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RAPID COMMUNICATION

Application of an indirect immunofluorescent staining method for detection of *Salmonella enteritidis* in paraffin slices and antigen location in infected duck tissues

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Abstract

AIM: To detect *Salmonella enteritidis* (*S. enteritidis*) in paraffin slices and antigen location in infected duck tissues.

METHODS: Rabbits were immunized with purified bacillus to obtain *S. enteritidis*-specific antibody, which were then extracted by the caprylic-ammonium sulphate method, purified through High-Q columns. An indirect immuno-fluorescent staining method (IFA) was established to detect the *S. enteritidis* antigen in paraffin slices. *S. enteritidis* was detected in each organ tissue of ducklings experimentally infected with *S. enteritidis*.

RESULTS: The gland of Garder, heart, kidney, spleen, liver, brain, ileum, jejunum, bursa of Fabricius from *S. enteritidis* experimentally infected ducklings were positive or strongly positive, and the *S. enteritidis* antigen was mainly distributed in the infected cell cytoplasm.

CONCLUSION: IFA is an intuitionist, sensitive and specific method in detecting *S. enteritidis* antigen in paraffin wax slices, and it is a good method in diagnosis and antigen location of *S. enteritidis*. We also conclude that the gland of Garder, heart, kidney, spleen, liver, ileum, jejunum are target organs in *S. enteritidis* infections of duck, and *S. enteritidis* is an intracellular parasitic bacterium.

Key words: Indirect immuno-fluorescent antibody staining; *Salmonella enteritidis*; Paraffin tissues; Detection; Localization of antigens

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INTRODUCTION

Salmonella enteritidis (*S. enteritidis*) is the infectious disease causing Zoonoses. *S. enteritidis* is one of the primary causes of human food poisoning throughout the world, and has become a pointed public health problem[1-4]. China is the biggest country in the raising and consumption of duck in the world, but the *S. enteritidis* bacillus infection is a severely important infectious disease in the duck industry[5]. *S. enteritidis* outbreaks have been found to be associated with the consumption of contaminated and undercooked poultry products, such as eggs and egg-containing products, and have become a serious economic and public health problem[6]. Conventional methods for isolation of *S. enteritidis* are too laborious, not sufficiently sensitive or correct[7,8]. To our knowledge, a serum method for detection and antigen location of *S. enteritidis* has not been reported. Thus, to establish a rapid, sensitive and highly specific

method for detection of *S. enteritidis* is necessary. We established the Indirect Immunofluorescent (IFA) method to detect and antigen locate *Salmonella enteritidis* to offer a clinical means of diagnosis and apathogenic mechanism of ecology.

MATERIALS AND METHODS

Bacteria strain

S. enteritidis MY1 strain (obtained from Avain Disease Research Center of Sichuan Agriculture University).

Rabbit anti-serum preparation

Briefly, 5 mL of *S. enteritidis,* an overnight culture grown in LB broth, was harvested by centrifugation after 18 h at 37℃. Then diluted at 4×10^9 cfu and inactivated by formaldehyde and equaled to complete Freund's adjuvant (CFA) made the antigen. Rabbits were immunized with 1 mL antigen to obtain *S. enteritidis*-specific antibody, which was then extracted by the caprylic-ammonium sulphate method[9], purified through High-Q columns.

The establishment of the IFA

According to references[10], the following factors were considered to establish the method: (1) antigen recovery, to partly recover the antigens based on a microwave using 0.01 mol/L pH 6.0 citrate buffer solution for 20 min, 200 μg/mL parenzyme for 20min and no recovery; (2) rinsing water: 0.01 mol/L pH 7.4 PBS, 0.01 mol/L pH 7.4 PBS (containing 0.05% Tween-20) and rinsing water separately; (3) confining liquid: using 10% horse serum and 10% BSA to confine; (4) the diluted content of anti-rabbit-SE IgG: 1:25; 1:50; 1:100 and 1:200, incubate time: 4℃ overnight and 37℃ for 40 min; (5) the diluted content of the FITC labeled goat anti-rabbit IgG: 1:25; 1:50; 1:10 and 1:200; (6) foiled liquid: partly used 0.01% Evans Blue and unfoiled.

