Occurrence of purulent arthritis broilers vertically infected with Salmonella enterica serovar Enteritidis in Korea


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ABSTRACT Salmonella enterica serovar Enteritidis (Salmonella Enteritidis) has been associated with morbidity and mortality in broiler chickens worldwide. The present study described purulent arthritis of broilers infected with Salmonella Enteritidis and investigated antibiograms and genetic characteristics of Salmonella Enteritidis isolates from epidemiologically related properties such as a hatchery and breeder farm in an attempt to elucidate the source of contamination. Clinical disease and mortality were observed in the affected broiler flock. Mortality was 5.8% until 12 d of age. The birds typically showed lameness with moderately swollen hock joints and footpads. The most prevalent lesions were severely purulent arthritis with polyserositis. Histopathology revealed moderate to severe inflammation in the synovial membrane of leg joints and visceral organs. When the antimicrobial susceptibility test was performed against 7 isolates of Salmonella Enteritidis from broilers, and relevant hatchery and breeder farms by the disk diffusion method using 18 antimicrobial agents, isolates from broiler and breeder farms had the same antibiogram characterized by multiple drug resistance to ampicillin, ceftiofur, cephalothin, gentamycin, nalidixic acid, streptomycin, sulfisoxazole, and tetracycline, whereas isolates from the hatchery were differently resistant to only nalidixic acid. Through the genetic analysis with pulsed-field gel electrophoresis using the restriction enzyme XbaI, Salmonella Enteritidis isolates from both broiler and breeder farms also showed the same PFGE pattern compared with the hatchery isolates resistant to nalidixic acid. As a result, the same PFGE profiles and antibiogram patterns among isolates from broilers and breeder farms provided direct evidence of vertical Salmonella Enteritidis transmission from the contaminated breeder farm to commercial broiler.

Key words: Salmonella Enteritidis, arthritis, antimicrobial resistance, pulsed-field gel electrophoresis

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INTRODUCTION

Salmonella enterica ssp. enterica serovar Enteritidis (commonly called Salmonella Enteritidis) infections often have very different consequences for newly hatched poultry than for more mature birds (Gast, 2003). In susceptible young chicks and poults, the infections can sometimes lead to moderate illness and death at high frequencies (O’Brien, 1988). Older birds are far less susceptible to the lethal effects of paratyphoid and may experience intestinal colonization and even systemic dissemination without significant morbidity or mortality (Gast and Holt, 1998). Salmonella Enteritidis also continues to be a major cause of foodborne infections, with poultry being a major source of this infection (Timoney et al., 1989; Barrow, 1991).

Vertical transmission of Salmonella Enteritidis to the progeny of infected breeder flocks could result from internal or external contamination of eggs (Gast, 2003). In addition, the penetration of the bacteria into or through the contaminated shell and shell membranes can also result in direct transmission of infection to the developing embryo or can expose the chick to Salmonella Enteritidis when the shell structure is broken during hatching (Nairn and Watson, 1972; Bhatia and McNabb, 1980; Davies and Breslin, 2001). Therefore, the resulting transovarian transmission of infection to progeny from breeder farms and eggborne transmission have long been known to play a major role in the spread of Salmonella Enteritidis infections in chickens.

Previous studies have demonstrated a high susceptibility of newly hatched chicks to Salmonella Enteritidis with descriptions of the pathological changes (Shivaprasad et al., 1990; Keller et al., 1997). After natural infection with Salmonella Enteritidis in broilers, indurated yolk sac remnants, pericarditis, necrotic foci, and petechiae in the liver were reported. Gorham et al. (1994) described the lesions in chickens infected at 1 and 7 d of age with the bacteria. Lesions were only observed in infected chicks at 1 d of age, consisting of
fibrinous pericarditis and perihepatitis and peritonitis. However, to our knowledge, little is known about the incidence of purulent arthritis in the broilers vertically infected with *Salmonella* Enteritidis.

