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A PCR- ELISA for the detection of *Salmonella* from chicken intestine

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ABSTRACT

In this study, a Polymerase Chain Reaction Enzyme Linked Immunosorbent Assay (PCR-ELISA) was described to detect *Salmonella* DNA from selective primary enrichment culture of chicken intestine. *Salmonella* genus-specific PCR product was produced using invA-specific primers and digoxigenin by commercial PCR ELISA DIG Labelling and ELISA DIG Detection kits. PCR-ELISA detection limits with *S.* Enteritidis 64K DNA, pure culture, and with the intestinal homogenate artificially-contaminated with this strain were found as 0.0079 µg ml^{-1}, 50 cfu ml^{-1}, and 70 cfu ml^{-1}, respectively. Sensitivity and specificity of the assay was determined as 100% with the tests performed with 41 *Salmonella enterica* serovar DNAs, with non-*Salmonella* strains, *Citrobacter* sp., *E. coli*, *Klebsiella* sp., *P. aeruginosa*, and *Streptococcus* sp. DNAs, and with 124 tetrathionate broth enrichment cultures of *Salmonella*-contaminated chicken intestinal samples. We suggest that this PCR-ELISA detection could be an alternative method to detect *Salmonella*-specific DNA from chicken intestine, and it would find use particularly in high-sample throughput laboratories in the poultry sector.

Key Words: *Salmonella*, PCR-ELISA, chicken, intestine, faeces

INTRODUCTION

Polymerase Chain Reaction (PCR) performed in the block-water heated thermal cycler and further PCR product detection methods have relatively low sensitivity, higher cost and are time-consuming. In this context, conventional PCRs and detection methods have become insufficient for sectors with routine and large-scale sample screenings for food-borne bacterial pathogens, such as *Salmonella*.

Recently, real time PCR modifications for *Salmonella* detection (Eyigor et al., 2003; Eyigor and Carli, 2003; Perelle et al., 2004; Hein et al., 2006) have been good alternatives to conventional PCR in solving the sensitivity and sample size problems. However, investment for real time PCR systems could be quite costly for laboratories with limited budget. In such laboratories, amplicons after conventional PCR can be detected in a relatively inexpensive way by an Enzyme Linked Immunosorbent Assay (ELISA), which is defined as PCR-ELISA. Gillespie et al. (2003) had previously developed and used this assay for the identification of *Salmonella enterica* somatic groups C1 and E1. Hong et al. (2003), Metzger-Boddien et al. (2004), and Perelle et al. (2004) developed similar assays for *Salmonella* detection in different food types, while Luk et al. (1997) performed this assay with human faeces.

In this study, we describe a PCR-ELISA for the detection of *Salmonella* from selective primary enrichment cultures of chicken intestine.

MATERIALS AND METHODS

Salmonella serovars and non-Salmonella strains

Salmonella enterica serovars Enteritidis 64K (*Salm*. Enteritidis 64K), Typhimurium LT2-CIP60–62, and Gallinarum 64K were obtained from M. Y. Popoff, Institut Pasteur, 28 rue du Dr Roux, 75015 Paris Cedex 15, France. In addition, 24 *Salmonella enterica* serovars Enteritidis, 13 *Salmonella enterica* serovar Agona, and one *Salmonella enterica* serovar Thompson and non-*Salmonella* strains (*Citrobacter* sp., *Escherichia coli*, *Klebsiella* sp., *Pseudomonas aeruginosa*, *Streptococcus* sp.) were provided from the Departments of Microbiology, Faculty of Veterinary Medicine and Medical School, Uludag University, Bursa, Türkiye.

DNA extraction

Crude DNA was extracted by a method described by Carli et al. (2001). Briefly, 1 ml of tetrathionate broth (TTB) (Oxoid 235780) 18 h cultures of *Salmonella* strains, which were incubated at 37°C, was centrifuged for 4 min at 4,600 x g. The pellet was suspended in 0.85% saline, was centrifuged, and resuspended in 20 µl of deionized water. This bacterial suspension was then boiled for 10 min and was centrifuged for 3 min at 18,000 x g. The supernatant was used as a template in PCR.

