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Full Length Research Paper

Characterization of biofilm formation by *Salmonella enterica* Serovar Pullorum strains

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Salmonella enterica **Serovar Pullorum causes acute or persistent infection in chickens, resulting in significant economic losses. The objective of this study was to evaluate the biofilm-forming ability of *S. enterica* Serovar Pullorum strains and association with the pathogenicity. A total of 62 *S. enterica* Serovar Pullorum isolates were measured for biofilm formation by crystal violet staining. Thirty-nine out of 62 strains were found to produce biofilm which were further confirmed by observation under light microscopy, field emission scanning electron microscopy. Analysis of the major components for biofilm formation showed that bacterial colonies were smooth phenotype and the architecture of biofilm was composed of exopolysaccharides and curli other than cellulose. In addition, animal experiments were performed to determine the 50% lethal doses (LD_{50}) of four *S. enterica* Serovar Pullorum isolates with or without biofilm-producing ability. Although the virulence of *S. enterica* Serovar Pullorum strains was independent of the ability of biofilm formation, prior growth as a biofilm for a biofilm producer of *S. enterica* Serovar Pullorum lead to enhanced virulence in chickens. The results suggested that biofilm may be one of the important virulence factors for *S. enterica* Serovar Pullorum infection.**

Key words: *Salmonella enterica* Serovar Pullorum, biofilm, curli, virulence.

INTRODUCTION

Pullorum disease caused by *Salmonella enterica* Serovar Pullorum is a common disease in chickens, which results in a high mortality rate in young chicks, and weight loss, decreased laying, diarrhea, and lesions and abnormalities of the reproductive tract in adult birds (Gast, 2003). Although rare in modern poultry industries of the developed countries, pullorum disease remains a serious problem in many developing countries (Orji et al., 2005; Anderson et al., 2006). *S. enterica* Serovar Pullorum is not a significant cause of disease in people and it is considered a host-adapted pathogen of birds (Mitchell et al., 1946; Tollefson and Miller, 2000).

Bacteria in biofilm are more resistant to chemical, physical and mechanical stresses, and host immune system, and cause persistent infection and repeated infection (Donlan and Costerton, 2002). A number of studies have demonstrated that *Salmonella* spp. from different sources may form biofilm (Joseph et al., 2001; Solomon et al., 2005; Malcova et al., 2008). Extracellular structures contributing to biofilm formation in *Salmonella* include curli fimbriae (Collinson et al., 1996), cellulose (Zogaj et al., 2001), BapA protein (Latasa et al., 2005), capsular polysaccharide (de Rezende et al., 2005), and other polysaccharides like LPS (Anriany et al., 2006). *Salmonella* biofilm may be associated with the virulence. Increased numbers of colony forming units were recovered from the spleens of mice 5 days after intraperitoneal injection with *S. enterica* Serovar Typhimurium grown as a biofilm, as compared to planktonic cells (Turnock et al., 2002). Another study also confirmed that only the virulent *S. enterica* Serovar Enteritidis produced aggregates and formed visible filaments attached to the glass tube (Solano et al., 1998). However, biofilm formation and the role of biofilm in the pathogenesis of *S. enterica* Serovar Pullorum remain unclear. Therefore, the aim of this study was to investigate biofilm formation of *S. enterica* Serovar Pullorum isolates and its association

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with the virulence of *S. enterica* Serovar Pullorum.

MATERIALS AND METHODS

Bacteria and growth condition

Sixty-two strains of *S. enterica* Serovar Pullorum used in this study were isolated from infected or dead chickens and stored at –70°C in LB medium containing 25% glycerol for long-term storage. Isolates were first grown on LB plates and incubated at 37°C overnight. A single colony of bacteria was picked to inoculate two milliliters of Trypticase Soy Broth (TSB) followed by incubation at 37°C overnight in a 200 rpm shaker-incubator and then used as inoculum in all experiments. TSB diluted 1:10 in distilled water was used as the medium to facilitate the formation of biofilm. LB agar plate without salt supplemented with 40 mg/L Congo red and 20 mg/L brilliant blue was used to determine the Congo red-binding property of the colonies. LB agar plate supplemented with 200 mg/L calcofluor (fluorescent brightener) was used to determine the cellulose production by comparing the fluorescence of the test strains under UV light (Anriany et al., 2006).

Quantification of biofilm formation

Biofilm formation was quantitated in 1/10 TSB (V/V) as described previously (O'Toole and Kolter, 1998). Briefly, the overnight broth cultures of each bacterium were diluted 1:100 in the diluted TSB. 100 µl of each bacterial suspension was added into 96-well U-bottomed polystyrene microtiter plates. Wells that contained only the medium served as negative control. Plates were incubated separately at 28, 37 and 40°C for 24 h under static conditions. Then non-adherent bacteria were removed and the wells were washed gently three times with 200 µl of distilled water. 100 µl of 0.4% crystal violet (v/v) was added into each well for 20 min. The liquid was then decanted. All loosely adhering bacteria and dye were gently washed off with distilled water for three times. The dye bound to the adherent cells was solubilized with 100 µl of anhydrous ethanol per well. The optical density (OD) was measured at 550 nm with a microplate reader (BIO-TEK, Elx800). The amount of biofilm cultured for 8, 24 and 48 h was determined using the optimal temperature. Then 62 strains of *S. enterica* Serovar Pullorum were examined under the established optimal growth conditions. The assays were performed in triplicates and repeated three times.