Identification, antibiogram analysis, pulsed-field gel electrophoresis (PFGE), phage typing, and plasmid profiling of the *Salmonella* isolates are essential for epidemiological surveillance and outbreak investigations (Guerra et al., 2000; Bender et al., 2001; Lee et al., 2007). Antimicrobial susceptibility has been used with success in tracking *Salmonella* outbreaks (Berge et al., 2004). Use of PFGE with endonuclease XbaI has been widely recognized as a sensitive means of fingerprinting *Salmonella* serovars (Liebana et al., 2001).

The objective of the present study was to describe the purulent arthritis caused by *Salmonella* Enteritidis in broilers and to analyze PFGE and antimicrobial resistance patterns of the *Salmonella* Enteritidis isolates from different sites including arthritis-affected broilers, hatchery, and their parent stocks to assess the epidemiological relationships.

**MATERIALS AND METHODS**

**Birds**

On April 28, 2009, a broiler integration company submitted 4 dead and 6 live broilers (12 d old) from a poultry farm that exhibited moderate mortality with clinical poor locomotion to the Avian Disease Division, National Veterinary Research and Quarantine Service, Anyang, South Korea. In the farm located in the mid-west region of South Korea (Chungnam province), a disease outbreak occurred, characterized by depression; reluctant movement; lameness or ataxia, or both; and moderate mortality (5.85% between 1 and 11 d old).

**Bacteriology**

For bacterial examination, samples of hock joints, footpads, livers, and air sacs were aseptically collected using sterilized cotton-tipped swabs. Collected swabs were streaked on both 5% sheep blood agar (Komed Co. Ltd., Sungnam, Korea) and MacConkey agar and were inoculated into 10 mL of trypticase soy broth as growth medium. The solid media plates and broths were then incubated at 37°C for 24 h under aerobic conditions.

**Pathology**

The surface of birds was wetted with disinfectant and necropsies were routinely performed. Tissues samples were taken (trachea, lung, heart, joint, liver, spleen, air sac, and brain), fixed in 10% neutral-buffered formalin, processed, and embedded in paraffin blocks. Sections were made at 5 µm and stained with hematoxylin and eosin.

**Isolation and Identification of Salmonella**

Two suspicious colonies per agar plate were picked and again streaked on MacConkey agar for pure culture and were then incubated overnight at 37°C. Samples on MacConkey agar were reacted with *Salmonella* O antiserum. Colonies showing typical agglutination by O antiserum were further serotyped with *Salmonella* H antiserum (Difco, Detroit, MI) according to the latest version of the Kaufman and White scheme (Grimont and Weill, 2007).

**Sample Collection and Identification of Salmonella spp. from a Hatchery and Breeder Farm for Epidemiological Study**

Cecal droppings, nest box swabs, egg sorting area swabs, and dust on the wall from breeder farms were taken for investigation. Hatchery samples were collected on the day of hatching and were obtained from the hatcher interior, chick sorting area, chick box, ventilation outlets, and waste area. Swab samples from the nest box and egg sorting area at the farm and chick sorting area, chick box, and ventilation outlets were collected by using a set of four 10 × 10 cm² gauze pads premoistened with sterile buffered peptone water (Difco) per site. The samples taken were brought directly to the laboratory under ambient conditions on the day of collection, and samples from each site were then divided into 2 portions, which were added with 225 mL of buffered peptone water, separately, and incubated at 37°C for 18 h. After preenrichment, 0.1 mL of broth was transferred into 10-mL of Rappaport-Vassiliadis (RV) broth (Merck, Darmstadt, Germany), which was prepared according to the instructions on the package. The RV broth was incubated overnight at 37°C. The RV broth samples were streaked onto Rambach agar (Merck) and incubated overnight at 37°C. Two typical colonies were picked and transferred to MacConkey agar for pure culturing and were identified as *Salmonella* serotypes as described above.

