An outbreak of gangrenous dermatitis in commercial broiler chickens

Guangxing Li¹², Hyun S. Lillehoj¹*, Kyung Woo Lee¹, Seung I. Jang¹, Pagès Marc³, Cyril G. Gay³, G. Donald Ritter⁴, Daniel A. Bautista⁵, Kathy Phillips⁵, Anthony P. Neumann⁶, Thomas G. Rehberger⁶ and Gregory R. Siragusa⁶

¹Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, USDA-ARS, Beltsville, MD 20705, USA, ²College of Veterinary Medicine, Northeast Agricultural University, Harbin 150030, China, ³Animal Production and Protection, USDA-ARS, Beltsville, MD 20705, USA, ⁴Mountaire Farms Inc., Millsboro, DE 19966, USA, ⁵Lasher Poultry Diagnostic Laboratory, University of Delaware, Georgetown, DE 16483, USA, and ⁶Danisco-Agtech Products, Inc., Waukesha, WI 53186, USA

The present report describes an outbreak of gangrenous dermatitis (GD) infection in a commercial poultry farm in Delaware involving 34-day-old broiler chickens. In addition to obvious clinical signs, some GD-affected broilers also showed severe fibrino-necrotic enteritis and large numbers of Gram-positive rods in the necrotic tissue. Histopathological findings included haemorrhage, degeneration and necrosis of parenchymatous cells, especially of skin, muscle, and intestine. Immunofluorescence staining revealed Clostridium-like bacilli in the skin and the intestine. Both Clostridium perfringens and Clostridium septicum genomic sequences were identified by polymerase chain reaction in bacterial cultures isolated from the skin, muscle, and intestine, and in the frozen tissues from the GD-affected birds. Serological analysis demonstrated that both affected and clinically healthy birds from the same house had high serum antibody titres against Clostridium perfringens, C. septicum, Eimeria, chick anaemia virus, and infectious bursal disease virus. These results are discussed in the context of the relationship between the different Clostridium spp. and the pathogenesis of GD.

Introduction

Gangrenous dermatitis (GD) is caused by the Gram-positive spore-forming anaerobic bacillus Clostridium perfringens type A (Weymouth et al., 1963; Char et al., 1986; Wages & Opengart, 2003b; Van Immerseel et al., 2004, 2009; McDevitt et al., 2006; Mataragas et al., 2008; Cooper & Songer, 2009) and Clostridium septicum (Frazier et al., 1964; Helfer et al., 1969; Fowler & Hussaini, 1975; Wages & Opengart, 2003a). Both C. perfringens and C. septicum may contribute synergistically to severe disease pathology associated with GD, but the role of other infectious agents in field GD cases remains to be clarified (Frazier et al., 1964; Saunders & Bickford, 1965; Hofacre et al., 1986). Since the first reported case of GD in 1930, this infection has been reported worldwide (Wages & Opengart, 2003a), and is responsible for significant economic losses for the global poultry industry (Bains & MacKenzie, 1975; Fowler & Hussaini, 1975). While the incidence of GD outbreaks has decreased with the advent of in-feed antibiotics, recent voluntary or legally-mandated withdrawal of antibiotic growth promoters (AGPs) and anti-coccidial drugs threatens the re-emergence of GD as a major emerging poultry disease (Neumann & Rehberger, 2009). According to the US Animal Health Association’s Committee on Transmissible Diseases of Poultry and other Avian Species, GD has consistently ranked as a top priority disease for the poultry industry in recent years (Mataragas et al., 2008).