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Primers and the probe design for ELISA

We used a *Salmonella inv*A gene-based primer pair with a previously determined specificity and sensitivity (Rahn et al., 1992, GenBank accession no. M90846). These primers, invA1 and invA2, have the following nucleotide sequences, respectively: 5'-GTG AAA TTA TCG CCA CGT TCG GGC AA-3' and 5'-TCA TCG CAC CGT CAA AGG AAC C-3'.

The probe for ELISA has the following sequence of 5'- CGA TAA ACT GGA CCA CGG TTT TTT TTT T- 3', and has a biotin molecule attached to its 3' end. Both primers and the probe were synthesized in Expedite DNA synthesizer (Perseptive Biosystems, USA) and were purified using reverse phase-High Pressure Liquid Chromatography (BioCAD700E, Perseptive Biosystems, USA).

Preparation of DIG-labelled Salmonella genus-specific PCR product

DIG-labelled *Salmonella* genus-specific PCR product was produced by PCR ELISA (DIG Labelling) (1636 120, Roche, Germany) using invA1 and invA2 primers and incorporating (DIG)-11'-dUTP to the product during PCR amplification process in mastercycler (Eppendorf, Germany). The 50 µl PCR mixture contained 24.75 µl of deionized water, 5 µl 10x reaction buffer without $MgCl_2$, 5 µl $MgCl_2$ (25 m mol l^{-1}), 2.5 µl of each primer (5 pmol μl^{-1}), 5 µl PCR-DIG labelling mix (2mM dATP, dGTP, dCTP; 1.9mM dTTP and 0.1mM DIG-dUTP), 0.25µl Taq DNA polymerase (5 $U\mu l^{-1}$), and 5 µl template DNA (250 µg ml^{-1}). PCR parameters were: initial denaturation, 5 min at 95°C; 35 cycles of 45 sec at 95°C, 45 sec at 50°C, and 45 sec at 72°C. PCR grade sterile deionized water and human genomic DNA (with its specific primers, included in PCR ELISA DIG Labelling Kit) were used as PCR negative (PCR ELISA blank/negative PCR control) and positive labelling controls, respectively.

Detection of DIG-labelled Salmonella genus-specific PCR product by PCR- ELISA

Reagent reconstitutions (for the DIG-labelled control PCR product, for biotin-labelled control capture probe, for anti-DIG peroxidase [anti-DIG POD] conjugate), working solution preparations (hybridization solutions for our biotin-labelled capture probe and for the control capture probe, washing solutions, anti-DIG-POD working solution, ABTS-substrate solution) for the ELISA detection after PCR were performed according to the manufacturer's instructions (PCR ELISA [DIG Detection] 1636 111, Roche, Germany). Briefly, 10 µl from the DIG-labelled control PCR product (positive labelling control), 5 µl from the 'PCR ELISA blank/negative PCR control', 10 µl of sterile deionized water (negative detection control) and 5 µl DIG-labelled control PCR product (supplied with the kit-positive detection control) were put into individual microfuge tubes. Ten microliters from the DIG-labelled *Salmonella* genus-specific PCR product was placed into another microfuge tube. Twenty microliters of denaturation solution was added to all the tubes and incubated at 20°C for 10 min, followed by the addition of 230 µl of hybridization solutions, mixing and transferring 200 µl from each reaction into streptavidine-coated microtiter plate (MTP) wells, which were incubated at 37°C for 3 h. Reactions were washed four times with 250 µl washing solutions, and 200 µl of the 1:2000 diluted anti-DIG POD was added to each well and incubated at 37°C for 30 min. Wells were re-washed four times with 250 µl washing solution, 200 µl of ABTS-substrate solution was added to each well and the plate was incubated in the dark at 37°C for 30 min to allow enzymatic reaction. Also, 200 µl of ABTS substrate solution was added to an empty well to measure intrinsic extinction of the ABTS solution. Absorbance of each sample was measured at 405 nm in UV-visible spectrophotometer (UV-1601 PC, Shimadzu Co., Japan). Background absorbance of the ABTS colour substrate was subtracted from the absorbance values measured from the samples.

