

## Isolation, identification and Analysis of Drug Resistance of *Salmonella Pullorum*

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### Abstract

The article provides data on the isolation and identification of the pathogen *S. Pullorum* from pathological material of chickens. With further study of tinctorial, cultural-morphological and biological properties of the pathogen. The susceptibility of *Salmonella pullorum* to broad-spectrum antibacterial drugs such as cephalosporins and carbapenems was further studied to determine the drug of choice for improving treatment and prevention of avian bacterial diseases. In order to diagnose *Salmonella pullorum* (*S. Pullorum*) diarrhea accurately and analyze its drug resistance. In this study, the pathogen of a chicken suspected of *S. Pullorum* was isolation, PCR amplification and drug sensitivity analysis of the pathogen from in chicken farm in Xinxiang, north China. The results showed that the bacteria strain was diagnosed as *S. Pullorum* base on isolation and identification, Gram staining and biochemical identification of the bacteria. Antibacterial drugs sensitivity test confirmed that the bacteria was sensitive to ceftiofur, ceftriaxone, meropenem and kanamycin, and the effect of sensitive antibiotics was obvious in clinical treatment. Altogether, the present experiment revealed a detailed measure for *S. Pullorum* prevention and control and that achieved good clinical results, which laid a fundamental information for farmers and veterinary workers on eradication of *S. Pullorum*.

**Key words:** *Salmonella Pullorum*, Isolation and identification, PCR amplification, Drug sensitivity test.



### Contents

1. Introduction .....	33
2. Materials and methods .....	33
3. Results and discussion .....	34
4. Conclusions .....	37
References .....	37

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## 1. Introduction

*S. Pullorum* is one of the most serious Enterobacteriaceae diseases among *Salmonella* (Li et al., 2019), and has a highly adapted host characteristic, which causes acute septicemia death includes chicks within 20-30 days and young chickens. The incidence rate and mortality rate are very high. Adult chickens can also be infected, which often causes the laying of eggs to reduce, reproductive tract malformation, and body weight decrease, which also leads to a significant decline in hatchability and hatching rate. However, it can also infect turkeys and occasionally other birds and birds, which has brought great harm to poultry industry in many countries and caused serious economic losses (Abakpa et al., 2014; Qiu et al., 2018).

At present, more and more attention has been paid to the quarantine and control of *S. Pullorum* in China, and has also formulated a series of prevention and control measures (Guo et al., 2018). In last decade studies, due to the blind use of antibiotics that the issue of antibiotic residues has become

increasingly serious in public health problems. The drug-resistant strains of *S. Pullorum* have increased rapidly, and the drug-resistance spectrum has become more and more extensive, showing multiple drug resistance and cross resistance (Song et al., 2020). Also, the emergence of multiple drug-resistant strains has therefore seriously affected the therapeutic effect of clinical antibiotics (Nhung et al., 2017). Due to the differences of climate and feeding mode, the drug resistance of *S. pullorum* in different areas and farms is also different, which seriously threatens the safety of animal derived food and human health (Zhao et al., 2020).

## 2. Materials and methods

Accordingly, in this experiment, *S. Pullorum* was isolated and identified from the liver and cecum of sick chickens in Xinxiang, north China. By using physical and chemical methods and molecular biological methods, Gram staining and culture characteristics observation, it was identified as *S. Pullorum*. At the same time, its drug sensitivity was ana-

lyzed to assess the presence of drug resistance. The findings of this study was to provide basic data in the prevention and treatment of the bacterial infectious diseases for future option of antibiotics.

### 2.1 Sample

A 21-day-old chicken suspected of *S. Pullorum* from a chicken farm of Xinxiang, Henan Province.

### 2.2 Reagents

SS medium, hektoen enteric agar (HE), eosin methylene blue medium, macconkey agar (MAC), m-broth medium, xylose lysine deoxycholate (XLD) agar, tryptone and Salmonella biochemical identification tube were purchased from Qingdao Haibo Technology Co., Ltd. (Qingdao, China); Bacterial genomic DNA extraction kit was purchased from Tiangen biochemical technology Co., Ltd. (Beijing, China); *S. Pullorum* multivalent staining plate antigen was purchased from Beijing Zhonghai biology Co., Ltd. (Beijing, China); Drug sensitive tablets were purchased from Hangzhou Chicheng Pharmaceutical Technology Co., Ltd. (Hangzhou, China); Gram staining solution was purchased from Beijing Solebo Technology Co., Ltd. (Beijing, China).