Biofilm formation in glass test tube

The overnight cultures of each bacterium were diluted 1:100 in the diluted TSB. Two milliliters of each bacterial suspension were added into borosilicate glass test tubes and incubated at 28°C for 48 h. Then the liquid was decanted and the tubes were washed gently three times with distilled water. 2 ml of 0.4% crystal violet (v/v) were added into each tube and stained at room temperature for 20 min.

Congo red /carbol fuchsin staining

The overnight culture (1:100 diluted in TSB) was inoculated into 3 ml of fresh TSB in a 6-well plate containing sterile polystyrene coverslip (20 ×20 mm). After incubation at 28°C for 24 or 48 h without agitation, the coverslips were removed carefully, treated with cetylpyridinium chloride (Sigma) (10 mM) for 30 s, rinsed with distilled water and air dried for 20 to 30 min. After fixation by gentle heating, the coverslips were stained with a mixture of saturated aqueous Congo red solution and 10% Tween-80 (2:1, V/V) for 30 min and rinsed with distilled water. After staining with 10% (v/v) Ziehl carbol fuchsin for 6 min and rinsing in distilled water, the coverslips were air dried and mounted on slides. All slides were visualized under a light microscope and photographs were taken (Harrison-Balestra et al., 2003).

Field emission scanning electron microscopy (FESEM)

Coverslips (5 × 5 mm) were cultured as described earlier for 24 h. Then the coverslips were fixed in 3% glutaraldehyde in 0.1 M phosphate-buffered saline at 4°C for 2 h. The samples were then dehydrated with increasing concentrations of ethanol (50, 70, 80, 90 and 100%) followed by isoamyl acetate (100%), each for 15 min. The samples were dried at critical point for 5 h, coated with gold palladium alloy, and observed under a S4800 field emission scanning electron microscope (Anriany et al., 2006).

RT-PCR

5 ml of diluted TSB and 50 ∝l of overnight broth cultures of each strain were added into disposable polystyrene cell culture flask and incubated at 28°C for 24 h. The bacterial cells were collected by vigorous vortexing with sterile glass beads. Total RNA samples were extracted using Total RNA Mini Purification Kit according to the manufacturer's protocol. Isolated RNA was treated with DNase I at 37°C for 30 min. After heat inactivation of DNase at 65°C for 10 min, the RNA was used as a template for reverse transcription (RT) with random primers and MMLV reverse transcriptase. The cDNA was amplified in separate PCRs with primers for a 156-bp *csgA* gene and a 234-bp *bcsA* gene. The amount of total RNA was equalized by assessing the RT-PCR products with primers for a 173-bp 16s rRNA reference gene. The primers used were as follows: *csgA*-F (5'-TATTATCCGCACCCTGGCCTAC-3') and *csgA*-R (5'-GCAATAGTTCCGGCCCG-3'), *bcsA*-F (5'-CAGTCAATGC CAAG CGAAGCG-3') and *bcsA*-R (5'-TTGGGACGACCCGGTCAGTC-3') 16S-F (5'-TCGATGCAACGCGAAGAACC-3') and 16S-R (5'-CGCTGGC AACAAAGGATAAGG-3'). PCR was performed under following conditions: 94°C for 5 min, 30 cycles at 94°C for 40 s, 56°C for 45 s, and 72°C for 30 s, followed by 72°C for 10 min. PCR products were examined by ethidiumbromide staining after 1% agarose gel electrophoresis.

Virulence assays

Experimental infection with different strains

The bacterial cells were prepared from a culture flask as described earlier. All bacterial cells including planktonic cell and biofilm were collected and the concentrations of bacterial cells were adjusted to 10^9CFU/ml. One-day-old SPF chickens, which are the most susceptible to *S. enterica* Serovar Pullorum (Williams and Tucker, 1980), were randomized into groups of five and were inoculated intraperitoneally (i.p.) with approximate 2×10^4 to 2×10^8 CFU of *S. enterica* Serovar Pullorum in 0.2 ml. Two weeks later, the 50% lethal doses (LD_{50}) values were determined by the method of Reed and Meunch (1938). The remaining chickens were euthanized and their livers and spleens were removed and homogenized in PBS. The homogenates were then serially diluted and plated onto MacConkey agar plates to determine the number of viable *S. enterica* Serovar Pullorum. The isolated bacteria were identified

