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Diagnosis of *Ornithobacterium rhinotracheale* Infection in Chickens by ELISA

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Specific *Ornithobacterium rhinotracheale* (ORT) antibodies were determined in serum samples of 24 clinically infected broiler flocks of different ages (1-42 d) and 11 broiler-breeding flocks (at ages between 26-56 w) by ELISA. Two commercially available kits were separately assessed. The BioCheck ELISA kit was used for testing 363 serum samples representing 12 broiler flocks, where 74 samples (20.3 %) were found to be positive and 49 (13.5 %) were suspected. The IDEXX ELISA kit was used for testing 148 serum samples representing different 12 broiler flocks, where 115 samples (77.7 %) were positive. Testing of additional 70 serum samples from 5 broiler- breeder flocks, associated with drop in egg production (1-4.5 %) at different ages, by BioCheck ELISA kit revealed that 78.5 % of the samples were positive and 21.4% were suspected. On the other hand, 338 serum samples representing 6 broiler-breeder flocks, associated also with egg drop, showed a 84.6 % rate of positive reaction, when tested by IDEXX ELISA kit. Positive serology correlated well with the clinical manifestations and isolation of the organism, which substantiates the reliability of the used kits in diagnosis of the disease.

Ornithobacterium rhinotracheale (ORT) infection is considered a serious problem facing the poultry industry, it can affect all avian species (wild and domestic birds) and is associated with respiratory diseases. Air sacculites and pneumonia are the most common features of infection. Other factors, especially respiratory viruses and bacterial infections aggravate these clinical signs. Infection can be transmitted horizontally by aerosol droplets and also vertically in eggs. ORT is a recently discovered bacterium of the rRNA superfamily V, which was first named in 1994 (Lopes et al., 2000). ORT is characterized by very slow growth under aerobic condition at 37°C, while full growth occurs under anaerobic incubation at 37°C for 48 hours on sheep blood agar (Back et al., 1996). Wojcinski (1996) recorded also that the ORT organism is a very slow grower on sheep blood agar after 48 hours incubation under CO_2 conditions and some cases may be overgrown by other robust bacteria like *E. coli*. The difficulty in isolation and identification of ORT led many authors to attempt the diagnosis of the disease serologically.

Van Empel et al. (1999) recorded that ELISA is useful for detection of antibodies to ORT in one-day-old birds and in egg yolk as well as in birds with clinical sings. They found that ELISA is more sensitive than the agglutination and that cross-reactions occur between ORT serovars in rapid slide agglutination test. Lopes et al. (2000) compared ELISA results with serum plate agglutination test, and found that the agglutination test detects specific antibodies for ORT in only 56% of experimentally infected turkeys during the first 2 weeks of infection, while ELISA detects up to 100% of infected birds at 8 weeks post infection. ELISA has been also used by Hafez et al. (2000), Sakai et al. (2000) and El-Gohary and Sultan (2002) for diagnosis of ORT infection in poultry.

In the present work ELISA was used for detection of antibodies against ORT in the serum of non-vaccinated broiler and broiler-

breeder flocks. The main objective was to evaluate the efficiency of two commercial kits in the diagnosis of the disease in chickens at different ages.

Material and Methods

Samples

A total of 919 serum samples **was** tested in the present work. Five hundred and eleven serum samples were collected from 24 clinically infected broiler flocks at ages of 1-42 day and 408 serum samples were obtained from 11 broiler-breeding flocks at ages between 26-56 week, associated with drop in egg production (1-4.5%).

ELISA kits

Two different commercial ELISA systems, BioCheck (Holland) and IDEXX (France) kits, were used for detection of specific ORT antibodies. The procedures recommended by the supplying companies were followed as described below.

BioChek ELISA

Serum samples from examined flocks (363 samples from 12 broiler flocks and 70 samples from 5 broiler-breeding flocks) were diluted (1:100), then 100 µl of diluted samples, both negative and positive controls were added to the micro titre wells (according to instruction in the kit) and incubated for 60 minutes, where any ORT antibodies present will bind and form an antigen antibody complex. Non-specific antibodies were then washed away by using 300 µl washing buffer for each well and 100 µl of conjugate reagent were added into all wells and incubated for 60 minutes. After another wash to remove unreacted conjugate, 100 µl of substrate solution were added. A yellow colour was developed if ORT antibodies were present after 30 minutes incubation and the intensity is directly related to the amount of antibodies in the samples. 100 µl of stop solution were added into each well to stop the reaction and the absorbance values of controls and samples were recorded by a microtitre plate reader. The relative amounts of antibodies in chicken samples can then be calculated by reference to the positive control. This relationship is expressed as S/P ratio (sample to positive ratio). The S/P ratio was calculated to obtain the titres by BioChek software. The titre less than 424 was considered negative, 424-1431 suspect, and the titre greater than 1432 was considered positive.

