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Original Article

Pathogenic potentials and shedding probability of *Salmonella enterica* serotype Kentucky in experimentally infected backyard chicken

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ABSTRACT

Objective: *Salmonella* is a widely-reported zoonotic bacterial pathogen and human infection is mostly attributed through direct or indirect contact with chickens. *Salmonella Kentucky* (*S. Kentucky*) is one of the motile serovars which has recently been identified from both poultry and human samples in Bangladesh. This study was conducted to assess its pathogenic potentials and shedding probability in backyard chicken.

Materials and methods: We infected 22 backyard chickens orally, each with 10^6 cfu of *Salmonella Kentuckey*, which were then observed for 23 days to enlist clinical signs, gross and histo-pathological changes. Polymerase chain reaction (PCR) for *Salmonella* was applied on some representative samples to identify the presence of *Salmonella*.

Results: Four chickens were sacrificed and the internal organs were examined to observe gross and microscopic tissue changes. Some reactive changes were seen in spleen during prolonged course of infection. The probability of *S. Kentucky* shedding was 77% (95%; CI 54-90%) on DPI 2, 41% (95%; CI 21-60%) on DPI 12 and 13% (95%; CI 3-31%) on DPI 21. The survival probability of the infected chickens was 50% (95%; CI 28-68%) on DPI 6, 32% (95%; CI 14-51%) on DPI 15 and 14% (95%; CI 3-31%) on DPI 23.

Conclusion: Zoonotic *S. Kentucky* strain of human non-typhoidal clinical cases of gastroenteritis has potentials to produce clinical signs such as reduced feed uptake, watery or pasty fecal droppings and lesions, such as catarrhal enteritis and typhlitis.

KEYWORDS

Backyard chicken; Histopathology; Infection study; Pathogenicity

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INTRODUCTION

Salmonella is one of the most important zoonotic pathogens of the world and one of its major sources is poultry. Certain serovars of *Salmonella* can cause infections in poultry, resulting in substantial economic losses through mortality and reduced production (Talha et al., 2001; Haider et al., 2004). *Salmonella enterica* serotype Kentucky (*S. Kentucky*) represents one of the non-typhoidal types of *Salmonella* that microbiologists and public health professionals encountered from time to time. *Salmonella enterica* serovar Kentucky (antigenic formula 8, 20:I:Z_6 is a serovar of the 0:8 (C_2:C_3). This serovar is commonly found in animals in the US (chicken, turkeys and cows), but rarely reported in human cases. It is the most common serovar identified in non-clinical non-human sources (CDC, 2011). Serovar kentucky is typically found in cattle and poultry. It is widely distributed. In the USA, it is commonly found in animals (specifically in cattle and poultry) and in meat. It has been identified both from layer (Li et al., 2007; Bouzidi et al., 2011) and broiler chickens. It was isolated from 25% poultry farms in 1997 in the USA, and nearly from 50% farms in 2006 (USDA, 2006).

In Australia, more than 12,000 cases of *Salmonella* infection were reported in 2010; five of them were related to *S. Kentucky*. Recently, a particular clone of *S. Kentucky* acquiring a virulence plasmid from avian pathogenic *Escherichia coli* (APEC) has been reported (Johnson et al., 2010). *S. Kentucky* has recently been identified from poultry and humans in Bangladesh and the human isolates from non-typhoidal clinical cases were genetically closely related to those from poultry (Barua et al., 2012).

Little work has been done to understand the biology of *S. Kentucky* in the avian host. In a comprehensive study, *S. Kentucky* was compared to other serovars for the presence of known virulence genes, invasiveness in chicken embryo hepatocytes, growth in laboratory media, biofilm formation, stress response, and pH response. However, its pathogenic potential and shedding probability and duration of shedding from infected/colonized chickens have never been reported in Bangladesh. The present study was carried out to determine the duration of shedding of non-typhoidal *S. Kentucky* originated from feces of human cases following experimental infection in backyard chickens. In this study, we also observed the pathogenic potentials of the said zoonotic pathogen in backyard chickens. Furthermore, we aimed to estimate the survivability, one of the measures of pathogenic potentials of the *S. Kentucky* strain in the experimentally infected backyard chickens and to explore the colonizing potential of *S. Kentucky* strain in various internal organs.