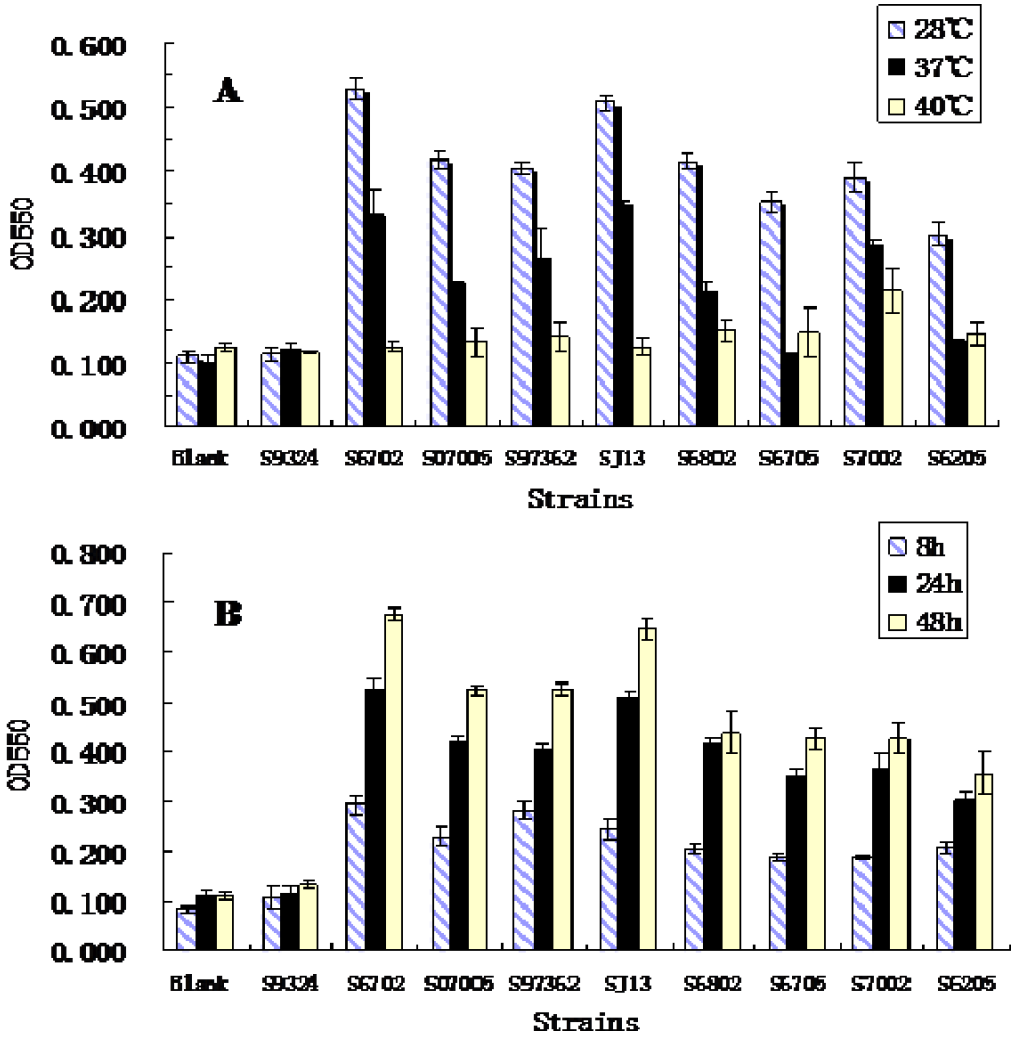

Figure 1. Biofilm formation of *S. enterica* Serovar Pullorum strains cultured at different temperature (A) and different duration of time (B). Bars indicated standard deviations.

by plate agglutinate test with *Salmonella* O_9 serum or PCR method.

Experimental infection with the same strain of different phases

50 ∝l of overnight broth cultures were added into disposable polystyrene cell culture flasks and incubated at 28°C for 24 h. Then the medium was removed and the planktonic cells were recovered by centrifugation. The cells which adhered to the flask surface were harvested by vortexing in 5 ml PBS using sterile glass beads. Colonies were grown on 1.5% LB agar plate for 5 h to reach exponential phase. Propagule cells were harvested by flushing the plates with PBS and the colonies were scraped off with a glass spreader. The bacterial concentrations were adjusted to 10^9CFU/ml.

One-day-old SPF chickens were randomized into groups of seven and were inoculated i.p. with approximate 10^7 to 10^8 CFU of *S. enterica* Serovar Pullorum in 0.1 ml. The mortality was monitored for two weeks. The remaining chickens were euthanized and bacteria in their livers and spleens were cultured as described above.

Statistical analysis

The biofilm formation was analyzed using two-tailed independent Student's t test. A 0.05 or lower p-value was considered significant.

RESULTS

Biofilm assay on polystyrene plates

S. enterica Serovar Pullorum strains were tested at different incubation temperature and time on polystyrene plates to determine the optimal condition for biofilm formation. The biofilm formation of *S. enterica* Serovar Pullorum strains reached the maximum OD_{550} value at 28°C as determined by crystal violet staining (Figure 1A). There was no significant difference in OD_{550} values between 24 and 48 h of incubation, suggesting *S. enterica* Serovar Pullorum strains formed mature biofilm