### 2.3 Primer Design and Synthesis

According to the conserved nucleotide standard sequence of *S. Pullorum invA* gene (Accession number: NC003197.1) published by GenBank, a pair of primers was designed by using primer 5.0 software analysis system. The sequences of the primers were *invA*-F, 5'-TCCTCCGCCCTGTCTACTT-3', *invA*-R, 5'-CCCTTTCGAATAACATCCT-3'.

### 2.4 Isolation and Purification of Bacteria

The liver and cecum of dead chicken was punctured aseptically with inoculating ring and inoculated on SS identification medium, eosin methylene blue medium, nutrient agar medium and MAC medium. The suspected bacterial colonies were selected and purified by scribing and shaking at 37 °C for 24 hours, and then was observed on the medium. The purified bacteria were stained with Gram staining.

### 2.5 Biochemical Identification

The biochemical tube was cut with gear in the ultra clean worktable, and the purified bacteria were aseptically inoculated into the biochemical tubes such as sucrose, maltose and mannitol. After 24–48 h of cultivation at 37 °C in the constant temperature incubator, the reaction state was observed and recorded in the fermentation tube.

### 2.6 Plate Agglutination Test

According to the instructions of multivalent staining plate agglutination, the slide agglutination test was carried out on the blood samples collected from a disease chicken, and the agglutination reaction was observed after two

minutes. Meanwhile, normal saline was used as negative control.

**2.7 Ocular Lesions** Under sterile conditions, the morphological features of liver, kidney, heart, spleen, lung and cecum were observed immediately.

### 2.8 PCR Procedure

The PCR reaction system were performed in a 25 µL reaction mixture PCR mixtures, containing 20 µM *invAF*, 20 µM *invAR*, 12.5 µL 2×Taq Master Mix, 9.5 µL of ddH<sub>2</sub>O, and 2 µL DNA from the isolated bacteria. PCR amplification procedures were 95°C for 5 min, followed by 30 cycles of 95 °C for 40 s, 59 °C for 30 s, and 72 °C for 1min 30 s; and a final extension of 72 °C for 10 min. After PCR amplification, The 10 µL PCR amplification product was analyzed by 1.5 % agarose gel electrophoresis. The PCR product size was 1143bp.

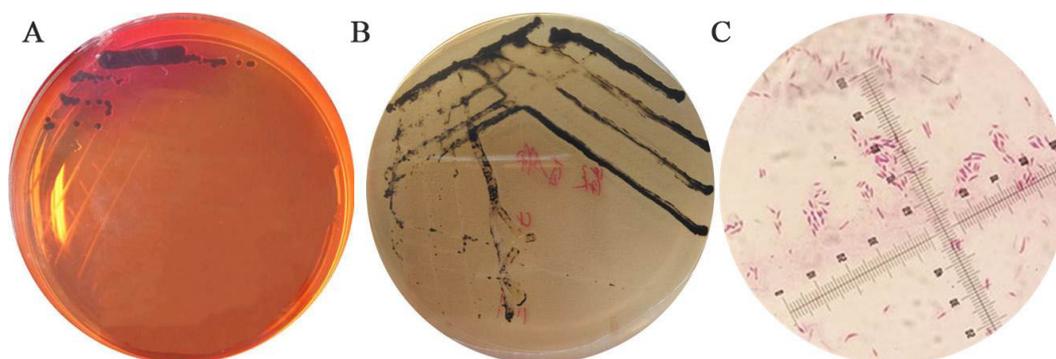
### 2.9 Antimicrobial Susceptibility Test

According to the National Committee of clinical laboratories standards (NCCLS) for Clinical and Laboratory Standards Institute (CLSI) (Sousa et al., 2020) the isolated *S. Pullorum* was purified and cultured. Take 100 µl bacterial solution and add to the nutrient agar plate. After the liquid is slightly dry and completely absorbed in the plate for 16 antimicrobial drugs on isolated bacterias. Under sterile environment, the drug sensitive tablets were clipped and attached to the culture medium with sterile tweezers, and then use tweezers to gently press them. All plates were wrapped upside down in an incubator at 37 °C for 16–18 h. The formation of bacteria-resistant zone was observed and calculated.

