PATHOGENICITY OF SALMONELLA ENTERITIDIS PHAGE TYPES 6A AND 7 IN EXPERIMENTALLY INFECTED CHICKS

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ABSTRACT

Pathogenicity of Salmonella enterica serovar enteritidis (S.E) phage types (PTs) 6A and 7 were determined in orally inoculated newly hatched specific pathogen free (SPF) chicks. Clinical signs and mortality were observed daily. Body weights, bacterial isolation, gross lesions and histological lesions were recorded on days 1, 3, 5, 7, 14 and 21 post inoculations (pi). Out of 155 newly hatched SPF White Leghorn chicks, five chicks were randomly separated to confirm the SPF status of the chicks before inoculation. The remaining chicks were divided into three sacrificed groups (A, B and C) of 30 chicks each and their respective three mortality groups (MA, MB and MC) of 20 chicks each. Groups A and MA, and groups B and MB were inoculated orally with 0.1mL containing 10^7 cfu of SE PT6A (UPM-0527) and SE PT7 (UPM-0530), respectively. The un-inoculated groups C and MC served as negative controls. Chicks in groups A and B showed clinical signs of depression, anorexia, ruffled feathers, vent pasting and diarrhea starting from day 1 pi. Lifting of wings from thorax was observed in group A and B from day 1 and 5 pi, respectively. There was significant difference (p<0.05) in body weight gain among the inoculated and the control groups on days 14 and 21 pi. Growth index values were 0.035, 0.036 and 0.037 for groups A, B and C, respectively. Mortality of 20% was recorded only in MA group. Goss lesions of unabsorbed yolk, airsaculitis, fibrinous pericarditis, fibrinous perihapatitis, enlarged kidneys, spleenomegaly and dehydration were observed in about 15% of sacrificed chicks in group A and 10% in group B. Mild to moderate lesions were observed under microscope. Salmonella was isolated from the cultured samples of group A and B throughout the experiment period with the individual variation of chicks and samples. It was concluded that newly hatched SPF chicks are susceptible to PT6A and PT7 infections. These SE PTs are mild to moderately pathogenic for SPF chicks. SE PT6A is more pathogenic than SE PT7.

Key words: Salmonella Enteritidis, SE PT6A, SE PT7, SPF chicks, colony forming units, pathogenicity.

INTRODUCTION

Salmonella is a genus of Gram-negative, non-spore forming and rod shape bacteria that can cause infections in humans and a wide range of animal species. Presently, there are more than 2500 serotypes which can be divided into two major groups, the host adopted and the wide-host range serotypes on the basis of pathogenesis and host adaptability. The host adopted serotypes have specific hosts and only cause systemic diseases in their respective hosts. In contrast, the wide-host range serotypes infect humans and a variety of animal species. This group is the principle concern of food-borne diseases in humans (Uzzau et al., 2000; Barrow and Wallis, 2000; Adams and Moss, 2008). Among the wide-host range serotypes, Salmonella enterica serovar enteritidis (SE) and Salmonella enterica serovar typhimurium (ST) are most prevalent worldwide (Galanis et al., 2006; EFSA, 2007). However, recently SE has replaced ST as a primary etiologic agent of Salmonella infections as well as the leading serotype responsible for food-borne human salmonellosis in most parts of the world (Fisher, 2004; Patrick et al., 2004; CDC, 2006; Galanis et al., 2006).

There is epidemiological connection between poultry and humans as SE phage types (PTs) commonly present in poultry have also been isolated from humans in a geographical location (Van Duijkeren et al., 2004; Akhtar et al., 2010). SE has been frequently isolated from broilers and layers in Europe and has been the most common serotype in humans. It has been reported in layers (58%), layer breeders (63%), broiler breeders (42%) and table eggs (72.9%) in Europe (EC, 2004; EU, 2007a; b) and accounted for 85% of Salmonella cases in the same region (Galanis et al., 2006).

