Short communication

Survey of Enterobacteriaceae contamination of table eggs collected from layer flocks in Australia

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A B S T R A C T

In the present study, eggs from commercial caged layer flocks at different stages of lay in Australia were collected. Enterobacteriaceae populations from eggshell surface and eggshell pore were enumerated and these populations characterized using API® Rapid 20E strips. The eggshell surface, eggshell pore and egg internal content samples were also processed for the isolation of Salmonella and these isolates were tested for the presence or absence of several virulence genes (prgH, sopB, spc, orfL, invA, sifA, sitC, misL). Results indicated that there was no significant difference in total Enterobacteriaceae count on the eggs of the flock from early, mid or late lay flocks. Enterobacteriaceae isolates were of 11 different genera which included: Cedecea, Citrobacter, Enterobacter, Escherichia, Klebsiella, Klyvera, Leclercia, Pantoea, Salmonella, Serratia and Yersinia. Out of all 153 identified Enterobacteriaceae isolates, the Escherichia genus was reported most frequently (60.78%). Results also indicated that overall there were 4.51% (14/310) Salmonella positive pooled samples. In this study, 14 Salmonella strains were isolated, serotyping confirmed that 12 out of them were Salmonella Infantis and the 2 others were Salmonella enterica subsp. enterica serovar 4,12: d: Polymerase chain reaction results indicated that all Salmonella Infantis isolates harboured invA, misL, orfL, prgH, sifA, sitC, sopB and spc genes which suggests that Salmonella Infantis strains isolated from eggshell surface may have the capacity to invade and survive in macrophages.

1. Introduction

Food borne illness costs Australia an estimated $1.2 billion per year (Hall et al., 2005). In Australia, Salmonella Typhimurium is the principal cause of egg-associated Salmonellosis outbreaks (OzFoodNet Working Group, 2010). Control of Salmonella shedding on farm still remains a challenge. Cage laying production systems are the major source of whole shell eggs in Australia (Chousalkar and Roberts, 2012). For the shelf life of an egg and from a food safety perspective, it is important to lower the level of bacterial contamination on eggs. Studies on microbial contamination of eggshells have been performed earlier (Musgrove et al., 2004; Musgrove et al., 2005b). It was observed that abnormalities in eggshells (thin shells, increased shell pore numbers) can potentiate the entry of food borne pathogens into the eggs (De Reu et al., 2008). With an increase in number of bacteria present on the eggshell surface, the chances of eggshell penetration and contamination of internal contents by bacteria increases (Smith et al., 2000). There is some evidence suggesting that eggshell transulence increases the incidence of bacterial penetration (Chousalkar et al., 2010). At oviposition, 90% of eggs are germ free (Board, 1966). However, the eggshell can be contaminated by any surface with which egg comes in contact (Board and Tranter, 1995). Faeces, water, caging material, nesting material, insects, hands, broken eggs, dust on egg belt, blood and soil are the most common sources of eggshell contamination (Board and Tranter, 1995; Ricke et al., 2001). Overall, food quality and sanitary processing conditions can be judged by coliforms, Enterobacteriaceae and Escherichia coli populations (Kornacki and Johnson, 2001; Ricke et al., 2001). The annual report of the OzFoodnet network (2010) reported 9533 cases of Salmonella infection in Australia. It is important to study the prevalence of Salmonella on the eggshell surface because contaminated eggshells may play an important role in cross contamination in the kitchen. Complex pathogenesis is a characteristic of Salmonella infections. The virulence capacity of Salmonella is encoded by multiple genes which are clustered together on Salmonella Pathogenicity Islands (SPI) (Hensel, 2004). Using the Type III secretion system, various pathogens deliver effector proteins into the cytosol of host cells (Marcus et al., 2000). SPI 1 and SPI 2 encode distinct type III secretion systems. By delivering effector proteins, SPI 1 helps the Salmonella to penetrate the intestinal epithelium and also it induces apoptosis in macrophages (Lostroh and Lee, 2001). SPI 2 is important in systemic disease as it contains genes which are essential for survival and replication of Salmonella within host macrophages and epithelial cells (Shea et al., 1996). SPI 3 encodes the high affinity Mg2+ uptake system (Marcus et al., 2000).
which is important for survival of *Salmonella* in macrophages, whereas SPI 4 is involved in secretion of toxins \cite{Gassama-Sow et al., 2006}. Hence, it is essential to investigate the presence or absence of virulence genes located on different SPIs. Currently, in Australia, there is limited information on the presence of virulence genes in *Salmonella* strains isolated from eggs. Keeping this perspective in mind, in the present study, visibly clean eggs collected from commercial egg farms from hens at various stages of lay were tested for the presence of *Salmonella* spp. The *Enterobacteriaceae* populations on the eggshell surface and in the eggshell pores were monitored. *Salmonella* isolates from eggs were tested for the presence of a wide range of virulence genes.