Both C. perfringens and C. septicum are widely distributed pathogenic bacteria that are commonly recovered from affected poultry and from the environment (Songer, 1996). Following infection of the host, typically 4 to 7 weeks of age in broiler chickens (Wages & Opengart, 2003a), clostridial toxins are responsible for a rapid and fatal toxemia (Tweten, 2001). Typical signs include growth depression, loss of appetite, ataxia, and oedema in the lower abdomen and inner thighs. Gangrenous lesions are often characterized by excessively red patches of skin devoid of feathers with underlying serosanguinous fluid accompanied by emphysema. The musculature is usually crepitant with gas present between muscle groups (Willoughby et al., 1996). Poultry farms that experience multiple episodes of C. perfringens/C. septicum infections are considered endemic disease sites that are more prone to subsequent and successive GD outbreaks. The present report describes a systematic investigation of a field outbreak involving clostridial infections, and summarizes multiple findings using bacterial culture, serology, pathology, histology, molecular biology, and indirect

*To whom correspondence should be addressed. Tel: +1 301 504 8771. Fax: +1 301 504 5103. E-mail: hyun.lillehoj@ars.usda.gov

Received 1 December 2009

ISSN 0307-9457 (print).ISSN 1465-3338 (online): 10.1080/03079457.2010.487517 DO}
immunofluorescence analyses that provide new insights into field GD infections.

Materials and Methods
Case background. In March 2009, a sudden onset disease outbreak with signs of GD and increasing mortality up to 0.50% per day occurred in 34-day-old broilers at a commercial poultry farm in Delaware, USA. The farm was situated on flat terrain and consisted of two broiler houses. One house that held 27,000 broiler chickens was identified as the affected house during the disease outbreak period of 5 days was 2.0%.

Tissue sampling and histological examination. Ten GD-likely birds, including one dead bird with obvious GD lesions, and five clinically healthy birds were selected from the affected house for tissue collection. Serum samples were collected by cardiac puncture for screening against Eimeria, C. septicum, and iron salts and a surrounding white zone of lecithinase activity, were isolated for further verification by PCR using the alpha toxin-specific primers designed for clostridial bacteria (Table 1). For the identification of clostridial DNA in various tissue extracts, DNA was isolated using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany). PCR was carried out using the previously published protocol: initial denaturation for 2 min at 94°C, 3 cycles of amplification for 30 sec at 94°C, 30 sec at annealing temperature, and 30 sec at 72°C, and final extension for 10 min at 72°C. Amplicons were visualized on ethidium bromide-stained 1% agarose gels.

Immunofluorescence staining of Clostridium in tissue samples. Indirect immunofluorescence assay was conducted as described (Fritschy et al., 1992) to identify clostridial bacteria in tissue sections using a bacteria-specific antibody. Cryosections of skin and intestinal jejunum were mounted on pre-cleaned glass slides, fixed in acetone for 20 min at 4°C, and blocked with 10% normal horse serum for 20 min at room temperature. Optimally diluted rabbit polyclonal antibody against Clostridium spp. (Abcam, Cambridge, Massachusetts, USA) was added, incubated for 2 h at room temperature, washed with phosphate-buffered saline (PBS), and stained with FITC-labelled goat anti-rabbit IgG antibody (1:200; Sigma) for 1 h at room temperature. Slides were mounted with Fluoromount-G (Southern Biotech, Birmingham, Alabama, USA) for evaluation by confocal microscopy.

Determination of serum antibodies. Serum samples from the live GD-affected and clinically healthy chickens were tested for antibodies against C. perfringens and C. septicum by a modified enzyme-linked immunosorbent assay (ELISA) (Min et al., 2002). Briefly, flat-bottomed 96-well microtitre plates (Corning Costar, Corning, New York, USA) were coated overnight at 4°C with 100 µl/well whole bacterial lysates of C. perfringens and C. septicum (10 µg/ml) in 0.1 M carbonate buffer, pH 9.6. Crude soluble clostridial antigens were prepared from C. perfringens and C. septicum cultures using the previously published protocol (Kulkarni et al., 2008). Plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T), blocked with 200 µl/well PBS containing 1% bovine serum albumin for 1 h at room temperature, washed with PBS-T, and incubated with 100 µl/well of appropriately diluted serum samples for 2 h at room temperature. After washing with PBS-T, horseradish peroxidase-conjugated goat anti-chicken IgG antibody (1:5,000; Sigma) was added, and bound antibodies were detected at OD405 after reaction with 0.01% tetramethylbenzidine (Sigma) in 0.05 M phosphate-citrate buffer, pH 5.0, using a microplate reader (Bio-Rad, Richmond, California, USA). Antibodies reactive with Eimeria recombinant profilin protein (3-1E) were detected by ELISA as described (Lillehoj et al., 2005). Antibodies against CAV, IBDV, and reovirus antibodies were determined using commercial ELISA kits (IDEXX Laboratories, Westbrook, Massachusetts, USA) according to the manufacturer’s instructions.

