

ISSN 1682-8356
ansinet.org/ijps



INTERNATIONAL JOURNAL OF
POULTRY SCIENCE

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Detection of *Salmonella* and *Escherichia coli* in Egg Shell and Egg Content from Different Housing Systems for Laying Hens

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Abstract: Polymerase Chain Reaction (PCR) assay and conventional microbiological methods were used to detect bacterial contamination of egg shells and egg content in different commercial housing systems, the open house and evaporative cooling systems. A PCR assay was developed for direct detection using a set of primers specific for the invasion by *invA* gene for *Salmonella* and *uidA* gene, encoding β -glucuronidase for *Escherichia coli*. PCR detected the presence of *Salmonella* in 2 (5%) samples of egg shells from the evaporative cooling system, while conventional cultural methods detected no *Salmonella* from the same samples. No *Salmonella* was detected from the open house system. The frequency of PCR positive samples for *E. coli* was 35% (16/40) isolated from the open house system and 42% (16/38) from the evaporative cooling system. Two samples screened for *E. coli* in egg content was also detected by PCR. In comparison, detection of *E. coli* by culture, done simultaneously with PCR, was 17.5% (7/40) sensitive in the open house system and 31.5% (12/38) sensitive in the evaporative cooling system. Results indicated that this PCR procedure is a sensitive method for *Salmonella* and *E. coli* detection. The contamination of *Salmonella* and *E. coli* was found at a high frequency in the evaporative cooling system compared with the open house system.

Key words: Egg, *Escherichia coli*, PCR, *Salmonella*

INTRODUCTION

Salmonella and *E. coli* are a major food-borne bacterial pathogen, with poultry and poultry products being a primary source of infection to humans (Sharma and Carlson, 2000). It has most often been associated with consumption of contaminated foods of animal origin, such as poultry, swine, dairy products and eggs (Gebreyes *et al.*, 2000; Rajashekara *et al.*, 2000; Daly *et al.*, 2002; Sahilah *et al.*, 2010). Poultry are considered an important source of food borne diseases and the illnesses were associated with the consumption of contaminated eggs. *Salmonella enteritidis* and *S. typhimurium* as well as other serotypes have been isolated from egg shells and egg content (Akhtar *et al.*, 1982; Mayes and Takeballi, 1983; Jones *et al.*, 1995; Rahman *et al.*, 2006). The prevalence of *E. coli* in poultry and eggs has also been reported (Doyle and Schoeni, 1987; Trampel *et al.*, 2007; Sahilah *et al.*, 2010). The most commonly used technique for *Salmonella* and *E. coli* detection is the conventional technique or microbiological technique. Conventional selective enrichment and serological tests for *Salmonella* and *E. coli* from eggs take five to seven days and are labor intensive. Thus, rapid and sensitive methods for detecting are in great demand in order to reduce time consumption and labor. PCR technology represents a

rapid procedure with high sensitivity and high specificity to detect *Salmonella* and *E. coli* in a wide variety of food. Several PCR assays have been developed by targeting various *Salmonella* genes, such as 16S rRNA (Iida *et al.*, 1993), *agfA* (Doran *et al.*, 1993) and *viaB* (Hashimoto *et al.*, 1995) and virulence-associated plasmids (Mahon and Lax, 1993). In addition, *invA* is one of the most often used genes to detect *Salmonella* sp. (Rahn *et al.*, 1992; Bulte and Jakob, 1995; Wang *et al.*, 1995). Many PCR assays have been developed using primers that target genes specific for *E. coli* O157:H7, with genes commonly targeted encoding for proteins such as intimin, *eaeA* (Gannon *et al.*, 1993; Paton and Paton, 1998), O157 lipopolysaccharide O-antigen synthesis genes, *rfbE* (Desmarchelier *et al.*, 1998) and β -glucuronidase, *uidA* (Cebula *et al.*, 1995; Heining *et al.*, 1999; Brasher *et al.*, 2002). The *uidA* gene, specific for *E. coli* was used for detection in this study.

