ISOLATION OF *SALMONELLA* SPP. FROM FAECAL SAMPLES OF CRACKED EGG FED HENS AND POLYMERASE CHAIN REACTION (PCR) CONFIRMATION

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**Summary**


At 56 weeks of age, 30 Hyline (W-377) laying pullets were randomly distributed in individual (1 pullet per cage) 45×45×35 cm cages. Cracked egg was used as protein supplement to the diet. Three groups of 10 Hyline laying pullets each were formed. Three isocaloric and isonitrogenous diets were prepared from different protein sources as followed: ration 1 (control) – soybean meal; ration 2 (experiment 1) – soybean meal + cracked egg (3.25%), ration 3 (experiment 2) – soybean meal + cracked egg (7.50%). A total of three feed and 30 faecal samples, were examined for the presence of *Salmonella* spp. The isolates were identified by both conventional methods and polymerase chain reaction (PCR). *Salmonella* spp. strains were isolated and identified in 2 out of the 3 diets (ration 3 – soybean meal + cracked egg 7.50% and ration 2 – soybean meal + cracked egg 3.25%), as well as in the faeces of 5 hens fed on ration 3 and 3 hens fed on ration 2. This study showed an increased prevalence of *Salmonella* spp. in the faeces of hens fed diets supplemented with different amounts of cracked egg.

**Key words:** cracked egg, culture, laying hens, *Salmonella* spp., PCR

**INTRODUCTION**

Feed is an important component in a pre-harvest *Salmonella* control programme. In particular, the type of feed appears to be strongly associated to the presence of *Salmonella* spp. (Farzan et al., 2006). It is also known that the physical properties of feed can influence pH, microbial populations, and volatile fatty acids in the digestive tract of broilers (Engberg et al., 2002).

Eggs and egg products are considered to be the major sources of confirmed salmonellosis (Lepoutre et al., 1994). Infected ovaries and oviducts of the hen are the major sources of contamination (Abdel Karem & Matter, 2001). Eggs can become contaminated also on the surface, either from faeces or the environment. In addition to *Salmonella* spp., there are also other pathogens, as *Listeria monocytogenes*, which can occur on egg shells and survive in egg wash water and may demonstrate higher thermal resistance than *Salmonella* spp. (Bartlett et al., 1993). Such surface microflora may contaminate broken-out liquid egg in both processing and commercial food preparation settings (Abdel Karem & Matter, 2001).
Cracked eggs are those that could not be sold at the market because of being damaged by the end of laying or during transportation. Ideally, all cracked eggs should be broken and pasteurized, but this is impractical (Todd, 1996). Hen eggs have become a principal source of Salmonella enteritidis, since this serotype can colonize the ovarian tissue of hens and thus be present within the contents of intact shell eggs (Humphrey, 1994). In recent years, S. enteritidis was found inside a small number of eggs. However, cracks on egg shell surfaces could increase the chances of penetration of the organism within the egg (Edema & Atayese, 2006).

As Salmonella spp. may survive in feed for as long as 16 months at 25 °C (Williams & Benson, 1978), feed may serve as a reservoir that may contaminate incoming flocks. Between 1 and 5% of all animal feed produced and 31% of animal by-products may be contaminated with Salmonella spp. (Allred et al., 1967). Therefore accurate, sensitive and rapid methods of Salmonella spp. detection must include a method to determine Salmonella spp. contamination in feed samples (Maciorowski et al., 2000).

Current conventional methods require 96 h for the detection and biochemical confirmation of Salmonella spp. in animal feed (Andrews et al., 1998). A more rapid method of detection or screening would reduce storage and treatment costs. Quality control technicians may potentially use the polymerase chain reaction (PCR) to rapidly screen feed samples (Maciorowski et al., 2000).

This study was planned to detect Salmonella spp. in feed with and without cracked egg supplements and in faecal samples of hens fed these feed by means of culture and PCR methods.

MATERIALS AND METHODS

Experimental animals and feed samples

One control and two experimental (1 and 2) groups were formed to determine the laying performance and digestibility of 30 White Hyline hens fed with diets containing 16% crude protein (CP). The trial lasted two months. Soybean meal was given to the control group as protein source. Three isocaloric and isonitrogenous diets were prepared from soybean meal and cracked egg supplement as followed: ration 1 (control group) – soybean meal, ration 2 (experimental group 1) – soybean meal + cracked egg 3.25% and ration 3 (experimental group 2) – soybean meal + cracked egg 7.50%. Protein contents of diets were analyzed one week after their preparation. The metabolic energy, Ca and P contents of diets were calculated. Prepared diets were stored in a cool environment. Crude protein of diets was determined according to AOAC (1995).

