methods have been explored and developed for the rapid detection of Salmonella Enteritidis. Generally, rapid detection methods are categorized into miniaturized biochemical kits, nucleic acid-based, biosensor-based, immunological-based methods and assays that are modifications of conventional
tests to speed up analysis (15). Nevertheless, this rapid method has some limitations, namely requirement of specialized laboratories, trained personnel, complicated apparatus, and expensive reagents (31). Therefore, a rapid, simple, cheap, specific and sensitive diagnostic kit for detection of Salmonella Enteritidis is extremely required.

The aim of this study was to develop a rapid, simple, cheap, specific and sensitive diagnostic kit for detection of S. Enteritidis in food. Indirect coagglutination technique was used to develop kit-prototype. Three main components of kit-prototype were Staphylococcus aureus Cowan I, rabbit IgG anti-chicken Fc IgY and chicken IgY anti-S. Enteritidis. Kit-prototype was compared with a multiplex polymerase chain reaction (mPCR) to determine sensitivity and specificity of kit-prototype. Artificially inoculated food sample was used to assign detection limit of kit-prototype in the food sample.

Material and Methods

Design of kit-prototype

Coagglutination is a technique to visualize the specific reaction of antigen and antibody. The agglutination reaction occurs in a short time so that it can be used as the basis of rapid test development. The positive result was marked by the formation of agglutinated particle, and the test result could be easily observed. Indirect coagglutination is a modification of coagglutination technique by using three main components, namely Staphylococcus aureus protein A (S. aureus Cowan I) as the carrier matrix, secondary antibody as the multiplex polymerase chain reaction (mPCR) to determine sensitivity and specificity of kit-prototype. Artificially inoculated food sample was used to assign detection limit of kit-prototype in the food sample.

Antisera production

Antisera were produced in chickens against killed whole cell of S. Enteritidis BCC B2691 (collection of The Indonesian Research Centre for Veterinary Science). Preparation of killed whole cell of S. Enteritidis BCC B2691 refers to Wibawan et al. (32). Bacterial cells were inoculated in 1000 mL brain heart infusion (BHI) broth and incubated at 37 °C for 18 – 24 h. Bacteria were harvested with centrifuge 10000×g for 15 min and washed three times with phosphate-buffered saline (PBS) at pH 7.4 by centrifugation and resuspension. The final concentration was adjusted to 10⁶ cells/mL. This suspension was heated at 80 °C in water bath for 1 h.

The production of antisera in chickens refers to Wibawan et al. (33) with a minor modification. Four female, Isa Brown layer chickens (20 weeks old) were divided into two groups, namely control group and injected group. The chickens were reared in an individual cage (40 x 40 x 80 cm) at 22 °C with free access to water and feed. Chickens in injected group were injected in the first week with 1 mL killed whole cell S. Enteritidis BCC B2691 (10⁹ CFU/mL, intra vena). The injection was repeated in the second week and the third week with 1 mL of antigen suspension (three times, intra vena) respectively. This procedure was approved by the Animal Ethics Committee of Bogor Agricultural University (permit number: 07 – 2015 IPB). The evaluation of specific antibody against S. Enteritidis in antisera was conducted with agar gel immunodiffusion test (AGID) (1) as qualitative measurement and with an indirect enzyme-linked immunosorbent assay (ELISA) (2) as a quantitative measurement. The serum was collected if antibody titer of injected group ≥ 15× than a control group.

Cross-reaction test was conducted by agglutination technique to assess the ability of antisera to react with various bacteria. S. Enteritidis ATCC 13076, S. Enteritidis strain Sm24/Rif12/Ssq, S. Enteritidis, S. typhi ATCC 35250, S. pullorum, S. typhimurium, Escherichia coli ATCC 35150 and Klebsiella sp. (collection of the laboratory of Microbiology, Faculty of Veterinary Medicine, Bogor Agricultural University) were used as antigens in the cross-reactions test. The agglutination procedure refers to Wibawan et al. (33). A volume of 25 µL of antisera and 25 µL of bacterial suspension in PBS at pH 7.4 were mixed on a glass microscope slide (76 × 25 mm), and the agglutination reaction was observed. Positive and negative control was included in each test. Cross-reaction tests were done to pre-absorbed and post-absorbed antisera.