The aim of this study was to compare egg contamination in commercial production from different housing systems, determining the prevalence of *Salmonella* and *E. coli* on egg shell and egg content using conventional microbiology detection compared with that detected using *invA* gene of *Salmonella* and *uidA* gene of *E. coli* by PCR technique in order to develop a protocol for sensitive and specific pathogen detection.

MATERIALS AND METHODS

Egg samples: Fresh chicken eggs were received from different housing systems for laying hens (from Animal Research Farm, Kasetsart University, Bangkok). In the entire experiment, 20 eggs were received from the open house system and 19 eggs were received from the evaporative cooling system. Unwashed eggs were collected in sterile bags and transported to the laboratory. Aseptic procedures were strictly adopted during sample collection. Sterile cotton swabs dipped in sterile peptone broth were used to swab the entire surface area of the eggshell then added to the peptone broth and subsequently incubated for 16 to 18 h at 37°C. In order to collect the egg contents, eggs were surface sterilized by immersion in 75% alcohol for 2 min, air dried in a sterile chamber for 10 min then cracked with a sterile knife. Each egg's content was mixed thoroughly and 1 ml of the mixed egg content was inoculated into 9 ml of peptone broth and incubated at 37°C for 16-18 h.

Conventional microbiology detection: After pre-enrichment, 1 ml of enriched cultures of all sample types were transferred to 9 ml of RVS and incubated at 42°C for 18-24 h. At the end of selective enrichment, the broths were plated onto XLD agar and incubated at 37°C for 24 h in order to isolate the suspected colonies. *S. typhimurium* was used as positive control. For *E. coli* detection, enriched cultures of all samples were plated onto sorbitol Mac-conkey agar and incubated at 37°C for 16-18 h to detect sorbitol positive colonies.

DNA preparation and PCR assay: Template DNA was prepared from the naturally contaminated egg product enriched by incubation for 16 to 18 h in peptone broth. After incubation, 1 ml of the pre-enrichment media was centrifuged for 2 min at 13,000 g. The bacterial cells were dissolved in 50 µl of H₂O and heated for 10 min at 100°C. The bacterial cells were centrifuged for 2 min at 13,000g. *Salmonella* specific *invA* gene sequences of *Salmonella*, 139 (forward primer: 5'-GTGAAATTATCGCCACGTTCCGGGCAA-3') and 141 (reverse primer: 5'-TCATCGCACCGTCAAAGGAACC-3') (Rahn *et al.*, 1992) were used as primers in this study. PCR was performed in a final volume of 25 µl containing 25 mM MgCl₂ 10 mM of dNTPs, 1.5 U of Taq DNA polymerase, 10 pmol of each primer and 1 µl of DNA template. The mixture was subjected to 30 cycles of amplification in a thermal cycler. The first cycle was preceded by denaturation for 2 min at 95°C. Each cycle consisted of denaturation for 30 s at 95°C, annealing for 30s at 64°C and elongation for 30s at 72°C. The last cycle was followed by a final elongation for 5 min at 72°C. The uidA gene primer; PT-2 (forward primer: 5'-GCGAAAACCTGTGGAATTGGG-3') PT-3 (reverse primer:

5'-TGATGCTCCATAACTTCCTG-3') (Cebula *et al.*, 1995) were used for *E. coli* detection. The mixture was subjected to 30 cycles of amplification in a thermal cycler. The first cycle was preceded by denaturation for 2 min at 95°C. Each cycle consisted of denaturation for 30 s at 95°C, annealing for 30 s at 58°C and elongation for 30 s at 72°C. The last cycle was followed by a final elongation for 5 min at 72°C. The PCR products were analyzed by a 1.2% (w/v) agarose gel electrophoresis.

