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Induction of arthritis in chickens by infection with novel virulent *Salmonella* Pullorum strains

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ABSTRACT

Salmonella enterica subsp. *enterica* serovar Gallinarum biovar Pullorum (*Salmonella* Pullorum) is a host-specific serovar causing systemic infection with high mortality in young chicks. Pullorum disease is characterized by white diarrhea. However, arthritis has become increasingly frequent recently, particularly in southern China. The aim of the present study was to determine the pathogenesis and arthritis induction of new *Salmonella* Pullorum isolates. We isolated and identified five *Salmonella* Pullorum strains from broilers with bacterial arthritis and lameness in a commercial poultry farm. Four of five isolates were resistant to at least three classes of antibiotics and were defined as multidrug-resistant *Salmonella* Pullorum. All isolates had the same CRISPR sequence type and belonged to a single major cluster. The isolates exhibited high capability of biofilm formation, which may facilitate their dispersal and survival in hostile habitats, and showed high virulence based on embryo lethality and inoculation of newly hatched chicks. Tissue distribution analysis confirmed that SP1621 was more adapted to colonize the joint when compared to the white diarrhoea-causing *Salmonella* Pullorum reference strain S06004. Reproducible arthritis and typical joint lesions were observed in SP1621-infected chicks, and histopathological examination showed necrotic synovitis and cartilage tissue hyperplasia of the joint. Koch's postulates were confirmed when the novel *Salmonella* Pullorum strain was re-isolated from the joint tissues of experimentally inoculated chicks. These novel *Salmonella* Pullorum isolates have unique ability to induce arthritis in chickens, representing expanded pathogenic diversity in China. These results suggest the need for strict control strategies and new vaccines to prevent the disease.

1. Introduction

Pullorum disease (PD), a septicemic bacterial disease of primarily avian species resulting from infection by *Salmonella* Pullorum, is among the most important diseases of poultry due to widespread outbreaks accompanied by high mortality (Barrow et al., 2012; de Souza et al., 2015). Although this disease has been eradicated from commercial poultry in many developed countries, it still persists in many countries in Africa, Asia and South America, leading to severe economic losses (Barrow and Freitas Neto, 2011). In China, *Salmonella* infection in poultry is common and the main prevalent serovar isolated from chickens is *Salmonella* Pullorum (Gong et al., 2014).

Newly hatched chicks show high susceptibility to *Salmonella* Pullorum infection that results in acute systemic disease (Wigley et al., 2001). The clinical signs in chicks affected by PD has been described previously (Shivaprasad, 2000). After natural infection with this organism, broilers may manifest depression, anorexia, somnolence and weakness. Dehydration, as well as diarrhea and adherence of droppings (white and viscous) to the vent feathers may also be observed. Other signs such as purulent arthritis and joint lesions associated with *Salmonella* Pullorum infection have occasionally been described (Ferguson et al., 1961; Salem et al., 1992; Shivaprasad, 2000). Other reported syndromes include claudication, swelling of the tibiotarsal joint and the radial, humeral and ulnar articulations. Moreover, locomotor disorders remain a challenge to the poultry industry, which represent not only a major economic concern but also a problem of animal welfare (Braga

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et al., 2016).

Infectious conditions such as arthritis, synovitis and claudication can be associated with diverse etiological agents. *Escherichia coli*, *Enterococcus cecorum*, *Staphylococcus aureus*, *Mycoplasma synoviae* and avian reovirus as causative agents in chickens were well described (Boerlin et al., 2012; Dijkman et al., 2013; Braga et al., 2016; Mosleh et al., 2016; Sellers, 2017).

Salmonella Pullorum has been suggested to be a cause of arthritis and has been frequently isolated from typically affected chicken joints in China (Ren et al., 2017). However, there are very few reports that reproduced the joint lesions in experimental chick infection model, and little is known about the incidence of arthritis or joint enlargement in broilers infected with *Salmonella* Pullorum.

Toward this end, in the current study, we isolated and identified novel arthritis-causing *Salmonella* Pullorum strains from chickens affected by two outbreaks of arthritis that occurred in commercial poultry farms. An embryo lethality assay and inoculation of newly hatched chicks were conducted to confirm the pathogenic potential of the isolates. The isolates were characterized in terms of antimicrobial susceptibility profiles, ability for biofilm formation and CRISPR sequence typing to determine their relationship to known strains. Chickens were also experimentally infected with one of the isolates intramuscularly and their symptoms were compared to those observed during the field arthritis outbreaks. The novel *Salmonella* Pullorum was re-isolated from the experimentally inoculated chickens to confirm its identity. Overall, these results can serve as a warning of the expanded diversity of *Salmonella* Pullorum in China as a potential aetiological agent for arthritis.

2. Materials and methods

2.1. Ethics statement

All experimental and animal management procedures were approved by the Animal Welfare and Ethics Committees of Yangzhou University (SYXK [Su] 2016–0020) and complied with the guidelines of the Institutional Administrative Committee and Ethics Committee of Laboratory Animals. Fertile HY-line white chicken embryos, obtained from Jiangsu Institute of Poultry Sciences (China), were hatched in the laboratory in an automatic incubator at 37.5 °C with 50%–60% relative humidity. The birds were housed in separate wire cages with sufficient formulated feed and water at ambient temperature. The chickens were checked to confirm the absence of *Salmonella* infection by bacteriological examination as described below and for any clinical signs of salmonellosis.

