ISOLATION OF *LISTERIA* SPP. FROM FAECAL SAMPLES OF CRACKED EGG FED CHICKENS AND RAPD ANALYSIS OF *LISTERIA MONOCYTOGENES* STRAINS

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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 x 45 x 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 obtained and examined for the presence of *Listeria* spp. Samples were enriched using *Listeria* Enrichment Broth and inoculated onto *Listeria* Selective Agar. The isolates were identified by both conventional methods and polymerase chain reaction (PCR). A 701 bp fragment of listeriolysin O sequence for *Listeria monocytogenes* was amplified using specific primers by PCR for confirmation of the identification. In ration 3 (soybean meal + cracked egg 7.50%) and ration 2 (soybean meal + cracked egg 3.25%), *L. monocytogenes* and *Listeria innocua*, were respectively isolated by culture. In the faeces from hens fed ration 3, *L. monocytogenes* strains that were positive by culture were also detected to be positive by the PCR. Random amplified polymorphic DNA (RAPD) were used to detect the genetic variability among *Listeria monocytogenes* isolates. All *L. monocytogenes* isolates were typed by RAPD using a random primer (OPA-11). In the molecular epidemiological analysis using RAPD assay, the isolates produced three different band profiles using OPA-11. The results of this study demonstrated that multiple genotypes of *L. monocytogenes* isolates existed in faeces of hens fed rations supplemented with cracked eggs.

**Key words:** cracked egg, laying hens, *Listeria* spp, PCR, RAPD

INTRODUCTION

Egg has a crude protein content (45.1%) similar to that of soybean meal (44.0%). Common sources of terrestrial animal protein are by-products such as meat and bone meal and poultry by-product meal, which generally contain 45–65% crude protein and are a good source of indispensable amino acids. However, the quality of these meals depends on both the quality of raw ingredients and the type of processing. A promising alternative to the independent use of plant or animal protein is
the utilization of a mixture of complementary ingredients in order to increase nutrient utilization and facilitate processing. Co-extruded soybean poultry by-product meal with egg supplement is one such mixture that has potential for use in animal diets (Tatli Seven et al., 2005). Co-extruded soybean poultry by-product meal with egg supplement was used as a substitute for fish meal in practical shrimp diets (Samocha et al., 2004).

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).

Current microbiological culture procedures for the detection of Listeria are labourious, cumbersome and time consuming (Bansal, 1996). The polymerase chain reaction (PCR) is an attractive alternative tool for detection of L. monocytogenes since it is specific, highly sensitive and eliminates the need for enrichment culturing (Wernars et al., 1991).

Several typing methods have been used to group L. monocytogenes isolates. However, most of these methods are not well suited for routine use in laboratories and are time-consuming (Farber & Addison, 1994). The usefulness of random amplified polymorphic DNA (RAPD) analysis for L. monocytogenes typing has been confirmed in several studies (Lawrence et al., 1993; MacGowan et al., 1993; Farber & Addison, 1994; Niederhauser et al., 1994; Boerlin et al., 1995; O’Donoghue et al., 1995; Cao et al., 2006). This technique is advantageous since no DNA sequence information is needed (Farber & Addison, 1994).

The aims of the present study were (1) to determine the prevalence of Listeria spp. in feed with and without cracked egg supplements and in faecal samples of hens fed these feeds by means of culture and PCR methods and (2) to investigate the genetic variability among L. monocytogenes isolates by RAPD using a random primer (OPA-11).

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 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. Their metabolic energy, Ca and P contents 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 (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 at 40 °C for 24 h.
All 30 dried excreta and 3 feed samples were tested for occurrence of *Listeria* spp. Samples were paralellly examined.

**Listeria isolation**

For *Listeria* isolation, samples of about 25 g of feed and 10 g faeces were transferred into 225 mL and 90 mL, respectively of *Listeria* Enrichment Broth (LEB, Oxoid). After 24 h incubation at 37 °C (primary enrichment), an aliquot of 0.1 mL of the culture was transferred into tubes containing 10 mL LEB. The tubes were incubated for 24–48 h at 37 °C (second enrichment). A loopful of each enrichment culture was streaked onto *Listeria* Selective Agar (LSA, Oxford Formulation) with *Listeria*-selective supplement and incubated at 37 °C for 24 h. The suspected colonies with dark brown color or black halo were transferred onto tryptic soya agar (Difco) and incubated overnight at 37 °C. The isolates were identified using conventional methods: Gram staining, synergistic lysis of erythrocytes (CAMP reaction), typical umbrella motility, and fermentation of mannitol, rhamnose and xylose (Seeliger et al., 1986).

**DNA extraction**

A $10^8$ CFU/ml of suspicious *Listeria* 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. Following this, 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 step, the same amount of phenol (saturated with Tris-HCl) was added to the suspension. The suspension was shaken vigorously by hand for 5 min and centrifuged at 11600×g for 10 min. The upper phase was carefully transferred into a new Eppendorf tube, and 0.1 volume 3 M sodium acetate and 2.5 volume absolute ethanol were added to the suspension, and was left at −20 °C overnight. After the precipitation stage, the suspension was then centri-
Isolation of Listeria spp. from faecal samples of cracked egg fed chickens and RAPD analysis of...