MATERIALS AND METHODS

Ethical approval: The experiment was carried out according to the Institutional Animal Ethics Committee (approval number CVASU/IAEC/27).

Description of the experimental chickens: Thirty-four externally healthy backyard chickens (3 males and 31 females), purchased from a local market were used as experimental chickens. They were different in plumage color and their average body weight was 700 gm to 2200 gm and was identified by their leg bands as G1 to G25 (for green-colored leg bands) and R26 to R34 (for red colored leg bands).

Management of the chickens: Antibiotics free locally available commercial feed (C.P. Bangladesh Co., Ltd) for 19-50 weeks layer chickens were provided to the birds. The crude ingredients of the feed were corn, soybean, rice polish and lysine that contained 15% crude protein, 4% calcium, 4% fat, 12% moisture and 2750 KCal/Kg energy (according to manufactuer). The feed and bacteria-free water were supplied to the chickens *ad-libitum*. Vitamin B complex (B com-vit®) at 1 mL/L, and vitamin A, D, E (Renasol AD_3E®) at 1 mL/4 L, calcium, phosphorus, cion, vitamin B_{12}, xylanase, phytase, lipase (Avical®) at 2 mL/L of drinking water were added as and when needed having consulted with a registered veterinarian. The vaccination history of the purchased chickens was unknown and no vaccines were administered before the infection given or following the infection. The chickens were kept for 7 days on the above ration and nutritional supplementations for acclimatization.

Screening chickens for presence of any motile *Salmonella*: Fecal samples from each chicken were collected before distributed into experimental and control groups directly from the cloaca by using sterilized cotton swabs which were thereafter kept in buffered peptone water for enrichment, and incubated at 37ºC for 18 h in laboratory. After enrichment, the culture was inoculated onto the surface of novobiocin added Modified Semisolid Rappaport Vassiliadis (MSRV) media, a selective medium for motile *Salmonella* which was prepared according to the manufacturer's instructions. Having inoculated, the MSRV plates were incubated at 41.5ºC for 24 h. In absence of a swarming turbid growth from the centre of inoculation, it was considered negative for the presence of any motile *Salmonella* serovar.

Experimental and control groups: *Salmonella* negative chickens were randomly divided into two groups-experimental group and a control, comprising 22 and 7 chickens, respectively, were housed separately by a fence into two pens on the same farm and managed on the same ration. All the chickens had free access to bacteria-free water.

***S. Kentucky* isolate used for the study:** Some motile *Salmonella* strains of human non-typhoidal clinical cases were provided by International Center for Diarrheal Disease Research, Bangladesh (icddr,b) for a previous study which were subsequently serotyped (Barua et al., 2012), characterized by PFGE and compared with those of poultry isolates from Bangladesh (Barua et al., 2012). An isolate of human non-typhoidal case origin showing close genetic relatedness to those of poultry origin was selected for the present study which was retrieved from the *Salmonella* repository at the Department of Microbiology and Veterinary Public Health, Chittagong Veterinary and Animal Sciences University (CVASU), Bangladesh. The strain was re-cultured on blood agar and tested using anti-*Salmonella* polyvalent serum produced by the Statens Serum Institute (SSI), Copenhagen, Denmark.

Procedures of giving infection and period of observation: Each experimental chicken was infected orally with 1 mL of inoculum containing 10^6 CFU of *S. Kentucky* by a 1 mL insulin syringe. Each control chicken was administered orally with 1 mL of the same sterile medium used for culturing *S. Kentucky* for making infection inoculum. Following infections all the experimental and control chickens were observed for a period of 23 days for shedding of motile *Salmonella* in feces.