Production of monospecific antisera

Production of monospecific antisera was done by absorption technique in accordance with Suwito (29) with a minor modification. S. typhi ATCC 35250, S. pullorum and S. typhimurium were used as absorbents. All Salmonella isolates that were used as absorbents were inoculated in 1000 mL of brain heart infusion (BHI) broth and incubated at 37 °C for 18 – 24 h. Bacteria were harvested with centrifuge 10000×g for 15 min and washed three times with phosphate-buffered saline (PBS) at pH 7.4 by
centrifugation and resuspension. The bacteria cells were added to 2 mL of antisera, mixed thoroughly, and allowed to react at 37 °C for 1 h, and at 4 °C for 18 – 24 h. The suspension was centrifuged at 10000×g for 20 min, and the supernatant was collected.

Preparation and staining of stabilized staphylococci

Staphylococcus aureus ATCC 12598 (Cowan I) was the strain of the organism, rich in protein A, used to bind antibody. Preparation of stabilized staphylococci was carried out by the method described by Yoshimizu and Kimura (36) with a minor modification. Staphylococcus aureus Cowan I was cultured in the brain heart infusion broth at 37 °C for 48 h. The cells were harvested by centrifugation (10000x g, 20 min) and washed three times with PBS (pH 7.4), and then resuspended in 0.5% formalin-PBS (v/v). After incubation for 3 h at 25 °C, the cells were washed three times with PBS, and resuspended in PBS at the concentration of 10% (v/v). The suspension was then heated at 80 °C for 1 h, washed three times with PBS, and resuspended in PBS at the concentration of 10% (v/v). The cells were stained by resuspending in 10% rose bengal. After incubation for 2 h at 25 °C, the cells were filtered in sterile cotton, centrifuged at 10000×g for 20 minutes, and resuspended in PBS-sodium azide 0.1% at the concentration of 10% (v/v).

Coupling of staphylococci and antibody

In this study, indirect coagglutination reagent consists of three components, namely Staphylococcus aureus Cowan I, rabbit IgG anti-chicken Fc IgY (conjugate) (Jackson Immunoresearch, USA) and monospecific antisera. Monospecific antisera were mixed with conjugate and the reaction was carried out at 25 °C for 2 h following thorough mixing. A volume of 100 µL of this suspension was mixed with 300 µL stabilized staphylococci suspension. After 3 h of incubation at 25 °C, the supernatant fluids of the reaction mixtures were separated by centrifugation (10000xg, 20 minutes). The pellet was resuspended in PBS-Tween 0.05% to be used as indirect coagglutination reagent.

Sensitivity and specificity of kit-prototype

Various concentrations (from 10⁶ CFU/mL to 1 CFU/mL) of S. Enteritidis in PBS buffer were tested using kit-prototype to determine sensitivity (detection limit) of kit-prototype in bacterial suspension. A total of 10 µL from each concentration of bacterial suspension was mixed with 10 µL of kit reagent on a glass slide. The agglutination reaction was examined for 10 sec.

A total of 37 bacteria isolates were used to determine specificity (cross-reactivity) of kit-prototype. All tested bacteria were cultured on blood agar medium for 24 h at 37 °C. Single colony from each tested bacteria was suspended in 10 µL of PBS, mixed with 10 µL kit reagent on a glass slide, and observed for 10 sec.

m-PCR was compared with kit-prototype to determine the specificity of kit-prototype. All 37 tested bacteria were tested by mPCR according to de Freitas et al. (9) with a minor modification. Fresh overnight cultures in the brain heart infusion (BHI) were used to nucleic acid extraction using PureLink® Genomic DNA Kits. PCR was conducted to detection gene ompC (204 bp) and gene Sdf I (304 bp). The primers sequence of gene ompC were 5’-ATC GCT GAC TTA TGC AAT CG-3’ (ompC F) and 5’-CGG GTT GCG TTA TAG GTC TG-3’ (ompC R). The primers sequence of gene Sdf I were 5’-TGT GTT TTA TCT GAT GCA AGA GG-3’ (Sdf I F) and 5’-TGA ACT ACG TTC TTG CTG G-3’ (Sdf I R). Amplification reactions were carried out with 5 µL 5X PCR buffer (Kapa®,) 0.5 µL dNTP (Kapa®, 10 mM), 1 µL primer (XIDT®, 10 pmol/µL), 0.15 µL Taq Polymerase (Kapa®, 5 U/µL), and 3.0 µL DNA template. Distilled water (DNAse-, RNAse-free) was added to bring the final volume to 25 µL. PCR protocol consisted of an initial denaturation step for 3 min at 95 °C followed by 35 cycles, with 1 cycle for 15 sec at 95 °C, 15 sec at 52 °C, and 10 sec at 72 °C, and a final elongation step for 5 min at 72 °C. Aliquot of PCR product was taken in the volume of 10 µL each and electrophoresed on the 1.5% agarose gel, stained with ethidium bromide (0.5 µg/mL) and visualized and photographed under UV illumination.