**Antimicrobial Susceptibility Testing**

All *Salmonella* Enteritidis isolates were investigated for their antimicrobial resistance by the agar disk diffusion test using the following disks (Difco): ampicillin (10 µg), amoxicillin-clavulanic acid (20-10 µg), cefoxitin (30 µg), cephalothin (30 µg), cefepime (30 µg), ceftiofur (30 µg), chloramphenicol (30 µg), florfenicol (30 µg), ciprofloxacin (5 µg), enrofloxacin (5 µg), nalidixic acid (NAL, 30 µg), apramycin (15 µg), neomycin (30 µg), streptomycin (10 µg), gentamicin (10 µg), tetracycline (30 µg), trimethoprim-sulfamethoxazole (1.25-23.75 µg), and sulfadiazine (250 µg) according to the guidelines of the Clinical and Laboratory Standards Institute (2006).
PFGE

Pulsed-field gel electrophoresis patterns of Salmonella Gallinarum isolates were analyzed according to the One-Day (24–28 h) Standardized Laboratory Protocol for Molecular Subtyping of Non-Typhoidal Salmonella by PFGE (Anonymous, 1997). A single colony of each isolate was streaked on tryptic soy agar and incubated overnight at 37°C. Using a cotton swab, a portion of the growth from the agar plate was transferred to 2 mL of cell suspension buffer (100 mM Tris:100 mM EDTA, pH 8.0) and the concentrations of cell suspensions were adjusted to 14 to 15% by a bioMerieux Vittek colorimeter (bioMerieux, Marcy l’Etoile, France). The 14 to 15% transmittance represents approximately 1.5 × 10⁹ cfu/mL (McFarland standard density no. 5). Immediately, 400 µL of the adjusted cell suspension was transferred to 1.5-mL microcentrifuge tubes with 20 µL of proteinase K, subsequently mixed with Tris-EDTA buffer (10 mM Tris:1 mM EDTA, pH 8.0), and pipetted into disposable plug molds. Three plugs were transferred to 50-mL polystyrene screw tubes with 5 mL of cell lysis buffer (50 mM Tris:50 mM EDTA, pH 8.0 with 1% sarkosyl) and 25 µL of proteinase K and incubated at 54°C in a shaker water bath for 2 h. Thereafter, the plugs were washed twice with 15 mL of sterile water and 3 more times with Tris-EDTA buffer (100 mM Tris:1 mM EDTA, pH 8.0 with 1% sarkosyl) and 25 µL of proteinase K and incubated at 54°C in a shaker water bath for 2 h. Then, the plugs were washed twice with 15 mL of sterile water and 3 more times with Tris-EDTA buffer at 50°C for 15 min. Chromosomal DNA was digested with 50 U of XbaI (Promega, Southampton, UK). Pulsed-field gel electrophoresis was performed on a CHEF Mapper AX system (Bio-Rad, Hercules, CA) in 0.5 × Tris-borate-EDTA buffer with recirculation at 14°C. Pulse times were ramped from 2.2 to 63.8 s during an 18-h run at 6.0 V/cm. After electrophoresis, the gels were stained with 2 µg of aqueous ethidium bromide per milliliter for 15 min and photographed by using 300 nm of UV light. Pulsed-field gel electrophoresis patterns were analyzed using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) with the Dice coefficient of similarity, the unweighted pair group method with arithmetic averages, and position tolerance of 1.3%. A PFGE type was defined as a group of isolates with a similarity of ≥90% and a subtype within each type was determined by 100% similarity.

RESULTS

Pathological Findings

All of the birds submitted were generally dehydrated and in poor physical condition. At necropsy, prominent lesions were observed in the footpads and hock joints. The affected unilateral or bilateral joints of the birds were swollen in various degrees, some showing fluctuation on palpation (Figure 1a). Within the articular cavity, there was an increase of synovial fluid that was turbid and whitish to yellowish in color (Figure 1b). Occasionally, the lesions spread up to the adjacent bursae or teninous sheaths. Moreover, yellowish fibrinous exudates on the pericardium and air sac, the serosal surface of liver and intestine, and within peritoneum (Figure 1c) were present with mild to moderate enlargement of the spleen and moderately swollen kidneys.