2.2. Sample collection, bacterial isolation and identification

Salmonella Pullorum strains isolated from broilers presenting lameness or arthritis symptoms were collected from commercial poultry farms in Guangdong Province, China. The samples consisted of swabs and tissues obtained from the synovial fluid and joint lesions, which were plated on xylose lysine Tergitol 4 (XLT4, Difco, USA) agar and incubated for 24–48 h at 37 °C. Small, dome-shaped, and translucent colonies were selected and further investigated by *Salmonella*-specific PCR (Gong et al., 2014) and the API 20E system (BioMerieux, France). Bacterial isolates were identified biochemically using a VITEK 2 COMPACT system (BioMerieux, France). The confirmed positive colonies were transferred to Luria-Bertani (LB) agar. Serotyping was performed using *Salmonella* O and H antiserum (Tianrun Bio-Pharmaceutical, China) in agglutination tests according to the manufacturer's instructions. Bacterial cells were suspended in phosphate-buffered saline (PBS), deposited on the grids, blotted dry and stained with 2% phosphotungstic acid. The negatively stained samples were observed using a Philips CM 100 transmission electron microscope (TEM). After bacterial identification, the strains were stored at −70 °C in cryogenic cultures (LB broth with 20% glycerol) until subject to the subsequent molecular, phenotypic and virulence tests described below.

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined by the disk diffusion tests on Mueller-Hinton agar plates according to the recommendation of the Clinical and Laboratory Standards Institute (CLSI, 2013). All isolates were tested against 16 antimicrobial agents (Oxoid, UK) as follows: ampicillin (AMP, 10 μg); amoxicillin/clavulanic acid (AMC, 20/10 μg); cefazolin (CZO, 30 μg); meropenem (MEM, 10 μg); aztreonam (ATM, 30 μg); kanamycin (KAN, 30 μg); gentamicin (GEN, 10 μg); streptomycin (STR, 10 μg); amikacin (AMK, 30 μg); tetracycline (TET, 30 μg); trimethoprim/sulfamethoxazole (SXT, 1.25/23.75 μg); ciprofloxacin (CIP, 5 μg); norfloxacin (NOR, 5 μg); nalidixic acid (NAL, 30 μg); chloramphenicol (CHL, 30 μg) and nitrofurantoin (NIT, 300 μg). *Escherichia coli* ATCC 25922 was used as a quality control strain.

2.4. CRISPR typing

The *Salmonella* Pullorum isolates were cultured in LB broth overnight at 37 °C with shaking. Genomic DNA was extracted with a TIANamp Bacteria DNA Kit (Tiangen, China) in accordance with the manufacturer's protocol and stored at −70 °C until used. CRISPR typing was performed based on a previous study (Xie et al., 2017). Polymerase chain reaction-amplified products were sequenced by the GenScript (Nanjing, China). To identify CRISPR spacers and obtain direct repeat information, the resulting sequences were submitted to the CRISPRfinder website (http://crispr.u-psud.fr/Server/) and visualized as described previously (Grissa et al., 2008; Horvath et al., 2008). A minimum spanning tree was generated with BioNumerics 7.5 software (Applied Maths, Belgium), and cluster analysis using Dice similarity coefficients was performed by the unweighted pair group method.

2.5. Biofilm formation

Biofilms formed by the *Salmonella* Pullorum isolates in LB broth were determined and visualized as described previously (Solano et al., 2002). A *Salmonella* Pullorum strain S6803 without biofilm-forming ability was used as control. For Congo red staining assays, isolates were grown on LB agar plates without NaCl supplemented with Congo red (40 μg/ml, Sigma, USA) and Coomassie brilliant blue (20 μg/ml) (Jonas et al., 2007). Colony color and morphology on Congo red agar plates were tested after 7 d of incubation at 28 °C. Crystal violet quantification of the biofilms formed by the *Salmonella* Pullorum strains was performed using a microtitre plate assay as described previously (Pratt and Kolter, 1998; Kawamura et al., 2011). Bacterial strains were incubated in 96-well polystyrene microtitre plates (Corning, USA) at 28 °C for 48 h. The non-adherent cells were decanted and the wells were gently washed with distilled water to which 200 μl of 0.1% crystal violet was then added. Adhesive cells were stained for 20 min and resolved by the addition of 200 μl of anhydrous ethanol after the wells were gently washed with distilled water. Absorbance at 595 nm was measured with an automated microplate reader (BioTek, USA), and the biofilm index was defined by the optical density value.