2. Materials and methods

2.1. Collection of eggs and preparation of *Salmonella* Infantis inoculums in egg samples

Visually clean eggs (n = 1860, 60 eggs from 31 flocks), collected from the cage fronts of commercial layer farms, were processed for isolation of *Salmonella* spp. from the eggshell surface, eggshell crush and internal contents. Selection of the flocks in the present study was based on the willingness of the producers to participate in the study. 60 eggs were collected from each flock. De Reu et al. \cite{2005a} reported that sampling of at least 40 eggs is necessary in order to get statistically reliable results for determination of bacterial contamination of eggshells. The farms included in this study had either HyLine or Isa Brown laying hens at different stages of lay. Eggs were candled to ensure they were intact eggs without cracks. All the 31 flocks were divided into three different categories based on the age of flock. Early lay (22–40 weeks), Mid lay (41–55 weeks) and Late lay (above 55 weeks) categories, including 10, 8 and 13 flocks respectively.

2.1.1. *Salmonella* Infantis strain (obtained from *Salmonella* reference laboratory, IMVS, Adelaide, Australia, this strain of *Salmonella* was isolated from the layer flock)

Bacteria stored at −80 °C in 50% glycerol were plated on Xylose lysine deoxycholate agar (XLD; Oxoid, Australia) and incubated overnight at 37 °C. A single colony was selected and characterized on the plates or buffered peptone water. An individual colony was selected and characterized on the plates or buffered peptone water. A 100 μl of this sample was transferred into Triple Sugar iron agar slopes (TSI; Oxoid, Australia). After incubation at 37 °C overnight and 100 μl of this sample was transferred into Rappaport Vasidalis (RV) broth (Oxoid, Australia) which was then incubated at 42 °C for 24 h. A loopful of the same sample was streaked on Xylose lysine deoxycholate agar (XLD; Oxoid, Australia) and Bismuth Sulphite agar (BSA; Oxoid, Australia) plates. Presumptive *Salmonella* colonies from BSA or XLD agar were selected and used to stab inoculate Triple Sugar iron agar slopes (TSI; Oxoid, Australia). After incubation at 37 °C, the inoculated TSI slopes were examined at intervals of 24 h up to 72 h, for typical *Salmonella* reactions. The presumptive *Salmonella* colonies were tested by slide agglutination reaction using Poly O and Poly H antigens (BD, Australia) along with API® Rapid 20E strips (BioMerieux, Australia). Slopes of isolates were sent to the Institute of Medical and Veterinary Sciences (IMVS), Adelaide, Australia for *Salmonella* serotyping.

2.2. Eggshell surface wash

Six pooled eggs were placed in 60 mL of sterile phosphate buffer saline (PBS) (Merck, Australia) in Whirl-Pak bags and rinsed by shaking for 2 min. Before rinsing, PBS was warmed to 37 °C to facilitate bacterial recovery. After a rinse sample was obtained, each egg was removed and transferred to a different sterile bag. Intact eggs were then stored at 4 °C overnight for future use. A 100 μl of the PBS rinsate was plated on violet red bile glucose agar (Oxoid, Australia) to enumerate *Enterobacteriaceae* counts. Also, 1 ml of rinsate was transferred to 4 ml of buffered peptone water (Oxoid, Australia) for *Salmonella* isolation. Plates or buffered peptone water were incubated at 37 °C overnight. After incubation, colonies on the plates were counted.

2.3. Shell crush methodology

For isolation of bacteria from eggshell pores, eggshells were processed as described by Musgrove et al. \cite{2005a,b}. Briefly, after eggshell surface processing, each eggshell surface was dipped into 70% alcohol for 30 s to kill any bacteria present on the outside of the shell and was allowed to air dry in a biosafety cabinet. The eggs were cracked open into a sterile container. The inside of the eggshells was then washed with sterile phosphate buffered saline to remove the adhering egg albumen because of the antimicrobial activity of albumen. Shell and shell membranes of six pooled egg samples were transferred to a sterile bag and crushed gently. To each bag, 60 ml of PBS was added. A 100 μl of PBS was plated on violet red bile glucose agar and also, 1 ml of PBS was transferred to 4 ml of buffered peptone water. Plates and buffered peptone water were incubated at 37 °C overnight. After incubation, colonies on the plates were counted.