The prominent microscopic lesions of the dead broilers were found in the joint, liver, heart, and spleen, which were compatible with the gross changes. In the hock joint, there were moderate to severe inflammatory responses characterized by the infiltration of mainly heterophils and macrophages and rarely lymphocytes and fibrin into joint spaces (Figure 1d) along with tendon sheaths. Diffuse fibrinous exudates were also found in the serosal membrane of the liver and intestine and in the epicardium of the heart.

Bacteriology

Identical, morphologically gram-negative, short rod bacteria were isolated from the hock joint, footprint, liver, heart, and air sac of the dead birds. One colony from each organ examined was selected as a representative isolate and was tested for phenotypic and serotypic properties. Isolates were motile and their serotype was Salmonella Enteritidis (1,9,12:g,m:−).

Isolation and Identification of Salmonella from a Breeder Farm

Table 1 presents the results of sampling at a breeder farm at 7 d after the broilers with purulent arthritis were infected with Salmonella Enteritidis. There, Salmonella Enteritidis isolates were found in the floor litter, egg sorting areas, and dust on the house walls. However, no Salmonella was found in the cecal dropings (20 birds) and nest boxes.

Isolation and Identification of Salmonella from a Hatchery

The results of sampling carried out in the hatchery that had hatched the broilers infected with Salmonella causing purulent arthritis are presented in Table 2. Salmonella Enteritidis was detected in the meconium, chick boxes, and chick sorting area. Salmonella Senftenberg was recovered from the hatcher interiors and waste areas. However, no Salmonella was isolated from the ventilation outlets in the hatchery.

Antimicrobial-Resistant Patterns of Salmonella Enteritidis

To identify the source of broiler-originated Salmonella Enteritidis, antimicrobial-resistant patterns from 7 representative isolates of Salmonella Enteritidis (2 isolates from broilers, 3 isolates from broiler breeder farm, 2 isolates from hatchery) were examined. Interestingly, we observed 2 distinct antimicrobial patterns as shown in Table 3. Two isolates from broilers affected
with purulent arthritis and 3 isolates from the breeder farm definitely had the same antimicrobial patterns, which were resistant to ampicillin, cephalothin, gentamicin, NAL, sulfisoxazole, streptomycin, tetracycline, and ceftiofur. However, the 2 isolates from the hatchery were resistant to only NAL. Based on the results of antimicrobial-resistant patterns, we suggested that Salmonella Enteritidis isolates from the broilers affected with arthritis might be cases of vertical transmission from the infected breeder farm.

**PFGE Patterns of Salmonella Enteritidis Isolates**

The PFGE of XbaI-digested chromosomal DNA from 7 strains, including the standard strain of Salmonella Enteritidis (ATCC 13076), gave stable and reproducible patterns consisting of 13 to 15 fragments (Figure 2). The predominant genotype accounting for 4 strains from both broiler and breeder farms was A1. The genetic similarities of the PFGE profiles, as demonstrated by the dendrogram, showed 2 clusters: the first consisted of 2 profiles (A1 and A2) from 1 broiler isolate, 3 breeder farm isolates, and 2 hatchery isolates in Korea; the second pattern comprised 1 profile (B) from the standard Salmonella Enteritidis strain. According to the fingerprinting patterns, we believed that Salmonella Enteritidis isolated from the affected broilers might have been spread by transovarian transmission from the breeder farm.

**DISCUSSION**

The prominent pathologic change observed at necropsy in this study consisted of moderate to severe bilateral arthritis caused by Salmonella Enteritidis. Pathological findings in broilers infected with Salmonella Enteritidis. a) Moderately swollen footpad (arrow). b) Gross observations of the foot reveal yellowish pus-like exudates in the subcutaneous area of the footpad (arrow). c) Moderately fibrinous air sacculitis and peritonitis in the abdominal cavity. d) Moderately heterophilic and mononuclear cellular inflammation within synovial membrane of hock joint. Hematoxylin and eosin stain. Bar = 30 µm. Color version available in the online PDF.