The SE infection in poultry and its outcome depends upon so many factors related to pathogen, host and environment. Among the pathogen factors, SE PT itself is one of the factors that affect the virulence and pathogenicity of SE infections in chicken. Different PTs are prevalent in different parts of world (van Duijkeren et al., 2002; Fisher 2004; Pang et al., 2007) and also they vary for their virulence and pathogenicity (Shivaprasad et al., 1990; Barrow 1991; Poppe et al., 1993; Alisantosa et al., 2000; Ahmad et al., 2011; Akhtar et al., 2011). Mostly, SE infection in poultry is symptomless and un-
noticed but can cause clinical disease and mortality under certain circumstances such as newly hatched chicks and under stress conditions (Barrow and Wallis, 2000; Gast, 2003). In newly hatched chicken SE can cause severe morbidity and high mortality whereas the older chicken may remain symptomless even with intestinal colonization and systemic dissemination (Barrow, 1991; Desmidt et al., 1997).

Few reports on the pathogenicity of SE infection in chickens have been published in which pathogenicity has been assessed on the basis of abnormal clinical signs, mortality, effects on growth and production of host, bacterial isolation, gross and microscopic lesions in various organs of the host (Shivaprasad et al., 1990; Barrow 1991; Gast and Benson 1995; Alisantosa et al., 2000; Dhillon et al., 2001; Ahmad et al., 2011; Akhtar et al., 2011). To our knowledge, no study on the pathogenicity of SE PT6A and PT7 has been published before. Although, SE PT6A and PT7 have been isolated from chicken and humans (Dominguez et al., 2002; dos Santos et al., 2003; Pang et al., 2005; Wales et al., 2007). The outbreaks in humans caused by SE PT6A and SE PT7 have also been reported (O’Brien 2002). SE PT7 has been reported as one of the main PTs consistently present in British poultry (Gillespie and Elson, 2005) and recently, has been isolated from commercial layer flocks in UK (Wales et al., 2007). This study was conducted to determine the pathogenicity of SE PT6A and PT7 isolates of Malaysia in newly hatched SPF chicks. The pathogenicity was determined on the basis of clinical signs of disease, mortality rate, body weight gain, bacterial isolation, and gross and microscopic lesions.

**MATERIALS AND METHODS**

**Experimental animals and pathogenicity determination:** Newly hatched SPF White Leghorn chicks (n=155) obtained from Malaysia Vaccine and Pharmaceutical Sdn Bhd (MVP) were brought to Experimental House, Faculty of Veterinary Medicine (FVM), Universiti Putra Malaysia (UPM) Serdang, Malaysia. Five chicks were randomly separated to confirm the SPF status of chicks. These chicks were individually weighed and killed by cervical dislocation after taking cloacal swab and blood for bacteriology. Necropsy was performed to observe gross lesions and collect samples of liver, spleen, caecal tonsils, mid-gut and caecal contents for bacteriology. Furthermore, tissue samples of liver, spleen, ileum, caeca, caecal tonsils and bursa of Fabricius were collected for histopathology (Ahmad et al., 2008).

**Isolates and their phage typing:** In 2005, SE was isolated from different samples collected from commercial chickens in different parts of Malaysia. The PTs of isolates were determined at the Laboratory of Enteric Pathogens Centre for Infectious Institute, 61 Collindale Avenue, London, United Kingdom. The PTs were designated and stocked at Pathology Laboratory, FVM, UPM for further research. The SE PT6A (UPM-0527) SE PT7 (UPM-0530) were isolated from liver of the apparently healthy commercial broiler chicken in Johor, Malaysia. The frozen bacterial stock were cultured on nutrient agar (Oxid, UK) and incubated at 37°C for 24 hours. The same procedure was repeated to refresh the culture. The refreshed isolates from Nutrient Agar were identified and confirmed. The confirmed isolates were then cultured onto blood agar (Oxid, UK) and incubated at 37°C for 24 hours. Fresh inoculums of 10^5 cfu/mL were prepared according to Mc Farland standard in normal saline to inoculate 0.1mL /chick (Ahmad et al., 2008).

**Experimental design:** The newly hatched SPF chicks were divided into six groups. Three sacrificed groups A, B and C of 30 chicks each served to study the clinical signs, body weight gain, bacteriology and pathology. Whereas, the three mortality groups MA, MB and MC of 20 chicks each were monitored for mortality. The chicks in groups A and MA, and groups B and MB were inoculated orally with 0.1mL containing 10^8 cfu of SE PT6A (UPM-0527) and SE PT7 (UPM-0530), respectively. The un-inoculated groups C and MC served as negative controls. The chicks in groups A and MA, B and MB and, C and MC were kept separately in 3 separate rooms and each group in a separate cage. The chicks were provided with fresh water and antibiotic free feed *ad libitum* throughout the trial. The chicks were monitored at least twice daily for clinical signs and mortality. In case of mortality in sacrificed or mortality group, chicks were necropsied and examined for gross lesions and collection of samples for bacteriology and histopathology similar as described below for the sacrificed groups. On days 1, 3, 5, 7, 14 and 21 post inoculations (pi), five chicks were taken randomly from each group A, B and C. The chicks were weighed. After taking cloacal swab and blood samples for bacteriology the chicks were sacrificed humanely by cervical dislocation and necropsy was performed to observe gross lesions and collection of samples for bacteriology and histopathology (Table 1).