2.4. Egg internal contents

The egg internal contents from pooled eggs were collected in the sterile containers and were thoroughly mixed. 1 ml of egg internal content was inoculated with 4 ml of buffered peptone water for further processing.

2.5. Isolation and identification of *Salmonella* spp.

The isolation of *Salmonella* was carried as described earlier by Cox et al. \cite{2002}. To isolate *Salmonella* spp., inoculated buffered peptone water (from shell surface, shell crush and egg internal contents) was incubated at 37 °C overnight and 100 μl of this sample was transferred into Rappaport Vasidalis (RV) broth (Oxoid, Australia) which was then incubated at 42 °C for 24 h. A loopful of the same sample was streaked on Xylose lysine deoxycholate agar (XLD; Oxoid, Australia) and Bismuth Sulphite agar (BSA; Oxoid, Australia) plates. Presumptive *Salmonella* colonies from BSA or XLD agar were selected and used to stab inoculate Triple Sugar iron agar slopes (TSI; Oxoid, Australia). After incubation at 37 °C, the inoculated TSI slopes were examined at intervals of 24 h up to 72 h, for typical *Salmonella* reactions. The presumptive *Salmonella* colonies were tested by slide agglutination reaction using Poly O and Poly H antigens (BD, Australia) along with API® Rapid 20E strips (BioMerieux, Australia). Slopes of isolates were sent to the Institute of Medical and Veterinary Sciences (IMVS), Adelaide, Australia for *Salmonella* serotyping.

2.6. Characterization of *Enterobacteriaceae*

Inoculated buffered peptone water (eggshell surface and eggshell crush) was plated on violet red bile glucose agar. After overnight incubation at 37 °C, an individual colony was selected and characterized using API® Rapid 20E strips (BioMerieux, Australia). Strips were inoculated, handled as per the manufacturer’s instructions and reactions were recorded using API webplus software (BioMerieux, Australia).

2.7. DNA extraction and Polymerase chain reaction (PCR) for *Salmonella* Infantis typing

*Salmonella* Infantis isolated from eggshell wash were grown in 5 ml Brain heart infusion broth (BHI, Oxoid, Australia) overnight at 37 °C with shaking. The cells were pelleted using a centrifuge at 1500 g for 10 min. DNA was extracted and purified using Wizard® Plus MiniPreps DNA purification system (Promega, Australia) as per manufacturers’ instructions. The extracted DNA suspended in nuclease free water was stored at −20 °C until further use. *Salmonella* Infantis isolates were tested for eight different virulence genes (Table 1). For PCR, each reaction mixture contained 1 x reaction buffer (Fisher Scientific, Australia), 1.8 mM MgCl2, 200 μM dNTPs, 1 μM of each primer, 1 U Taq polymerase, and 50 μg DNA template made up to 20 μl with nuclease free water. Samples were amplified using a Bio-Rad Thermal Cycler with an initial denaturation step at 95 °C for 5 min followed by 30 cycles of amplification (denaturation at 95 °C for 30 s, annealing temperature as per primer for 30 s and extension at 72 °C for 1 min 30 s), with a
as described earlier by Musgrove et al. (2005a,b). Limit of detection for tents (Smith et al., 2000). Spiking method indicated that the limit of detection for eggshell penetration and contamination of internal contents (60%).

3. Results and discussion

A high bacterial load present on the eggshell surface could increase the chance of eggshell penetration and contamination of internal contents (Smith et al., 2000). Spiking method indicated that the limit of detection for Salmonella Infantis by culture method was approximately 1 log CFU/ml. In the present study, shell rinse and crush methods were used to recover Enterobacteriaceae from commercial shell eggs as described earlier by Musgrove et al. (2005a,b). Limit of detection was not calculated for Enterobacteriaceae isolation/count from eggs. It is possible that the limit of detection of Enterobacteriaceae isolates from 11 different genera could be variable and further investigations are necessary to determine the detection limits of these different genera. We found a relatively low average Enterobacteriaceae count on the eggshell (1.46 log CFU/eggshell), which is in agreement with De Reu et al. (2009) who reported 1.51 log CFU/eggshell count in eggs from furnished cages. Musgrove et al. (2005b) reported an average Enterobacteriaceae count of 2.29 log CFU/eggshell from the eggs which were collected at a commercial egg processor. The difference in the bacterial counts might be due to the variation in sampling as, in the present study, shell rinse and crush methods were used to recover Enterobacteriaceae from furnished cages. Musgrove et al. (2005b) reported an average of 2.29 log CFU/eggshell count in eggshell wash. However, the study was not calculated for any signification difference in Enterobacteriaceae count (on eggshell and in shell pore) across early, mid and late lay (Table 2). There is a dearth of literature regarding Enterobacteriaceae counts in eggshell pores or effect of flock age on Enterobacteriaceae count on eggs, which precludes comparison of our findings with those of other workers. However, there are a number of studies which have investigated the effect of flock age on bacterial contamination of eggshells. De Reu et al. (2005b) and Protais et al. (2003) reported that there was no significant difference in eggshell contamination between beginning and end of the laying period in furnished cages or aviaries. Huneau-Salaün et al. (2010) found that eggshell contamination increased significantly with increasing age of flock but Mallet et al. (2006) reported that contamination decreased with age. However, both of these authors attributed the variation in their results to seasonal or environmental effects rather than flock age.