## 3. Results and discussion

### 3.1 Bacterial isolation and culture

The isolated strains were colorless, transparent to light orange, round, transparent and needle size colonies on MAC medium. The colony morphology were pink or with black center, round, with neat edge and smooth surface on XLD medium (Fig. 1A). Nutrient broth can be seen in the uniform turbidity of floccules, gently shaking into white sediment. On HE agar, there were colorless and translucent blue-green colonies, a few yellow colonies with a diameter of 2 mm, and most of the colonies showed black luster. The isolated bacteria were colorless, transparent or small colonies with black center with the diameter of 2–3 mm on SS medium (Fig. 1B). Gram staining was negative. Under the microscope, the bacteria was red and straight rod-shaped with blunt round ends, with the size of 0.8–1.6 µm × 1.9–4.5µm (Fig. 1C).



A – On XLD medium, the isolated bacteria presented a round with black center; B – The strains were transparent or small colonies with black center on SS medium; C – The bacteria were most of single and short shaped by Gram staining.

**Fig. 1.** A gram stained oil microscopic examination of the strain (100×)

### 3.2 Biochemical characteristics

The isolated strains were inoculated into the biochemical identification kit for identification. The biochemical reaction characteristics are shown in Table 1. It can be seen that the isolate bacterium does not liquefy gelatin, can ferment glucose and produce gas on trisaccharide iron agar, and can not

metabolize lactose, can ferment sorbitol and glucose, does not produce indole. Methyl red test, MR test and indigo matrix test were positive. VP test and ONPG test were negative. These test results are consistent with the characteristics of *S. Pullorum*.

**Table 1**

Biochemical experiment results of the isolated strain

Items	Isolates	Model strain	Test items	Isolates	Model strain
Salicin	-	-	ONPG	-	-
Glucose	+	+	Methyl red	+	+
Urease	-	-	H <sub>2</sub> S	+	+
Amino acid decarboxylase					
Indole test	-	-	Lysine	+	+
Potassium cyanide	-	-	Gelatin	-	-
V-P test	-	-	MR	+	+
Tartrate	+	+	Sorbitol	+	+

Note: "+" means positive; "-" means negative

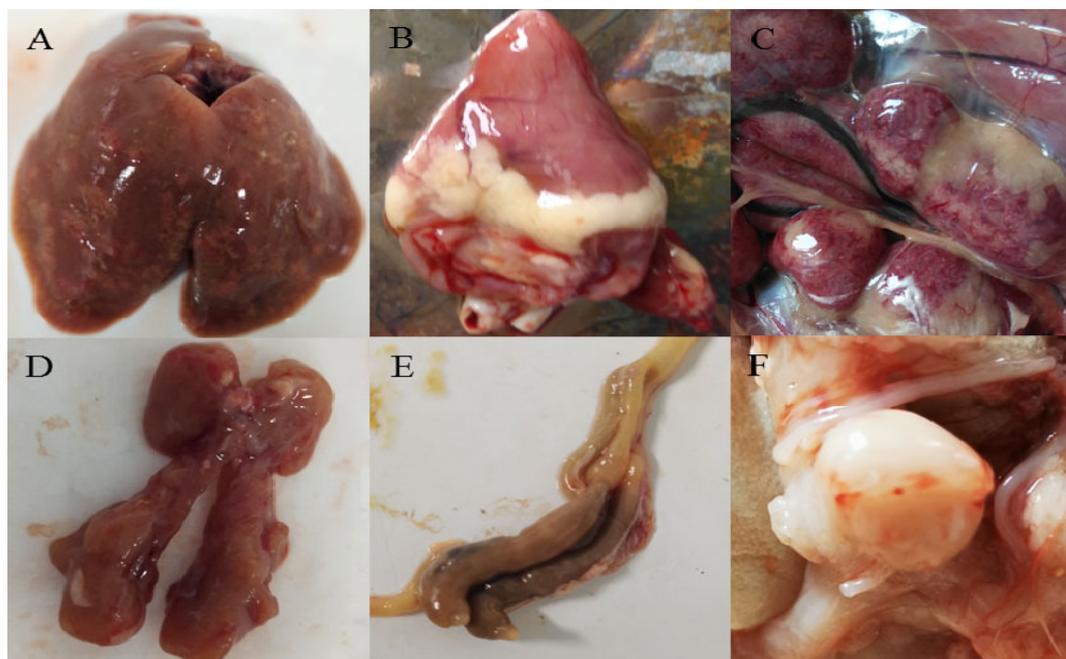
### 3.3 Plate Agglutination Assay

Through the plate agglutination test, the strains isolated from the disease samples showed agglutination, while the negative control was no agglutination.