Artificial infected cases detection

Experimental infections were performed with a *S. enteritidis* MY1 strain. In brief, a group of 24 (specific antibody of SE negative) Bei Jing duck were inoculated with 0.2 mL (2×10^9 cfu) of *S. enteritidis* at the age of 7 d in the crop. Twenty-four uninoculated 7-d-old ducks were used as control animals.

After 24 h post-inoculation some of the ducks began to die resulting in organ harvesting. The organs were detected by IFA and conventional isolation respectively.

Treatment included first, using a non-selective pre-enrichment medium of buffered Peptone Water, incubated at 37℃ for 18 h. Then, using the selective enrichment media-Selenite Cystine Broth, incubating at 37℃ for 18 h. And then, transferring to SS agar, two differential media, Macconkey agar and Triple sugar iron agar were streaked and incubated at 37℃ for 24 h. Suspected colonies were picked up for biochemical and serological tests. The following tests were used: glucose, maltose, arabopyranose, mannitol, glycitol, hydrogen sulfide, MR, lysine decarboxylase, argininedecarboxylase, ornithinedecarboxylase, *etc.* Serotypes were identified by multivalence serum of $O_{A\text{-}F}$ and $O_{1, 9, 12}$ factor serum. The results were referred to[11,12].

The other organs were submerged in 4% formaldehyde, after 24 h; the organs were then embedded in paraffin. Then the tissue sections were made at 4 μm and stained with an indirect immunofluorescent technique.

Specific detection

The established method was applied to detect the livers from the dead ducks infected by *S. pullorum, S. gallinarum, E. coli Riemerella anatipestifer, P. multocida* and Duck plague virus (obtained from the Avian Disease Research Center of Sichuan Agriculture University).

The standard of judging results

The standard of the results are according to the presence, quantity, the depth and lighting of the coloured cells under the fluorescent microscope. There are no flavoviren cells judged negative, infra 5% weakly positive, 5%-50% positive, super 50% strong positive.

RESULTS

Results of anti-SE antibody

The purified anti-rabbit-SE IgG has an Antigen Jade Enlarge (AGE) potency to 1:32, through the SDS-PAGE, there are duplicate bands, and the molecular weight equals to the l-chain and H-chain of IgG. This suggests the IgG is highly pure.

The optimum conditions of IFA

The optimum conditions of this IFA were as follows: Ten minutes antigen retrieval by microwave with 0.01 mmol/L citrate buffer solution (pH 6.0); 10 min antigen retrieval by pancreatin; incubate in 10% bovine serum albumin at 37℃ for 30 min; dilute the primary antibody (1:25) and incubate for 40 min at 37℃; and then incubated at 37℃ for 30 min with diluted FITC-labeled-secondary antibody (1:100) which contains 0.01% Evans.

Distribution in organs of the infected ducks

We detected the organs by IFA after 24 h, a large part of the organs had positive results, and the results were as follows (Figure 1): Heart: Presented as a strong positive, Cardiac muscle fiber had positive cells, and the positive signal distributing the cytoplasm (Figure 1A). Kidney: Presented positive, the mesenchyme between the tube of the Kidney had positive cells, and the positive signal distributing the cytoplasm (Figure 1B). Liver: Presented positive, the mesenchyme between the hepatic cord had positive cells, and the positive signal distributing the cytoplasm (Figure 1C). Bursa of Fabricius: presented positive, the area of the medulla of the follicle had positive cells, and the positive signal distributing the cytoplasm (Figure 1D). Brain: Presented positive, positive cells presented in capillary vessels, and the positive signal distributing the cytoplasm (Figure 1E). Gland of Garder: Presented as a strong positive, the mesenchyme of the tube of the gland had positive cells, and the positive signal