Table 1. PCR primers used in the identification of C. perfringens and C. septicum z-toxin genes.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ to 3’)</th>
<th>Melting temperature (°C)</th>
<th>Product size (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpaF</td>
<td>GTTGATAGCGCCAGGACATGTGAAG</td>
<td>56.1</td>
<td>402</td>
</tr>
<tr>
<td>cpaR</td>
<td>CATGATGTCTCTGTCCCGCATC</td>
<td>55.5</td>
<td>300</td>
</tr>
<tr>
<td>csaF</td>
<td>CAGTGTCGCGGCACTAGTACC</td>
<td>58.0</td>
<td>269</td>
</tr>
<tr>
<td>csaR</td>
<td>CCACCTGCCCCAACTTCTC</td>
<td>58.4</td>
<td>300</td>
</tr>
</tbody>
</table>

Statistical analysis. Serological data were subjected to an unpaired Student’s t test, and P <0.05 was taken as the level of significance.

Results
Gross pathological examination. Gross skin lesions with red to dark discolorations, usually featherless or with ruffled feathers, and around moist areas of skin were primarily seen in the breast, abdomen, wings, and thighs. Extensive blood-tinged oedema was present beneath the
affected skin areas. The underlying musculature also exhibited extensive discoloration with or without oedema and gas between muscle bundles. In some GD-affected chickens, emphysema and serosanguinous fluid were present in subcutaneous tissues, without loss of integrity in the overlying skin. The small intestine, particularly the jejunum and the ileum, displayed distended areas containing excessive bile-stained contents and gas; however, other areas exhibited oedematous and congested tissue with inflamed and thickened intestinal walls. The intestinal lumen revealed slight to moderate haemorrhage, occasionally forming a pseudomembrane on the mucosal surface, and, in severe cases, fibrino-necrotic enteritis with a diphtheritic membrane tightly adhered to the necrotic intestinal tissue. Additionally, livers were moderately swollen and softened. Randomly scattered, discrete, small, grey-brown foci of necrosis appeared on the hepatic surface accompanied by a distended bile duct and gall bladder. Kidneys were moderately to severely swollen, and appeared red from haemorrhage. The thymus and spleen were also enlarged, with small haemorrhagic petechiae on the thymus, whereas the bursa of Fabricius was small with white or yellow discoloration. Some GD birds showed unab sorbed, swollen, and hard yolk sacs. In contrast, clinically healthy birds from the same location where the GD birds were housed did not develop any lesions.

Histological examination of affected tissues. Skin sections showed extensive subcutaneous oedema, emphysema, and haemorrhage with heterophil infiltration (Figure 1a). Epidermal integrity was compromised and a red-stained pseudomembrane appeared on the surface of necrotic tissue that contained infiltrating inflammatory cells and fibrin exudates (Figure 1a,b). Sections of breast muscle showed the tissue in various stages of degeneration and necrosis, characterized by fibre lysis, lack of a distinctive cytomembrane structure, weak staining of the cytoplasm, cell rupture, and prominent erythrocyte and leukocyte infiltration. Numerous large bacilli were observed. These microscopic lesions of skin and muscle were very similar to those described in previous reports of *C. septicum*-associated GD (Willoughby et al., 1996; Wages & Opengart, 2003a).