In the present study, cracked eggs which stayed outside market and were not consumed by humans were used. Each treatment group was randomly assigned to one of the three diets with composition given in Table 1. Feed and water were provided ad libitum throughout the 2-month period. On day 53 of the experiment, chickens of each group (10 birds in each) were randomly placed in individual metabolic cages (20×40 cm) that enabled the excreta collection. Excreta samples of each chicken were collected for 7 days, dried immediately at 40 °C for 24 h. All 30 dried excreta samples were finally mixed and were tested for occurrence of Salmonella spp. Samples from the three feeds were also tested for salmonellae. Samples were paralellly examined.
Bacteriological analysis

*Salmonella* organisms were isolated according to standard methods (ISO 6579, 1993). Samples of 25 g feed and 10 g faeces were placed in a stomacher bag containing 225 mL and 90 mL, respectively, of preenrichment medium-buffered peptone water (BPW, Oxoid, Basingstoke, UK), treated in a Stomacher (Interscience, 78860 St Nom, France) for 2 min and incubated for 18−20 h at 37°C. The preenriched cultures, 0.1 mL and 1 mL, respectively, were then transferred to Rappaport-Vassiliadis broth (Oxoid) and selenite broth (Difco Laboratories, Detroit, MI) and incubated at 42 °C and 37 °C, respectively. After 24 and 48 h of incubation, one loopful from each of enriched broths was streaked onto plates of Salmonella Shigella (SS) agar (Difco) and xylose lysine deoxycholate (XLD) agar (Difco) and incubated at 37 °C for 24 h. The plates were examined for the presence of typical colonies of *Salmonella* spp., i.e. transparent colonies with black centres on SS agar and red colonies with black centres on XLD agar (Antunes *et al.*, 2003). Suspected colonies were confirmed by conventional biochemical methods (Lautrop *et al.*, 1979; Nissen, 1984).

DNA extraction

A $10^8$ CFU/ml of suspicious *Salmonella* spp. culture growth on selective agar was transferred into an Eppendorf tube containing 300 µL sterile distilled water. Bacterial suspension was mixed thoroughly by vortexing and incubated at 56 °C for 30 min. Then the samples were treated with 300 µL of TNES buffer (20 mM Tris pH 8.0 + 150 mM NaCl + 10 mM EDTA + 0.2 % SDS) and 200 µg/mL proteinase K. Following 30 min of boiling, the same amount of phenol (saturated with Tris-HCl) was added to the suspension. The suspension was hand-shaken vigorously for 5 min and centrifuged at 11600×g for 10 min. The upper phase was carefully transferred into a new Eppendorf tube, 0.1 volume 3 M sodium acetate and 2.5 volumes absolute ethanol were added to the

<table>
<thead>
<tr>
<th>Ingredients (%)</th>
<th>Ration 1 (Control)</th>
<th>Ration 2 (Soybean meal + 3.25 % cracked egg)</th>
<th>Ration 3 (Soybean meal + 7.50 % cracked egg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>60.50</td>
<td>62.60</td>
<td>62.50</td>
</tr>
<tr>
<td>Soybean meal, 44%</td>
<td>24.70</td>
<td>20.35</td>
<td>17.10</td>
</tr>
<tr>
<td>Vegetable oil</td>
<td>2.00</td>
<td>1.00</td>
<td>–</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>10.00</td>
<td>10.00</td>
<td>9.50</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>2.10</td>
<td>2.10</td>
<td>2.70</td>
</tr>
<tr>
<td>Salt (NaCl)</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Vitamin</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Mineral</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Cracked egg, 45.1%</td>
<td>–</td>
<td>3.25</td>
<td>7.50</td>
</tr>
<tr>
<td>Metabolisable energy (kcal/kg)</td>
<td>2710</td>
<td>2740</td>
<td>2750</td>
</tr>
<tr>
<td>Crude protein, %</td>
<td>16.00</td>
<td>15.92</td>
<td>16.15</td>
</tr>
<tr>
<td>Calcium, %</td>
<td>3.40</td>
<td>3.50</td>
<td>3.55</td>
</tr>
<tr>
<td>Phosphorus, %</td>
<td>0.70</td>
<td>0.68</td>
<td>0.72</td>
</tr>
</tbody>
</table>

Table 1. Composition (%) of experimental diets
Isolation of Salmonella spp. from faecal samples of cracked egg fed hens and polymerase chain ...

... suspension, and was left at –20°C overnight. After the precipitation stage, the suspension was centrifuged at 11600×g for 10 min and the upper phase was discharged. The pellet, obtained after centrifugation was washed twice with 95% and 70% ethanol respectively, each step followed by 5 min centrifugation. Finally, the pellet was dried, resuspended in 50 µL of sterile distilled water, and stored at –20 °C until further use.

A reference S. enteritidis strain (ATCC 4931) (kindly provided by Dr. A. A. Mohamed Hatha, Department of Biology, The University of the South Pacific, Private Mail Bag, Suva, Fiji) was used in PCR tests as positive control and distilled water was used as the negative control.

