Salmonella enterica serovars Enteritidis, Pullorum/Gallinarum, and Dublin are infectious pathogens causing serious problems for pig, chicken, and cattle production, respectively. Traditional serotyping for Salmonella is costly and labor-intensive. Here, we established a rapid multiplex PCR method to simultaneously identify three prevalent Salmonella serovars Enteritidis, Pullorum/Gallinarum, and Dublin individually for the first time. The multiplex PCR-based assay focuses on three genes tcpS, lygD, and flhB. Gene tcpS exists only in the three Salmonella serovars, and lygD exists only in S. Enteritidis, while a truncated region of flhB gene is only found in S. Pullorum/Gallinarum. The sensitivity and specificity of the multiplex PCR assay using three pairs of specific primers for these genes were evaluated. The results showed that this multiplex PCR method could accurately identify Salmonella Enteritidis, Pullorum/Gallinarum, and Dublin from eight non-Salmonella species and 27 Salmonella serovars. The least concentration of genomic DNA that could be detected was 58.5 pg/µL and the least number of cells was 100 CFU. Subsequently, this developed method was used to analyze clinical Salmonella isolates from one pig farm, one chicken farm, and one cattle farm. The results showed that blinded PCR testing of Salmonella isolates from the three farms were in concordance with the traditional serotyping tests, indicating the newly developed multiplex PCR system could be used as a novel tool to accurately distinguish the three specific Salmonella serovars individually, which is useful, especially in high-throughput screening.

Keywords: Salmonella Enteritidis, Salmonella Pullorum/Gallinarum, Salmonella Dublin, multiplex PCR, accurate discrimination

INTRODUCTION

Salmonella is a prominent food-borne pathogen, capable of causing serious illness in humans, including gastroenteritis, typhoid fever, septicemia, and sometimes even death (Tatavarthy and Cannons, 2010). It is reported almost 75% of Salmonella infections in human cases are caused by contaminated food products, including pork, poultry, and beef (Hald et al., 2004).
Although more than 2,600 Salmonella serovars exist (Ranieri et al., 2013; Xiong et al., 2016), S. Enteritidis, S. Pullorum/Gallinarum, and S. Dublin are the main serovars causing animal diseases (Nielsen et al., 2013; Saeki et al., 2013; Zhu et al., 2015). S. Enteritidis could cause severe infection in humans (Rodrique et al., 1990; Nesbitt et al., 2012), and was the main serovar in the contaminated food products and infected individuals in southern Brazil between 1999 and 2008 (Paíão et al., 2013). S. Gallinarum only infects birds and has two biovars Gallinarum and Pullorum, causing fowl typhoid and “white diarrhea,” respectively (Soria et al., 2012; Xiong et al., 2016). Particularly, S. Gallinarum could transmit to the reproductive system and result in salmonellosis (Keller et al., 1997). S. Dublin causes widespread losses in cattle husbandry, mainly as a result of increased levels of abortion, mortality, and morbidity, and a reduced milk yield, and has attracted considerable attention from cattle industries worldwide (Carrique-Mas et al., 2010; Nielsen and Dohoo, 2013). Human infections are most caused by the consumption of milk or beef products (Nielsen, 2013). Thus, timely detection of the three prominent Salmonella serovars, S. Enteritidis, S. Pullorum/Gallinarum, and S. Dublin, is very essential and urgent.

Traditional serotyping for Salmonella is based on the identification of the somatic (O) and flagellar (H) antigens by using specific sera following the White–Kaufmann-Le Minor scheme (Majchrzak et al., 2014). Many useful data could be obtained by Salmonella serotyping. Thus, an accurate diagnostic method for Salmonella serovars is highly important for public health. Despite its wide use, traditional Salmonella serotyping has many disadvantages, which is expensive, time-consuming and labor-intensive (Ranieri et al., 2013). Recent studies showed that polymerase chain reaction (PCR) can be a useful method to detect pathogens for its high specificity and sensitivity (Abdissa et al., 2006; Moyo et al., 2007). PCR-based method for Salmonella serotyping is a rapid and economical tool (Karns et al., 2015). Gene $lygD$ in Sdf locus has been found only in S. Enteritidis and could be used to distinguish this serovar specifically (Zhu et al., 2015). Previously, we have proved that $flhB$ gene can be used to detect S. Pullorum/Gallinarum because a unique region was deficient only in this serovar (Xiong et al., 2016).