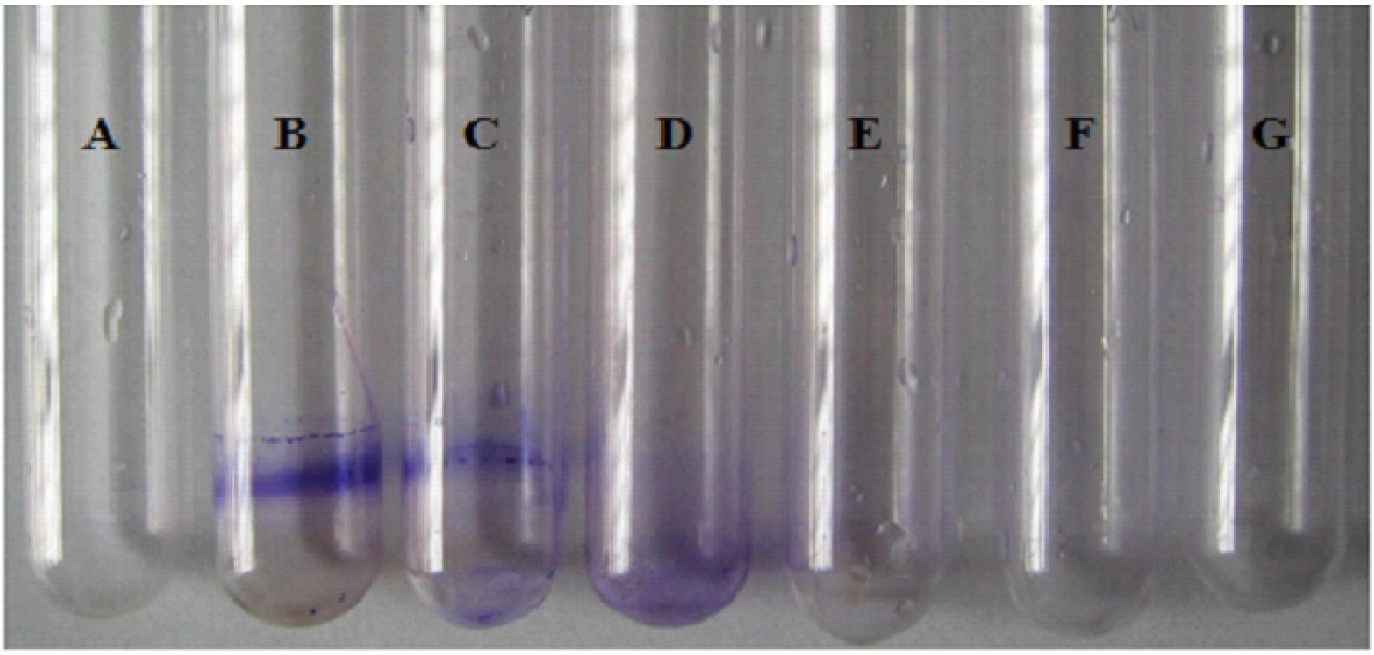

Figure 2. Biofilm formation of *S. enterica* Serovar Pullorum strains with different biofilm-forming ability cultured on glass test tube and stained by crystal violet. Samples included blank control (A), S6702 (B), S7002 (C), SJ13 (D), S7102 (E), S9324 (F), SJ11 (G).

at 24 h (Figure 1B). A total of 62 strains of *S. enterica* Serovar Pullorum were examined and 63% of these strains (39/62, OD_{550}: 0.192 ± 0.014 to 0.528 ± 0.021) formed biofilm. The OD_{550} values of other strains showed no difference from negative control (0.110 ± 0.017, P > 0.05). Therefore, six strains were chosen for further study because of their different biofilm-forming abilities (no biofilm strains: S9324 (0.114±0.014) and SJ11 (0.130±0.017); biofilm-producing strains: S7102 (0.253±0.017), S7002 (0.379±0.029), SJ13 (0.520±0.017), and S6702 (0.528±0.021).

Biofilm assay on glass test tube

Selected *S. enterica* Serovar Pullorum strains were tested on glass surface. Strain S7002 and S6702 produced rings at the liquid-air interface on the glass test tube walls, while strain SJ13 produced color staining at the bottom of the tube. However, Strain S9324, SJ11 and S7102 produced no visible color staining at the liquid-air interface or on the wall of glass tube (Figure 2).

Morphological observation of biofilm

Under a light microscope, only a few dispersed bacterial cells of strains S9324 and SJ11 adhered to the coverslip surface and showed purple staining (Figure 3A and 3B). Bacterial cells of strain S7102 and S7002 exhibited microcolonies and cluster on the coverslip (Figure 3C and 3D). Bacterial cells of strain SJ13 formed exopoly-saccharides on the coverslip which was stained pink (Figure 3E). The bacterial cells of strain S6702 formed structures of biofilm that fully covered the coverslip (Figure 3F). The biofilm formed on the coverslip was further determined by FESEM. Consistent with Congo red/carbol fuchsin staining, strain S9324 and SJ11 exhibited only a few dispersed bacteria cells on the coverslip surface (Figure 4A and 4B). In contrast, the other four strains exhibited increased clusters of bacteria cells with curli fimbriae and had meshwork-like structures surrounding the cell surfaces (Figures 4C to F).

Congo red plate

The colonies of six strains on Congo red plate were all smooth after growth at 28°C for 4 days. The colonies of strain S9324 and SJ11 were nearly white and those of strain S7102 and S7002 were pink. The colonies of S6702 were red, while the colonies of SJ13 were white in the center with red around (Figure 5A). Cellulose production was detected by comparing the fluorescence of the test strains under UV light. The fluorescence of all the six strains was weak and similar, indicating that cellulose was not the major component of biofilm formed by these *S. enterica* Serovar Pullorum (Figure 5B). To further confirm the role of curli and cellulose in biofilm formation, total RNAs isolated from six strains were subjected to RT-PCR analysis using primer sets designed for genes 16s rRNA, *csgA* and *bcsA.* A 173-bp