Fuged at 11600×g for 10 min and the upper phase was discarded. The pellet, obtained following the centrifugation at high speed for 10 min, 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.

All purifications and PCR reactions used L. monocytogenes as positive controls and distilled water as negative control.

**Primers**

Primers used in this study were designed by Border et al. (1990). The sequences of primer pairs were as followed: LM1 (5’-CCT AAG ACG CCA ATC GAA - 3’) and LM2 (5’-AAG CGC TTG CAA CTG CTC - 3’). These primers amplify a 701 bp fragment on listeriolsin O sequence of L. monocytogenes.

**PCR**

The PCR was performed in a Touchdown Thermocycler (Hybaid, Middlesex, England) in a total volume of 50 µL, containing 5 µL of template sample DNA, 10× PCR buffer (10 mM Tris-HCl, pH 9.0; 50 mM KCl, 0.1% Triton X-100), 25 mM MgCl₂, 1 µM forward and reverse primers (LM1 and LM2), 250 µM of each deoxynucleoside triphosphate, Taq DNA Polymerase (1.25 U; 5 U/mL, Fermentas, Lithuania). Amplification was obtained with an initial denaturation step at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1 min. The final cycle was at 72 °C for 10 min (Paziak-Domaniska et al., 1999). The PCR products were detected by electrophoresis in 1.5% agarose in 1× Tris-borate-EDTA (TBE) buffer. Gels were stained with ethidium bromide (0.5 µg/mL). The DNA fragments were visualized by UV illumination and photographed with Polaroid film. The molecular sizes of the PCR products were compared with a 100 bp DNA ladder.

**RAPD analysis**

The RAPD reaction mixture was prepared in a total volume of 25 µL consisting of 2.5 µL 10× PCR buffer (750 mM Tris-HCl, 200 mM (NH₄)₂SO₄, 0.1% Tween 20), 3.5 mM MgCl₂, 200 µM deoxynucleoside triphosphates, 1.25 U of Taq DNA polymerase (Fermentas, Lithuania), 1 µM of OPA-11 primer (5’-CA AT CG CC GT-3’), 11 µL of sterile distilled water and 2.5 µL of template DNA. Each sample was amplified through 45 cycles of denaturation (1 min at 95 °C), primer annealing (2 min at 35 °C), and extension (1 min at 72 °C). A last cycle of extension was applied at 72 °C for 10 min (Vogel et al., 2001). Twenty microlitres of the product were resolved by electrophoresis on a 2% agarose gel in TBE buffer and visualised by staining with ethidium bromide for 30 min. Gels were photographed by Polaroid Gel Cam and images were scanned. A 1 kb DNA ladder (Promega, Maddison, USA) was used as a molecular size standard.

**Statistical analysis**

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

**RESULTS**

**Culture results**

L. monocytogenes was isolated from ration 3 (soybean meal + cracked egg
but not from faeces of hens from the control group (soybean meal) and those fed ration 2 (soybean meal + cracked egg (3.25%). In addition, faecal samples of hens fed on ration 3 (supplemented with cracked egg 7.50%) were positive for *L. monocytogenes* (5 hens) and *L. welshimeri* (1 hen). Faeces of four hens given soybean meal + cracked egg 3.25% and samples from this ration were positive for *L. innocua*. Also, the faeces of one hen fed soybean meal + cracked egg 3.25% were positive for *L. welshimeri* (Table 2).

The differences between the prevalence of *L. monocytogenes* strains isolated from the control group and groups given either soybean meal + cracked egg 7.50% or soybean meal + cracked egg 3.25% were statistically significant (*P*=0.002). No significant difference was detected in the prevalence of *L. monocytogenes* isolates from rations 2 and 3.

The prevalence of *L. innocua* strains isolated from the control group and groups given soybean meal supplemented with cracked egg at 7.50% and 3.25% differed significantly (*P*=0.01). However, there was no significant association between the numbers of *L. innocua* isolated from rations 2 and 3.

No significant difference was detected in the numbers of *L. welshimeri* isolates between groups given soybean meal + cracked egg 7.50% or 3.25% compared to the control group.

**PCR results**

The biochemical test identification results were confirmed by PCR based upon the use of specific primer pairs derived from listeriolysin O sequence of *L. monocytogenes*. A 701 bp fragment was amplified from all tested *L. monocytogenes* strains identified by conventional methods (Fig. 1).

**RAPD results**

The results of RAPD typing are showed in Fig. 2. In the RAPD analysis using OPA-11 primer of 6 *L. monocytogenes* isolates, three distinct band profiles were obtained. In only two faecal samples, similar band profile with feed was obtained.

**DISCUSSION**

In the present investigation we have characterized *Listeria* spp. isolated from feed containing cracked egg and faecal samples from hens given these feed and have investigated the genetic heterogeneity of *L. monocytogenes*.