2.6. Chicken embryo lethality assay

The virulence of the *Salmonella* Pullorum isolates in 12-day-old embryos was determined via allantoic cavity inoculation based on a previously reported procedure (Guo et al., 2017a). Bacterial cultures grown overnight at 37 °C in LB broth was harvested by centrifugation and subsequently washed and diluted to 10^3 colony forming unit (CFU) per milliliter with PBS (pH 7.2). Ten 12-day-old embryonated chicken eggs per group were inoculated with 0.1 ml of the suspension containing 10^2 CFU of each strain into the allantoic cavity using an 18-

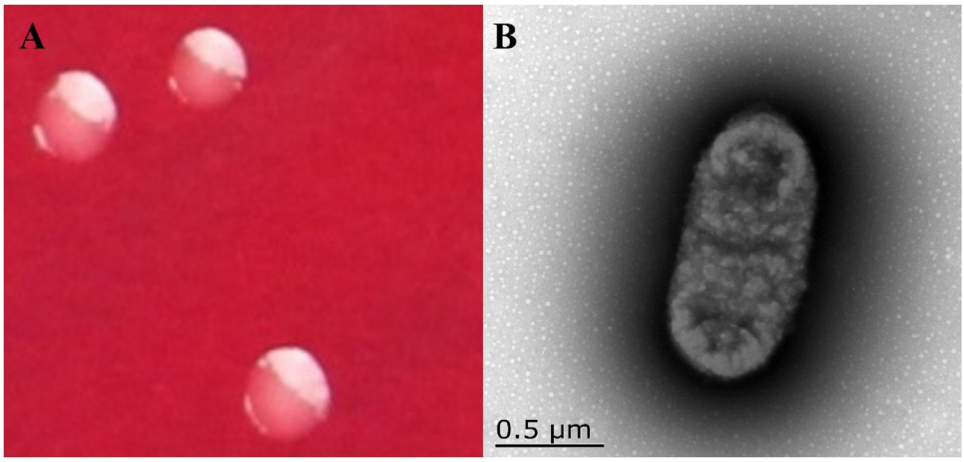

Fig. 1. Colonial morphologies on XLT4 agar (A) and transmission electron micrograph of the novel *Salmonella* Pullorum isolate (B). Isolates formed small red translucent colonies without black centers after 24 h of incubation and appeared aflagellate under TEM (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

gauge needle. PBS injected embryos were used as control. The eggs for the embryo lethality assay were candled at regular intervals after inoculation to detect dead embryos based on the integrity of the venous system and movement of embryo.

2.7. Pathogenicity to chickens

2.7.1. Assessment of virulence in newly hatched chicks

The virulence of *Salmonella* Pullorum isolates was determined by intramuscular administration of the isolate in 1-day-old HY-line white chicken. Birds inoculations were performed as described previously (Guo et al., 2017b) to evaluate the 50% percent lethal dose (LD_{50}) values. The strains were grown, harvested, resuspended and adjusted to achieve a dose of $\sim 10^5$ to $\sim 10^{10}$ CFU in a volume of 100 µl for intramuscular inoculation of chicks (10 chicks per dose). The chickens were monitored for up to 3 weeks for virulence assessment.

2.7.2. Histopathology and bacterial dissemination in affected organs

Seventy-five three-day-old chicks randomly divided into three groups of equal size (25 chicks per group) and inoculated intramuscularly with the wild-type strains SP1621 ($\sim 10^6$ CFU), S06004 ($\sim 10^6$ CFU; Li et al., 2013; Geng et al., 2014; Guo et al., 2017b) and PBS, respectively. The chicks were observed twice daily for signs of disease and mortality. Twenty-one days after inoculation, five chickens from each group were euthanized and tissues were aseptically collected. Gross pathological lesions were observed at the time of necropsy. Representative tissue samples of the liver, spleen, heart, cecum and hock joint were fixed in 10% neutral formalin. Tissue sections were prepared, deparaffinized, stained with hematoxylin-eosin (H&E), and then examined under light microscopy. To investigate the dissemination and colonization of the SP1621 infection in chickens, the liver, spleen, cecum and hock joint obtained from each group of five birds were weighed, homogenized, serially diluted, and subsequently plated on XLT4 agar for the enumeration of *Salmonella* strains present in each tissue. The remaining 20 chicks in each group were observed for 2 months post-inoculation. X-ray radiographs of chickens with claudication symptoms were taken using a high-frequency X-ray system (YEMA, USA). Infection with each strain was performed in three times.

2.8. Confirmation of the identity of Salmonella Pullorum re-isolated from infected chicks

Isolation and identification of *Salmonella* Pullorum from the joint tissue of experimentally challenged chicks was carried out as described in Section 2.2. The samples were cultured on XLT4 agar plates, and suspected *Salmonella* colonies (small, red and translucent) were subcultured onto LB agar plates to obtain a pure culture for further identification. Growth that appeared on the agar plate was tested by the slide agglutination method using *Salmonella* O and H antiserum. Biochemical identification was performed using the VITEK 2 COMPACT system to confirm that the re-isolated *Salmonella* had the same characteristics as the challenge strain.

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, USA). One-way analysis of variance followed by Dunnett's multiple comparison tests was used to determine the statistical significance of the differences between multiple experimental groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Serotyping and antibiotic resistance profiles of the bacterial isolates

The isolates taken from the swollen joints of dead chickens were gram-negative, and formed small, red and translucent colonies without black centres when grown on XLT4 agar (Fig. 1A). The isolates were serotyped as *Salmonella enterica* Gallinarum with somatic antigen O:9 and 12. Electron micrographs revealed a typical *Salmonella* morphology with short rods approximately 0.5 µm in diameter and 1–2.5 µm in length (Fig. 1B). The isolates had no flagella, which is typical for other *Salmonella* serovars without mobility. Biochemical assays showed that the *Salmonella* isolates could ferment ornithine, mannitol and dextrose but not sucrose, dulcitol, or maltose, which confirmed their classification as the *Salmonella* Pullorum biovar. Four isolates were resistant to at least three classes of antibiotics and were thus defined as multidrug resistant bacteria (Table 1). Specifically, the antimicrobial susceptibility test revealed the highest rate of resistance to AMP and NAL (100%) followed by STR (80%).