IDEXX ELISA

The tested serum samples (148 samples from 12 broiler flocks and 338 samples from 6 broiler-breeding flocks) were diluted into (1:500) with sample dilution buffer, 100 µl of each diluted sample with both negative and positive controls were dispensed into 96- well of antigen coated plates. After 30 minutes incubation, the plates were washed out by distilled water 4 times and 100 µl of conjugate were dispensed into each well in the plates and incubated for 30 minutes at room temperature. The washing step was repeated then 100 µl of substrate solution were dispensed into each well and the plates were incubated for 15 minutes at room temperature, then 100 µl of stop solution were added into each well to stop the reaction and the absorbance values were measured and recorded by ELISA reader. The amounts of antibodies in samples were calculated by using IDEXX software to obtain sample to positive (S/P) ratio. The S/P ratio greater than 0.4 (titre greater than 844) should be considered positive and indicates exposure to the infection.

Isolation and identification of ORT bacteria

Samples from different organs as tracheas, lungs, sinuses, eyes, joints, air sacs, pericardium, bone marrow and liver were obtained for primary isolation. They were cultivated on 10% defibrinated sheep blood agar containing 10 ug/1ml of gentamicin sulphate to inhibit the overgrowth of other bacteria according to Back et al. (1996), incubated at 37°C for 24-48 hours in microaerophilic condition using candle jar (Vandamme et al., 1994 and Travers et al., 1996). Pure suspected colonies of ORT were sub-cultured on semi-solid slope agar, peptone water and brain heart infusion broth containing NAD and 1-2% swine or horse serum as enrichment to enhance the growth and multiplication of ORT according to Opengart (1996). ORT strains were tested biochemically according to Charlton et al. (1993). In addition, identification using Api system (Api 20E and API ZYM) was also done. The anti-biogram was done according to the technique described by Back et al. (1997).

Results

1. Application of BioChek ORT ELISA kits

a. Results of antibody detection of ORT in serum samples from broilers: The serological examination of 363 serum samples representing 12 broiler flocks by using BioChek kits was done for detection of ORT antibodies in correlation with disease manifestations and ORT isolation. The results (Table1) indicated that 74 (20.3%) of 363 sera were positive for ORT antibodies, while 49 (13.5%) were suspected. ORT antibodies were detected in 7 out of the 10 flocks (70%)

showing clinical symptoms and were positive for ORT isolation. On the other hand, antibodies could be detected in one apparently healthy flock, which was negative for ORT isolation. Five of the 7 positive flocks suffered also from ND. In most cases, antibodies titres were detected at ages between 27 and 35 days. Only in one flock positive results was obtained from 4 day old chicks.

Table 1. Detection of antibodies to *Ornithobacterium rhinotracheale* (ORT) by ELISA in serum samples from broilers using BioChek kit.

Flocks	No of tested samples	ELISA Results				Disease manifestations	Isolation results
		Positive		Suspect			
		No	%	No	%		
F1	40	1	2.5	0	0	Respiratory	+
F2	40	4	10.0	0	0	G. retardation	+
F3	45	9	20	15	33.3	Respiratory	+
F4	38	9	23.7	12	31.6	Arthritis and/or Respiratory	+
F5	56	0	0	6	2.4	Respiratory	-
F6	20	0	0	0	0	G. retardation	-
F7	40	34	85	2	5	Nervous with high mortality	+
F8	14	4	28.5	8	57.1	Arthritis and/or Respiratory	+
F9	16	13	81.2	3	18.7	Respiratory	+
F10	12	0	0	1	8.3	Respiratory	-
F11	30	0	0	0	0	Healthy	-
F12	12	0	0	5	41.5	Healthy	-
Total	363	74	20.3	49	13.5		

F= flock, += ORT was isolated from one or more birds in the flock, -= None of the birds examined in the flock yielded ORT in culture.
G. retardation= growth retardation

b. Results of antibody detection of ORT in serum samples from broiler- breeder flocks associated with (1-4%) drop in egg production: The results of 70 serum samples collected from 5 broiler-breeder flocks (before entering production, during the egg production period and at the end of production), associated with (1-4%) drop in egg production and tested by BioChek ELISA kits, revealed that ORT antibodies were detected in 55 (78.5%) of the 70 sera, while 15 (21.4%) were suspected (Table 2). The positive mean titres were higher at the beginning of egg production and at the end of production period.

Table 2. Detection of antibodies to *Ornithobacterium rhinotracheale* (ORT) by ELISA in serum samples from broiler-breeders using BioChek kit.

Flock*	Age (weeks)	No. of tested samples	ELISA results				
			Positive*		Suspect		Mean titre
			No	%	No	%	
F1	32	10	7	70	3	30	6215
F2	33	10	7	70	3	30	4565
F3	49	20	14	70	6	30	2581
F4	54	20	18	90	2	10	7751
F5	57	10	9	90	1	10	7166
Total		70	55	78.5	15	21.4	-----

* The tested flocks were associated with drop in egg production. Titre greater than 1432 was considered positive, 424-1431 suspect and less than 424 negative.