**Clinical signs, mortality and body weight:** The clinical signs of disease and mortality were observed in all the six groups at least twice daily and were recorded if any. However, the mortality in MA, MB and MC groups was considered to assess the mortality rate. The chicks in sacrificed groups A, B and C were weighed individually before sacrificed on days 1, 3, 5, 7, 14 and 21 pi.

**Bacteriology:** The samples from cloacal swabs, blood, liver, spleen, caecal contents, caecal tonsils and mid-gut contents were collected from each chick (of sacrificed
groups A, B and C) individually for bacterial isolation and identification. The samples were individually collected in Rappapart Vassi dias (Oxid, UK) and incubated at 37°C for 18-24 hours. Then were streaked onto Brilliant Green Agar (BGA) and Xylose-Lysine-Desoxycholate Agar (XLD) and incubated at 37°C for 24 hours. Suspected black colonies from XLD and pink colonies from BGA were further inoculated for biochemical characterization by Triple Sugar Iron (TSI) and urease test. The TSI positive and urease test negative samples were considered positive for SE (Ahmad et al., 2008).

**Histopathology:** The tissue samples from liver, spleen, bursa of Fabricius, ilium, caecal tonsil and caecum were aseptically collected from every chick in a bottle containing 10 % buffered formalin and kept for 24-36 hours for fixation. Tissues were further processed using standard histological techniques (Islam et al., 2006). Briefly, the tissues were trimmed post fixation with scalpel blade into about 5mm pieces and were placed into embedding cassettes. The tissues were processed over night. For processing, the embedding cassettes containing tissues were placed into an automatic tissue processor (Leica, ASP300, Germany) for dehydration in a graded alcohol (30 %, 40 %, 50 %, 70 %, 90 %, and 100 %) and then cleared by two changes of 100% xylene. Then, the tissue samples were embedded in paraffin wax using semi-automatic tissue embedder (Leica EG1160, Germany). The paraffin embedded tissues were then trimmed by microtome (Leica RM2155, Germany) at the thickness of 15um followed by 5um. After trimming the tissues were sectioned at a thickness of 4um. The ribbons of sectioned tissue were floated on the surface of warm water at 56°C in a thermostatically controlled water bath (Leica H1220, Germany). The tissue sections were taken on a glass slide from water and allowed to dry for 15-20 minutes by placing the slides in vertical position on a simple wooden rack. After drying the slides were placed on a hot plate (the tissue section not touching the plate) at 57°C for 10-15 minutes for adhesion of the tissue and the de-waxing. The slides were labeled and stained using Haematoxylin and Eosin (HE). After staining, the slides were allowed to dry over night. Then cover slips were fixed with DPX and slides were examined under the light microscope.

**Statistical analysis:** The data was analysed using Tukey’s (HSD) pairwise multiple comparison procedure to determine the nature of significant effects on body weight gains (Daniel, 1991). The growth index for all the three groups was calculated as described by Waldbauer (1968).