In the present study, we established a rapid multiplex PCR method to distinguish the three prevalent Salmonella serovars Enteritidis, Pullorum/Gallinarum, and Dublin individually for the first time. The approach was based on designing three pairs of primers targeting tcpS, $lygD$, and $flhB$ genes. The sensitivity and specificity of the multiplex PCR assay were determined, and the PCR assay was used to detect three sets of Salmonella isolates from one pig farm, one chicken farm, and one cattle farm. The newly developed multiplex PCR with three pairs of primers could be used as a novel tool to timely identify the three specific Salmonella serovars, and reinforce the traditional Salmonella serotyping method, particularly in high-throughput screening.

MATERIALS AND METHODS

Bacterial Strains

Strains of Salmonella and non-Salmonella organisms, including S. Enteritidis, S. Gallinarum, S. Pullorum, S. Dublin, S. Meleagridis, S. Uganda, S. Anatis, S. London, S. Rissen, S. Typhimurium, S. Derby, S. Choleraesuis, S. Sinstorf, S. Indiana, S. Newlands, S. Dumfries, S. Muenster, S. Yoruba, S. Kentucky, S. Agona, S. Senftenberg, S. Thompson, S. Blockley, S. Inchpark, S. Farsta, S. Dabou, S. Virchow, Mycobacterium tuberculosis, Brucella abortus, Listeria monocytogenes, Campylobacter jejuni, and Escherichia coli, were commercially available or previously isolated in our routine monitoring (Table 1).

Bacterial Growth and Genomic DNA Isolation

The bacterial culture and DNA isolation were performed as previously described (Xiong et al., 2016). Briefly, all strains used in the study were inoculated in Brain Heart Infusion broth (Becton, Dickinson and Company, Sparks, MD, USA) or Luria-Bertani broth (Oxoid, Basingstoke, Hampshire, England) at 37°C overnight with an agitation speed of 180 rpm. Bacterial DNA was harvested with a TIANamp Bacterial DNA kit (TianGen, Beijing, China). The purity and concentration of the extracted DNA were determined using a NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE, USA), and DNA samples were stored at −20°C until use.

In silico Analysis

To establish a sequence- and PCR-based Salmonella serotyping method for discrimination of S. Enteritidis, S. Pullorum/Gallinarum, and S. Dublin individually, the basic local alignment search tool (BLAST) algorithm (NCBI, non-redundant nucleotide collection) was applied. The tcpS, $lygD$, and $flhB$ nucleotide sequences were used against the nucleotide collection (nt/nt) database, respectively. The aligned sequence number was set to the maximal value 20,000 with other parameters set to the default values. The three pairs of primers for the targets were designed using Primer Premier 5 (Premier, Palo Alto, CA, USA).

PCR Procedure

PCRs were conducted in a 25 µL reaction volume, consisting of 100 ng of genomic DNA template, 1 × polymerase buffer, 1 U of Taq polymerase (Takara Biotechnology Co., Dalian, China), 200 µM of deoxynucleoside triphosphate, and 80 nM of tcpS/$lygD/flhBinner$ primers. PCRs were performed with a T100 Thermal Cycler (Bio-Rad, Hercules, California, USA) as follows: 94°C for 5 min, 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min, followed by 72°C for 10 min. The amplified PCR products were analyzed on 1% agarose gel in 1 × TAE buffer.

Specificity of the Multiplex PCR

The specificity of the three pairs of primers in the multiplex PCR system was evaluated by detecting genomic DNA from
29 different Salmonella strains, which included 27 Salmonella serovars and eight non-Salmonella species (Table 1).

**Sensitivity of the Multiplex PCR**

The genomic DNA of S. Enteritidis strain C50041, S. Pullorum strain S06004 and S. Dublin strain SL5928 were 10-fold continuously diluted from 58.5 ng/µL to 585 fg/µL, respectively. Each dilution (2 µL) was used in the multiplex PCR assay. This assay was to determine the minimum limit of DNA that can be detected by the multiplex PCR method.