Real food sample test

Egg yolk was employed as the food sample for detection of S. Enteritidis by using kit-prototype. Before artificial inoculation, egg yolk was pasteurized to eliminate other contaminant bacteria by heating at 61.1 °C for 3.5 min as described by Froning et al. (13). Egg yolk samples were inoculated with S. Enteritidis in various concentrations (10⁴ CFU/mL, 10⁵ CFU/mL, 10⁶ CFU/mL, 10⁷ CFU/mL and 1 CFU/mL). Egg yolk inoculated with 10⁶ CFU/mL of S. typhimurium was used as negative control. A total 25 mL of egg yolk sample was transferred into 225 mL of sterile buffered peptone water (BPW) 0.1% as pre-enrichment media, and incubated 18 – 20 h at 37 °C (28).

A total of 1 mL sample suspension from pre-enrichment media was transferred into selective enrichment media Rappaport-Vassiliadis (RV) broth and incubated for 24 h at 42 °C. A total of 3 mL of RV suspension was centrifuged at 10000x g for 5 min. The pellet was used as a sample for DNA extraction in mPCR procedure, and as a sample for coagglutination procedure using kit-prototype.

Results

Antisera production

The cross-reaction test of pre-absorbed antisera showed that there was a cross-reaction occurring between antisera with a bacterium which belongs to the genus Salmonella. Cross reaction does not occur between antisera with another bacterium which belongs to the family Enterobacteriaceae. The cross-reaction test of post-absorbed antisera showed that there was no significant cross-reaction towards other bacteria. Table 1 presents the results of cross-reactivity of pre-absorbed and post-absorbed antisera to the different species of bacteria.
**Table 1.** Cross-reactivity results of pre-absorbed and post-absorbed antisera to the different species of bacteria

<table>
<thead>
<tr>
<th>Bacterial antigen</th>
<th>Cross-reactivity of antisera</th>
<th>Pre-absorbed</th>
<th>Post-absorbed</th>
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<tbody>
<tr>
<td>S. Enteritidis BCC B2691</td>
<td></td>
<td>+</td>
<td>+</td>
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<tr>
<td>S. Enteritidis ATCC 13076</td>
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<td>S. Enteritidis</td>
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<td>S. Enteritidis strain Sm24/Rif12/Ssq</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>S. Typhi ATCC 35250</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>S. Pullorum</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>S. Typhimurium</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Escherichia coli ATCC 35150</td>
<td></td>
<td>-</td>
<td>-</td>
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<tr>
<td>Klebsiella sp.</td>
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</table>

**Development of indirect coagglutination kit-prototype**

The development of indirect coagglutination kit-prototype was performed by using three main components, namely *S. aureus* Cowan I, rabbit IgG anti-chicken Fc IgY and monospecific antisera. The process of matrix construction can be done optimally so that this reagent can differentiate a positive and negative control clearly without self-agglutination. Figure 2 presents coagglutination reaction pattern for the positive and negative result by using indirect coagglutination kit-prototype.

**Sensitivity and specificity of kit-prototype**

The lowest concentration of *S. Enteritidis* in the buffer, which was able to be detected correctly by the kit-prototype was $10^6$ CFU/mL. Kit-prototype can detect 1 CFU/mL of *S. Enteritidis* in the artificially inoculated food sample if applied after selective enrichment procedure. The artificially inoculated food sample that was inoculated with $10^8$ CFU/mL of *S. typhimurium* showed a negative result. The result of mPCR in the artificial food sample showed the typical result with kit-prototype (Figure 3).

**Discussion and conclusions**

Salmonellosis is a foodborne illness, which catches the attention because of its threats to the food security (7). *S. Enteritidis* was the most isolated serotype in the incidents of salmonellosis (30). The rise of *S. Enteritidis* infection does not only occur in developing countries (11), but also in the developed countries like Canada (23), USA (6) and European countries (10). The existence of this pathogen in food threatens human health extremely even though contaminated food is of a small quantity. Early detection...
of S. Enteritidis in food is important for food security, and as an early warning of the outbreak (7). Recently, several methods have been explored and developed for the rapid detection of S. Enteritidis (28). Rapid test has been developed with some principals, for example miniature of biochemical kits (18), immunological-based (3), biosensor-based (37) and DNA hybridization (38). Nevertheless, some rapid tests have shortages such as requirement for expensive, highly developed technology to be used in a laboratory, and also well-trained personnel. The coagglutination technique has been developed to detect some bacteria (36). This technique is sensitive, specific, fast, easy, cheap and reliable (18).