3.2. Molecular characterization of isolates by CRISPR typing

Two CRISPR loci (CRISPR 1 and CRISPR 2) were present in the *Salmonella* Pullorum isolate genomes. Overall, all five isolates had the same spacer content in CRISPR 1 (Ent1-Ent3-Ent4) and CRISPR 2 (EntB0-GallB1-GallB2-EntB8-EntB9var2) loci (Fig. 2). Using the published CRISPR sequences of *Salmonella* Pullorum strains (Xie et al., 2017), phylogenetic analysis of the five isolates was carried out based on diversity of CRISPR types. The analysis showed that the five isolates were closely genetically related and all belonged to a single major cluster.

Table 1
Source, antimicrobial resistance and virulence of *Salmonella* Pullorum isolates.

Isolate	Source	Antimicrobial resistance pattern	Chicken embryo lethality [a]	LD_{50} (CFU) [b]
SP1611	Broiler breeder with arthritis	AMP-STR-NAL	100%	4.9×10^7
SP1621	Broiler breeder with arthritis	AMP-STR-NAL-CIP-TET	100%	7.2×10^7
SP1622	Broiler with arthritis	AMP-STR-NAL-CZO	100%	1.7×10^7
SP1631	Broiler with arthritis	AMP-NAL	100%	4.9×10^7
SP1632	Broilers with arthritis	AMP-STR-NAL-CIP-TET	100%	4.2×10^7

[a] Survival rate of chicken embryos inoculated with isolates was 0%.
[b] One-day-old chicks were infected intramuscularly with the isolates.

3.3. Biofilm formation capability of the isolates

Biofilm formation capacity was assessed by incubating the bacteria in LB broth under static conditions, followed by observing colony morphology on Congo red agar plates as well as with a crystal violet biofilm assay. The biofilm formation in standing LB broth was visualized as a ring of bacteria adhering to the glass wall with a floating pellicle containing a tight bacterial network at the air-liquid interface (Fig. 3A). Red colony phenotypes were observed for isolates incubated on Congo red-supplemented agar, while the control strain showed a pink morphotype (Fig. 3B). Crystal violet quantification analysis of biofilms further confirmed the strong biofilm-forming ability of *Salmonella* Pullorum isolates on polystyrene surfaces (Fig. 3C).

3.4. Virulence of the isolates for chicken embryos

The overall percent survival of chicken embryos inoculated with the isolates was 0% (Table 1). No dead embryos were observed after 12 h, and mortality appeared between 2 and 3 days post-inoculation. None of the chicken embryos inoculated with PBS died during the 3 days of observation. The embryos in the control group showed no gross lesions or typical symptoms (such as curling and stunting), and the amniotic fluid was clear. By contrast, the amniotic fluid of embryos inoculated with bacteria was turbid, and dead embryos revealed prominent subcutaneous oedema and hemorrhages. There was no significant difference in embryo lethality rates among the five isolates.

3.5. Virulence and tissue distribution in newly hatched chicks

To assess the lethality of *Salmonella* Pullorum isolates in newly hatched chicks, 1-day-old birds were inoculated intramuscularly with a series of does (rang from 10^5 to 10^{10} CFU per dose). The LD_{50} following infection ranged from 1.7×10^7 CFU to 7.2×10^7 CFU (Table 1). No significant differences were observed among strains, with all demonstrating 100% mortality at the highest dose (10^9 and 10^{10} CFU). The LD_{50} is similar to that previously reported for S06004, which is known to be a highly virulent strain in chickens (Geng et al., 2014).

To evaluate the pattern of the systemic spread of infection for the isolate SP1621 in chicken, its tissue distribution was compared to that of the well-characterized strain S06004. There were no statistically significant differences observed in the quantitation (CFU) of viable bacteria in the liver and spleen of SP1621-inoculated birds compared to those infected with S06004, although a slight downward trend could be observed for SP1621 (Fig. 4). Although SP1621 exhibited a lower ability to colonize the cecum, it was present at significantly higher levels in the joint tissues, whereas S06004 was not detected in the joint tissues.