API Rapid 20E was used to identify the various members of Enterobacteriaceae at genus and species level. Identified isolates belonged to 11 different genera which included: Cedecea, Citrobacter, Enterobacter, Escherichia, Klebsiella, Klyuyera, Leclercia, Pantoea, Salmonella, Serratia and Yersinia (Table 3). Out of all isolates identified, isolates from Escherichia genus were reported most frequently (60.78%), followed by Salmonella (9.15%), Enterobacter (8.49%) and Serratia (5.22%) (Fig. 1). All other genera were identified less frequently (<5%). Isolates reported at least once were: Cedecea spp., Citrobacter freundii, Enterobacter carcinogenes, Enterobacter spp., E. coli, Escherichia fergusonii, Klebsiella pneumoniae, Klyuyera spp., Leclercia adecarboxylata, Pantoea spp., Pantoa spp. 3, Salmonella Infantis, Salmonella enterica subsp. enterica serovar 4,12:d:, Serratia odorifera, Serratia plymuthica, Serratia spp., Yersinia enterocolitica. Also, three E. coli isolates were identified from eggshell pores. Our findings regarding the presence of various genera of Enterobacteriaceae on eggshells are in agreement with Musgrove et al. (2004) who also reported Escherichia, Salmonella, Enterobacter, Serratia, Yersinia, Klebsiella, Pantoea, Klyuyera and Citrobacter on eggshell surface. However, Cedecea spp. and Leclercia adecarboxylata were not reported in their study. Out of these, Leclercia adecarboxylata is infrequently isolated from eggshells. This microorganism was rarely reported in humans (Hess et al., 2008). There are very few reports of Cedecea isolation from eggshells and

### Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Forward primer (F) (5′–3′)</th>
<th>Reverse primer (R) (5′–3′)</th>
<th>Annealing temperature (°C)</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>prgH</td>
<td>Invasion of macrophages</td>
<td>F-GCCCGACGCGCCGACGTTAGAAA</td>
<td>R-TGGAATAGCCCCCTAGGCTGCTGC</td>
<td>55</td>
<td>755</td>
<td>Hughes et al. (2008)</td>
</tr>
<tr>
<td>sopB</td>
<td>Invasion of macrophages</td>
<td>F-GAGAGCTAAGCCGGCGACTT</td>
<td>R-TTGGGTATGCTGGCTGAGG</td>
<td>55</td>
<td>804</td>
<td>This study</td>
</tr>
<tr>
<td>invA</td>
<td>Invasion of macrophages</td>
<td>F-CTGCGGGGTGTTTGTCTCTCTTCT</td>
<td>R-AGTTCGCGCTTGCCTGCCTGTC</td>
<td>60</td>
<td>1062</td>
<td>Hughes et al. (2008)</td>
</tr>
<tr>
<td>sitC</td>
<td>Invasion of macrophages/</td>
<td>F-GCTATATGCTGCAAGCCGAGTGCTTGC</td>
<td>R-CGGGGCCGAAATAAAGGCCTGCTGAC</td>
<td>64</td>
<td>740</td>
<td>Hughes et al. (2008)</td>
</tr>
<tr>
<td>spiC</td>
<td>Survival in macrophages</td>
<td>F-CTCTGCGATGACTATTGAT</td>
<td>R-AGTTTATGGTGATTGCGTAT</td>
<td>56</td>
<td>300</td>
<td>Hughes et al. (2008)</td>
</tr>
<tr>
<td>sitA</td>
<td>Survival in macrophages</td>
<td>F-CTGGCGGTAGCATGATG</td>
<td>R-GTTGCCTTTTCTTGCGCTTTCCACCCATCT</td>
<td>62</td>
<td>448</td>
<td>Hughes et al. (2008)</td>
</tr>
<tr>
<td>misL</td>
<td>Survival in macrophages</td>
<td>F-GTGGCGGTGAGCGCCGCCGAGAAGC</td>
<td>R-GCGCTGTTAACGCTAATAGT</td>
<td>58</td>
<td>540</td>
<td>Hughes et al. (2008)</td>
</tr>
<tr>
<td>orfL</td>
<td>Survival in macrophages/</td>
<td>F-CTGCGGCGCGGCGCGGAAATAAAGGCCTGCTGAC</td>
<td>R-GGCCGTGTAAGCTGAAAGATTT</td>
<td>56</td>
<td>331</td>
<td>Hughes et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>colonisation</td>
<td></td>
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<td></td>
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</table>