### 3.4 Autopsy assay

The surface of liver was densely covered with small gray miliary necrotic foci (Fig. 2A). The heart was severely deformed with round appearance, punctate hemorrhage and large granuloma (Fig. 2B). There was yellow effusion in the abdominal cavity, and the kidney was swollen and congest-

ed (Fig. 2C). The spleen was swollen and necrotic with brittle texture and gray white nodules on the surface (Fig. 2D). Hemorrhagic catarrhal inflammation of the small intestine, gray white protuberant nodules on the mucosal surface of the colon. The cecum and cecal tonsils were enlarged with bleeding spots and small ulcers, and the contents were caseous (Fig. 2E). There was gray tumor like necrosis in the lung. The cysts are large with bleeding points (Fig. 2F).



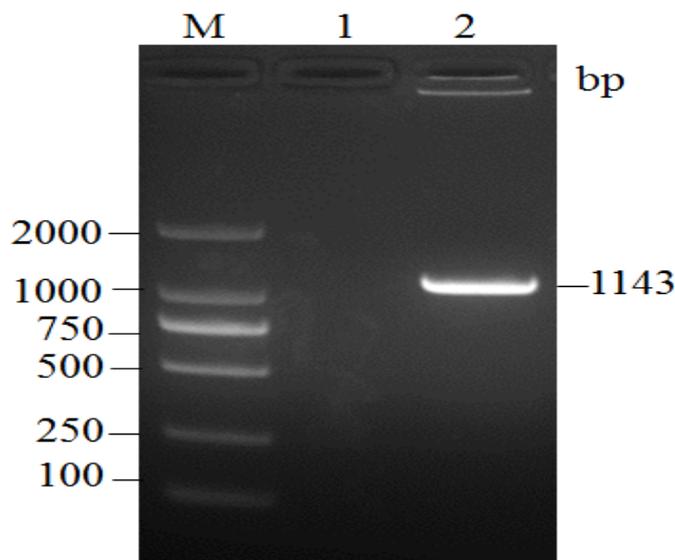
A – Liver enlargement and necrosis; B – Granuloma on the surface of pericardium; C – The kidney is swollen and congested; D – The spleen is swollen, necrotic and brittle; E – Cecal enlargement; F – Massive hemorrhage of Fabricius cyst

**Fig. 3** Gross pathological changes of different organs and intestinal tract

### 3.5 PCR results

The PCR results generated the specific 1143-bp band, while the negative control showed not amplification bands.

The results showed that the isolated strain was *S. Pullorum* (Fig. 6).



M – DL2000 DNA Marker; 1 – Negative control; 2 – The isolated strain

**Fig. 4.** Electrophoretic map of *invA* gene of isolates identified by conventional PCR products

### 3.6 Drug sensitivity test results

The drug sensitivity of the isolated bacteria was determined by the drug sensitive disk method. The results showed (Table 2) that the isolated bacteria were resistant to

amoxicillin, tetracycline, ampicillin and cotrimoxazole, but sensitive to ceftiofur, ceftriaxone, mezlocillin and kanamycin.

**Table 2**

Drug sensitivity test results to 16 antibacterial drugs of isolated *S. Pullorum*

Drugs	Judgment standard R I S	Diameter of bacteriostatic zone/mm	Sensitivity
Amoxicillin	≤14 15-20 ≥21	0	R
Polymyxin	≤10 11-15 ≥16	16	S
Sulfamethazine	≤14 15-19 ≥20	6	R
Tetracycline	≤14 15-18 ≥19	7	R
Ceftriaxone	≤13 14-20 ≥21	36	S
Gentamicin	≤12 13-14 ≥15	28	R
Neomycin	≤12 13-16 ≥17	15	I
Ampicillin	≤15 16-20 ≥21	0	R
Mezlocillin	≤17 18-20 ≥21	33	S
Ciprofloxacin	≤18 19-21 ≥22	6	R
Ceftiofur	≤14 15-17 ≥18	24	S
Fudaxin	≤14 15-17 ≥18	20	S
Neomycin	≤12 13-16 ≥17	29	R
Sulfamethoxazole	≤23 24-32 ≥33	9	R
Kanamycin	≤13 14-17 ≥18	22	S
Streptomycin	≤13 14-16 ≥17	9	R