RESULTS AND DISCUSSION

A total of 20 egg samples from the open house system and 19 samples from the evaporative cooling system were tested by PCR assay and conventional methods to detect the prevalence of *Salmonella* and *E. coli*. When samples were enriched in peptone broth at 37°C for 16 to 18 h, the enriched samples were subjected to selective media (RVS broth and XLD agar) for *Salmonella* detection. The results in Table 1 show that none of the microbiological methods detected any positive samples of *Salmonella* both from the open house system and the evaporative cooling system. To confirm the prevalence of *Salmonella* and *E. coli*, enriched culture was subjected to extract DNA by heat lysis for PCR detection. Because *Salmonella* and *E. coli* detection in various samples contained high numbers of microflora or contaminant microorganisms, pre-enrichment of bacterial material was carried out prior to the PCR assay, especially using the heat lysis method to prepare DNA to reduce the growth of competitive bacteria and rendering a less labor-intensive procedure for the *Salmonella* and *E. coli* DNA extraction. Most research attempts to establish a method, which can reduce the time required for *Salmonella* identification procedures from various samples (Guo *et al.*, 1999; Ferretti *et al.*, 2001; Schneder *et al.*, 2002; Salehi *et al.*, 2005). Hence, the ability of *Salmonella* specific primers to detect *Salmonella* species rapidly and accurately is enhanced by selecting the *invA* gene of *S. typhimurium* (Rahn *et al.*, 1992; Bulte and Jakob, 1995; Wang *et al.*, 1995). The *invA* gene codes for protein in the inner membrane of bacteria, necessary for invasion to epithelial cells (Darwin and Miller, 1999). The PCR based methods with genus-specific primers of the *invA* gene (284 bp) are selected according to its speed, specificity and sensitivity because no amplified DNA fragments were obtained from non-*Salmonella* species, a reliable technique for *Salmonella* identification. In this study, part of the *invA* gene was amplified (Fig. 1) and detected 2 (5%) samples of *Salmonella* only in egg shells from the evaporative cooling system (Table 1). Thus, the PCR assay and the conventional microbiological method showed a different level of sensitivity to detect *Salmonella* sp.

Table 1: Detection of *Salmonella* and *E. coli* strain by PCR and conventional microbiology technique

Sample	No. of samples	Conv. method	PCR positive results by PCR
Open house system			
<i>Salmonella</i> sp.			
Egg-shell	20	-	-
Egg content	20	-	-
<i>E. coli</i>			
Egg-shell	20	7	13
Egg content	20	-	1
Evaporative cooling system			
<i>Salmonella</i> sp.			
Egg-shell	19	-	2
Egg content	19	-	-
<i>E. coli</i>			
Egg-shell	19	11	15
Egg content	19	1	1

Conv. = Conventional

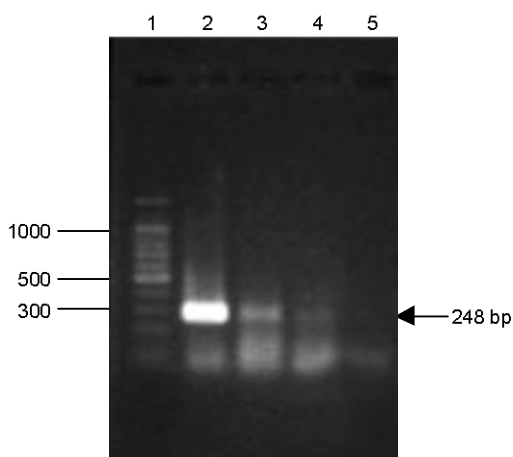


Fig. 1: Representative PCR amplification of the *InvA* gene on 1.2% agarose gel electrophoresis. The expected size for this gene is 284 bp. Lane 1: 100 bp marker. Lane 2: Positive control *S. typhimurium*. Lane 3-4: *Salmonella* sp. isolated from egg shell from the evaporative cooling system. Lane 5: Negative control

For *E. coli* detection, many PCR assays have been developed using primers that target genes specific for *E. coli* O157:H7 (Gannon *et al.*, 1993; Paton and Paton, 1998; Desmarchelier *et al.*, 1998; Cebula *et al.*, 1995). The *uidA* gene (Cebula *et al.*, 1995) which encodes for β -glucuronidase in *E. coli* O157: H7 at 252 bp, was used in this study. Nonspecific bands with *E. coli* ATCC 25922 of *uidA* gene (252 bp) showed specific products (Fig. 2) against 15 samples from egg shell and 1 sample from egg content (total 16 samples, 42%) in the evaporative cooling system (Table 1). The PCR technique also detected *E. coli* from 13 samples of egg shell and 1 sample of egg content (total 14 samples, 35%) from the open house system. Interestingly, two samples detected