Screening *Salmonella* after infection: After giving infection, the chickens were observed daily initially for clinical signs, morbidity, mortality, fecal changes, feed and water intake etc. Cloacal swabs were collected daily first few days, then twice a week for the isolation of motile *Salmonella*. A collected cloacal swab was then cultivated into buffered peptone water for 18 h at 37°C. Then the culture was inoculated onto MSRV supplemented with novobiocin, and incubated at 41.5°C for 24 h. Straw colored colonies at the site of inoculation surrounded by white or grey hollow zone indicated a positive result. From such growth onto MSRV was streaked onto Brilliant Green Agar (BGA) and incubated at 37°C. Growth of pink colored colony on BGA indicated the presence of motile *Salmonella*. Such colonies on BGA were tested for agglutination reaction with anti-*Salmonella* polyvalent serum produced by SSI. Positive result was encountered by clumping within 2 min. For preservation culture from BGA 2-3 colonies were inoculated into peptone water, incubated at 37°C for 24 h and for each time 300 µL of the broth-culture was preserved with 15% buffered glycerol and kept at -80°C. Some of the selected cultures were further identified by PCR using *Salmonella* specific primers. The sequences of the primer set used to detect *Salmonella* were (F) 5´-AGC CAA CCA TTG CTA AAT TG-3´ and (R) 5´-GGT AGA AAT TCC CAG CGG GTA CTG-3´, published elsewhere for the identification of *Salmonella*.

Clinical pictures, gross and histopathology: The clinical signs following infections were noted for each infected chicken. On day 2 post infection (DPI 2), 2 infected chickens (G2, G25) showing some clinical signs and 2 control chickens (G11, G15) were sacrificed showing no clinical signs. Additional two–from infected group, one diagnosed persistently and the other intermittently with *S. Kentucky* were also sacrificed on DPI 15. At DPI 15, 2 infected chickens (G23, R33) positive with motile *Salmonella* (G23, R33) were sacrificed. On DPI 23 all of the survived chickens were sacrificed. The dead and sacrificed chickens were thoroughly examined by postmortem examination to observe gross lesions in different organs. Inoculums from liver, spleen, cecal tonsils and cecal contents of the dead or sacrificed chickens were examined by bacteriological tests as in broth and culture for the colonization of motile *Salmonella*. Furthermore, tissue samples from liver, spleen, caeca and cecal tonsils were collected for histopathology.

Liver, lungs, spleen, intestine were collected in 10% neutral buffered formalin and kept for 3-5 days. The tissues were trimmed into thin sections and washed over night in running tape water to remove formalin. They were dehydrated in 50, 70, 80, 95, 100, 100 and 100% ethanol for 1 h to prevent shrinkage of cells. The tissues were cleaned in chloroform for 3 h to remove ethanol (two changes; 1.5 h in each) followed by impregnation in melted paraffin (56-60°C) for 3 h. Then the tissues were sectioned with a microtome at 5-µm thickness. A small amount of gelatin was added to the water bath for better adhesion of the section to the slide. The sections were allowed to spread on warm water bath at 40-42°C. Then the sections were taken on grease free clear slides. The slides containing section were air dried and kept in cool place until staining**.** Sections from all the collected organs were stained according to routine hematoxylin and eosin staining procedure, as described by Fischer et al. (2008).

Statistical analysis: All data were entered into a spreadsheet (Excel, 2003, Microsoft Corporation) and transferred to STATA (Intercooled STATA 9.2) (STATA Corporation, Texas, USA) statistical software for data management and analysis. Kaplan-Meier curves were constructed by plotting the duration of shedding of *S. Kentucky* in cloacal samples of each infected chicken.

RESULTS

Out of 22 infected chickens *S. Kentucky* was recovered only from 15 fecal samples. Twelve chickens shed the organism (*Salmonella Kentucky)* persistently while the other three were shed intermittently *Salmonella* positive. The durations of *S. Kentucky* shedding in feces from the infected chickens and their ultimate fates are shown in **Table 1**. Regardless of shedding nature-persistent or intermittent, the last day at which fecal sample from a chicken diagnosed positive with *S. Kentucky* was considered its total shedding time. Overall, in this experiment 138 chicken-days at risk were observed and the probability of *S. Kentucky* shedding was 77% (95%; CI 54-90%) on DPI 2, 41% (95%; CI 21-60%) on DPI 12 and 13% (95%; CI 3-31%) on DPI 21 (**Figure 2**). The shedding probability of *S. Kentucky* in the feces of control chickens were 0%.

Until the end of the observation only three chickens remained alive, four were sacrificed and 15 died. The survival probability of the infected chickens was 50% (95%; CI 28-68%) on DPI 6, 32% (95%; CI 14-51%) on DPI 15 and 14% (95%; CI 3-31%) on DPI 23 (**Figure 3**). The survival probability of the control chickens were 100%.