Intestinal sections showed moderate to severe histopathology with more intense damage in the jejunum and ileum compared with the duodenum. Moderate injury was characterized by epithelial cell degeneration and necrosis, goblet cell hyperplasia, congestion and oedema of the lamina propria, and inflammatory cell infiltration in the submucosal layer and lamina propria. In more severe necrotic lesions, individual epithelial cells, as well as intact villi, were detached from the basement membrane into the intestinal lumen. Occasionally, a pseudomembrane was observed that was composed of degenerated epithelial cells, heterophils, mononuclear cells, fibrin, and Gram-positive bacilli that were adhered to the intestinal tissue. Severe lesions also exhibited pronounced inflammatory cell infiltration in the lamina propria and submucosal layer, often obscuring normal intestinal structures (e.g. enteric glands). These infiltrates

---

**Figure 1.** Histopathology of GD-affected skin lesions stained with haematoxylin and eosin. 1a: Skin with thickened epidermis showing necrotic pseudomembrane and markedly enlarged subcutaneous tissue filled with fluid, gas, and infiltrating heterophils. Magnification x10. 1b: Higher x40 magnification of (1a).

**Figure 2.** Immunofluorescence staining of *Clostridium* spp. in the skin and intestine of GD-affected birds. 2a: Negative control staining without anti-*Clostridium* primary antibody. Magnification x10. 2b: *Clostridium* bacilli in the skin. Magnification x40. 2c: *Clostridium* bacilli in the intestine. Magnification x10.
consisted of lymphocytes, heterophils, and macrophages. Intermittently, lymphoid follicles in the submucosal layer were enlarged with large germinal centres with increased lymphocyte accumulation. Evidence of epithelial regeneration was also apparent due to epithelial cell proliferation, transformation of columnar epithelial cells into cuboidal cells, and decreased numbers of goblet cells resulting in the formation of a multilayered pseudostratified epithelium.

In the liver, hepatic congestion, vacuolar degeneration, and necrosis were evident. Multifocal coagulative necrosis of hepatocytes with infiltration by a mixed population of inflammatory cells including lymphocytes, heterophils, and macrophages was observed. Bacilli appeared in the distended central vein, and the bronchioles exhibited haemorrhage with leukocyte infiltration into the submucosal layer and sero-cellular exudates in the lumen. The lungs showed significant congestion and haemorrhage accompanied by distension of and erythrocyte extravasation into the alveoli. Nephritic lesions were observed in the kidneys with signs of degeneration, necrosis, and haemorrhage in interstitial spaces associated with inflammatory cell infiltration. The bursa of Fabricius showed moderate to severe lymphoid atrophy characterized by extensive follicular lymphocyte necrosis with more prominent connective tissue.

Indirect immunofluorescence staining of clostridial bacteria. Two out of nine GD birds showed positive immunostaining in the skin and jejunum sections using rabbit anti-clostridial antibody (Figure 2b,c). No staining was seen in the absence of the Clostridium primary antibody (Figure 2a).

Bacterial isolation and PCR confirmation of Clostridium spp. Clostridium-like bacteria were isolated from the skin/muscle samples of four GD-affected birds and from the intestine samples of six GD-like birds. All colonies were morphologically similar to ATCC C. perfringens (#13124) and C. septicum (#12464) reference strains (Manassas, Virginia, USA). Both C. perfringens and C. septicum isolates were verified by PCR after growth on Perfringens agar plates using C. perfringens-specific and C. septicum-specific α-toxin probes (Figure 3a,b). Five out of 10 GD birds showed positive transcript amplification in the skin/muscle tissues, while only one affected bird showed an amplified band in the intestine (data not shown). No clostridial gene was amplified in the tissue samples from clinically healthy chickens.

Serum antibodies to C. perfringens, C. septicum, Eimeria, CAV, IBDV, and reovirus. As shown in Table 2, all sera from GD-affected birds and clinically healthy chickens had high antibody titres against C. perfringens, C. septicum, and Eimeria (3-1E profilin protein). Furthermore, all showed positive antibody titres against CAV and IBDV (data not shown). With the exception of a single serum sample from a GD-affected bird, all samples were negative for reovirus antibodies (data not shown).

