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Original article

Enzyme-linked immunosorbent assay with a *Salmonella enteritidis* antigen for differentiating infected from vaccinated poultry

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Abstract – The specificity and sensitivity of indirect ELISA, based on the use of four different antigenic extracts obtained from a clinical isolate of *Salmonella enteritidis*, were compared with those obtained with the gm-flagellin based ELISA (IDEXX). A total of 116 serum samples from salmonellae free, naturally infected and vaccinated hens were studied. The results showed that the indirect ELISA, based on lipopolysaccharide (LPS), O-polysaccharide (PS) or membrane sediment (SD) antigens, enable the identification of a greater number of infected birds and discriminated field antibody responses from vaccinal ones better than the commercial IDEXX test. The indirect ELISA that used a O-polysaccharide rich fraction (PS) proved to be the most specific and sensitive test, suggesting that this indirect ELISA could be used to confirm IDEXX results, especially when the differentiation between vaccinated and infected poultry is required.

***Salmonella* / serological diagnosis / poultry / ELISA**

Résumé – ELISA basé sur l'utilisation d'un antigène de *Salmonella enteritidis* pour la différentiation entre la volaille infectée et vaccinée. La spécificité et la sensibilité d'une nouvelle méthode ELISA indirecte, basée sur l'utilisation de quatre extraits antigéniques différents obtenus d'un isolat clinique de *Salmonella enteritidis*, ont été comparées avec celles d'un autre ELISA basé sur l'utilisation de la gm-flagelline (IDEXX). Un total de 116 échantillons de sérum provenant de poules saines (non-infectées avec Salmonella), infectées ou vaccinées, a été analysé par les deux techniques. Les résultats ont clairement démontré que la méthode ELISA indirecte, utilisant les antigènes LPS, une fraction riche en polysaccharide-O (PS) ou le sédiment de membranes SD (LPS-proteins complex), permettait l'identification d'un plus grand nombre d'animaux infectés, et une meilleure différentiation

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entre les poules infectées et les poules vaccinées que le test commercial IDEXX. La méthode ELISA utilisant la fraction polysaccharidique-O (PS) s'est révélée être le test de diagnostic sérologique le plus spécifique et le plus sensible. Ces résultats suggèrent que cet ELISA peut être utilisé pour la confirmation des résultats obtenus par le test IDEXX, notamment quand la différentiation entre la volaille vaccinée et non-vaccinée est nécessaire.

***Salmonella* / diagnostic sérologique / volaille / ELISA**

1. INTRODUCTION

Salmonella enteritidis is recognised as a frequent and important pathogen for poultry and has been isolated from broiler, breeder and commercial egg laying flocks [1, 19]. Both poultry meat and eggs are often mentioned to cause salmonellosis in man [26]. Monitoring flocks for *S. enteritidis* infection is typically accomplished via bacteriological or serological methods. Bacteriological examination for the detection of flock infections on a practical basis is laborious, time-consuming, and expensive, especially when a statistically reliable number of faecal samples per flock is tested to confirm that a flock found bacteriologically negative for *S. enteritidis* is truly free of *S. enteritidis* [11]. Bacteriological examination may also yield false negative results when *S. enteritidis* is overgrown by other *Salmonella* serotypes present in the flock [28]. Identification of infected birds is also difficult because *S. enteritidis* can induce a chronic carrier state, whereby apparently healthy birds excrete the organisms intermittently [31].

For these reasons, many authors have pointed out the need for a suitable serological assay for use as a screening technique to detect *S. enteritidis* infection in poultry [2, 3, 14, 20, 21, 27]. Many techniques for the serological identification of infected flocks have been described; these include agglutination tests (rapid whole blood slide test, tube agglutination test, and antiglobulin test) and enzyme-linked immunosorbent assays (ELISAs) with different antigens, such as lipopolysaccharide (LPS), SEF 14 fimbrial antigen, flagellin, outer membrane proteins, and crude extracts (obtained by heat treatment) of *S. enteritidis* [4, 6–10, 12, 15–17, 21, 23–25].

The use of vaccines for the control of *Salmonella* infections is gradually increasing [33] but it interferes with serological screening, since conventional tests cannot discriminate vaccinal antibody responses from natural infection. This paper evaluates the specificity, sensitivity and discrimination capacity of an indirect ELISA based on the use of four different antigens obtained from a clinical isolate of *S. enteritidis*, and compares the results to those obtained with the gm-flagellin based ELISA implemented in the Dutch *S. enteritidis* eradication program [27, 28], for detecting infection in naturally infected chickens.