<table>
<thead>
<tr>
<th>Flock size (no.)</th>
<th>Flock age (wk)</th>
<th>Sample site</th>
<th>Serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>35,000</td>
<td>54</td>
<td>Fresh feces</td>
<td>Salmonella Enteritidis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Litter</td>
<td>Salmonella Enteritidis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Egg sorting area</td>
<td>Salmonella Enteritidis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nest boxes</td>
<td>Salmonella Enteritidis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wall dust</td>
<td>Salmonella Enteritidis</td>
</tr>
</tbody>
</table>

1 Negative results in Salmonella culture.
eral or unilateral swollen hock joint or footpad, or both, with purulent exudates. Histopathology also indicated purulent arthritis. The pathological findings indicated that the conditions were due to bacterial infection. Several studies have shown that the most common etiological agents of purulent arthritis in chickens and turkeys are *Escherichia coli* and *Staphylococcus* and have referred to an induced septicemia (Bremell et al., 1992; Gomis et al., 1997; Goldenberg, 1999). In this study, it was thought that purulent arthritis of the broilers may be caused by septicemic infection of *Salmonella Enteritidis* because the organism isolated from the joints and other visceral organs of the infected broilers was definitely identified as *Salmonella Enteritidis*. The other lesions in the visceral organs in the present study were commonly fibrinous peritonitis, perihepatitis, and pericarditis, resembling the fatal findings of young chicks that were vertically or experimentally infected with *Salmonella Enteritidis*, as described previously (Gast, 1994; Gorham et al., 1994).

Although *Salmonella Enteritidis* has been shown to be spread by horizontal transmission mechanisms including direct bird-to-bird contact and ingestion of contaminated feces or litter, ovarian or vertical transfer of pathogens from breeding hens to progeny has been an important aspect of the epidemiology of *Salmonella* species infection within the poultry industry. The structure of the chick supply and distribution chain is such that a single infected breeding flock may have a significant effect on the level of infection in commercial flocks (Jones et al., 1991; Riemann et al., 2000; Davies et al., 2003). In this study, epidemiological investigation is actually needed to identify the source of *Salmonella Enteritidis* infection. In recent years, many DNA-based genotyping techniques have been used to delineate epidemiological relationships between various isolates (Anonymous, 1997; Laconcha et al., 1998; Seo et al., 2006). However, the combination of several methods including antibiogram and genetic typing may provide a powerful discriminatory tool for the epidemiological analysis of unrelated and related *Salmonella* strains (Bender et al., 2001; Liebana et al., 2001; Cardinale et al., 2005). Here, we detected several bacterial isolates from a breeder farm and hatchery that were directly related to chick supply and distribution. Moreover, this study showed that both antibiogram and PFGE profiles were capable of differentiating between strains of *Salmonella Enteritidis* and these molecular tools also demonstrated that *Salmonella Enteritidis* from both the affected broilers and their parent flock had the same antimicrobial pattern and PFGE profiles. Therefore, the results of this study suggest that the bacteria causing purulent arthritis in the commercial broilers was directly transferred from the infected broiler breeders, not from the hatchery as previously reported.

Antimicrobial resistance in *Salmonella* spp. is a serious health problem worldwide, and increased cases of multiple antibiotic resistance have been described in *Salmonella* isolates from various countries including Korea (Evans and Davies, 1996; Chang, 2000). Similarly, 5 of 7 *Salmonella Enteritidis* tested clearly showed resistance to 7 drugs. The concern is not only for the spread of resistant clones but the possibility of the transfer of resistance genes between human- and animal-associated bacteria (van den Bogaard and Stobberingh, 2000).