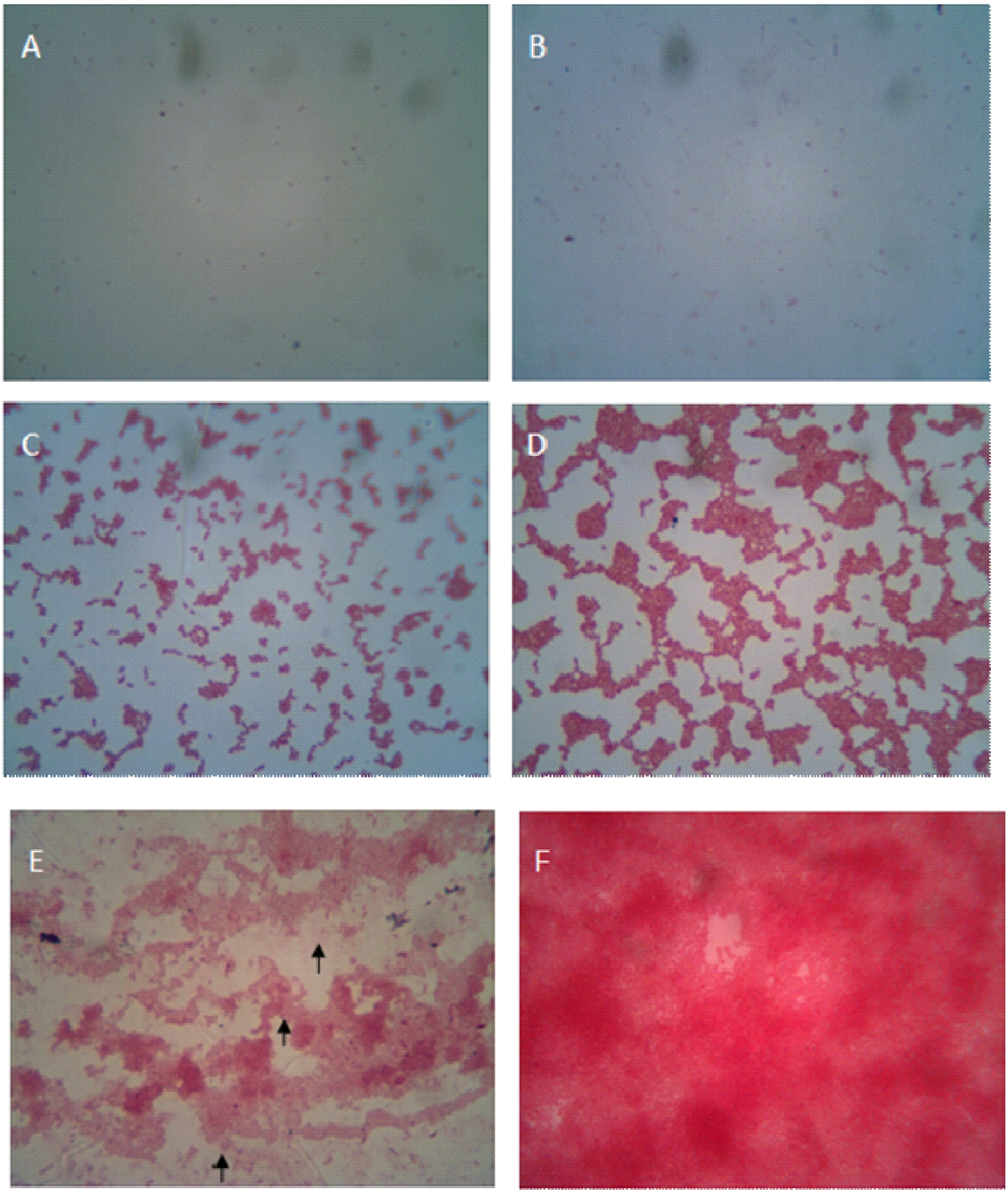

Figure 3. Congo Red/carbol Fuchsin staining of *S. enterica* Serovar Pullorum. The biofilm was formed on coverslips and examined under light microscope under the oil emersion lens (×1,000 magnifications). Congo red stained the exopolysaccharides pink (indicated by arrows). *S. enterica* Serovar Pullorum cells were stained purple. Samples included S9324 (A), SJ11 (B), S7102 (C), S7002 (D), SJ13 (E), S6702 (F).

fragment with a similar strength was observed in RT-PCR products of all the strains with primers 16s-F/R. No fragment was observed in the RT-PCR products of strain S9324 and SJ11 with primers *csgA*-F/R, while a 156-bp fragment was observed in the RT-PCR products of the other four strains, of which S7102 produced a weak fragment. A 234-bp fragment with a similar strength was observed in the RT-PCR product of all the six strains with primers *bcsA*- F/R (Figure 6. At same time, no fragment was obtained in the PCR product of all the six strains