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**RESULTS AND DISCUSSION**

**Clinical signs:** No abnormal clinical signs of disease were observed in chicks before inoculation and in the control group (C) throughout the experiment. About 15-20% of the chicks inoculated with SE PT7 and SE PT6A and the 100% chicks those died during experiment showed the clinical signs from day 1 pi. The chicks inoculated with SE PT6A and SE PT7 showed the abnormal clinical signs of depression, anorexia, ruffled feather, vent pasting, diarrhea and lifting of wings from thorax with respiratory distress. The inability to move was observed in SE PT6A inoculated chicks on days 5, 7 and 14 pi, but only on day 5pi in SE PT7 inoculated chicks (Figure 1). There was variation in different clinical signs and the severity during the experiment period (Table 2). The clinical signs similar to the present study have been reported previously (Shivaprasad et al., 1990; Barrow, 1991; Poppe et al., 1993; Desmidt et al., 1997, Akhtar et al., 2011), but with variation in severity and expression time pi. SE PT4 caused clinical signs in young chicks (Poppe et al., 1993; Desmidt et al., 1997), but not in adult chicks (Desmidt et al., 1997). It is not necessary that every PT may cause clinical signs even in young chicks. In contrast to our study no clinical signs were observed in day old chicks challenged with SE PT8 and PT13 (Poppe et al., 1993). However, Shivaprasad et al., (1990) has observed the clinical signs in 18 months hens orally inoculated with SE PT8. The expression, severity or even the absence of clinical signs depends upon so many factors. The pathogen related factors such as the PTs, isolates, dose rates and route of inoculation, and host related factors such as age, breed, health and immune status of host (Barrow, 1991; Poppe et al., 1993; Desmidt et al., 1997; Ahmad et al., 2008; Akhtar et al., 2011). The difference in clinical signs in this study and previous studies may be due the pathogen and host related factors.

**Mortality:** The 20% (4/20) mortality was observed only in the SE PT6A mortality group (MA). One chick died each time at days 3, 9, 10 and 14 pi. No mortality was observed in un-inoculated controls (C and MC) and SE PT7 inoculated mortality group (MB) throughout the experiment period. Similar mortality of 20% with SE PT 13A inoculated orally at dose rate of 10^5 cfu (Barrow, 1991) and 21% at dose rate of 10^7 Gorham et al (1994) in young chicks has been reported. The high mortality rates of 96% with SE PT4, 55% with SE PT6 and 60% with SE PT8 were reported in 1-day-old chicks (Barrow, 1991). The low mortality rates such as 10% by SE PT3A, 5% by PT35 (Akhtar et al., 2011) and 8% by SE PT5 (Dhillon et al., 2001) have been reported in previous studies. It has been reported that even SE PT4 infections causing abnormal clinical signs of disease may not necessarily cause death in chicks (Shivaprasad et al., 1990; Eigaard et al., 2006).
The difference in mortality rates in the present study and previous studies may be due to the different PTs used for the studies and the dose of inoculums as different PTs of SE and even same PTs vary in their virulence (Shivaprasad et al., 1990; Barow 1991; Poppe et al., 1993; Gast and Benson 1995; Alisantosa et al., 2000; Dhillon et al., 2001; Akhtar et al., 2011).

**Body weight gain:** There was no significant difference (p>0.05) in weight gain among the chicks inoculated with SE PT6A (Group A), SE PT7 (Group B) and the un-inoculated control group (Group C) till day 7 pi. The adverse significant (p<0.05) effect of infection was observed on days 14 pi and 21 pi. The weight gain was more adversely affected by SE PT6A infection as compared to SE PT7 infection (Table 3). Control group (C) showed the highest growth index (0.037) among the three sacrificed groups followed by groups B (0.036) and A (0.035). SE infections could have adverse affects on body weight gain of infected chicks which depends upon PTs and time pi (Gorham et al., 1994; Dhillon et al., 1999; Alisantosa et al., 2000; Eigarrd et al., 2006). The present study indicated that weight gain was reduced even though no mortality was recorded in chicks inoculated with SE PT7. Alisantosa et al., (2000) reported the similar findings in chicks inoculated with SE PT8. The results of the present study suggest the presence of sub-lethal infection that may lead to poor growth and production.

**Bacteriology:** No *Salmonella* was isolated from chicks before inoculation and the control group. SE was isolated from all the cultured samples from dead chicks. There was individual variation for the isolation of *Salmonella* from chicks and different samples from sacrificed groups A and B. The overall isolation of SE was higher for group A (SE PT6A) as compared to group B (SE PT7) (Table 4). Similar to the present study, isolation of *Salmonella* has been reported from different samples and tissues with the variation in isolation (Dhillon et al., 1999; Alisantosa et al., 2000; Akhtar et al., 2011). However, these researches have conducted studies on different SE PTs. The present organ colonization by SE not in every challenged bird is in conformity with the previous studies (Duche-Suchaux et al., 1995; Asheg et al., 2001). Detection of SE in blood, liver and spleen in sacrificed SE inoculated chicks confirmed the systemic infection, whereas in dead chicks supported that death is originated from Salmonellosis (Duche-Suchaux et al., 1995). SE has been reported a better invaders of the intestine than the other serotypes of *Salmonella* (Aabo et al., 2002). However, SE can invade and localize in different organs (Alisantosa et al., 2000; Akhtar et al., 2011). Bailey (1987) has reported several factors that affect the susceptibility of chickens to *Salmonella* colonization. Genetically, *Salmonella* Pathogenicity Islands (SPI) SPI-1 and SPI-2 genes are the most important SPI required for the colonization of the intestinal tract, liver, and spleen (Rychlik et al., 2009). The results of bacteriology revealed that silent carriers infected with SE PT6A and SE PT7 may spread infection to other flocks and may become serious threat for public health.