Clinico-pathological findings of the *S. Kentucky* infected backyard chickens are summarized in **Table 2**. Reduced-feeding was observed in the 68% infected chickens and 4 chicks stopped feeding -of them 3 died on DPI 2. In the first week of infection, particularly in the first two days, watery or pasty fecal droppings were seen in the 55% infected chickens. Splenomegaly was observed in four chickens, but the predominant gross change was catarrhal enteritis (36%) followed by typhlitis (32%). No clinical signs were observed in the control chickens. Visceral organs were noticed normal in appearance after postmortem of control chickens.

No abnormal histopathological changes were observed in liver, spleen and cecal tonsils from two chickens sacrificed on DPI 2 (**Figure 4-5**). However, one of the two chickens sacrificed on DPI 15 revealed a reactive spleen with a marked increase in lymphatic follicles. Infiltration of mononuclear macrophages and plasma cells in primary lymphatic follicles in the spleen and caseous nodules in the caecal tonsil sections were also seen (**Figure 4**). The other bird sacrificed on DPI 15 showed hemorrhages in the submucosa of caecal tonsil and fatty changes in liver No abnormal gross or histopathological changes were observed in the sacrificed control birds. PCR positive results from some representative samples are displayed in **Figure 1**. The size of PCR amplicon of each *S. Kentucky* isolate was 429-bp.

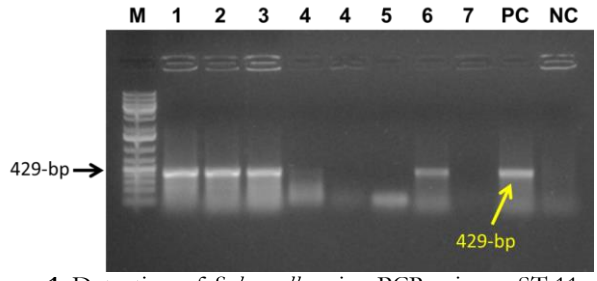

Figure 1. Detection of *Salmonella* using PCR primers ST 11 and ST 15. Lane M: 100-bp, Lanes 1-7 test samples, Lane PC=positve control, Lane NC=negative control. PCR products from preserved samples of experimentally infected chickens.

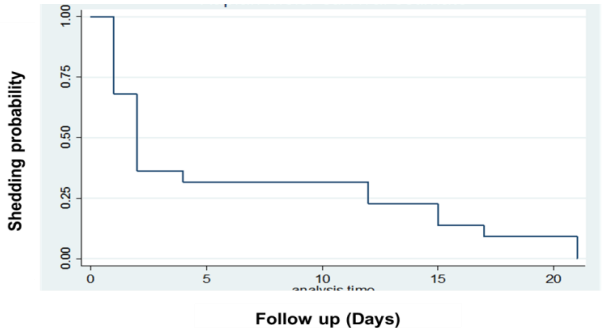

Figure 2. Shedding probability of backyard chickens infected with *S. Kentucky*

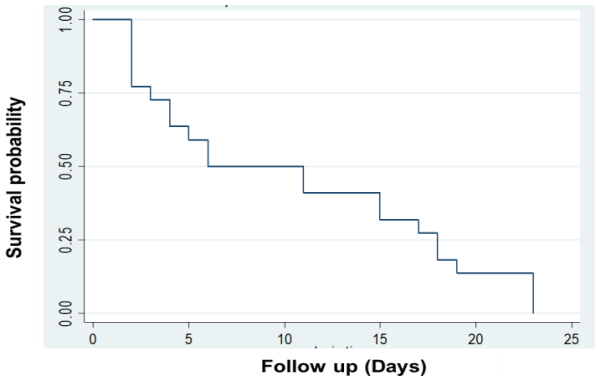

Figure 3. Survival probability of backyard chickens infected with *S. Kentucky*

Table 1. Duration and nature of *S*. Kentucky persistency in feces and internal organs of the infected backyard chickens