Detection limit of PCR-ELISA with Salmonella DNA, pure culture, and artificially contaminated samples

To determine the detection limit of PCR-ELISA with *Salmonella* DNA, template DNAs taken from serial dilutions (1:2, 1:10, 1:50, 1:100, 1:500, 1:1000, 1:10.000) of *Salm*. Enteritidis 64K DNA stock (79 µg ml^{-1}) was used in the PCR and in ELISA.

For the detection limit determination of PCR-ELISA with pure *Salmonella* culture, *Salm*. Enteritidis 64K stock culture ($5x10^6$ cfu ml^{-1}) was subjected to ten fold dilutions up to 10^{-7}. Each diluted sample was subjected to crude DNA extraction as described above and the extract was used as template in PCR-ELISA.

The detection limit of PCR-ELISA with artificially contaminated samples was determined as follows: One gram of *Salmonella*-free chicken ileocecal samples (determined by a bacteriological method and PCR) were minced and added into 10 ml of TTB. Tenfold dilutions from $7x10^6$ cfu ml^{-1} to 7 cfu ml^{-1} of *Salm*. Enteritidis 64K were inoculated into TTB with intestinal homogenate and were incubated at 37°C for 18 h. PCR was performed by using 1 ml from each culture as described above.

Sensitivity and specificity determination of PCR-ELISA
For sensitivity tests, PCR-ELISA was performed with the DNAs extracted from 41 *Salmonella enterica* serovars. PCR-ELISA specificity was determined by carrying out the test with DNAs from non-*Salmonella* strains, *Citrobacter* sp., *E. coli*, *Klebsiella* sp., *P. aeruginosa*, and *Streptococcus* sp.

PCR-ELISA with clinical samples
One hundred and twenty four intestinal samples from five day-old meat type chickens, which had been previously diagnosed by bacteriology as infected with *Salmonella*, were used. One gram from each intestinal homogenate was inoculated into TTB, and incubated at 37°C for 18 h. Then, 1 ml of this TTB culture was used in template preparation for PCR as described above.

RESULTS

Detection limit of PCR-ELISA
Detection limits of the assay with *Salm.* Enteritidis 64K DNA, pure culture, and with artificially contaminated samples were found as 1:10.000 (0.0079 µg ml^{-1}) with OD_{405} of 0.0186 (Table 1), 50 cfu ml^{-1} with OD_{405} of 1.097 (Table 2), and 70 cfu ml^{-1} with OD_{405} of 1.488 (Table 3), respectively.

Table 1. Detection limit of PCR-ELISA with pure *Salm.* Enteritidis 64K DNA dilutions

Salmonella 64K DIG-labelled PCR product dilution	DNA concentration (µg ml-1)	OD_{405}*	Visible blue colour in well
0	79	0.792	yes
1:2	39.5	0.541	yes
1:10	7.9	0.407	yes
1:50	1.58	0.078	yes
1:100	0.79	0.019	yes
1:500	0.158	0.018	yes
1:1000	0.079	0.0115	yes
1:10000	**0.0079**	**0.0186**	**yes**
1:100000	0.00079	0.0055	no

* OD_{405} = OD_{405} of sample- OD_{405} of negative detection control (0.0049)

Sensitivity and specificity determination of PCR-ELISA
All 41 *Salmonella enterica* serovars yielded positive and all non-*Salmonella* strains gave negative results in the sensitivity and specificity tests, respectively.