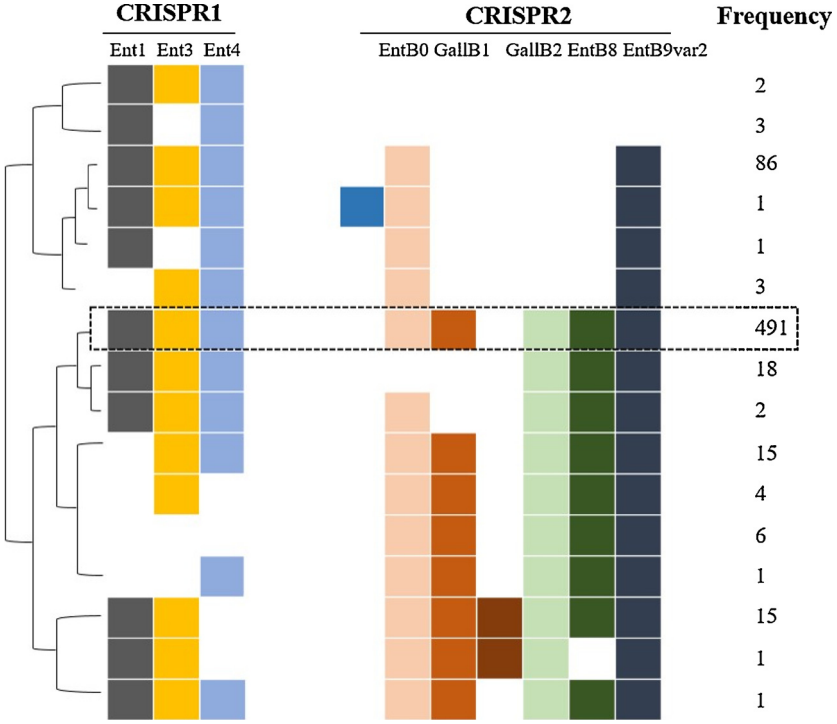

Fig. 2. Phylogenetic relationship of the five isolates among 649 *Salmonella* Pullorum strains based on CRISPR1 and CRISPR2 patterns. A graphic representation of the spacer content for CRISPR alleles identified in the strains is shown. The coloured box designates each spacer sequence.

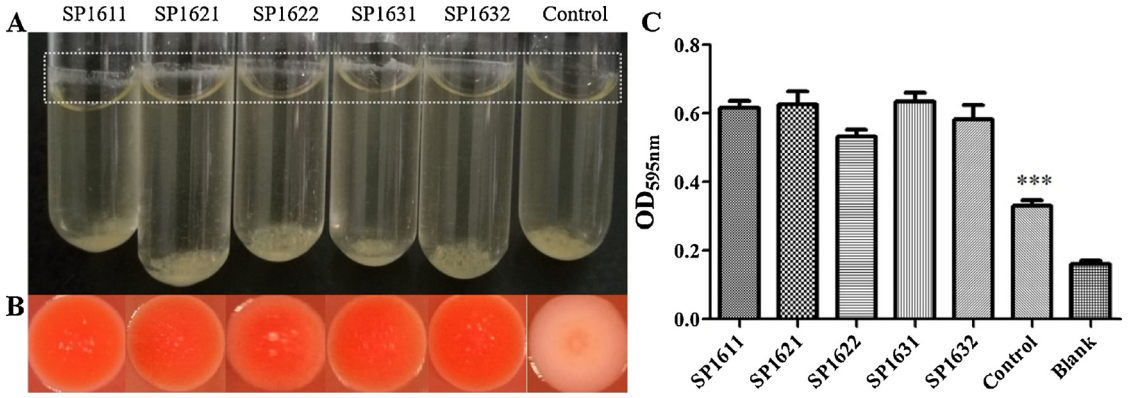

Fig. 3. Morphologic observation and quantitative analysis of biofilm formation in *Salmonella* Pullorum isolates. (A) Biofilm formation in standing LB broth was visualized as a ring of bacteria that adhered to the glass wall. (B) The distinct morphotypes of isolates observed on Congo red agar plates. (C) Quantification of biofilm formation using the microtitre plate assay (***, $P < 0.001$ vs. the control strain) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.6. Frequency and clinical findings related to arthritis symptoms

Clinically, all of the chicks in the infected groups were generally emaciated and in poor physical condition. Three weeks post-inoculation, chicks in the group challenged with strain SP1621 had typical of clinical cases of arthritis symptoms with the disease occurrence of 30%–35% (6/20–7/20) (Table 2). Infected chickens showed a swollen hock joint, which resulted in different degrees of limited mobility depending on the joint lesion site (bilateral or unilateral); birds with bilateral lesions showed lameness and often remained in ventral recumbence (Fig. 5A). The X-ray image of a representative arthritic chick shows joint swelling and soft tissue destruction (Fig. 5B). However, none of the chicks had white diarrhea. The main clinical signs in the S06004 inoculated group included dehydration, diarrhea and pasting of the vent feathers, while no symptoms of arthritis were detected.

Table 2
Presence of arthritis and swollen joints in chickens.

Strain	Swollen joint (per 20 birds)[a]	White diarrhea (per 20 birds) [a]	Time of earliest observation of arthritis (weeks p.i.)	Number of birds showing lameness
SP1621	6.67 ± 0.33[b]	0	3	1.33 ± 0.67[b]
S06004	0	3.0 ± 1.15[b]	–	–
Control	0	0	–	–

[a] Five birds were euthanized from each group at 3 weeks post-inoculation; thereafter, the remaining 20 birds were examined.
[b] Data are presented as mean ± standard error of the mean values from independent experiments.