S. Enteritidis strain C50041, S. Pullorum strain S06004, and S. Dublin strain SL5928 were cultured overnight and the bacterial concentration was evaluated by the plate count assay. The bacterial culture was washed with phosphate buffered saline (PBS) twice, 10-fold serially diluted to the concentrations 2 × 10⁷ to 2 × 10² CFU/mL, and boiled for 10 min to collect the genomic DNA. In the multiplex PCR method, each dilution (5 µL) was used as templates to determine the minimum cells of Salmonella that can be detected.

**Isolation of Salmonella Strains from Different Farms**

Additional clinical Salmonella strains with unknown serovars were obtained from three farms, one pig farm, one chicken...
farm, and one cattle farm in Yangzhou, China. The *Salmonella* isolates were collected from feces, floors and lairage, and identified as previously described methods (Cai et al., 2016; Li et al., 2016; Xiong et al., 2016). In brief, each sample was suspended in 50 mL buffered peptone water (Difco, BD, Sparks, MD, USA) and followed by incubation at 37°C for 16–18 h. This broth culture (0.1 mL) was subcultured in 10 mL of Rappaport–Vassiliadis enrichment broth (Difco, BD) at 42°C for 24 h. After incubation, the RV broth was streaked onto xylose lysine tergitol 4 (Difco, BD), and cultured at 37°C for 24–48 h. The presumptive *Salmonella* colonies were picked from all plates and followed by biochemically confirmation using an API-20E test kit (bioMérieux, Marcy l’Etoile, France).

**Application of the Multiplex PCR Method on Clinical Samples**

The multiplex PCR method was applied to detect the genomic DNA of *Salmonella* isolates from one pig farm (24 *Salmonella* isolates), one chicken farm (24 *Salmonella* isolates), and one cattle farm (11 *Salmonella* isolates). The obtained results of the developed PCR method were compared with traditional *Salmonella* serotyping approach.

**Traditional Serotyping of Salmonella Isolates from Different Farms**

The traditional serotyping for all isolated *Salmonella* strains from the pig, chicken and cattle farms were conducted by slide agglutination assay using the specific antisera (Tianrun Bio-Pharmaceutical, Ningbo, China) following the White-Kaufmann-LeMinor instructions (Grimont and Weill, 2007).

**RESULTS**

**In silico Analysis and Primer Design**

*In silico* analysis showed that tcpS exists only in *S. Enteritidis*, *S. Pullorum/Gallinarum*, and *S. Dublin*, and has 98–100% sequence similarity across the three *Salmonella* serovars in the database. Although tcpS in one *E. coli* strain showed 89% DNA sequence identity to the *Salmonella* tcpS, it does not contain the C-terminal region of tcpS or a match to the tcpS-R primer site (data not shown). *lygD* gene exists only in *S. Enteritidis*, and shares 98–100% sequence similarity among this serovar in the database (data not shown). Our previous study showed that *flhB* gene of *S. Pullorum/Gallinarum* lacks a unique region *flhBinner* compared with other serovars, and could be used to identify *S. Pullorum/Gallinarum* (Xiong et al., 2016). Therefore, three pairs of oligonucleotide primers distinguishing three specific *Salmonella* serovars were designed based on the three targets tcpS, *lygD* and *flhBinner* (Table 2).

**Specificity of the Multiplex PCR Assay**

The specificity of the multiplex PCR method was determined by detecting 29 *Salmonella* strains and eight non-*Salmonella* species. The results showed that *S. Enteritidis* generated three specific bands for tcpS, *lygD* and *flhBinner*, and *S. Dublin* generated two specific bands for tcpS and *flhBinner*, while *S. Pullorum/Gallinarum* generated only one specific band for tcpS. In contrast, only one band of *flhBinner* was generated in the other 23 *Salmonella* serovars, and no amplification product was observed in all non-*Salmonella* pathogens (Figure 1).