2. MATERIALS AND METHODS

2.1. Serum samples

A total of 116 serum samples from reproductive hens (7–11 months old) were studied. Serum samples in group 1 (n = 72) were obtained from *S. enteritidis* naturally infected hens as confirmed by rectal swab culture. Serum samples in group 2 (n = 22) were taken from salmonellae free hens and samples in group 3 (n = 22) were taken from hens vaccinated at the age of 4 months (*S. enteritidis* commercial bacterine; Laboratorios Hypra, Gerona, Spain).

Positive and negative control serum sample pools were prepared by pooling ten individual serum from infected hens and ten

individual serum from salmonellae free hens respectively.

2.2. Preparation of ELISA antigenic extracts

Antigenic extracts were prepared from *S. enteritidis* 4520, a clinical isolate obtained from Ramon Díaz, Microbiology Department of the Clínica Universitaria de Navarra (Pamplona, Spain). *S. enteritidis* 4250 was incubated in Trypticase Soya Broth (TSB, BioMérieux) pH 7.2, at 37 °C up to the exponential phase (OD_{590} = 0.4). Cells were then inactivated by addition of phenol (final concentration, 0.5%), washed with saline solution and centrifuged at 4 000 *g* for 20 min. The supernatant was harvested and ultracentrifuged (100 000 *g*, 6 h), then the sediment (SD) and supernatant (SS) were collected and lyophilised. A crude lipopolysaccharide extract (LPS) was obtained from the harvested cells after the first centrifugation by the hot water-phenol method of Westphal et al. [30]. An O-polysaccharide rich fraction (PS) was obtained from the extracted LPS by resuspending it in 2% acetic acid (2 mg of dried weight·mL^{-1}) and hydrolysing it at 100 °C for 1 h. After centrifugation (100 000 *g*, 6 h) the supernatant was dialysed for 2 days at 4 °C against several changes of distilled, deionised water, and lyophilised.

The protein and LPS contents of these four antigenic preparations were measured by the Lowry [18] and ketodesoxyoctonate (KDO) assays [22, 29], respectively.

2.3. Bacteriological examination

Reproductive hens were bacteriologically examined for the presence of salmonellae by incubation of rectal swabs in enrichment media (Rappaport Vassiliadis and Selenite Cystine broth) followed by inoculation of Xylose Lysine Agar (XLD) and Salmonella Shigella Agar (SS).

2.4. Determination of positive/negative cut-off value

A total of 22 serum samples from *Salmonella*-free birds were examined by ELISA using four different antigen extracts (SS, SD, LPS, PS). The cut-off point for the antigens was selected as the mean plus two standard deviations in order to prevent the detection of false positives. The dilution of sera was 1/1000.

2.5. ELISA procedure

IDEXX test — The gm-flagellin based ELISA (IDEXX) procedure was carried out as described by the manufacturer (cut off value C/N: 0.75) (Idexx laboratories, Inc., Westbrook, Maine, USA).

Indirect ELISA — The following conditions were determined in preliminary experiments: Polystyrene microtitre plates (Bioreba, Spain) were coated with 50 µL of their respective antigenic extract solutions (5 µg·mL^{-1}) in coating buffer (0.05 M carbonate/bicarbonate buffer, pH 9.6) and incubated at 4 ºC for 12 h. The solution was then tipped off and the microplates were washed three times in PBST (PBS plus 0.05% Tween 20). Then, 50 µL of test and control sera diluted 1/100 and 1/1000 in PBS Tween buffer containing 5% skimmed milk were added per well. The plates were then incubated for 4 h at 37 ºC and washed as before. A solution of peroxidase conjugated to goat anti-chicken IgG (Nordic Labs, Tilburg, The Netherlands), diluted 1/5000 in PBS containing 0.05% Tween 20, was added in 50 µL volumes to each well. The conjugate was incubated for 30 min at 37 ºC and washed. Finally, plates were developed by incubation for 20 min at room temperature with 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid diammonium salt 0.01% (Sigma) in 0.1 M citrate buffer (pH 4.0) containing hydrogen peroxide 0.01%. The colorimetric values were determined by measuring the OD at 405 nm.

The percentage specificity and sensitivity of IDEXX and indirect ELISA based on the four antigenic extracts, were calculated with the Episcope 1.0 software (Agriculture University, Wageningen, the Netherlands) with a 95% confidence level.

3. RESULTS

3.1. Chemical characterisation of the antigenic extracts

Table I shows the percentage of proteins/LPS of the four antigenic extracts used in this study, obtained from *S. enteritidis* 4520. The protein contamination of the LPS fraction was only 1.3%. The polysaccharide extract (PS) contained LPS (17.9%) and a trace of proteins. SD and SS antigenic preparations were the richest in proteins.

Table I. Percentage of proteins/LPS of the four antigens obtained from *Salmonella enteritidis* 4520.