<table>
<thead>
<tr>
<th>Region</th>
<th>Hatchery capacity (× 1,000 eggs/wk)</th>
<th>Sample site</th>
<th>Distribution</th>
<th>Serotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iksan</td>
<td>160</td>
<td>Meconium</td>
<td>Hatcher interiors</td>
<td><em>Salmonella Enteritidis</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chick sorting area</td>
<td><em>Salmonella Enteritidis</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ventilation outlets</td>
<td>-1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Waste area</td>
<td><em>Salmonella Senftenberg</em></td>
<td></td>
</tr>
</tbody>
</table>

1Negative results in *Salmonella* culture.

Figure 2. Representative XbaI restriction pattern. Dendrogram was generated by BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium; showing the relationships between pattern types of *Salmonella Enteritidis* isolated from different poultry facilities).
Table 3. Antimicrobial resistance pattern and pulsed-field gel electrophoresis (PFGE) pattern of Salmonella Enteritidis obtained from broiler, breeder farm, and hatchery

<table>
<thead>
<tr>
<th>Isolate stock name</th>
<th>Origin</th>
<th>Source</th>
<th>Antimicrobial resistance pattern&lt;sup&gt;1&lt;/sup&gt;</th>
<th>PFGE pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>09520</td>
<td>Broiler with arthritis</td>
<td>Hock joint</td>
<td>AMP-CEF-GEN-NAL-SMX-STR-TET A1</td>
<td>NT</td>
</tr>
<tr>
<td>09521</td>
<td>Broiler with arthritis</td>
<td>Liver</td>
<td>AMP-CEF-GEN-NAL-SMX-STR-TET NT</td>
<td>NT</td>
</tr>
<tr>
<td>09536</td>
<td>Breeder farm</td>
<td>Litter</td>
<td>AMP-CEF-GEN-NAL-SMX-STR-TET A1</td>
<td>A1</td>
</tr>
<tr>
<td>09537</td>
<td>Breeder farm</td>
<td>Egg sorting area</td>
<td>AMP-CEF-GEN-NAL-SMX-STR-TET A1</td>
<td>A1</td>
</tr>
<tr>
<td>09538</td>
<td>Breeder farm</td>
<td>Wall dust</td>
<td>AMP-CEF-GEN-NAL-SMX-STR-TET A1</td>
<td>A2</td>
</tr>
<tr>
<td>09534</td>
<td>Hatchery</td>
<td>Meconium</td>
<td>NAL A2</td>
<td>NT</td>
</tr>
<tr>
<td>09535</td>
<td>Hatchery</td>
<td>Chick sorting area</td>
<td>NAL A2</td>
<td>NT</td>
</tr>
</tbody>
</table>

<sup>1</sup>AMP = ampicillin; CEF = cephalothin; GEN = gentamicin; NAL = nalidixic acid; SMX = sulfisoxazole; STR = streptomycin; TET = tetracycline.

The most frequent serovars isolated from humans in Korea were Salmonella Enteritidis, Salmonella Typhimurium, and Salmonella Typhi. These serovars were responsible for over 63% of foodborne illnesses recorded during 2004 to 2005 (Kim et al., 2007). Recently, it has been reported that the prevalence of Salmonella spp. in food, especially in poultry products (up to 2.2%), is high in Korea (Chung et al., 2003). Investigations have shown that the prevalence of Salmonella Enteritidis-infected chicken and eggs in retail outlets is 18.5% and have suggested that infected chickens or raw eggs could be a major source of salmonellosis in Korea (Chang, 2000). Unfortunately, Salmonella control in integrated broiler operations is complicated because there are numerous potential sources of Salmonella contamination including chicks, feed, rodents, wild poultry, and the processing plant environment (O’Brien, 1990; Smith and Fratamico, 1995; Davies et al., 2001). However, Davies et al. (2001) reported that infection within a breeding company can be effectively controlled by application of good hygienic procedures after culling of infected flocks. Clearly, the data obtained in our study support the critical need to control Salmonella including Salmonella Enteritidis in breeder farms and demonstrate important points for the control of infection in a large-scale poultry operation in Korea.

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forming eggs is not unique to *Salmonella enteritidis*. Avian Dis. 41:535–539.