**Sensitivity of the Multiplex PCR Assay**

To evaluate the sensitivity of the multiplex PCR method, genomic DNA of *S. Enteritidis*, *Pullorum*, and *Dublin* were consecutively diluted and used as templates. The targeted fragments could be amplified at the lowest concentration of 58.5 pg/µL (Figure 2A), suggesting that the five isolates and the other 11 isolates were *S. Enteritidis* and *S. Pullorum/Gallinarum* respectively. Among the isolates from the cattle farm, only one sample generated two specific bands of tcpS and *flhBinner*, suggesting that this isolate was *S. Dublin* (Table 3).

**DISCUSSION**

*Salmonella* remains the most frequently isolated bacteria among food-borne pathogens, and over 19,000 cases were reported in the USA in 2013 (Crim et al., 2014). Thus, a simple method to detect and monitor *Salmonella* serovars in farms is urgently required.
### TABLE 2 | Multiplex PCR primers used for identification of *Salmonella* Enteritidis, S. Pullorum/Gallinarum, and S. Dublin.

| Primers | Primer sequence (5’ → 3’) | Size (bp) | Accession no./Nt segments | Salmonella serovars |
|---------|-----------------------------|-----------|--------------------------|-------------------|
| tcpS F  | ATGTCTATAAGCACCACAATG       | 882       | KM408432.1 1–882          | SE, SP/SG, SD      |
| tcpS R  | TCATTTCAATAATGTTCAAGC       |           |                          |                   |
| lygD F  | CATTCTGACCTTTAAGCCGCTAATGAG| 339       | CP007175.1 1468298–1468636| + – –              |
| lygD R  | CCAAAAAGCGAGACCTCAAACCTACTCAAG |           |                          |                   |
| flhBinner F | GCGGAGCTATTGTAATACCGAGCGG | 155       | CP014983.1 2041558–2041712| + – +              |
| flhBinner R | TCTAAGCTGGAGACCGATTTGACGG |           |                          |                   |

SE, S. Enteritidis; SP/SG, S. Pullorum/Gallinarum; SD, S. Dublin.

### FIGURE 1 | Specificity of the multiplex PCR method for the identification of *Salmonella* serovars Enteritidis, Pullorum/Gallinarum, and Dublin. The multiplex PCR assays, using genomic DNA from various *Salmonella* and non-*Salmonella* strains, were conducted using the designed primers targeting tcpS (882 bp), lygD (339 bp), and flhBinner (155 bp). The three specific PCR products could be amplified in S. Enteritidis. tcpS and flhBinner could be amplified in S. Dublin, while only tcpS gene could be amplified in S. Pullorum/Gallinarum. Detailed strain information is given in Table 1.

Several approaches based on antigens and DNA analysis have been developed to detect *Salmonella* in foodstuffs, including enzyme-linked immunosorbent assay, PCR analysis, and next generation sequencing (Ricke et al., 2013; Park et al., 2014).

Traditional serotyping could provide subtyping information that allows worldwide comparison. This has promoted the characterization of many international *Salmonella* outbreaks (Werber et al., 2005; Elviss et al., 2009). Furthermore, comparison
with historical data was also available based on serotyping because of its wide use for almost 70 years. Verifying the causative pathogens is usually the essential first step in many important epidemiological investigations. Traditional serotyping could be a tough task because it requires necessary expertise and numerous antisera to interpret the agglutination results (Hong et al., 2008). Traditional serotyping methods are also labor-intensive, complicated, expensive, and time-consuming. More importantly, morphological descriptions and biochemical tests may produce ambiguous results (de Freitas et al., 2010). Although whole genome sequencing is becoming more accessible and has been used as a genotyping method, it could be costly and time-consuming, and not practical for sequencing numerous isolates simultaneously. Therefore, rapid PCR-based detection systems for Salmonella have been developed in recent years (Persson et al., 2012).