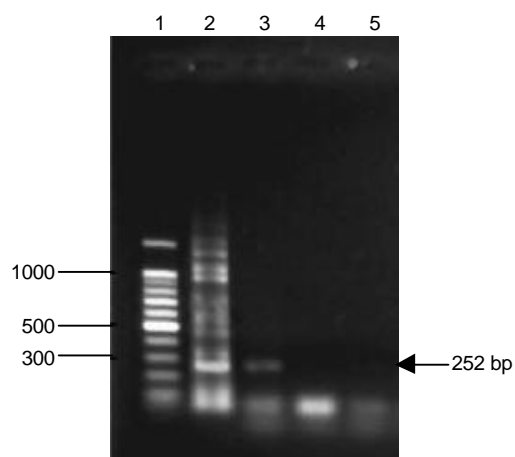


Fig. 2: Representative PCR amplification of the *uidA* gene on 1.2% agarose gel electrophoresis. The expected size for this gene is 254 bp. Lane 1: 100 bp marker. Lane 2: Positive control *E. coli* ATCC 25922. Lane 3-4: *E. coli* isolated from egg shell from the evaporative cooling system. Lane 5: Negative control

E. coli in egg content, indicating exogenic contamination by penetration of the shell by bacteria deposited on the surface of the egg after it has been laid (Haines, 1938; Harry, 1963; Schoeni *et al.*, 1995; De Reu *et al.*, 2006a). By conventional microbiological method, Mac-conkey agar was used to isolate *E. coli* from egg samples. The samples tested positive for *E. coli* 7 (17.5%) of samples of egg shell from the open house system. The egg samples from the evaporative cooling system tested positive for *E. coli* from 11 samples from egg shell and also isolated 1 sample from egg content (total 12 samples, 31.5%) (Table 1). Thus, *E. coli* isolates was recovered less than the PCR-based method.

PCR is a sensitive and specific method with a superior ability to detect *Salmonella* (Mahon *et al.*, 1994; Santos *et al.*, 2001; Schrank *et al.*, 2001; Tournon *et al.*, 2005; Rozila *et al.*, 2007; Robles *et al.*, 2009; Bonetta *et al.*, 2010) and *E. coli* (Holland *et al.*, 2000; Jothikumar and Griffiths, 2002; Oberst *et al.*, 2003; Heijnen and Medema, 2009) in the presence of other competing bacteria from a wide variety of samples. Moreover, the method was also much quicker than conventional techniques taking less than 24 h to obtain a result as opposed to 4 to 5 d. Thus, the PCR assay targeting the *invA* and *uidA* genes can potentially be used to detect *Salmonella* and *E. coli*, respectively in egg samples. In addition, when comparing the initial egg shell and egg content contamination between the two housing systems for laying hens, only samples from the evaporative cooling system tested positive for *Salmonella* sp.. In *E. coli* detection, a higher number of *E. coli* isolates were also detected from the evaporative cooling system. This may have resulted because the evaporative cooling system

is more humid than the open house system providing more suitable conditions for bacterial growth. A study of the influence of temperature and atmospheric humidity on bacterial eggshell contamination found that the Gram-negative bacteria on the egg shell decreased significantly in the room environment but not in the evaporative cooling environment (De Reu *et al.*, 2006b). This was probably due to the lower humidity at room temperature. Therefore, the contamination by pathogenic bacteria could be found more frequently on egg samples in the evaporative cooling system than in the open house system.

ACKNOWLEDGEMENTS

We are most grateful to the Animal Research Farm, Kasetsart University, Bangkok for the egg samples. This work was supported by the Department of Animal Science, Faculty of Agriculture, Kasetsart University, Bangkok. The Integrative Research Center for Animal Science, Aquaculture and Animal Health, ASA-AH, KU-Research University, is also recognized for providing laboratory facilities.

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