Chicken leg band ID	Last day of *Salmonella* isolation	Nature of isolation	Outcome (on DPI)	*Salmonella* culture positive from
G1	2	Con	D(5)	-
G2	2	Con	**S(2)**	CT, SP
G3	1	-	D(4)	LI
G4	-	-	D(11)	-
G5	2	Con	D(6)	-
G6	2	Con	D(2)	CT, SP
G7	-	-	D(11)	CT
G8	-	-	D(2)	-
G16	12	Con	D(19)	-
G18	12	Con	A	-
G19	21	Int	A	-
G20	2	Con	D(2)	LI, SP
G21	2	Con	D(6)	LI, CT
G22	-	-	D(3)	-
G23	15	Int	**S(15)**	CT, In
G25	2	Con	**S(2)**	CT
R28	17	Con	D(17)	SP,LI
R29	-	-	D(18)	CT
R30	21	Int	A	-
R31	-	-	D(18)	Li
R33	15	Con	**S(15)**	CT, Int
R34	4	Con	D(4)	CT, Li,SP

A=Alive; Con=Continuously; CT=Cecal tonsil; D=Dead; DPI=Day post infection; I=Intermittently; In=Intestine; Li=Liver; Lu=Lungs; S=Sacrificed; SP=Spleen

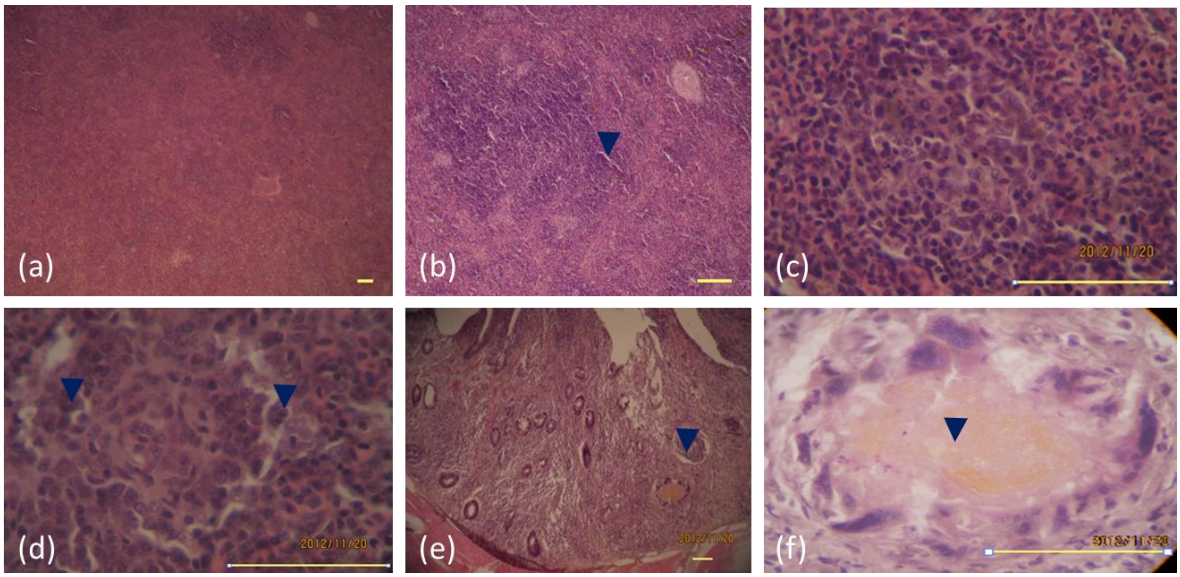

Figure 4. Histopathological changes observed in spleen and caecal tonsil of an *S*. Kentucky infected backyard chicken. (a) A section of spleen of an *S*. Kentucky infected chicken showing reactive spleen with a marked increase in lymphatic follicles, (b) A closer view of a, (c) Infiltration of mononuclear macrophages and plasma cells in primary lymphatic follicles, large foamy lymphoblasts and numerous plasma cells in germinal centers of spleen, (d) A closure view of c, (E) Cecal tonsil of an *S*. Kentucky infected chicken showing caseous nodules (arrow-marked) in the sub mucosa and depletion of lymphocytes, (f) A closer view of the caseous nodule shown in (e).