Comparative genomic analysis is becoming common to validate novel serovar-specific genes because of the improved BLAST program and continuously supplemented genomic data (Zhai et al., 2014). This approach is more economical, convenient, and effective than traditional methods. For example, serovar-specific sequences (STM4495 and SEN1392) for identifying S. Enteritidis and S. Typhimurium were obtained by comparative genomics (Liu et al., 2012). At present, vagC, located in the Salmonella virulence plasmid, is considered a better target for PCR detection of S. Dublin (Persson et al., 2012). However, false-positive results still occur, such as misidentification of a S. Muenchen serovar as S. Dublin (Zhai et al., 2014). Previously, we have found Salmonella flhB gene could be used to identify S. Pullorum/Gallinarum from others because a unique region was deficient only in this serovar (Xiong et al., 2016). Here, we took advantage of three Salmonella genes, tcpS, lygD, and flhB, which were predicted by comparative genomic analysis, to design primers that can accurately distinguish Salmonella serovars Enteritidis, Pullorum/Gallinarum, and Dublin. This allowed the development of a reliable and rapid multiplex PCR method to screen these three serovars individually. To the best of our knowledge, it is the first one-step multiplex PCR method to detect these three prominent Salmonella serovars individually.

The multiplex PCR method produced positive results in S. Enteritidis, S. Pullorum/Gallinarum, and S. Dublin only, with negative results obtained in other Salmonella serovars and eight non-Salmonella bacteria (Figure 1). Besides, the PCR method is very rapid and takes about 3 h to complete. Thus, the PCR results agreed with the BLAST analysis, and the proposed
### TABLE 3 | *Salmonella* strains isolated from three different farms to examine the application of the developed multiplex PCR method.

| Source      | Serovar (no. of isolates) | Isolate no. | PCR results | Source      | Serovar (no. of isolates) | Isolate no. | PCR results |
|-------------|---------------------------|-------------|-------------|-------------|---------------------------|-------------|-------------|
|             | tcpS lygD flhB inner      | tcpS lygD flhB inner |             |             | tcpS lygD flhB inner      | tcpS lygD flhB inner |             |
| Pig farm    |                           |             |             |             |                           |             |             |
|             | Enteritidis (3)           | Pi9         | + + +       | Ch14        | + − −                   |             |             |
|             |                           | Pi17        | + + +       | Ch16        | + − −                   |             |             |
|             | Derby (9)                 | Pi1         | − − +       | Ch20        | + − −                   |             |             |
|             |                           | Pi2         | − − +       | Ch21        | + − −                   |             |             |
|             |                           | Pi5         | − − +       | Entertidis (5) | + + +                   |             |             |
|             |                           | Pi7         | − − +       | Ch6         | + + +                   |             |             |
|             |                           | Pi12        | − − +       | Ch8         | + + +                   |             |             |
|             |                           | Pi13        | − − +       | Ch17        | + + +                   |             |             |
|             |                           | Pi18        | − − +       | Ch22        | + + +                   |             |             |
|             |                           | Pi22        | − − +       | Ch24        | + + +                   |             |             |
|             | Typhimurium (5)           | Pi3         | − − +       | Ch1         | − − +                   |             |             |
|             |                           | Pi10        | − − +       | Ch4         | − − +                   |             |             |
|             |                           | Pi14        | − − +       | Ch9         | − − +                   |             |             |
|             |                           | Pi20        | − − +       | Thompson (3) | − − +                   |             |             |
|             |                           | Pi24        | − − +       | Ch2         | − − +                   |             |             |
|             |                           | Pi8         | − − +       | Ch15        | − − +                   |             |             |
|             | London (2)                | Pi16        | − − +       | Cattle farm | − − +                   |             |             |
|             |                           | Pi2         | − − +       | Dubin (1)   | Ca7         | + − −                   |             |             |
|             |                           | Pi19        | − − +       | Newlands (8) | + − −                   |             |             |
|             | Rissen (5)                | Pi4         | − − +       | Ca1         | − − +                   |             |             |
|             |                           | Pi6         | − − +       | Ca2         | − − +                   |             |             |
|             |                           | Pi11        | − − +       | Ca3         | − − +                   |             |             |
|             |                           | Pi15        | − − +       | Ca5         | − − +                   |             |             |
|             |                           | Pi19        | − − +       | Ca6         | − − +                   |             |             |
|             | Chicken farm              | Pi3         | − − +       | Ca8         | − − +                   |             |             |
|             | Pulorum (11)              | Ch3         | + − −       | Ca9         | − − +                   |             |             |
|             |                           | Ch6         | + − −       | Ca11        | − − +                   |             |             |
|             |                           | Ch7         | + − −       | Ca4         | − − +                   |             |             |
|             |                           | Ch10        | + − −       | Muenster (2) | − − +                   |             |             |
|             |                           | Ch12        | + − −       | Ca10        | − − +                   |             |             |
|             |                           | Ch13        | + − −       |             |                         |             |             |