2. Application of IDEXX ORT ELISA kits

a. Results of antibody detection of ORT in serum samples from broilers: When the IDEXX ELISA kits were used for the testing of 148 serum samples representing other 12 broiler flocks in different ages, it was observed that 115 out of 148 samples (77.7%) were positive for ORT antibodies (Table 3). The antibodies against ORT were detected in 10 of the 11 flocks, which suffered from respiratory manifestations or growth retardation. Isolation of the organism was successful in 10 flocks. In most cases, antibodies titres were detected after 21 day of age and the number of positive samples increased in older ages.

b. Results of antibody detection of ORT in serum samples from broilers- breeder flocks associated with (1-4%) drop in egg production: IDEXX kits were used for testing 338 serum samples representing 6 brioler-breeder flocks, which were also associated with drop in egg production. The results shown in (Table 4) revealed that 286 (84.6%) of 338 sera were positive and also, the positive mean titres were higher at the beginning of egg production and at the end of production.

Table 3. Detection of antibodies to *Ornithobacterium rhinotracheale* (ORT) by ELISA in serum samples from broilers using IDEXX kit.

Flock	No of tested samples	Interpretation of ELISA results (positive)		Disease manifestations	Isolation results
		No	%		
F13	20	29	100	Respiratory.	+
F14	10	10	100	G. retardation	+
F15	10	10	100	Respiratory	+
F16	20	20	100	G. retardation.	+
F17	20	1	5.0	G. retardation.	+
F18	24	10	41.6	Respiratory, G. retardation	+
F19	12	12	100	Respiratory	+
F20	10	10	100	Respiratory	+
F21	12	12	100	Respiratory	+
F22	10	0	0	Respiratory, G. retardation	+
F23	10	10	100	Respiratory	-
F24	10	0	0	Healthy	-
Total	148	115	77.7		

F= flock, += ORT was isolated from one or more birds in the flock, -= None of the birds examined in the flock yielded ORT in culture. G. retardation= growth retardation.

Table 4. Detection of antibodies to *Ornithobacterium rhinotracheale* (ORT) by ELISA in serum samples from broiler-breeders using IDEXX kit.

Flock*	Age (weeks)	No. of tested samples	ELISA results		
			Positive		Mean titre
			No	%	
F6	26	40	38	95.0	4619
F7	31	90	65	76.2	1880
F8	37	37	28	75.6	3653
F9	50	49	37	75.5	4495
F10	56	62	59	95.1	6526
F11	57	60	59	98.3	3896
Total		338	286	84.6	----

*The tested flocks were associated with drop in egg production. Titre greater than 844 was considered positive.

Discussion

Van Empel et al. (1999) recorded that the serovar specificity of the ELISA was a disadvantage but commercial kits had been developed, which were able to detect antibodies against at least nine of the 12 known serovars. Antibody titres peaked at 1 to 4 weeks after a field infection but declined rapidly, so serum samples for flocks screening should be taken at different ages. Hafez et al. (2000) compared self-made ELISA based on SDS-antigen extraction of serovar B and one commercial ELISA-kit (Biocheck. Gouda, the Netherlands) for their ability to detect antibodies against 12 ORT serovars (A-L). Antibodies against all serovars were detected by both ELISA systems with similar results and some minor variations. Lopes et al. (2000) used the outer membrane proteins of ORT in an indirect ELISA as an antigen to detect infection in turkeys exposed to different serovars of ORT. The results suggested that ELISA was able to detect the exposure to ORT in later stages of infection and this assay could be used in serological surveillance of ORT infection for poultry in field.

In the present investigation, specific ORT antibodies were detected in serum samples of 24 clinically infected broiler flocks of different ages (1-42 d) and 11 broiler-breeding flocks (at ages between 26-56 w) by using two different ELISA systems, BioCheck and IDEXX kits. Unfortunately, the two types of kits were not available at the same time to compare between them; accordingly they were used for testing separate flocks.

The results of ELISA conformed well with results of isolation. All ORT positive flocks by ELISA, except flock F23, were confirmed by ORT isolation. The failure of isolation of ORT in this flock may be due to the massive application of antibiotics in this flock. On the other hand, the flock F22 was ORT ELISA negative at age of 21 days in spite of ORT positive isolation. This may be explained as that the ORT infection was recent and ORT antibodies did not develop yet. That is why it was recommended that serum samples for flock screening should be taken frequently (Hafez et al., 2002). Generally, ELISA can detect antibodies against ORT in serum at 1 to 4 weeks post infection (Van Empel et al. (1996). Also the antibiotic therapy could affect the serological response to ORT as studied by Popp and Hafez (2002), who recorded that the immediate treatment of infected chicken by certain antibiotics did not influence the antibody response but when the treatment started 7 day post infection, this resulted in lower antibody response and these findings explain why the antibodies response was low in some ORT positive flocks.

Furthermore, the present study indicates that the prevalence of ORT antibody is high in the commercial broiler-breeder population in different ages during production period and associated with drop in egg production, a finding which is in agreement with that reported by Hafez (1996) as well as El-Gohary and Soltan (2002). The fact that the positive serology in all cases examined correlated well with the clinical manifestations and isolation of the organism, substantiates the reliability of the used kits in diagnosis of the disease.

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