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**Table 2.** Culture results of *Listeria* spp. from feed and faeces of chickens fed on different rations

<table>
<thead>
<tr>
<th>Feed samples</th>
<th><em>L. monocytogenes</em></th>
<th><em>L. innocua</em></th>
<th><em>L. welshimeri</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ration 1 (control: without cracked egg)</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Ration 2 (soybean meal + 3.25 % cracked egg)</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Ration 3 (soybean meal + 7.50 % cracked egg)</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Faecal samples*</th>
<th>No. Positive/No. Tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ration 1 (control: without cracked egg)</td>
<td>0/10</td>
</tr>
<tr>
<td>Ration 2 (soybean meal + 3.25 % cracked egg)</td>
<td>0/10</td>
</tr>
<tr>
<td>Ration 3 (soybean meal + 7.50 % cracked egg)</td>
<td>5/10</td>
</tr>
</tbody>
</table>

* Number of positive/number of tested samples
Isolation of Listeria spp. from faecal samples of cracked egg fed chickens and RAPD analysis of ...
nearly 26% of chilled carcasses. Jones & Musgrove (2007) suggested that L. innocua can better withstand the shell egg washing environment than other Listeria species and that 21% of tested samples was positive for Listeria (33 shells and 5 contents). Of the Listeria isolates recovered (38 total), the following identifications were made: Listeria grayi (2.6%), L. welshimeri (13.2%), and L. innocua (84.2%). L. innocua was the only isolate found in the positive egg contents pools. L. monocytogenes has been reported to survive in frozen liquid egg products and powdered egg products for up to 180 days (Brackett & Beuchat, 1992). Such finding raise concern regarding the safety of commercial egg products (Erdoğrul, 2004).

Skovgaard & Morgen (1988) showed that approximately 82% of the feed samples harbourd Listeria spp. and 62% L. monocytogenes, and 67% harboured Listeria spp. (51% L. monocytogenes) in faecal samples. In Germany, a rate of 8% L. monocytogenes in faecal samples from chickens has been reported (Weber et al., 1995). In a study carried out in Egypt, a prevalence of 8.3% and 13.3% L. monocytogenes in egg shells and faecal samples from chickens respectively, has been reported (Hussein, 1997). Tastan (1995) revealed that faecal samples from chickens were positive for Listeria spp., of which 8%, 6% and 2% were identified as L. monocytogenes, L. welshimeri, and Listeria murayi, respectively. Arslan & Muz (2001) reported that 4% L. welshimeri, 4% L. innocua and no L. monocytogenes were isolated from 50 feed specimens. Kalender (2003) found that 11.65% of faecal samples from chickens were positive for Listeria spp., of which 4.36%, 4.85%, 0.97%, 0.97% and 0.48% were identified as L. monocytogenes, L. innocua, L. welshimeri, Listeria seeligeri and L. murayi, respectively. Our results are contradictory to those of Cox et al. (1997), who observed a very low incidence of L. monocytogenes on whole incoming birds (one of 115) and no L. monocytogenes in 115 caecal samples from two slaughterhouses. The presence of Listeria spp. in faeces was associated with the prevalence of these bacteria in feed (Bunić, 1991; Sanaa et al., 1993). In this study, the majority of Listeria spp. isolated were L. monocytogenes and L. innocua. L. monocytogenes and L. innocua are probably present in larger numbers in feed than other Listeria spp, and therefore had higher frequencies of detection in animal faeces.

A RAPD assay using a random primer (OPA-11) was also employed in this study. All the strains were succesfully typed by RAPD method using only OPA-11 primer. Lawrence & Gilmour (1995) identified 7 different types among 93 L. monocytogenes isolates from raw poultry products by RAPD. By RAPD, Byun et al. (2001) classified 54 L. monocytogenes isolates from the beef, pork, chicken and bacon imported from the USA, Denmark, Belgium, China, New Zealand and from domestic meats during 1997−1999 into 10, 6 and 6 types using three random primers (PB1, PB4 and HLWL74, respectively). Our results indicated a large amount of genetic heterogeneity of L. monocytogenes isolated from faeces of hens with added cracked egg (7.50%) in feed. The use of different and more than one RAPD primers may improve differentiation power of the RAPD process. One random primer was used and 3 different profiles were observed in this study. Our results are not consistent with those of Lawrence & Gilmour (1995) and Byun et al. (2001). Although the number of L. monocytogenes isolates in this study was
rather low, the results of RAPD analysis showed a considerable genetic diversity among them.

In summary, this study reported the isolation of Listeria spp. from faeces of hens with added cracked egg in Turkey for the first time and emphasized cracked egg-containing feeds as being the most important vehicles for chicken listeriosis. Listeria spp. were isolated from both faeces of hens and from their feed. The numbers of listerial isolates from the faeces have been suggested to be associated with the prevalence of Listeria spp. in feed supplemented with cracked egg. Moreover, the isolation of identical profile of L. monocytogenes from feed (soybean meal-cracked egg 7.50%) and two faecal samples, suggests that such feed may be possible sources for listeriosis cases in hens. However, further studies are needed to clarify the potential pathogenic role of Listeria in feeds.

REFERENCES


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