Primers

The primers used were: 16SF1 (5’-TGTGTGGTATAAACCGCA-3’) and 16SIII (5’-CACAAATCCATCTCTGGA-3’) (Promega) derived from 16S rRNA gene (Lin & Tsen, 1996).

PCR

The reaction mixture was prepared in a total volume of 50 µL containing 5 µL of 10x PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100), 5 µL of 25 mM MgCl₂, 250 µM of each deoxynucleoside triphosphate, 2 U of Taq DNA Polymerase (Fermentas, Lithuania), 10 pg of each primer and 5 µL samples of extracted bacterial DNA. PCR involved 35 cycles of denaturation (94 °C, 1 min), primer annealing (58 °C, 1 min) and primer extension (72 °C, 1 s). The primer extension step (72 °C, 10 min) followed the final amplification cycle (Fluit et al., 1993). For all experiments, a Touchdown Thermocycler (Hybaid, Middlesex, England) was used. PCR reaction products (15 µl) were analysed by electrophoretic separation on 1.5% agarose gels stained with ethidium bromide. The gel was visualized by UV illumination and photographed with Polaroid films.

Statistical analysis

Yates-corrected X² tests (Yates, 1982) were used to detect differences between proportions of Salmonella spp. isolated from feed samples. These tests were carried out using Epi info version 6 (Dean et al., 1994).

RESULTS

Culture Results

Salmonella spp. were isolated and identified in 2 out of the 3 rations: ration 3 – soybean meal + cracked egg 7.50% and ration 2 – soybean meal + cracked egg 3.25%. Salmonella organisms were also isolated and identified in the faeces from 5 hens fed on ration 3 and 3 hens fed on ration 2. Salmonellae were not isolated from feed (soybean meal) and from the faeces of control hens (Table 2).

The difference between the prevalence of Salmonella spp. isolated from the control group and groups given soybean meal + cracked egg 7.50% and soybean meal + cracked egg 3.25% was statistically significant (P = 0.04). No significant difference was detected in the prevalence of Salmonella spp. isolated from soybean meal + cracked egg 7.50% and soybean meal + cracked egg 3.25%.

PCR Results

In this study, PCR amplification of genomic bacterial DNA using 16SF1 and 16SIII primer pairs gave bands of 572 bp, corresponding to the expected size of amplified genomic Salmonella spp. DNA (Fig. 1). All Salmonella spp. isolates that
Table 2. Culture results of *Salmonella* spp. from feed and faeces of chickens fed on different rations

<table>
<thead>
<tr>
<th>Rations</th>
<th>Samples</th>
<th>Feed samples</th>
<th>Faecal samples*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ration 1 (control: not supplemented with cracked egg)</td>
<td>Negative</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>Ration 2 (soybean meal + 3.25 % cracked egg)</td>
<td>Positive</td>
<td>3/10</td>
<td></td>
</tr>
<tr>
<td>Ration 3 (soybean meal + 7.50 % cracked egg)</td>
<td>Positive</td>
<td>5/10</td>
<td></td>
</tr>
</tbody>
</table>

* Number of positive/number of tested samples.

**DISCUSSION**

Feed is believed to be an important vector for the transmission of *Salmonella* spp. to poultry (Ha *et al.*, 1998). *Salmonella* spp. has been detected in poultry feed, animal feed or feed ingredients in a number of studies (Williams, 1981; Cox *et al.*, 1983; Stuart, 1984; Veldman *et al.*, 1995). Of the individual feed ingredients, meat and bone meal or animal by-products are reported to have the highest incidence of *Salmonella* spp., with estimates of contamination between 31% and 86% of feeds sampled (Allred *et al.*, 1967; Williams *et al.*, 1969). However, salmonellae have also been detected with lower incidence in plant protein sources including soybean oil meal, and thus, plant protein was also shown as an important source of...
Salmonella spp. transmission (Vanderwal, 1979).

In fact, 77–80% of salmonellosis outbreaks have been associated with grade A shell eggs, or egg-containing foods (Cabo et al., 2004). Salmonellae may occasionally be present on eggshells even after washing, and any Salmonella organisms reaching the membranes can be transferred to an egg mixture through breaking, and will rapidly grow under improper storage conditions (Todd, 1996).

Todd (1996) conducted a risk analysis on cracked eggs and found that they were 3 to 93 times more likely to cause outbreaks of salmonellosis than uncracked shell eggs. Poppe et al. (1998) detected that pools containing eggs that were both cracked and dirty, were more frequently contaminated with Salmonella spp. than all other pools of eggs. It is reported that 2.99%, 1.15%, 2.17%, 1.17%, 3.87%, 1.15% from cracked, whole, dirty, clean, cracked and dirty, whole and clean eggs, respectively, were positive for Salmonella spp. and that the overall Salmonella spp. contamination rate of the table eggs was from 0.07 to 0.4% (Poppe et al., 1998). Jones & Musgrove (2007) showed a low Salmonella spp. prevalence in eggshells (1.1%).

del Cerro et al. (2002) reported that faeces, caecal swabs and eggs from chickens were positive for Salmonella spp. by culture in 39%, 18% and 64.5%, respectively and positive in 39%, 13.6% and 60% by PCR, respectively.