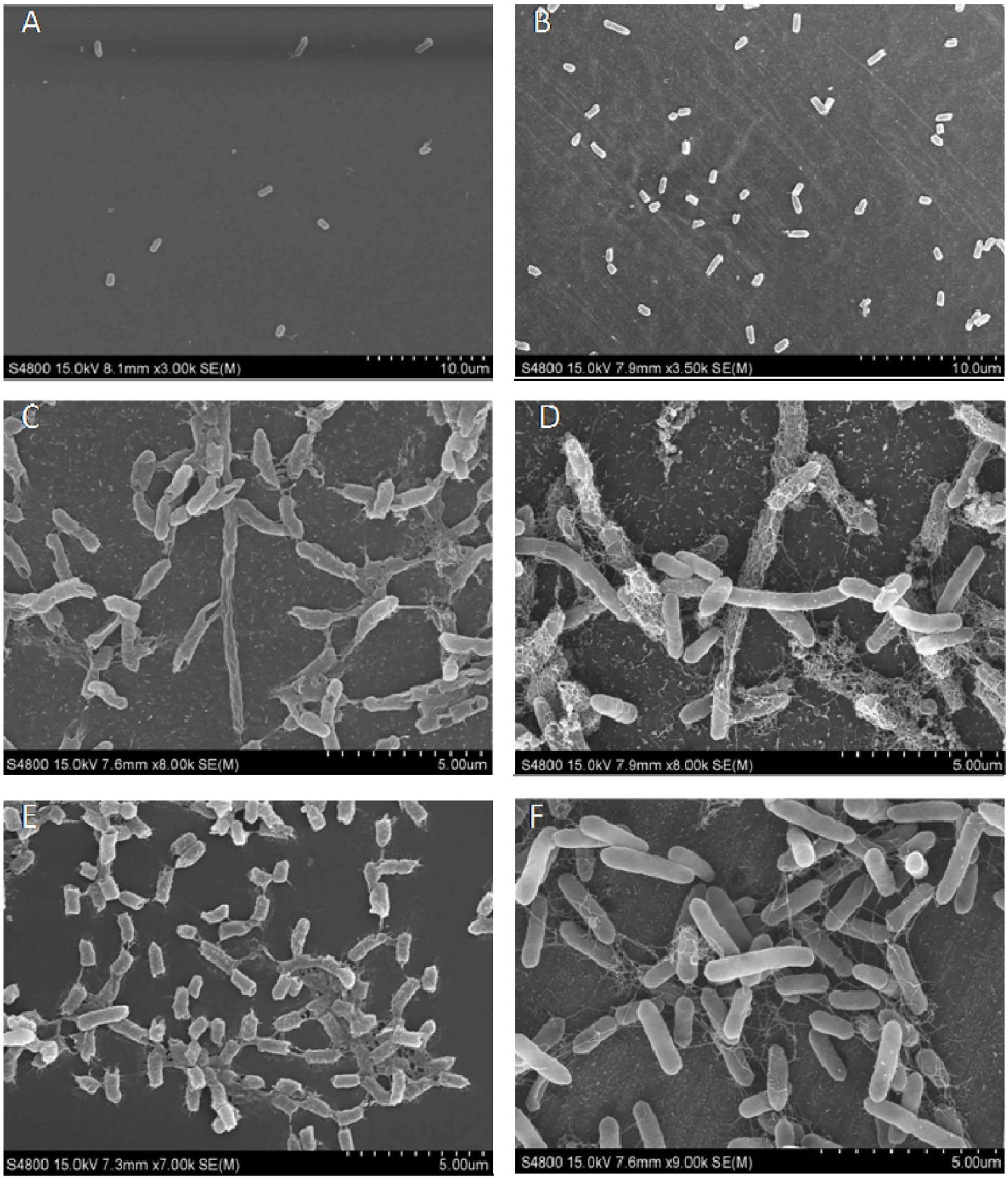

Figure 4. Field emission scanning electron micrographs of bacteria cells of *S. enterica* Serovar Pullorum strains grown on coverslips at 28°C for 24 h. Samples included S9324 (A), SJ11 (B), S7102 (C), S7002 (D), SJ13 (E), S6702 (F).

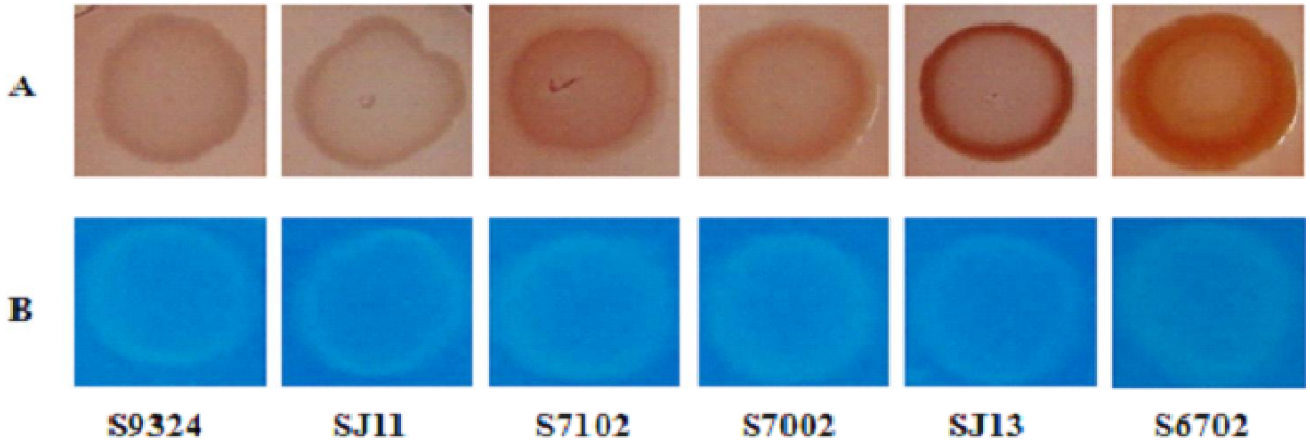

Figure 5. The phenotypes of *S. enterica* Serovar Pullorum strains cultured on Congo red plate (A) and LB plate containing calcofluor (B).