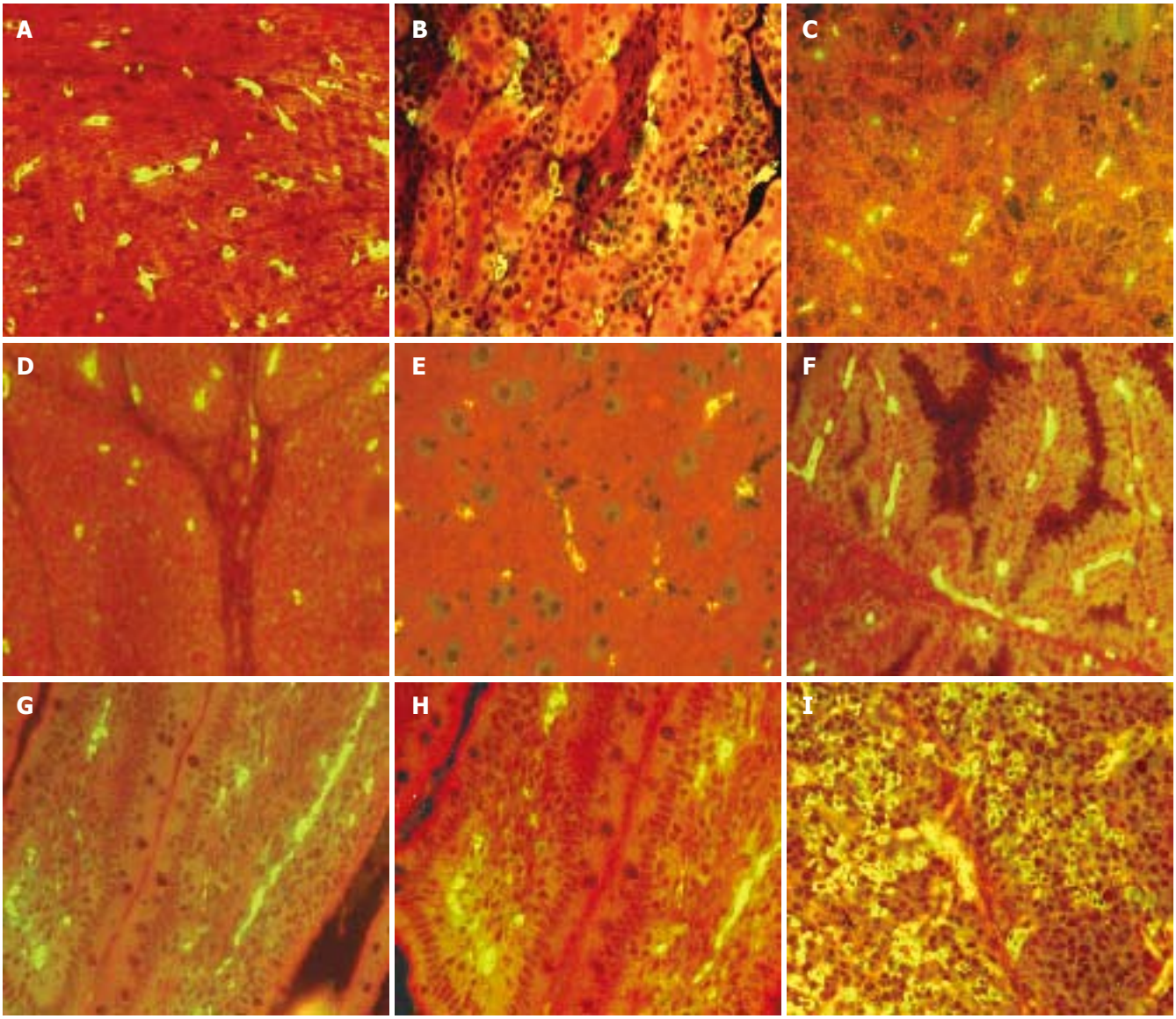

Figure 1 Organ tissues from SE-inoculated ducks immunofluorescent stain for SE. **A**: Positive staining baclli are adhering to the Cardiac muscle fiber (Heart); **B**: Positive staining baclli are adhering to the mesenchyme between the tube of Kidney (Kidney); **C**: Positive staining baclli are adhering to the mesenchyme between the Hepatic cord; **D**: Positive staining baclli are adhering to the area of medulla of the Follicle; **E**: Positive staining baclli are adhering to vascular endothelial cell; **F**: Positive staining baclli are adhering to the mesenchyme of the tube of the gland; **G, H**: Positive staining bacllis are adhering to the interstitial tissue of the lamina propria in villi; **I**: Positive staining baclli are adhering to red medulla. (Images were acquired by using 40 x objective).