Although numerous studies related to isolation of Salmonella spp. from egg samples in Turkey have been performed (Arda, 1968; Inal & Ozyer, 1992; Var, 1993; Erol, 1994; Arg-Kuçuker et al., 1995; Altay & Yardmci, 2001; Erdoğrul et al., 2002; Cakiroglu & Gümüşsoy, 2005; Ata, 2006), none of them has reported the incidence of Salmonella spp. isolates from cracked eggs. Studies carried out in different parts of Turkey have found no Salmonella spp. in egg samples (Arda, 1968; Inal & Ozyer, 1992; Erol, 1994; Arg-Kuçuker, 1995; Cakiroglu & Gümüşsoy, 2005; Ata, 2006). In a study conducted by Var (1993) to determine the presence of Salmonella spp. in 448 egg samples from chickens, ducks and quails, 3 egg samples were positive for Salmonella paratyphi. Ata (2006) found out that egg samples obtained from 50 layer flocks were negative for Salmonella spp. whereas cloacal swaps obtained from 6 flocks were positive for Salmonella spp. In other countries such as Spain, Canada and the USA, the prevalence has been reported to be considerably higher (del Cerro et al., 2002) or low (Poppe et al., 1998; Jones & Musgrove, 2007).

When evaluating the prevalence of Salmonella spp. in feed samples in ration 2 (soybean meal + cracked egg 3.25%) and ration 3 (soybean meal + cracked egg 7.50%) and the faecal samples from hens given these feeds, a significant increase in the prevalence of recovered Salmonella isolates compared to controls was found out. This may be explained by variations in the proportions of the cracked egg supplement. The difference between the prevalence of Salmonella spp. isolated from the control group and groups given either soybean meal + cracked egg 7.50% or soybean meal + cracked egg 3.25% was statistically significant (P<0.04). No Salmonella spp. were detected in feed and faecal samples on any of control groups.

There were a reduction in the prevalence of Salmonella spp. recovered from the faeces of hens fed on a ration containing 3.25% cracked eggs compared to hens fed on 7.50% cracked eggs. No significant difference was detected in the numbers of
Salmonella spp. isolated from groups fed on soybean meal + cracked egg 7.50% or soybean meal + cracked egg 3.25%.

Traditional microbiological techniques such as the ISO 6579 for detecting this pathogen in food require up to 5 days to obtain a result, including the pre-enrichment and the selective enrichment in liquid culture and the biochemical and serological confirmation of colonies grown on agar plates (Anonymous, 2002). The PCR method is a useful tool to overcome these time-consuming procedures (Arnold et al., 2004).

The primers 16SF1 and 16SIII were proved to be specific for the PCR detection of all Salmonella isolates with various serogroups (Lin & Tsen, 1996). For these reasons, we used the primers 16SF1 and 16SIII derived from the 16S rRNA gene and found that all Salmonella spp. isolates identified by conventional tests gave positive bands with PCR.

Maciorowski et al. (2000) found that indigenous Salmonella spp. were detected in five (63%) of eight samples of poultry diets by conventional methods and that with commercial PCR, Salmonella spp. could not be detected in any of the samples after only 7 h of enrichment but could be detected in 2 dietary samples after 13 h of enrichment and 4 dietary samples after 24 h of enrichment. Löfström et al. (2004) developed PCR procedure for routine analysis of viable Salmonella spp. in 14 different feed samples and 8% of the samples were positive by PCR, compared with 3% with the conventional method.

In a study carried out in Turkey, Altay et al. (2002) investigated Salmonella spp. presence in 75 feed samples collected from different poultry farms and feed plant in Bolu, Ankara, İzmit and Afyon region and found 2 (2.67%) of them positive for salmonellae.

Our results are in contradiction with those of Altay et al. (2002) and Löfström et al. (2004) who found low prevalence of Salmonella spp. from feed samples but in agreement with Maciorowski et al. (2000) who found high prevalence of Salmonella spp. from feed samples.

This study reported the isolation of Salmonella spp. from faeces of hens fed diets with added cracked egg for the first time in Turkey and emphasized that cracked egg containing feeds were the most important vehicles for chicken salmonellosis. The occurrence of Salmonella spp. in both feed and faecal samples suggested that feed could be a possible source of salmonellosis in hens. However, further studies are needed to clarify the potential pathogenic role of Salmonella spp. in feeds.

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