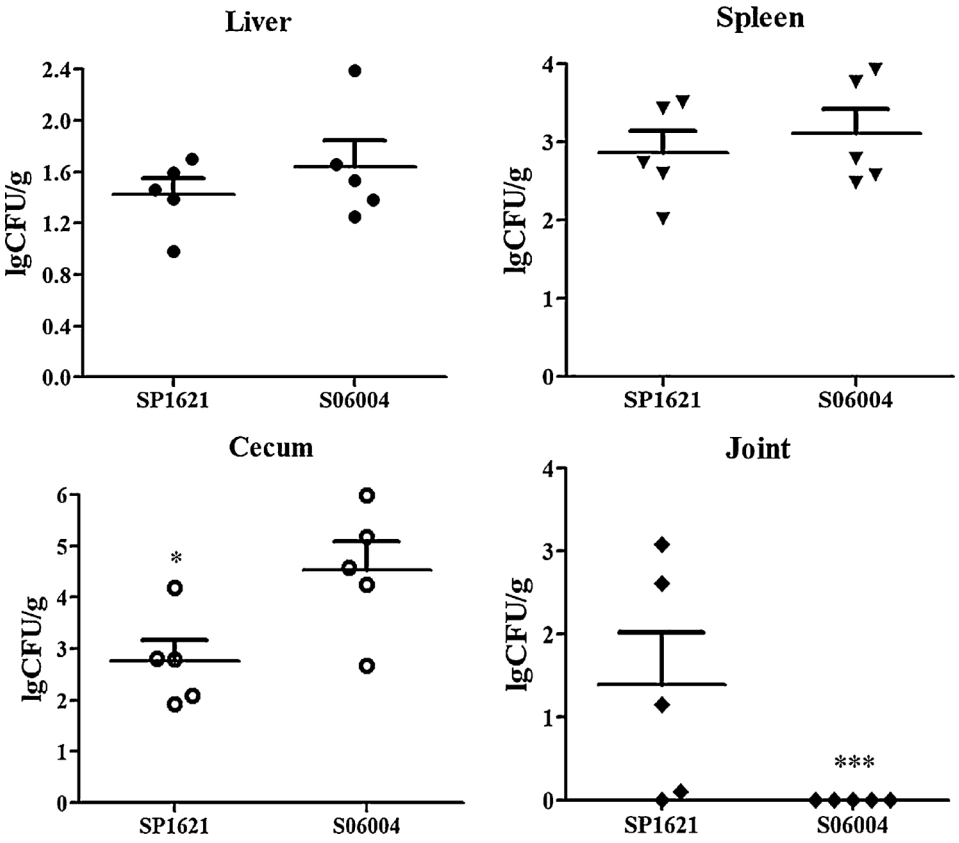

Fig. 4. Tissue distribution of a selected strain SP1621 in chicks following infection. The liver, spleen, cecum and hock joint were excised from each group of five chicks. SP1621 was recovered from the cecum at lower numbers (*, $P < 0.05$) and spread to joint tissues at significantly higher levels relative to S06004 (***, $P < 0.001$), a well- characterized strain using as a reference.

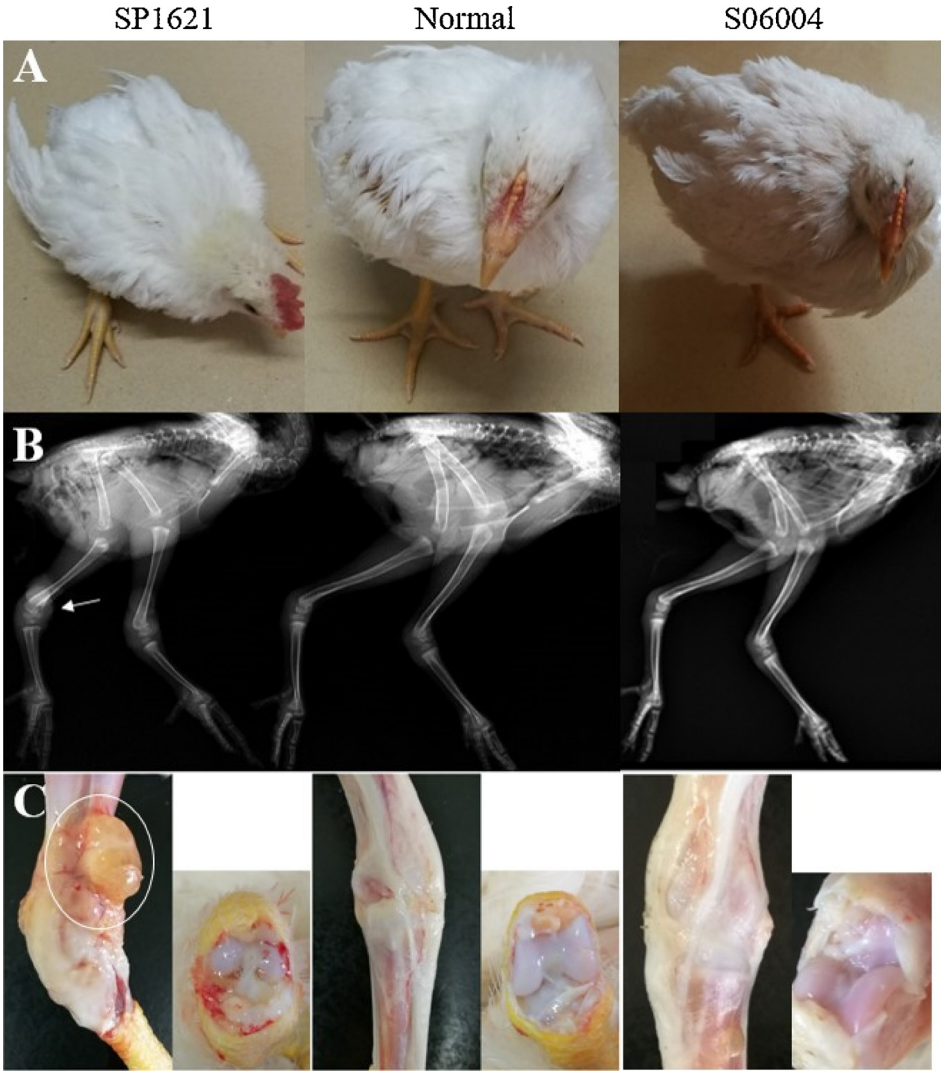

Fig. 5. Clinical signs and joint lesions from broilers affected by the novel *Salmonella* Pullorum isolate strain characterized by severe locomotor disorders (A); swelling and destruction of the hock joints (B); viscous exudate around the joint and accumulation of a serous yellowish fluid in the articular cavity (C).