PCR-ELISA with clinical samples
Seventy two Salmonella- positive samples determined by PCR-ELISA were also found to be culture positive, while 52 PCR-ELISA negative samples were negative by bacteriology. These results revealed that the relative sensitivity and specificity of PCR -ELISA compared to bacteriology as 100 %.

DISCUSSION

PCR-ELISA used in this study has several advantages over conventional PCR, as previously reported by others (Luk et al., 1997; Gillespie et al., 2003; Hong et al., 2003; Metzger-Boddien et al., 2004; Perelle et al., 2004.) The system neither requires gel electrophoresis, ethidium bromide staining and UV detection of the amplicon, nor southern hybridization of the PCR product with probes and the detection of the hybrids with specialized equipments. Another advantage of the PCR-ELISA compared to conventional PCR, particularly for private sector requiring simultaneous large-scale sample screenings, such as poultry companies, is its cost-effectiveness due to a 96-well microplate format used.

Table 2. Detection limit of PCR-ELISA with pure *Salm.* Enteritidis 64K culture dilutions

Number of *Salmonella* 64K (cfu ml^{-1})	OD_{405}*	visible blue colour in well
5×10^6	0.680	yes
5×10^5	0.633	yes
5×10^4	1.905	yes
5×10^3	1.574	yes
5×10^2	0.576	yes
5×10^1	**1.097**	**yes**
5	0.009	no

* OD_{405} = OD_{405} of sample- OD_{405} of negative detection control (0.0007)

Table 3. Detection limit of PCR-ELISA on intestinal samples artificially contaminated with *Salm.* Enteritidis 64K culture dilutions

Number of *Salmonella* 64K (cfu ml^{-1})	OD_{405}*	visible blue colour in well
7×10^6	0.410	yes
7×10^5	0.932	yes
7×10^4	1.238	yes
7×10^3	1.772	yes
7×10^2	1.538	yes
7×10^1	**1.488**	**yes**
7	0.007	no

* OD_{405} = OD_{405} of sample- OD_{405} of negative detection control (0.0021)

The detection limit results of our PCR-ELISA with pure culture and artificially contaminated intestinal samples were almost identical to each other. This finding shows us that TTB ingredients and intestinal sample contents do not have an inhibitory effect either on our optimized PCR, or on the consequent ELISA part of the procedure.

Even though several studies had reported detection limits of PCR-ELISAs for *Salmonella* (Luk et al., 1997; Hong et al., 2003; Metzger-Boddien et al., 2004; Perelle et al., 2004), it is impossible to make accurate comparisons between our data and theirs due to changes in parameters such as sample type, primers, probes, DNA extraction methods, and different possible PCR inhibitors.

Relative sensitivity and specificity of PCR -ELISA with chicken intestinal samples compared to bacteriology was 100 % in our study. This finding is in agreement with the findings of Luk et al. (1997) and Hong et al. (2003), who had used stool samples from patients with diarrhoea and Rappaport broth as the primary enrichment medium, and drag swabs from poultry farms and tetrathionate brilliant green enrichment broth, respectively. We think that PCR-ELISA relative sensitivity compared to bacteriology yields similar and consistent results, when faecal or intestine-related samples are used. This agreement between the two methods is most likely related to the compatibility of the preenrichment medium used for bacteriology/PCR and the sample type. In contrast, higher inconsistencies with various food matrices, such as poultry meat (Hong et al., 2003) and minced beef, fish and raw milk (Perelle et al., 2004) have been reported. We think that the inconsistencies observed with food samples are in the PCR part of the PCR-ELISA, and are possibly related to the use of media specific for standard bacteriology, but not for PCR. In these types of studies, predetermination of appropriate pre/primary enrichment medium according to sample type, bacteriology, and to PCR (Carli et al., 2001; Gunaydin et al., 2007) is recommended.

To our knowledge, this is the first report on the detection of Salmonella from chicken intestinal samples by PCR-ELISA. We suggest that this assay would find use in large-scale sample screenings for the poultry sector.

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