*The serotyping of the Salmonella isolates was determined based on the traditional serotyping tests according to the White-Kauffmann-Le Minor scheme.*
application of the multiplex PCR method was verified by screening the three prominent Salmonella serovars in samples isolated from pig, chicken, and cattle farms. The results described in this study provide a proof of concept and demonstrate the feasibility of using this PCR method to screen S. Enteritidis, Pullorum/Gallinarum, and Dublin. Future studies will investigate different approaches to isolate DNA directly from infected animals and determine if it can be applied in the field.

This multiplex PCR method could be used for a rapid screening of the three specific Salmonella serovars and simplify the procedures of traditional serotyping. Besides, the combination of traditional serotyping methods and the developed PCR-based approach would promote the efficiency for the serovar identification of Salmonella strains.

CONCLUSION

In summary, an efficient multiplex PCR method targeting three prominent Salmonella serovars, S. Enteritidis, S. Pullorum/Gallinarum, and S. Dublin, was identified for the first time. The multiplex PCR method was based on three genes of tcpS, lygD, and flhB, and the specificity and sensitivity of the multiplex PCR method were determined. The multiplex PCR system was exploited to test extensive sets of Salmonella strains isolated from three farms, thus validating the effectiveness and specificity of the assay. The results suggest that the developed rapid and efficient multiplex PCR assay could be used as a novel and high-throughput diagnostic tool for simultaneous identification of the three specific Salmonella serovars.

REFERENCES

Abdissa, A., Asrat, D., Kronvall, G., Shittu, B., Achiko, D., Zeidan, M., et al. (2006). High diversity of group A streptococcal emm types among healthy schoolchildren in Ethiopia. *Clin. Infect. Dis.* 42, 1362–1367. doi: 10.1086/503422

Cai, Y., Tao, J., Jiao, Y., Fei, X., Zhou, L., Wang, Y., et al. (2016). Phenotypic characteristics and genotypic correlation between Salmonella isolates from a slaughterhouse and retail markets in Yangzhou, China. *Int. J. Food Microbiol.* 222, 56–64. doi: 10.1016/j.ijfoodmicro.2016.01.020

Carrique-Mas, J. J., Willmington, J. A., Papadopoulou, C., Watson, E. N., and Davies, R. H. (2010). *Salmonella* infection in cattle in Great Britain, 2003 to 2008. *Vet. Rec.* 167, 560–565. doi: 10.1136/vr.c4943

Crim, S. M., Iwamoto, M., Huang, J. Y., Griffin, P. M., Gilliss, D., Cronquist, A. B., et al. (2014). Incidence and trends of infection with pathogens transmitted commonly through food-foodborne diseases active surveillance network, 10 U.S. sites, 2006–2013. *MMWR. Morb. Mortal. Wkly. Rep.* 63, 328–332. Available online at: https://www.cdc.gov/mmwr/preview/mmwrhtml/mm6315a3.htm

de Freitas, C. G., Santana, A. P., da Silva, P. H., Gonçalves, V. S., Barros Mde A., Torres, F. A., et al. (2010). PCR multiplex for detection of Salmonella Enteritidis, Typhi and Typhimurium and occurrence in poultry meat. *Int. J. Food Microbiol.* 139, 15–22. doi: 10.1016/j.ijfoodmicro.2010.02.007

Elviss, N. C., Little, C. L., Hucklesby, L., Sago, S., Surman-Lee, S., de Pinna, E., et al. (2009). Microbiological study of fresh herbs from retail premises uncovers an international outbreak of salmonellosis. *Int. J. Food Microbiol.* 134, 83–88. doi: 10.1016/j.ijfoodmicro.2009.01.015

Grimon, P. A. D., and Weill, F. X. (2007). *Antigenic Formulae of the Salmonella Serovars*, 9th Edn. Paris: WHO Collaborating Center for Reference and Research on Salmonella; Institut Pasteur.