Chickens in the control group were normal without any clinical symptom of salmonellosis.

3.7. Gross lesions and histopathology

On post-mortem examination, chickens challenged by SP1621 showed enlargement of the liver, with numerous necrotic foci detected in some cases. Pericarditis and enlargement of the spleen were also observed in some cases. A viscous exudate, slight yellow to amber in colour, emerged after removing the skin around the hock joint (Fig. 5C). When the joint was dissected, there was accumulation of serous yellowish fluid in the articular cavity (Fig. 5C). Histopathological evaluation (Fig. 6) showed conspicuous abnormality of the heart tissue with inflammatory cell infiltrates. The pathological changes detected in the challenged chicks included a significant reduction in lymphocyte numbers of the spleen with unevenly sized focal necrosis in the liver. No prominent damage was observed in the cecum after SP1621 infection. Necrotic synovitis and cartilage tissue hyperplasia were observed in the swollen joints. No significant changes were detected in the un-inoculated group.

4. Discussion

Salmonella is recognized as one of the most common microbial pathogens in poultry. However, limited knowledge is available regarding the distinct pathogenesis mechanisms associated with chicken *Salmonella* infections (Foley et al., 2013). Insight into chicken host-pathogen interactions is important not only to elucidate *Salmonella* Pullorum bacterial pathogenesis but also from an epidemiological perspective (Lalsiamthara and Lee, 2017). In this study, we infected broilers with a novel *Salmonella* Pullorum isolate to determine whether the pathogen has particular ability to induce arthritis. The experimental reproduction of typical joint lesions and its successful re-isolation from joint tissues of the experimentally challenged chickens suggest that the novel *Salmonella* Pullorum is the causal agent for arthritis in broilers.

Chicks in the group infected with the novel *Salmonella* Pullorum strain SP1621 had typical of clinical cases of arthritis symptoms with the disease occurrence of 30%–35%. In fact, according to our epidemiological investigation, the prevalence of the arthritis symptoms during the outbreaks in poultry farms was ranged from 3% to 40% (high prevalence of morbidity usually occurred in native chicken breeds and the free-ranged chickens raised by individual farmers). Studies have revealed considerable differences between chicken lines in the systemic salmonellosis and the responses in the gastrointestinal tract to *Salmonella* infection. To overcome resistance to oral infection and facilitate systemic infection, we used intramuscular inoculation in this study and thus we achieved relatively high occurrence of arthritis. It will be interesting to study whether or not arthritis can be caused by

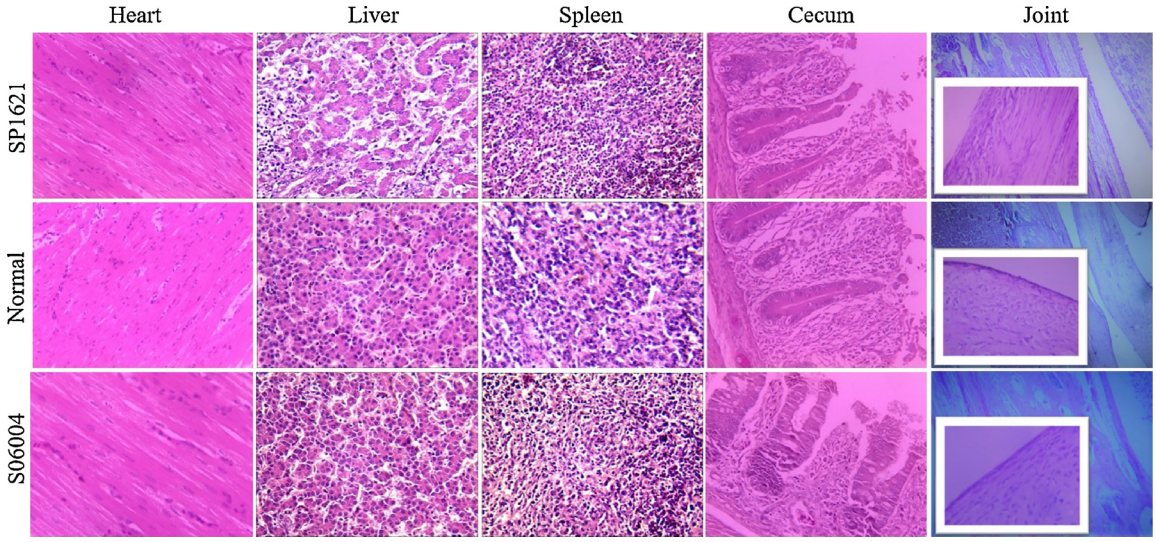

Fig. 6. Histopathological analysis after infection with the novel *Salmonella* Pullorum SP1621. Twenty-one days after inoculation, tissue samples were collected and subjected to H&E staining for histopathology. Pathological lesions were observed in the heart, liver, spleen and joint tissues on the infected chicks (400 ×).

oral infection in different chicken breeds.