Discussion

The present report describes detailed findings on gross pathology, histology, bacteriology, immunological and molecular diagnosis, and serology of a field outbreak of GD. In summary, post-mortem examination of gross lesions showed necrotic enteritis and obvious discolouration of the skin around the breast, abdomen, and wings as well as in the underlying muscle, which was filled with serosanguinous fluid. Histological examination of tissue sections revealed haemorrhagic lesions with degeneration and necrosis of parenchyma cells in the skin, muscle, and intestine. Immunohistological and PCR analyses confirmed the presence of clostridial bacteria in various tissues, and PCR was used to verify the transcripts of Clostridium α-toxin. Serological testing showed that all birds had serum antibody titres against C. perfringens, C. septicum, Eimeria, CAV and IBDV regardless of GD clinical status.

For decades, AGPs and anti-coccidial drugs have been used worldwide in routine management of broiler chicken production. This was necessary to promote animal growth, and reduce avian coccidiosis, but these drugs also exerted anti-clostridial effects and reduced the incidence of outbreaks of clostridial infections, including GD and necrotic enteritis (Williams, 2005; Neumann & Rehberger, 2009; Van Immerseel et al., 2009). However, with growing consumer demands for antibiotic-free poultry products and increasing regulations on the use of in-feed AGPs (Williams, 2005), most non-therapeutic anti-bacterial feed additives have been banned in Europe since 1999 (European Union legislation, Council Regulation 2821/98). As a
result, outbreaks of clostridial infections in commercial poultry farms have recently become more prevalent (Wages & Opengart, 2003a, b; Mataragas et al., 2008). Moreover, recent GD outbreaks in commercial farms where AGPs and anti-coccidial drugs were used necessitate a thorough investigation of host–pathogen interaction to examine the role of various internal and external factors relevant to Clostridium infections (Mataragas et al., 2008).

Both the post-mortem and histopathological findings that we described in this report confirm those from a previous report of C. septicum-associated GD (Willoughby et al., 1996). Furthermore, in some GD-affected birds, severe histopathological signs characteristic of GD and necrotic enteritis were observed in the jejenum and ileum. Recent progress in defining the virulence factors of C. septicum and C. perfringens is contributing to a better understanding of the aetiology and pathogenesis of both of these Clostridium-related diseases. α-Toxin, which is known as the principal virulence factor of C. septicum, is a lethal, necrotizing, membrane pore-forming protein that binds to glycosylphosphatidylinositol-anchored proteins (Huang et al., 2004; Kennedy et al., 2005; Kennedy et al., 2009a, b). C. perfringens type A also produces α-toxin, in addition to a range of other toxins (NetB, β2, enterotoxin) and enzymes that contribute to its virulence (Bueschel et al., 2003; Jost et al., 2005; Williams, 2005; McDevitt et al., 2006; Keyburn et al., 2008; Cooper & Songer, 2009; Martin & Smyth, 2009; Van Immerseel et al., 2009). It is possible that other virulence factors, such as additional hydrolytic enzymes (Shimizu et al., 2006; Myers et al., 2006) and as yet unidentified toxins, may also play a role in the complex pathogenesis of clostridial infections. Moreover, a variety of predisposing factors have been shown to promote clostridial infections in chickens. For instance, intestinal damage due to Eimeria infection, especially E. maxima, was suggested to be a contributing factor to GD, as this was previously shown in necrotic enteritis (Baba et al., 1992; Jackson et al., 2003; Hong et al., 2006; Collier et al., 2008; Park et al., 2008). In other instances, immunosuppressive diseases such as CAV, IBDV and reovirus, were associated with Clostridium infections (Rosenberger et al., 1975; Williams, 2005). In this study, sera from GD-affected chickens as well as clinically healthy chickens all showed high antibody titres against other pathogens such as Eimeria, CAV, and IBDV. This finding indicates a potentially complex aetiology for GD infections. Indeed, it has been reported that host immunosuppression induced by various pathogens poses a significant risk factor for GD in broiler chickens (Ritter, 2006; Hoerr, 2007). Other predisposing factors, such as high flock stocking density, low diet quality, and increased moisture in the litter, have all been suggested to promote field clostridial disease outbreaks (Kaldhusdal et al., 1999; Williams, 2005). Collectively, these findings support a possibility that the outbreak of GD in broiler chickens may occur when both C. septicum and C. perfringens are translocated to remote target tissues (e.g., skin, joint) as a secondary response to the gut damage caused by local infection.