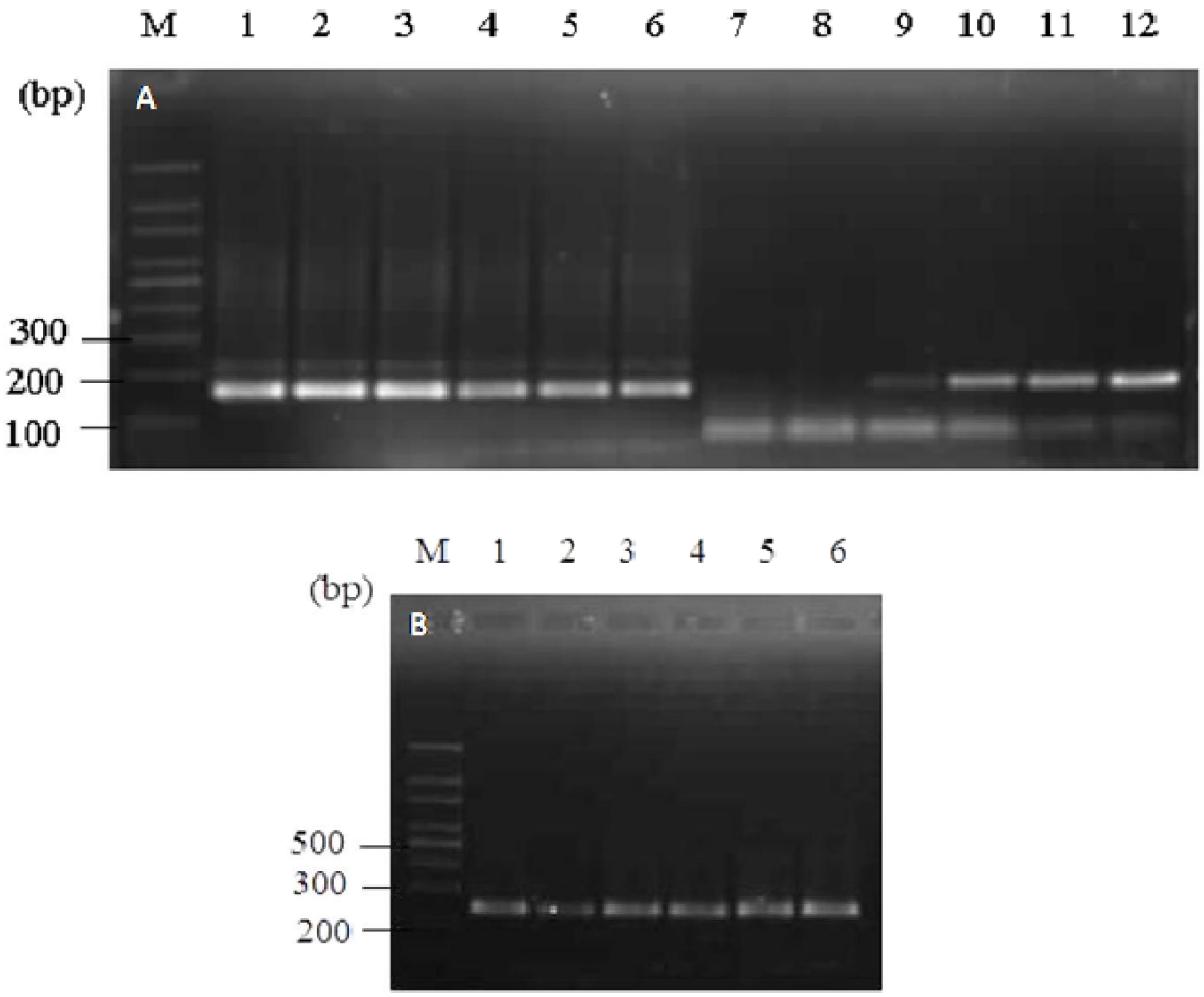

Figure 6. Expression of cs*gA* and *bcsA* genes of *S. enterica* Serovar Pullorum strains detected by RT-PCR. RT-PCR was performed using the following nucleic acid as templates: total RNA from S9324 (Lanes 1, 7), SJ11 (Lanes 2,8), S7102 (Lanes 3,9), S7002 (Lanes 4,10), SJ13 (Lanes 5,11), and S6702 (Lanes 6,12). Reaction sets contained the following primers: 16s- F/R (Lanes A1-A6), *csgA*- F/R (Lanes A7-A12), *bcsA*- F/R (Lanes B1-B6). 100 bp ladder marker was labeled as Lane M.

Table 1. Determination of LD_{50} of test strains.

Strain†	Group	Dose (CFU/chicken)	Mortality /total chickens	Mortality (%)	LD_{50}
S9324	1	2.04×10^{8}	5/5	100	$10^{7.41}$
	2	2.04×10^{7}	1/5	20	
	3	2.04×10^{6}	1/5	20	
	4	2.04×10^{5}	0/5	0	
	5	2.04×10^{4}	0/5	0	
SJ11	1	1.82×10^{8}	5/5	100	$10^{7.16}$
	2	1.82×10^{7}	2/5	40	
	3	1.82×10^{6}	1/5	20	
	4	1.82×10^{5}	0/5	0	
	5	1.82×10^{4}	0/5	0	
S7002	1	2.18×10^{8}	4/5	80	$10^{6.84}$
	2	2.18×10^{7}	3/5	60	
	3	2.18×10^{6}	3/5	60	
	4	2.18×10^{5}	0/5	0	
	5	2.18×10^{4}	0/5	0	
S6702	1	2.38×10^{8}	1/5	20	$>2.38\times10^{8}$
	2	2.38×10^{7}	1/5	20	
	3	2.38×10^{6}	1/5	20	
	4	2.38×10^{5}	0/5	0	
	5	2.38×10^{4}	0/5	0	

†strain without ability of biofilm formation: S9324 and SJ11; strain with ability of biofilm formation: S7002 and S6702.

with these primers when total RNA was used as the template, indicating no chromosomal DNA contamination in the total RNA.