Hald, T., Vose, D., Wegener, H. C., and Kouppev, T. (2004). A Bayesian approach to quantify the contribution of animal-food sources to human salmonellosis. *Risk. Anal.* 24, 255–269. doi: 10.1111/j.0272-4332.2004.00427.x

Hong, Y., Liu, T., Lee, M. D., Hofacre, C. L., Maier, M., White, D. G., et al. (2008). Rapid screening of *Salmonella enterica* serovars Enteritidis, Hadar, Heidelberg and Typhimurium using a serologically-correlative allelotyping PCR targeting the O and H antigen alleles. *BMC Microbiol.* 8:178. doi: 10.1186/1471-2180-8-178

Karns, J. S., Haley, B. J., and Van Kessel, J. A. (2015). Improvements to a PCR-based serogrouping scheme for *Salmonella enterica* from dairy farm samples. *J. Food Prot.* 78, 1182–1185. doi: 10.4315/0862-028X.JFP-14-475

Keller, I. H., Schifferli, D. M., Benson, C. E., Aslam, S., and Eckroade, R. J. (1997). Invasion of chicken reproductive tissues and forming eggs is not unique to *Salmonella enteritidis*. *Avian Dis.* 41, 535–539. doi: 10.2307/1592142

Li, Y., Cai, Y., Tao, J., Kang, X., Jiao, Y., Guo, R., et al. (2016). *Salmonella* isolated from the slaughterhouses and correlation with pork contamination in free market. *Food Control* 59, 591–600. doi: 10.1016/j.foodcont.2015.06.040

Liu, B., Zhou, X., Zhang, L., Liu, W., Dan, X., Shi, C., et al. (2012). Development of a novel multiplex PCR assay for the identification of *Salmonella* enterica Typhimurium and Enteritidis. *Food Control* 27, 87–93. doi: 10.1016/j.foodcont.2012.01.062

Majchra, J., Krzyzanowska, A., Kubiak, A. B., Wojtasik, A., Wolkowicz, T., Szych, J., et al. (2014). TRS-based PCR as a potential tool for inter-serovar discrimination of *Salmonella* Enteritidis, S. Typhimurium, S. Infantis, S. Virchow, S. Hadar, S. Newport and S. Anatum. *Mol. Biol. Rep.* 41, 7121–7132. doi: 10.1007/s11303-014-3592-9

Moyo, S. J., Maselle, S. Y., Matee, M. I., Langeland, N., and Mylvaganam, H. (2007). Identification of diarrheagenic *Escherichia coli* isolated from infants and children in Dar es Salaam, Tanzania. *BMC Infect. Dis.* 7:92. doi: 10.1186/1471-2334-7-92

Nesbitt, A., Ravel, A., Murray, R., McCormick, R., Savelli, C., Finley, R., et al. (2012). Integrated surveillance and potential sources of *Salmonella enteritidis* in human cases in Canada from 2003 to 2009. *Epidemiol. Infect.* 140, 1757–1772. doi: 10.1017/S0950268811002548

Nielsen, L. R. (2013). Review of pathogenesis and diagnostic methods of immediate relevance for epidemiology and control of *Salmonella* Dublin in cattle. *Vet. Microbiol.* 162, 1–9. doi: 10.1016/j.vetmic.2012.08.003

Nielsen, L. R., and Dohoo, I. (2013). Time-to-event analysis of predictors for recovery from *Salmonella* Dublin infection in Danish dairy herds between 2002 and 2012. *Prev. Vet. Med.* 110, 370–378. doi: 10.1016/j.prevetmed.2013.02.014

AUTHOR CONTRIBUTIONS

ZP and XJ designed the experiments; DX and LS performed the PCR assays; DX, JT, HZ, and ZZ isolated the samples from the chicken farm; SG participated in the data analysis and interpretation; DX, ZP, and XJ wrote the paper. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