Table 1 The results of man-made clinical cases detection using isolation and IFA methods

Detection methods	Results of different organs (faeces) by different methods (positive/total)								
	Heart	Liver	Spleen	Lung	Kidney	Intestine	Brain	Thymus	BF
Isolation	2/24	24/24	24/24	2/24	24/24	24/24	3/24	0/24	8/24
IFA	24/24	24/24	22/24	0/24	24/24	20/24	2/24	0/24	5/24

distributing the cytoplasm (Figure 1F). Small intestine: Numerous *S. enteritidis* organs were observed lying free within the interstitial tissue of the lamina propria in the villi, and the strong positive signals observed in the cytoplasm (Figure 1G, H). Spleen: Presented strong positive, a number of positive cells with particle immunofluorescent antigen distributed in the red medulla of the spleen (Figure 1I).

The results of conventional isolation

All the positive samples detected by the isolation method can be detected by this IFA (Table 1).

The results of specific detection

We detected the livers from the dead ducks infected by *S. pullorum, S. gallinarum, E. coli Riemerella anatipestifer, P.multocida* and Duck plague virus, *S. enteritidis*. The result

Table 2 The results of the specific detection, the positive labeled with (+), negative (-)

Strains	*S. pullorum*	*S. gallinarum*	*E. coli*	*R.A*	*P.multocida*	*DPV*	*SE*
Results	-	-	-	-	-	-	+

showed only the *S. enteritidis* infected, present positive reaction; the others have negative reaction. This suggested good establishment for specificity of the IFA (Table 2).

DISCUSSION

Comparing the differences between IFA and other methods

In the present study, there is little to report about the clinical diagnosis beside conventional methods of isolation for *S. enteritidis*, but there is much about other species. Gast *et al*[6] collected various organs of the infected birds and isolated the bacteria, but it is too laborious, not sufficiently sensitive and correct and can not be used with the rapid diagnoses. Kim *et al*[13] detected the *S. enteritidis* by enzyme immunoassay and had a good result. Desmidt *et al*[14] observed the *S. enteritidis* in the ceca of chickens by immunohistochemistry, which can be used to locate sub-cells of organ tissues of *S. enteritidis* infected chickens. Coope[15] detected the *S. enteritidis* by Dot-Blot and Western-Blot, which is rapid and specific, but could not detect the location in sub-cells of organ tissues. Compared to the above method, the IFA method established has the ability to detect and locate cells and organ tissues of *S. enteritidis* infection, thus offering a clinical means of diagnosis and a pathogenic mechanism of ecology.

The distribution of S. enteritidis in organ tissues

We checked all the ducks when they began to die. At 24 h post-inoculation, the positive signals were observed in the small intestine and spleen; at 36 h, in the heart, kidney, livers, bursa of Fabricius, and Gland of Garder; at 72 h, the positive signals were at the peak; at 6 d post-inoculation, the signals began to reduce. From the result (Figure 1) of the study, we know that the IFA can detect the antigens in almost all organ tissues. The small intestine (ileum, jejunum), heart, liver, kidney, spleen, the Gland of Garder have strong positive signals (Figure 1A-C, I), and presented in the cytoplasm of phagocytic cells of the macrophile system. This may be caused by phagocytic cells after entering the host. Meanwhile, we can detect a positive signal in the brain, a positive signal presented in the vascular endothelial cell, which may be caused by *S. enteritidis* invading the organs and *S. enteritidis* in blood. At the anaphase of infection, the infection appeared bacteremic, the *S. enteritidis* needed to transit the vascular endothelial cells, and uniformly distributed in the cytoplasm of the vascular endothelial cells. We also detected small positive signals in the bursa of Fabricius.

The value of S. enteritidis infections from other viral infectious diseases

Immuno-fluorescent labeled bacilli were associated with the epithelial surface of the intestinal villi at 4 h. At 24 a strong positive signal was detected intracellularly in the small intestine (Figure 1G, H). This resulted in a positive signal which only presented in the lamina propria of the villi. This is a big difference from Duck plague virus (DPV)[16] and Duck Hepatitis Virus (DHV)[17], which was located in the lamina propria. This variation might be related to a different microenvironment[18] in different parts of the gut, dividing into a microcolony at that site. The microenvironment has an important effect in the colonization of the gut, primarly as follows. Gastric acid is the first defense of the intestinal infection, the bacterium can hardly survive (pH < 1.5). The slime layer of the intestinal mucosa has a barrier function to the *S. enteritidis*; moreover pankrin, bile salt and small intestinal juice can depress the bacterium. Some studies suggest that the gene encoding the invasive protein is depressed because of the bile[19,20]. Meanwhile, the involvement of mucosal IgA in the protection against *S. enteritidis* involving the epithelial surface has been reported[21,22]. The cathodolyte antibacterial peptide secreted by pit cells in the small intestine belongs to the Toalexin family, which can increase the permeability of the cytomembrane of the bacterium, and not profit to survive[23].