Diagnosis of clostridial infections traditionally involves bacterial isolation and characterization by common bacteriological and biochemical techniques. Since clostridia are anaerobes, these methods are time and labour intensive. Development of sensitive and antigen-specific immunoassays, such as ELISA, will be necessary for the rapid diagnosis of field Clostridium infections. Detection of expressed genes of Clostridium in various tissues from affected birds using molecular biological techniques, such as PCR, offers an efficient and sensitive tool that enables the speciation of Clostridium. Furthermore, development of quantitative real-time PCR for C. perfringens and C. septicum will prove to be even more useful to compare the bacterial loads in the affected tissues of broiler chickens where conventional bacteriological methods fail to isolate fastidious pathogens. Quantitative real-time PCR has the potential to detect as low as 50 fg genomic DNA, or about 20 bacterial cells, in pure cultures of C. perfringens (Wise & Siragusa, 2005; Albini et al., 2008). Furthermore, PCR can be established to type multiple C. perfringens and C. septicum field strains using multilocus sequence typing analysis that would facilitate epidemiological studies of commercial flock infections (Siragusa et al., 2006; Chalmers et al., 2008a, b; Neumann & Rehberger, 2009). However, high-throughput assays for the detection of clostridial field infections are currently not commercially available.

In conclusion, the present report summarizes multiple findings from a GD outbreak in a local broiler farm, and the results suggest a complex host–pathogen interaction. Further studies to identify the role of various factors discussed in this paper on GD pathogenesis will be necessary to develop rational disease control strategies against clostridial infections. Alternative control methods, including avoidance of immunosuppressive diseases, optimization of feed nutrients, utilization of prebiotic and probiotic feed additives, and/or developing novel vaccines, should be explored to minimize the use of in-feed antibiotics (Williams, 2005; Corthesy et al., 2007; Kulkarni et al., 2008; Park et al., 2008; Tsai et al., 2008; Zekarias et al., 2008; Cooper et al., 2009).

Acknowledgements

The present project was supported by a Trust agreement between ARS-USDA and Danisco/Agtech. The authors

<table>
<thead>
<tr>
<th>Serum sample</th>
<th>GD-affected birds&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Healthy birds&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Standard error of difference</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. perfringens</td>
<td>0.521</td>
<td>0.486</td>
<td>0.014</td>
<td>0.015</td>
</tr>
<tr>
<td>C. septicum</td>
<td>0.517</td>
<td>0.505</td>
<td>0.010</td>
<td>0.131</td>
</tr>
<tr>
<td>Eimeria&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.841</td>
<td>0.916</td>
<td>0.088</td>
<td>0.206</td>
</tr>
</tbody>
</table>

All values represent the means with subtracted background OD<sub>450</sub>. <sup>a</sup>Sera were diluted at 1:1600, 1:3200, and 1:6400 for the C. perfringens, C. septicum, and Eimeria antigen detection, respectively, using ELISAs. Each sample was assayed in duplicate. <sup>b</sup>Eight GD-likely birds and five healthy birds were used. <sup>c</sup>Profilin antigen was used for Eimeria.

Table 2. ELISA analysis of the serum antibodies from GD-affected and healthy broiler birds<sup>c</sup>.
thank Marjorie Nichols and Stacy Torreyson for technical assistance, and Dr Erik P. Lillehoj for editorial comments. G.L. was a short-term overseas visiting scholar supported by the China Scholarship Council from Northeast Agricultural University, Harbin, People’s Republic of China.

References