This work was supported by the National Key Research and Development Program Special Project (2016YFD0501607), the Special Fund for Agroscientific Research in the Public Interest (201403054), the National Natural Science Foundation of China (nos. 31320103907 and 31230070), the Project for Agricultural Products Quality and Safety Supervision (Risk Assessment) (18162130109236), the Research and Development Program of Jiangsu (BE2015343), the “Six Talent Peaks Program” of Jiangsu Province (NY-028), the Yangzhou University Science and Technology Innovation Team, and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).
Paião, F. G., Arisitides, L. G., Murate, L. S., Vilas-Bôas, G. T., Vilas-Boas, L. A., and Shimokomaki, M. (2013). Detection of Salmonella spp, Salmonella Enteritidis and Typhimurium in naturally infected broiler chickens by a multiplex PCR-based assay. *Braz. J. Microbiol.* 44, 37–41. doi: 10.1590/S1517-83822013005000002

Park, S. H., Aydin, M., Khatiwara, A., Dolan, M. C., Gilmore, D. F., Bouldin, J. L., et al. (2014). Current and emerging technologies for rapid detection and characterization of *Salmonella* in poultry and poultry products. *Food Microbiol.* 38, 250–262. doi: 10.1016/j.fm.2013.10.002

Persson, S., Jacobsen, T., Olsen, J. E., Olsen, K. E., and Hansen, F. (2012). A new real-time PCR method for the identification of *Salmonella* Dublin. *J. Appl. Microbiol.* 113, 615–621. doi: 10.1111/j.1365-2672.2012.05378.x

Ranieri, M. L., Shi, C., Moreno Switt, A. I., den Bakker, H. C., and Wiedmann, M. (2013). Comparison of typing methods with a new procedure based on sequence characterization for *Salmonella* serovar prediction. *J. Clin. Microbiol.* 51, 1786–1797. doi: 10.1128/JCM.03201-12

Ricke, S. C., Khatiwara, A., and Kwon, Y. M. (2013). Comparison of typing methods with a new procedure based on sequence characterization for *Salmonella* serovar prediction. *J. Clin. Microbiol.* 51, 1786–1797. doi: 10.1128/JCM.03201-12

Soria, M. C., Soria, M. A., and Bueno, D. J. (2012). Comparison of 2 culture methods and PCR assays for *Salmonella* detection in poultry feces. *Poult. Sci.* 91, 616–626. doi: 10.3382/ps.2011-01831

Tatavarthy, A., and Cannons, A. (2010). Real-time PCR detection of *Salmonella* species using a novel target: the outer membrane porin F gene (ompF). *Lett. Appl. Microbiol.* 50, 645–652. doi: 10.1111/j.1472-765X.2010.02848.x

Werber, D., Dreesman, J., Feil, F., van Treck, U., Feil, G., Ethelberg, S., et al. (2005). International outbreak of *Salmonella* Oranienburg due to German chocolate. *BMC Infect. Dis.* 5:7. doi: 10.1186/1471-2334-5-7

Wigley, P., Hulme, S., Powers, C., Beal, R., Smith, A., and Barrow, P. (2005). Oral infection with the *Salmonella enterica* serovar Gallinarum 9R attenuated live vaccine as a model to characterise immunity to fowl typhoid in the chicken. *BMC Vet. Res.* 1:2. doi: 10.1186/1746-6148-1-2

Xiong, D., Song, L., Geng, S., Tao, J., An, S., Pan, Z., et al. (2016). One-step PCR detection of *Salmonella* Pullorum/Gallinarum using a novel target: the flagellar biosynthesis gene flhB. *Front. Microbiol.* 7:1863. doi: 10.3389/fmicb.2016.01863

Zhai, L., Kong, X., Lu, Z., Lv, F., Zhang, C., and Bie, X. (2014). Detection of *Salmonella enterica* serovar Dublin by polymerase chain reaction in multiplex format. *J. Microbiol. Methods* 100, 52–57. doi: 10.1016/j.mimet.2014.02.014

Zhu, C., Yue, M., Rankin, S., Weill, F. X., Frey, J., and Schifferli, D. M. (2015). One-step identification of five prominent chicken *Salmonella* serovars and biotypes. *J. Clin. Microbiol.* 53, 3881–3883. doi: 10.1128/JCM.01976-15

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.