The pathogenicity of *S. enterica* Serovar Pullorum strains with different biofilm-forming ability

To check the correlation of the biofilm-forming ability of *S. enterica* Serovar Pullorum strains with the virulence, four strains with or without biofilm-forming ability were used to challenge 1-day-old chickens via i.p. route. The results showed that the LD_{50}s of non-biofilm-forming strain S9324 and SJ11 were $10^{7.41}$ and $10^{7.16}$ CFU, respectively, and the LD_{50} of biofilm-forming strain S7002 was $10^{6.84}$CFU, while the LD_{50} of the most strong biofilm producer S6702 was more than 2.38×10^{8}CFU. At 14 day postinoculation, the bacteria were isolated from livers or spleens only in a few chickens (2/23) inoculated by strain S6702 (Table 1).

The pathogenicity of strain S6702 of different phases

To investigate the effects of biofilm on virulence, the strong biofilm producer S6702 of three different phenotypes were used to challenge 1-day-old chickens via i.p. route. The results showed that the mortality rate caused by strain S6702 in biofilm phase was higher than that in planktonic state or propagule phase. At 14 days postinoculation, the bacteria were isolated from the spleen in one out of seven survived chickens only when strain S6702 was challenged in biofilm phase (Table 2).

DISCUSSION

As a fowl-specific pathogen, *S. enterica* Serovar Pullorum may persist for a number of months in the spleen, leading to infection of the reproductive tract and eggs or to progeny via vertical transmission (Shivaprasad, 2000). Intramacrophage survival is crucial in persistent infection which requires Salmonella pathogenicity island 2 type III secretion system (Wigley et al., 2001, 2002), Also, a nonspecific suppression of cellular responses occurring at the onset of laying assists *S. enterica* Serovar Pullorum in infecting the reproductive tract, leading to its transmission to eggs (Wigley et al., 2005). However, the roles of other virulence factors in *S. enterica* Serovar Pullorum are unclear. Some studies indicate that biofilm

Table 2. The mortality rate of S6702 in three different phases.

Phenotype	Group	Dose(CFU/chicken)	Mortality /total chickens	Mortality (%)
Logarithmic phase	1	0.95×10^{8}	2/7	28.6
	2	0.95×10^{7}	1/7	14.3
Planktonic phase	1	0.80×10^{8}	2/7	28.6
	2	0.80×10^{7}	1/7	14.3
Biofilm phase	1	0.99×10^{8}	4/7	57.1
	2	0.99×10^{7}	3/7	42.9

formation of bacteria is a common cause of persistent infection and repeated infection (Donlan and Costerton, 2002; Jayaraman, 2008). In this study, we demonstrate that more than half of tested *S. enterica* Serovar Pullorum isolates (39/62) were biofilm producers on polystyrene plate surface. The biofilm formation of six selected strains was further confirmed by Congo red / carbol fuchsin staining using FESEM. Biofilm of *Salmonella* is mainly composed of curli and cellulose, and *Salmonella* strains were grouped into distinct morphotypes according to Congo red binding:

(1)Red, dry and rough indicating curli and cellulose production (RDAR). (2)Brown, dry and rough, indicating a lack of cellulose synthesis (BDAR).

(3) Pink, dry and rough, indicating a defect in curli expression (PDAR).

(4) Smooth, brown and mucoid, indicating a lack of cellulose synthesis but overproduced capsular polysaccharide (SBAM).

(5) Smooth and white, indicating a lack of both curli and cellulose production (SAW) (Romling et al., 1998; Solomon et al., 2005; Malcova et al., 2008).In our study, all selected strains of *S. enterica* Serovar Pullorum showed smooth phenotype when grown on Congo red plate. The strains without biofilm-forming ability showed nearly white colonies, while biofilm producers showed pink to red colonies. Congo red/carbol fuchsin staining of biofilm cells revealed that there were exopolysaccharides in biofilm structure. FESEM images of biofilm revealed that there were curli fimbriae in biofilm structure. RT-PCR results showed that there was a significant difference in expression of *csgA* gene between biofilm producers and non-biofilm forming strains, while there was no difference in expression of *bcsA* gene in all six strains. In addition, the fluorescence result revealed that there was similar expression of cellulose in all six strains. These data indicated that exopolysaccharides and curli but not cellulose played major role in biofilm formation for these *S. enterica* Serovar Pullorum strains. Since the morphotypes of *S. enterica* Serovar Pullorum strains on Congo red plate are different from other *Salmonella* spp., other components of biofilm need to be further studied.

The biofilm formation of several pathogens is reported to be associated with chronic infections, including *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Streptococcus pneumoniae*, *Staphylococcus aureus* and enteropathogenic *Escherichia coli* (Hall-Stoodley and Stoodley, 2009*)*.

In another case, bioflm formation may not have a direct correlation with virulence of *S. aureus, Burkholderia pseudomallei, E. coli,* and *Riemerella* anatipestifer (Kristian et al., 2004; Taweechaisupapong et al., 2005; Rijavec et al., 2008; Hu et al., 2010). In the current study, the animal experiment demonstrated that although the mortality in chicken induced by a biofilm-producing strain of *S. enterica* Serovar Pullorum was not always higher than that of a non-bioflim-producing strain, prior growth as a biofilm for a biofilm producer of *S. enterica* Serovar Pullorum lead to enhanced virulence in chickens. Consistent with this, the bacteria were isolated from liver or spleen only when strain S6702 in biofilm phase was used to challenge chickens, suggesting that *Salmonella* Pullorum in biofilm phase might cause persistent infection.

Conclusion

More than half of *S. enterica* Serovar Pullorum strains can produce biofilm *in vitro* with curli as the major component. Although the biofilm ability of *S.enterica* Serovar Pullorum is not directly correlated with their virulence *in vivo*, prior growth as a biofilm enhances virulence of *S. enterica* Serovar Pullorum.

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