DISCUSSION

The results of this study indicate that *S. Kentucky* of human non-typhoidal clinical case origin in Bangladesh might produce clinical signs, such as off-feeding, watery and pasty feces, green colored faeces (**Table 2**) in backyard chickens. The zoonotic isolate of *S. Kentucky* might also colonize in liver, spleen, cecal tonsil and intestine of the infected chickens with variable intensities. The survivability of the infected chickens at 23 DPI was 14% (**Figure 3**), suggesting that ~10% *S. Kentucky* infected backyard chickens might be chronically colonized/infected to shed the organism through feces, thus contaminating eggs and the environment.

Table 2. Clinico-pathological findings in the backyard chickens infected with *S. Kentucky*

Feature	Occurrence frequency	Comments
Clinical signs (n=22)		
Watery or pasty feces	12/22	Seen early in the infection
Green-colored diarrheic feces	1/22	Seen persistently
Off-feeding	4/22	In the first 2 days
Reduced feeding	15/22	Seen early in the infection
Closed eyes	1/22	Seen before death
Watery oral discharge	1/22	
Death	15/22	Occurred between 2-19 days
Gross changes (n=22)		
Catarrhal enteritis	8/22	-
Typhlitis	7/22	-
Congested spleen or splenomegaly	4/22	-
Congested or bronze-colored liver	4/22	-
Thickened intestinal wall	3/22	-
Hemorrhagic enteritis	2/22	-
Empty crop	2/22	-
Glandular hemorrhages in proventriculus	2/22	-
Others: each with 1 frequency	-	Included necrotic enteritis, thickened proventriculous wall, pododermatitis, petechial hemorrhages in gizzard, unilateral granulomatous thoracic airsacculitis, intestinal lumen packed with *Ascaridia galli*
Histopathological changes (n=4)		
None in liver, spleen, cecal tonsil	2/4	At DPI 2
Marked increase in lymphocytic follicles in spleen	¼	At DPI 15
Infiltration of mononuclear macrophages and plasma cells in primary lymphocytic follicles of spleen	¼	At DPI 15
Caseous nodules in the submucosa of cecal tonsil	¼	At DPI 15
Multinucleated foreign body type ginat cells in caseous nodule	¼	At DPI 15
Lymphocytic depletion in cecal tonsill	¼	At DPI 15
Reactive cell infiltration on the tip of the duodenal villi	¼	At DPI 15
Focal accumulation of mononuclear cells in liver sinusoids	¼	At DPI 15
Hemorrhages in the submucosa of cecal tonsil	¼	At DPI 15
Fatty change in liver	¼	At DPI 15

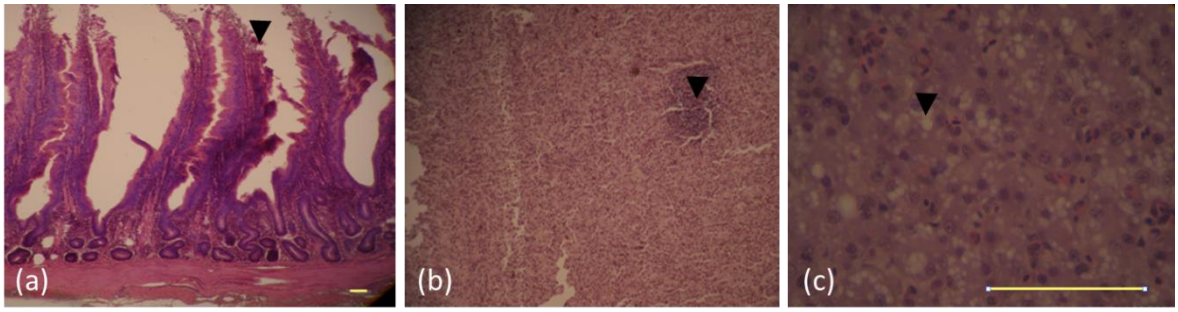

Figure 5. Histopathological changes observed in duodenum and liver of an *S.* Kentucky infected chickens (stained with H&E). (a) Reactive cell infiltration in tip of the duodenal villi, (b) A liver section showing focal accumulation of mononuclear cells in sinusoids, (c) A liver section demonstrating fatty changes.

