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

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Summary

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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* (5 hens) and *Listeria welshimeri* (1 hen) were detected by culture. Birds fed ration 2 exhibited positive culture results for *L. innocua* (4 hens) and *L. welshimeri* (1 hen). All *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 *L. 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 ^{o}C for 24 h.

Table 1. Composition (%) of experimental diets

Ingredients (%)	Ration 1 (Control)	Ration 2 (Soybean meal + 3.25 % cracked egg)	Ration 3 (Soybean meal + 7.50 % cracked egg)
Maize	60.50	62.60	62.50
Soybean meal, 44%	24.70	20.35	17.10
Vegetable oil	2.00	1.00	–
$CaCO_3$	10.00	10.00	9.50
Dicalcium phosphate	2.10	2.10	2.70
Salt (NaCl)	0.20	0.20	0.20
Vitamin	0.20	0.20	0.20
Mineral	0.20	0.20	0.20
DL-methionine	0.10	0.10	0.10
Cracked egg, 45.1%	–	3.25	7.50
Metabolisable energy (kcal/kg)	2710	2740	2750
Crude protein, %	16.00	15.92	16.15
Calcium, %	3.40	3.50	3.55
Phosphorus, %	0.70	0.68	0.72

All 30 dried excreta and 3 feed samples were tested for occurrence of *Listeria* spp. Samples were paralelly 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 ºC. 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 ºC 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-

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 listeriolysin 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 $MgCl_2$, 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-Domańska *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 $(NH_4)_2SO_4$, 0.1% Tween 20), 3.5 mM $MgCl_2$, 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 ºC), primer annealing (2 min at 35 ºC), and extension (1 min at 72 ºC). A last cycle of extension was applied at 72 ºC 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 X^2 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

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

	L. monocytogenes	*L. innocua*	*L. welshimeri*
Feed samples			
Ration 1 (control: without cracked egg)	Negative	Negative	Negative
Ration 2 (soybean meal + 3.25 % cracked egg)	Negative	Positive	Negative
Ration 3 (soybean meal + 7.50 % cracked egg)	Positive	Negative	Negative
*Faecal samples**			
Ration 1 (control: without cracked egg)	0/10	0/10	0/10
Ration 2 (soybean meal + 3.25 % cracked egg)	0/10	4/10	1/10
Ration 3 (soybean meal + 7.50 % cracked egg)	5/10	0/10	1/10

* Number of positive/number of tested samples

7.50%), 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.*

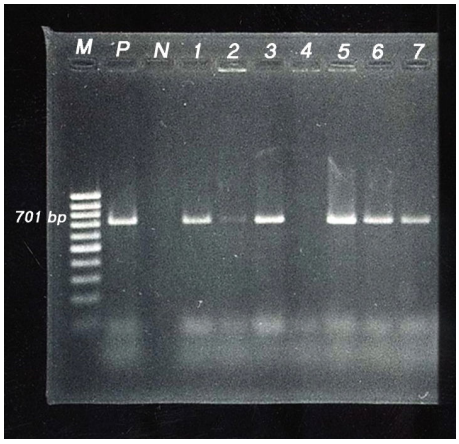

Fig. 1. An agarose gel stained with ethidium bromide, with PCR products of *L. monocytogenes* isolates from feed and faecal samples (M: 100 bp DNA ladder, P: positive control, N: negative control; 2: *L. monocytogenes* from samples of ration 3 (soybean meal + cracked egg 7.50%); 1, 3–6: isolates from faecal samples of hens.

In these trials, the presence of *L. monocytogenes* in feed samples in ration 3 (soybean meal + cracked egg 7.50%) and in faecal samples from hens fed on this ration was in a significant proportion. This may be explained by variations in the proportions of supplemented cracked egg. No *L. monocytogenes* were detected in feed and faecal samples in any of control groups. Difference between the numbers of *L. monocytogenes* strains 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=0.002).

Samples of ration 3, containing soybean meal and cracked egg 7.50% and 5 faecal samples, identified as positive for *L. monocytogenes* from biochemical tests, were confirmed by PCR.

In 1999, Cox and colleagues stated that *L. monocytogenes* had not been detected in whole eggs, but found in broken eggs instead (Cox *et al.,* 1999). Moore & Madden (1993) detected *Listeria* spp. in 72% of in-line shell filtres tested in egg product plants and the genera isolated were *L. innocua* (62%) and *L. monocytogenes* (38%). In another study, approximately 36% of raw liquid whole egg samples from further processing plants were found to be positive for *L. innocua* (Leasor & Foegeding, 1989). *L. innocua* has also been isolated from egg samples and wash water in shell egg processing plants in Canada (Farber *et al.,* 1992). Lawrence & Gilmour (1994) detected that 15% of raw poultry samples tested were positive for *L. monocytogenes*, and Cox *et al.* (1997) recovered *L. monocytogenes* from

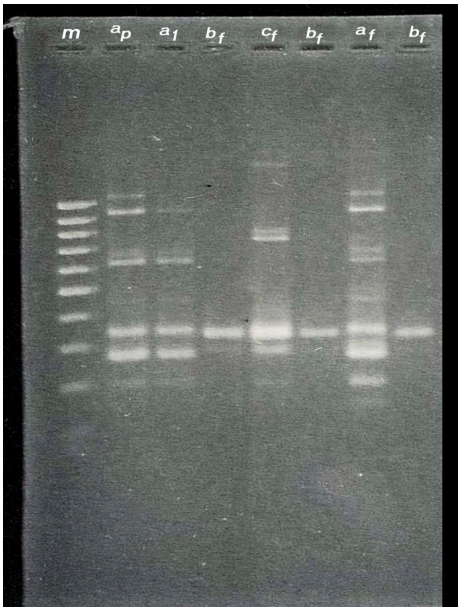

Fig. 2. Random amplified polymorphic DNA (RAPD) analysis of *L. monocytogenes* isolates from feed and faecal samples (m: 100 bp DNA ladder, lanes a_p: profile of positive control, a_1: profile of *L. monocytogenes* isolates from feed; a_f, b_f, c_f: profiles of *L. monocytogenes* isolates from faecal samples).

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 harboured *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 (Buncić, 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.

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