**Gross lesions:** Unabsorbed yolk, airsaculitis, fibrinous heart, enlarged and fibrous liver, enlarged kidneys, spleenitis and dehydration were observed in 15% and 10% of the chicks infected with SE PT6A and SE PT7, respectively. Almost all the gross lesions were observed in dead chicks (Figure 2). The results of gross lesions indicated that SE PT6A is more invasive than SE PT7 which may be assigned to the pathogenicity of PTs. Similar lesions have been observed with variation in severity, number of chicks and organs showing lesions in previous studies conducted with SE PTs 4, 8 and 23 (Alisantosa et al., 2000). We have also recorded 10% gross lesions in another experiment conducted on PTs other than the studied in present trial (Akhtar et al., 2011). The gross lesions present in chicks that died during study suggest the septicemia that caused fatal avian SE infections (Gorham et al., 1994).

**Histopathology:** Lesions were observed in both the inoculated groups A and B with the variation in severity of lesions and variation in number of tissues showing lesions. However, lesions were not observed in every examined tissue.

In ileum, inflammation with the cellular infiltration, congestion, degeneration and reactive goblet cells were observed from day 3pi in group A and from day 5pi in group B. Sloughing of enterocytes into intestinal lumen, bacterial infiltration and attachment to the lining of cells and colonization was also observed in group A on day 21pi. Caecum and caecal tonsils showed mild inflammation, congestion, degenerative changes and reactive goblet cells from day 5pi and afterwards in both the groups A and B. In bursa of Fabricius, both the inoculated groups A and B showed lesions from day 1pi and afterwards. Mild to moderate congestion, depletion of lymphocytes, cellular infiltration and necrosis was observed. Chronic bursitis was also observed with follicular atrophy and cystic formation. Liver showed degeneration, fatty changes and multifocal necrosis in hepatocytes in both A and B groups throughout the experiment. Spleen showed mild to moderate inflammation, congestion, cellular degeneration and necrosis from day 1 pi in group A and from day 3 pi in group B and afterwards. Inflammation, congestion and degeneration were most common, however, necrosis was not observed in every tissue that showed lesions.

There was variation of lesions among the different organs of a group. More severe lesions were observed in group A as compared to group B. The sloughing of superficial layers of villi reveals the damage to the integrity of intestinal epithelium that results into
translocation of bacteria to other tissues. These morphological changes are triggered by proteins secreted by Salmonella into the cytosol of the epithelial cells via type III secretion system (TTSS) encoded by genes of the Salmonella pathogenicity island-1 (SPI-1) (Zhou and Galan, 2001). Several regulatory proteins that are involved in Salmonella invasion have been characterized (Lucas and Lee, 2000). It has been reported that after oral inoculation, SE not only colonize and invade the intestine but also invade the other organs including liver and spleen and results in lesions in invaded tissues (Alisantosa et al., 2000; Ahmad et al., 2008; Akhtar et al., 2011). Potential of SE PTs to invade different organs and severity of lesions may explain their respective pathogenicity. Inflammatory reactions were witnessed by the presence of inflammatory cells (Henderson et al. 1999). The similar microscopic lesions have been reported previously but for SE PTs other than used in the present study (Gorham et al., 1994; Dhillon et al., 1999; Alisantosa et al., 2000). Also we have reported the similar lesions in our previous experiments (Akhtar et al., 2011).

**Conclusion:** It is concluded that newly hatched chicks are susceptible to SEPT6A and SEPT7 infections. SE infections may or may not cause clinical disease and mortality. SE infection may cause direct economical losses by mortality and poor growth. Silent carriers are a serious threat that may infect chicks and humans. SE PT6A is more pathogenic than SE PT7.

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