Arthritis is a complex syndrome of musculoskeletal disorders consisting of a variety of conditions or diseases that destroy the joints, bones, cartilage, muscles and other connective tissues that hamper or halt physical movement (Chan et al., 2015). Humans infected with *Salmonella* show a high rate of acute gastroenteritis developing into reactive arthritis (Wilson and Whitehead, 2006). Similarly, sublethal infection with *Salmonella* Enteritidis could induce long-lasting gut inflammation and histological changes in the knee joint in BALB/c mice (Noto Llana et al., 2009). Although reactive arthritis involves sterile joint inflammation triggered by extra-articular infection, and salmonellosis in mice can cause bacteraemia, the pathogen could not be recovered from the inflamed joints of mice with *Salmonella*-induced reactive arthritis (Noto Llana et al., 2009). In the current study, the liver and spleen were identified as the main sites of *Salmonella* Pullorum bacterial localization, and dissemination of the novel isolate could be detected in the synovial exudates and articular cavity from inoculated chickens. Moreover, no conspicuous damage was detected in the cecum, such as epithelial cell necrosis or disintegration of the intestinal villus, after SP1621 infection. These results indicate that arthritis induced by the novel *Salmonella* Pullorum isolate is not a type of reactive arthritis.

Formation of a multicellular biofilm is an effective adaptation for *Salmonella*. The structurally dynamic and complex system of a biofilm provides a protective environment to facilitate survival in hostile habitats and can further contribute to the dispersal of pathogenic organisms (Pascoe et al., 2015). Biofilms can also play a key role in infection and provide a constant source of infecting pathogens. The ability of *Salmonella* to form biofilms is considered important for its successful colonization and infection in chicken (Ledeboer et al., 2006). Moreover, biofilm formation by *Staphylococcus aureus* has been associated with evading host defenses and is linked to the pathogenesis of staphylococcal bone and joint infections (Valour et al., 2015). Interestingly, all of the isolates tested in the present study showed strong biofilm-forming ability. However, it is currently unclear if formation of a biofilm is required for *Salmonella* Pullorum to cause arthritis and colonize the joints.

Although we detected a significant difference in the colonization rates between the new arthritis-causing isolate SP1621 and the reference white diarrhoea-causing isolate S06004 in the cecum and joint, they showed the same rates in colonization of the liver and spleen. This tissue distribution pattern suggests that the inherent differences between the dissemination of SP1621 and S06004 in chickens is not due to differences in the initial adherence and invasion of the bacteria to host cells. Apoptosis has been shown to be an important regulation mechanism in the pathogenesis of arthritis. Thus, it is likely that the differences in the systemic colonization of the novel *Salmonella* Pullorum isolate SP1621 are multifactorial, including a combination of differences in survival, replication and apoptosis induction.

The heart was consistently affected, followed in frequency by the spleen and liver. In an index case of pullorum disease, severe articular and periarticular swelling of hock joint was observed. Hydropericardium and large white-gray nodules in the heart were also observed. *Salmonella* Pullorum was isolated from the hock-joint fluids by direct culture on MacConkey agar, but the samples from heart, liver and spleen were negative after enrichment in selenite broth (Salem et al., 1992). This case report suggests that there is a potential association between the cause of arthritis and sterile heart lesions. According to the current epidemiological evidence, it is interesting to note that the initial case was arthritis and lameness, not early mortality. If true, it will be a topic for future study.

Antimicrobial therapy is still a widely applied strategy to control *Salmonella* infection in poultry, and *Salmonella* Pullorum strains isolated from chickens frequently show resistance to antimicrobial agents (Pan et al., 2007). Similarly, the *Salmonella* Pullorum isolates identified in this study showed high rates of resistance to several drugs, and most isolates exhibited resistance to more than two classes of antibiotics. These multidrug-resistance strains were mainly characterized by resistance to AMP (beta-lactam class, 100%), NAL (quinolone class, 100%) and STR (aminoglycosides, 80%). In particular, the high rise of resistance to NAL is a cause of concern, with extremely high resistance rates reported among the *Salmonella* isolates obtained from poultry in China (Fei et al., 2017). This situation is probably the result of the increasing use of these antibiotics, suggesting the need for the rational and cautious use of antimicrobials in poultry. It is also important to develop and test the efficacy of other non-antibiotic treatment methods such as vaccination and symbiotic feed additives.

In summary, we successfully isolated and identified five *Salmonella* Pullorum strains from outbreaks of arthritis in poultry farms in China, which all showed high virulence. Furthermore, the novel *Salmonella* Pullorum isolate induced reproducible arthritis symptoms seen in the field utilizing a chicken model. These results suggest that the pathogenicity of *Salmonella* Pullorum in China is diverse, which may increase the complexity and difficulty of salmonellosis control. These findings highlight the urgent need to conduct further detailed studies to elucidate the mechanism of infection and gain knowledge to serve as a guide for the development of new vaccines for the control of *Salmonella*

Pullorum.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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