### Table 2

<table>
<thead>
<tr>
<th>Bacterial count</th>
<th>Early lay</th>
<th>Mid lay</th>
<th>Late lay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterobacteriaceae count (log CFU/eggshell)</td>
<td>1.78 ± 0.33</td>
<td>1.46 ± 0.22</td>
<td>1.22 ± 0.15</td>
</tr>
<tr>
<td>Enterobacteriaceae count (shell surface)</td>
<td>0.32 ± 0.17</td>
<td>0.44 ± 0.28</td>
<td>0.29 ± 0.10</td>
</tr>
</tbody>
</table>

Mean ± SEM.
Jones et al. (1995) reported that 7.8% of the eggshells from eggs sampled were contaminated in hens individually attacked by Salmonella spp. who undertook a microbiological survey of commercial eggs in Australia. In the present study, fourteen eggshell wash samples were found positive for Salmonella. Serotyping confirmed that twelve samples were Salmonella Enteritidis whereas two were S. enterica subsp. enterica serovar 4,12:d. isolates. All of the eggshell pore and egg internal content samples were negative for Salmonella. Overall there were 4.51% (14/310) Salmonella positive pooled samples. Similar findings were reported by Stepien-Pyszniak (2010) who reported 3.2% prevalence of Salmonella on eggshells. Cox et al. (1973) reported that less than 10% of the eggshells were contaminated in hens individually artificially infected with S. enterica serovars Senftenberg, Thompson and Typhimurium, whereas Jones et al. (1995) reported that 7.8% of the eggshells from eggs sampled before processing were contaminated. In all these three studies, eggs were processed individually for Salmonella isolation. In the present study, Salmonella spp. were not detected in any of the egg internal contents. This finding is in agreement with Daughtry et al. (2005) who undertook a microbiological survey of commercial eggs in Australia to determine the prevalence of Salmonella contamination. During Daughtry’s study, Salmonella spp. was not isolated from the internal contents of any of the 20,000 eggs sampled.

All of the Salmonella Infantis isolates from the present study possessed invA, misL, orfL, prgH, sipA, sitC, sopB and spiC virulence genes. These genes play an important role in invasion of macrophages and are also essential for survival of Salmonella within macrophages. Out of these genes, prgH, sopB, invA and sitC are located on Salmonella pathogenicity island (SPI) 1 whereas spiC and sipA are located on SPI-2. The misL and orfL genes are located on SPI-3 and SPI 4 respectively (Skyberg et al., 2006; Cassama-Sow et al., 2006). SPI 1, SPI 2 and SPI 3 play an important role in invasion of macrophages and survival of Salmonella within macrophages (Hughes et al., 2008) whereas SPI 4 is involved in secretion of toxins (Cassama-Sow et al., 2006) and survival of Salmonella in macrophages. For Salmonella Typhimurium, SPI 1 and SPI 2 are important in causing systemic and gastrointestinal tract infection in young chicks (Jones et al., 2007). Salmonella Infantis isolates in the present study possessed genes located on SPI 1 (prgH, sopB, invA, sitC) and SPI 2 (spiC, sipA) which suggest that these isolates may have the capacity to cause systemic and gastrointestinal infection in old chicks. However, further animal trials are essential to confirm these findings. PCR results also indicated that all the Salmonella Infantis isolates possessed orfL and misL genes which are involved in survival of Salmonella in macrophages. However it is essential to note that possession of a single or a few virulence genes does not endow a strain with pathogenic status unless that strain has acquired the appropriate virulence gene combination to cause disease in a specific host species (Gilmore and Ferretti, 2003). Salmonella Infantis strains isolated from eggshells may have capacity to invade and survive in macrophages. However, further studies such as a macrophage invasion assay are essential to confirm these findings.

Even though, in the present study, the prevalence of Salmonella on eggshells was low, proper handlings of eggs in the kitchen is essential as improper handling may cause cross contamination of other food materials leading to food poisoning outbreaks (Slinko et al. 2009). It is essential to adopt safe food handling practises in the food service sector, so that cross contamination can be avoided which will, potentially, reduce the risks of Salmonella food poisoning cases.

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