Antigen [a]	Prot/LPS
LPS	1.3/99.0
PS	0.6/17.9
SD	31.6/50.7
SS	23.8/27.9

[a] LPS, lipopolysaccharide; PS, O-polysaccharide rich fraction; SD, membrane sediment fraction; SS, soluble fraction.

3.2. Comparison between IDEXX test and ELISA using different *S. enteritidis* antigenic extracts

First, the cut-off points were established, in order to determine the sensitivity and specificity of the indirect ELISA using the different antigenic extracts. These were selected as the mean plus two standard deviations, after studying a total of 22 serum samples from *Salmonella*-free birds, by indirect ELISA. The cut-off points were as follows: OD_{405nm} LPS, 0.559; PS, 0.336; SD, 0.557; SS, 0.401.

Table II shows the mean OD_{405nm} registered for each sample group after being examined by indirect ELISA and the commercial IDEXX test. Serum samples in group 1 (n = 72), which were obtained from *S. enteritidis* infected hens, as confirmed by rectal swab culture, presented a more variable response when examined by IDEXX than when examined with LPS, PS or SD antigens.

Table II. ELISA comparative results (mean OD_{405nm}; SD) using the four different antigenic extracts for each serum sample group (serum dilution 1/1000).

Group[a]	n[b]	IDEXX	LPS [0.559][c]	PS [0.336]	SD [0.557]	SS [0.401]
1	40	+	0.946 (0.590)	0.752 (0.610)	0.906 (0.282)	0.460 (0.274)
1	16	–	0.799 (0.106)	0.640 (0.352)	0.642 (0.255)	0.444 (0.208)
1	16	+/–	0.676 (0.127)	0.471 (0.425)	0.494 (0.360)	0.392 (0.225)
2	22	–	0.245 (0.157)	0.178 (0.790)	0.333 (0.112)	0.253 (0.740)
3	22	+	0.792 (0.257)	0.536 (0.270)	0.970 (0.164)	0.370 (0.125)

[a] Groups: 1, naturally infected with *S. enteritidis* (this group was divided in three depending on the IDEXX results, positive, negative or intermediate, for a cut off value of 0.75); 2, Salmonella free; 3, vaccinated with a commercial bacterine of *S. enteritidis*.
[b] Number of serum samples.
[c] Cut off values.

Table III. Comparative values of sensitivity and specificity (%) of IDEXX and indirect ELISA (four different antigenic extracts).

		Sensitivity[a]	Specificity[a]	Sensitivity[b]	Specificity[b]
Indirect	LPS	70.8	100	70.8	59.9
ELISA	PS	**76.4**	**100**	**76.4**	**88.6**
	SD	72.2	100	72.2	52.3
	SS	75.0	68.2	75.0	47.7
IDEXX		**55.6**	**100**	**55.6**	**50.0**

[a] Comparative results obtained with serum samples from "*Salmonella* free hens" vs. samples from "infected hens".
[b] Comparative results obtained with serum samples from "*Salmonella* free hens + vaccinated hens" vs. samples from "infected hens".

Furthermore, when examined by IDEXX, serum samples in group 3 (n = 22) which were taken from vaccinated hens were all considered as *S. enteritidis* positive, while 18.2, 22.7, and 4.5% were positive when examined by indirect ELISA with the LPS, PS or SD antigens were used, respectively.

3.3. Sensitivity and specificity of IDEXX and the indirect ELISA

When the results of serum samples from salmonellae free hens and samples from infected hens were compared, the IDEXX sensitivity was found to be inferior (55.6%) to that of indirect ELISA, with antigen PS showing the greatest sensitivity (76.4%). On the other hand, the specificity of IDEXX and indirect ELISA based on LPS, PS or SD was 100% (Table III).

When serum samples of vaccinated hens were taken into account, in order to serologically differentiate field antibody responses from vaccinal ones (Table III), the sensitivity of IDEXX was again evidently lower than that of the indirect ELISA. In this comparison, the specificity of IDEXX was similar to that obtained with indirect ELISA based on LPS, SD or SS, with PS again yielding the best results (sensitivity 76.4%, specificity 88.6%).

4. DISCUSSION

These results showed that the indirect ELISA, based on LPS, PS or SD antigens, enables the identification of a greater number of infected birds and discriminates field antibody responses from the vaccinal ones better than the commercial IDEXX test. Overall, it is suggested that indirect ELISA based on the O-polysaccharide rich fraction (PS) could be used to confirm IDEXX results, especially when the differentiation of vaccinated birds is required.

In many control programs of microbial pathogens the development of serological screening tests for the discrimination of the vaccinated state from vaccine failure and natural infection is priority [5, 13, 32]. The use of vaccines in the control of *Salmonella* infections is gradually increasing [33] but conventional tests cannot discriminate vaccinal antibody responses from natural infection. This is one area where improvements are required, and more information is needed; obviously, further work needs to be done.

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