### Discussion

At present, *S. Pullorum* is an important zoonosis pathogen in Enterobacteriaceae, and it is also the most serious infectious disease in poultry industry (Gut et al., 2018). According to previous reports (Huang et al., 2016) *S. Pullorum* has a global distribution, which can occur all year round, mostly in autumn. Especially in developed industrialized countries, the infection rate is rising (Salem et al., 2015; Wigley, 2017; Guo et al., 2018). In addition, it is of great significance to control the spread of Salmonella in the animal husbandry farm.

This experiment was conducted to detect the chicken infected with *S. Pullorum* from Xinxiang City, Henan Province. The liver and cecum of the diseased chicken was aseptically collected. The diagnosis was confirmed by the isolation and cultivation of pathogenic bacteria, staining microscopy, biochemical test, drug sensitivity test and PCR assay.

The results showed that the diarrhea death of the chickens was caused by *S. Pullorum* infection in a chicken farm. At the same time, antimicrobial susceptibility test showed that the isolated wild strain was highly sensitive to ceftriaxone, amoxicillin, tetracycline, ampicillin and cotrimoxazole, which was basically consistent with the results reported by Akinola et al. (2019). Different findings have been reported in the study of Tamang et al. (2014) and Ramirezherandez et al. (2021), which may be related to different serotypes and regional differences of Salmonella. Parvin et al. (2020) used multiplex PCR method to detect Salmonella from frozen muscle and found that resistant to tetracycline, which was consistent with our experimental results.

The drug resistance of bacteria is closely related to the drug resistance of chicken farm (Guo et al., 2014). In drug prevention and treatment of bacteria diseases, we should choose less or no drugs at ordinary times, and select highly

sensitive drugs in combination with the results of drug sensitivity test. Similarly, we should also consider the absorption of each drug in the body to determine the appropriate route of administration (Gharieb et al., 2015; Ahmed et al., 2019). Some researchers use probiotics “probiotics”, so as to competitively inhibit *Salmonella* parasitism in the intestinal tract, and have also achieved good results, indicating that it is of great reference value in the prevention of *S. Pullorum* (Torres et al., 2016; Chen et al., 2020).

Although domestic and foreign scholars have carried out extensive research in various drug resistances of *Salmonella*, there are still many problems need to be solved (Nhung et al., 2017). In order to reduce the threat of *Salmonella* to human health and animal borne diseases, we must continue to conduct in-depth research on the pathogenic mechanism of *S. Pullorum*. Especially for the study of vertical transmission mechanism, which will help us find out some effective strategies to control animal infection diseases. At the same time, there is still a long way to strengthen the research of *S. Pullorum* vaccine, especially new vaccines, such as subunit vaccine, live vector vaccine and nucleic acid vaccine. The tolerance of *S. Pullorum* is a problem that we must take seriously and rising public health concerns. The coexistence of multidrug resistance and virulence genes in animals has a certain effect on the treatment of patients to humans (Ahmed et al., 2020). At present, strengthening quarantine and eliminating positive chickens is still effective measures to prevent and control the disease on the farm. It is known that the positive incidence rate and incidence rate of chickens are controlled by using vaccines and microecological preparations. The elimination of high incidence of chickens is an important step in preventing and controlling the disease, and provides conditions for the final purification of the disease.

#### 4. Conclusions

In this study, a case chicken with diarrhea from a large-scale chicken farm in Xinxiang, north China that was diagnosed as *S. Pullorum*, and potentially pathogenic. The results of drug sensitivity test showed that the isolated strain was amoxicillin, tetracycline, ampicillin and cotrimoxazole. Furthermore, our results can be made at the early stage of in animal feeding, reducing unnecessary losses, and has a certain application value for clinical prevention and control of *S. Pullorum*.

#### Conflict of interest

The authors declare that there is no conflict of interest.

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