The intestine is an important barrier for prevention against invasion by bacteria. The lymphoid tissue called Peyer macular under the muscoa[24] consists of T cells, B cells, macrophages and dendritic cells, following the swallowing of the pathogenic bacterium.

The immune reaction also played an important role in preventing *S. enteritidis* lying free in the endothelial cells of the intestine. After swallowing the *S. enteritidis* by macrophage and heterophil granulocyte, both happened to outbreak by breathing, which produced oxygen free radical, for instance, O_2-, H_2O_2, OH-, and O_2- and so on. The oxygen free radical can kill and wound bacteria; the cell factors (IL-1, IL-6, TNF-α[25]) composed and released have a pathopoiesis effect and a bactericidal effect; when the *S. enteritidis* invades the enterocyte, it can induce the secretion of IL-8[26], which can kill and wound bacteria. $CD4^+$ T cells also have an effect on the immune defense[27].

The cooperation between the host cells and S. enteritidis infections

Infection with bacteria is usually started by oral ingestion of the pathogen and is followed by bacterial colonization of the gut and invasion of internal tissues. Intracellular replication is essential for the virulence.

Attachment to host tissues is the first important step to establish a bacterial infection. The attachment is primarily referred to as the inv gene[28]. Once attached to the host cells, the bacterium manifests athletic phenomenon of invasion of the cells, phagocytosis by cell invagination. After fusion of macrophage lysosomes with phagosomes, the *S. enteritidis* appears in the phagocytotic vesicle of the cytoplasm[29], and may destroy the cell invagination. In the present study, there are different reports about the *S. enteritidis* entering the cell, the bulk of the investigations

support the view that *S. enteritidis* enters the cell by a virulence factor. Higashide and McGhie suggest that when *S. enteritidis* enter the cell, firstly SspC excreted by TTSS insert into the plasma membrane, then Sip and SipA bind to the actin of the cytoskeleton and rearrange the frame, which enhances the entrance of the bacterium[30,31]. Scientists report recently that the dendritic cell (DC) residing in the mucosa of the small intestine can use the dendrite to open the tight junction of the cells, and directly ingest the bacteria from the mucosa[32]. From the result (Figure 1), the positive signal distributed in the cytoplasm of infected cells of the organ tissues, we conclude that *S. enteritidis* is an intracellular parasitic bacterium.

COMMENTS

Background

Salmonella enteritidis (*S. enteritidis*) is the infectious disease causing Zoonoses. *S. enteritidis* is one of the primary causes of human food poisoning throughout the world. China is the biggest country in growing and consumption in the world, but the *S. enteritidis* bacillus infection is the severely important infectious disease in raising duck's industry. But the pathogenic mechanism of ecology is unknown.

Research frontiers

The *S. enteritidis* can be isolated from various organs of the infected birds. It is possible that the *S. enteritidis* invades the tissues of these organs; electron microscopic studies of chicks have shown passage of *Salmonella* through ileocecal mucosa. The observation of attachment and invasion of the ceca of infected chicks have been reported. Also, in mammalian animal models, the colonization of *S. enteritidis* has been studied further. However, there are few studies about ducks.

Innovations and breakthroughs

The present study was to establish a serum method for detection and antigen location of *S. enteritidis* which has not been reported.

Applications

The results can offer a clinic means of diagnosis and pathogenic mechanism of ecology.

Peer review

This is a methodology manuscript. The author of this manuscript establishes a method of IFA and detected the organs of the *S. enteritidis* infected ducks. The author conclude that IFA is a sensitive and specific method in detecting *S. enteritidis* antigen in paraffin slice; it's a good method in diagnosis and antigen location of S. enteritidis; the Gland of Garder, heart, kidney, spleen, liver, and small intestine are target organs in *S. enteritidis* infections of duck; *S. enteritidis* is an intracellular parasitic bacterium.

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