The most widely reported zoonotic serovar of *Salmonella Enteritidis* infections in poultry are characterized by vascular damage, eruptions at specific locations on the mucosal surface of the gastrointestinal tract, lesions in the lymphoid organs, and degenerative sequelae involving the parenchymatous organs (Dhillon et al., 2001; Kogut et al., 2003; Takata et al., 2003; Deng et al., 2008). In a susceptible host, *S.* Enteritidis replicates primarily in the mucosa of the digestive tract after oral challenge and then spreads to the spleen, liver, and various other organs and

tissues (Dibb-Fuller et al., 1999). *S. Kentucky* is an emerging serovar, and unlike *S. Enteritis*, a little is known on the lesions attributable to natural or artificial infections with this serovar in any kinds of poultry including backyard chickens.

In the present study, *S. Kentucky* was isolated from some but not all infected backyard chickens, indicating that some birds might clean the infections and some becomes persistently infected. Osman et al. (2010) reported a variable re-isolation rate of *S. Kentucky* from infected chickens- 60, 20, 60 and 20% on DPI 1, 2, 3 and 7, respectively. In the present study, the isolation rate gradually decreases with the progression of the infection, again suggesting that most infected backyard chickens could clean the infection and only 13% might harbor the infection until DPI 21. The proportion of the chickens remain infected throughout their lives could not be predicted from this study because of short study period.

Most Salmonella, except for serovars pullorum and Gallinarum (Wilson et al., 2000) and possibly other strains *e.g.*, *S. Kentucky* (Ogunleye and Carlson, 2012) are capable of asymptomatically residing in the intestinal tracts of poultry.

Backyard chickens roam freely on the homesteads of the owners and collect their most feeds from the outside environment. Having kept in a house under intensive system of rearing, their normal homeostasis thus might have been interfered and stressed, allowing other organisms to infect them. Thus the high mortality observed during the experimental window of 23 days might not have been attributed to *S. Kentucky* alone rather with other concomitant infections and rearing factors which could not be controlled.

Because only 4 chickens were sampled for histopathological examinations; from this study, it is hard to conclude the probable tissue changes *S. Kentucky* can elicit in different internal organs. However, besides this limitation it might be assumed that, probably, at the beginning of infection this serovar might not produce any significant changes in the internal oranges. But in chronic infections the following changes can be observed: infiltration of mononuclear macrophages and plasma cells in primary lymphocytic follicles of spleen, caseous nodules in the submucosa of cecal tonsil, multinucleated foreign body type giant cells in caseous nodule of spleen, lymphocytic depletion in cecal tonsil, reactive cell infiltration on the tip of the duodenal villi, focal accumulation of mononuclear cells in liver sinusoids, hemorrhages in the submucosa of cecal tonsil and fatty change in liver.

S. Kentucky might continuously or intermittently shed from the infected backyard chickens to contaminate the environment and the shedding probability of *S. Kentucky* of human non-typhoidal clinical case origin from infected backyard chickens might be 77, 41 and 13% on DPI 2, 12 and 21. Many Salmonella serotypes can be acquired by the fecal-oral route and then be shed into the feces (Traub Dargatz et al., 2006). Many birds can be infected since the ingestion, colonization and shedding events typically cause no harm to the birds and salmonella is ubiquitous in the environment. Salmonella can therefore contaminate poultry meat prior to (from fecal shedding) or during processing (from intestinal leakage), resulting in one of the leading causes of salmonella infections in humans (CDC, 2011).

CONCLUSION

Zoonotic *S. Kentucky* strain of human non-typhoidal clinical cases of gastroenteritis has potentials to produce clinical signs such as reduced feed uptake, watery or pasty fecal droppings and lesions, such as catarrhal enteritis and typhlitis in backyard chickens. The shedding probability of this strain from infected chickens might be 77, 41 and 13% at DPI 2, 12 and 21, respectively. The survival probability of the infected chickens with the strain might be 50% on DPI 6, 32% on DPI 15 and 14% on DPI 23. The roles of other factors contributing to the observed survivability might not be ruled out. No noticeable histopathological changes are probably seen in any internal organs after DPI 2, but changes evidenced of reactive spleen might be seen in prolonged case of infection.

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CONFLICT OF INTEREST

There is no conflict of interest to declare.

AUTHORS' CONTRIBUTION

SA and PKB implemented the study design. SA and SD carried out the laboratory experimentation. PKB supervised the overall research work. SA, BKN and MOQ drafted and revised the manuscript. All the authors contributed in writing and reviewing the manuscript, and approved the final manuscript.

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