The coagglutination kit developed in this study was based on indirect coagglutination technique by using three main components, namely Staphylococcus aureus protein A (S. aureus Cowan I) as the carrier matrix, secondary antibody as the connector between the S. aureus protein A and primary antibody, and the primary antibody as the detector of antigen in the sample. The use of indirect coagglutination technique was aimed at increasing kit sensitivity. The increased sensitivity could be reached due to several detecting components attached to one cell of S. aureus Cowan I. Specificity was also an important feature in these assays. Kit specificity was dependent on the specificity of primary antibody used (21, 27). Pre-absorb antisera showed a cross reaction with S. typhi, S. pullorum and S. typhimurium. Thus, the three bacteria were used as absorbents in the absorption procedure to produce monospecific antisera. There was no cross-reaction towards other bacteria after the process of absorption, which indicated the process of absorption was done optimally (12).

The construction of indirect coagglutination kit-prototype can be performed optimally to clearly differentiate positive and negative controls. A positive reaction was identified by the development of fine powdery particles (agglutinated particle), which can be observed by naked eyes. The formed reagent had a good quality because there was no self-agglutination reaction. Self-agglutination was a formation of powdery particles, which did not occur because of a specific reaction of antigen and antibody. Self-agglutination was affected by some chemical interactions occurring among the kit components, such as hydrophobic interaction, ion interaction, and covalent bonds among the sulphydryl groups in the immunoglobulin (8, 26).

Kit-prototype was compared with mPCR technique. The result showed that there was no cross-reaction towards other bacteria. This assay has a high specificity to S. Enteritidis, which is in line with some research performed by Mathai et al. (19) and Rahman et al. (25). The determination of the kit sensitivity was done by using bacteria suspension in the buffer proceeded with the application to the artificially inoculated food sample. Obtained results indicated that the kit could detect S. Enteritidis in food at the very low quantity. The application of indirect coagglutination kit to detect S. Enteritidis that was developed in this study has advantages of being sensitive, specific, cheap and easy to apply. The detection time of this kit was 10 seconds. The application of this kit can reduce detection time down to 4 days. There was no complicated antigen preparation for the test so numerous and costly equipment was not necessary. Indirect coagglutination kit-prototype was able to differentiate a positive from negative control without self-agglutination reaction. This assay has a high specificity for S. Enteritidis without significant cross-reactivity towards other bacteria. Kit-prototype has high sensitivity and specificity. The application of this kit was able to render a fast result (reaction can be observed in 10 sec), be used in a sample without extraction in the preparation of antigen, and reduces detection time of S. Enteritidis in food down to 4 days.

References


Razvoj brzog dijagnostičkog kита za otkrivanje *Salmonella Enteritidis* u hrani koristeći tehniku indirektne koagulacije

**Sažetak**

Cilj ovog istraživanja je u tome da razvije brz, jednostavan, jeftin, osjetljiv i specifičan test za otkrivanje *Salmonella Enteritidis* u hrani. Prototip testa je razvijen koristeći tehniku indirektne koagulacije sa tri osnovne komponente: *Staphylococcus aureus* Cowan I, kuničevi IgG specifični za kokošiji Fc IgY i kokošiji IgY anti-*S. Enteritidis*. Za dobijanje specifičnih antitijela protiv *S. Enteritidis* korištene su kokoši nosilje rase ISA Brown. Monospecifični antiserum je pri-premljen apsorptivnom metodom. *Staphylococcus aureus* Cowan I je konjugiran sa kuničevim antitijelima specifičnim za kokošiji Fc IgY i monospecifičnim antiserumom anti-*S. Enteritidis*. Dobijeni prototip testa je uspoređen sa multiplex-PCR da bi se ustanovila osjetljivost i specifičnost. Umjetno inokuliran uzorak hrane je iskorišten da bi se ustanovio limit detekcije prototipa testa. Prototip je uspio da razlikuje pozitivnu kontrolu od negativne kontrole bez autoaglutinacijske reakcije. Ovaj test posjeduje veliku specifičnost za *S. Enteritidis* bez značajne unakrsne reakcije sa drugim bakterijama. Testom je uspješno detektovao $10^8$ CFU/mL *S. Enteritidis* u puferu u količini od $10^4$ CFU/mL i 1 CFU/mL S. $10^5$ CFU/mL u uzorku hrane nakon selektivnog procesa obogaćivanja. Primjena ovog testa je omogućila postizanje brzih rezultata (reakcija se može posmatrati za 10 sekundi), primjenu na uzorku bez ekstrakcije antigena i skraćenje vremena otkrivanja *S. enteritidis* u hrani do 4 dana.