

## Seroprevalence and Identification of *Ornithobacterium rhinotracheale* from Broiler and Broiler Breeder Flocks in Thailand

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**SUMMARY.** Ornithobacteriosis is an infectious disease of avian species that has been reported in almost all countries around the world, except Thailand. The objectives of this study were to determine the seroprevalence of *Ornithobacterium rhinotracheale* (ORT) and to isolate and identify ORT in broilers and broiler breeders in Thailand. Chicken antibodies had been randomly checked from 17 farms (19 flocks) of broilers and 23 farms (28 flocks) of broiler breeders. The seropositive flocks were 63% and 100% in broilers and broiler breeders, respectively. The sera analysis showed that the individual 280 broiler sera antibody responses were 67.5% negative, 12.9% suspected, and 19.6% positive. The individual antibody responses of 510 broiler breeder sera revealed 12.2% negative, 38.0% suspected, and 49.8% positive samples. The bacteria were isolated and identified by polymerase chain reaction (PCR). Bacterial isolation and identification revealed that nine isolates of the 12 PCR analysis samples showed positive results to PCR analysis. All the positive PCR samples were collected from the broiler breeder farms.

**RESUMEN.** *Nota de Investigación*—Seroprevalencia e identificación de *Ornithobacterium rhinotracheale* a partir de pollos y reproductoras de engorde en Tailandia.

La Ornithobacteriosis es una enfermedad infecciosa de las especies aviarias que se ha reportado en casi todos los países del mundo con la excepción de Tailandia. Los objetivos de este estudio fueron determinar la seroprevalencia del *Ornithobacterium rhinotracheale* (por sus siglas en Inglés ORT), lo mismo que aislar e identificar el ORT en pollos y reproductoras de engorde en Tailandia. La presencia de anticuerpos fue evaluada aleatoriamente en 17 granjas de pollos de engorde y en 32 granjas de reproductoras de engorde. Se encontró una positividad del 63% en los pollos y del 100% en la reproductoras. Los análisis del suero mostraron que de las 280 muestras de suero de pollos, el 67.5% fueron negativas, el 12.9% sospechosas y el 19.6% positivas. Las respuestas individuales de anticuerpos de 510 muestras de suero de reproductoras de engorde mostraron que el 12.2% fueron negativas, el 38.0% sospechosas y el 49.8% positivas. La bacteria fue aislada y identificada por medio de la reacción en cadena por la polimerasa. El aislamiento y identificación de las bacterias mostró que de 12 muestras analizadas, nueve fueron identificadas como positivas. Todas las muestras positivas fueron obtenidas de las granjas de reproductoras de engorde.

**Key words:** seroprevalence, *Ornithobacterium rhinotracheale*, PCR, chickens

**Abbreviations:** ELISA = enzyme-linked immunosorbent assay; ORT = *Ornithobacterium rhinotracheale*; PCR = polymerase chain reaction

A major problem of the poultry industry around the world is respiratory tract infection, which creates a major economic impact due to the increase in treatment cost and losses through the deaths of animals and higher condemnation rates. Respiratory diseases of poultry arise from the various causes, including bacteria (9), viruses (2), and fungi (1). *Ornithobacterium rhinotracheale* (ORT) is the recently described species of bacterium that is a slow growing, gram-negative, pleomorphic bacterium (29). Up to now, at least 18 serotypes of ORT could be distinguished (30). Serotyping in chickens has revealed that the majority of isolates are of serotype A and that 95% of strains belong to the four major serotypes A, B, D, and E. Turkey isolates seemed to be more heterogeneous, being distributed among the serotypes (32). ORT causes respiratory tract infection in many avian species (19,34), which can be isolated from the air sacs and pneumonic lesions of poultry around the world (19,24,25,28,30). Also, the bacteria can be isolated in many avian species, including peacocks, ostriches, quails, guinea fowls, and ducks (10,12,29,30,32). The disease was first discovered in 1991 by Jan DuPreez (28). ORT can be a primary or secondary etiologic agent depending on strain virulence, adverse environmental factors, the immune state of the flock, and the presence of other infectious agents (30). ORT can cause highly contagious diseases in poultry, although the severity of the

clinical signs, the duration of the disease, and mortality have been found to be extremely variable (30). Losses of 5%–10% are frequently seen, and even losses up to 90% are not rare from this disease (33). In Hungary, substantial mortality (0.5%–2% per day) associated with ORT infection in 4- to 6-wk-old broiler flocks has been reported (23). Gross pathologic lesions usually include serous-catarrhal inflammation of the mucous membrane in the upper respiratory tract with less often unilateral or bilateral pneumonia, as well as serofibrinous airsacculitis and pericarditis (11). ORT infections have been reported in many countries around the world, including Belgium (35), Canada (17), the United States (10), France (18), Israel (7), England (4), Slovenia (37), Mexico (22), Peru (16), Korea (36), Japan (28), Jordan (13), Turkey (14,27), Egypt (12), and Taiwan (26). In Thailand, ORT isolation has not been reported yet. In addition, serologic evidence of ORT infection has been obtained (4) and the presence of ORT can be confirmed from maternal immunity derived from the infected chickens. The aim of this study was to determine the prevalence of ORT infection and also to isolate and identify ORT in broilers and broiler breeders in Thailand, which is the first step necessary before control measures can be implemented.

### MATERIALS AND METHODS

**Broiler and broiler breeder serum samples.** Serum samples were collected from the 17 broiler farms (19 flocks) and 23 broiler breeder

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Table 1. ELISA results of ORT from broiler farms.

Farm code	Province	Age (days)	No. of samples	ELISA results		
				Negative	Suspect	Positive
Vet-24	Phetchabun	40	12	1	2	9
Vet-25	Buriram	44	10	0	2	8
Vet-26	Chaiyaphum	42	10	0	8	2
Vet-27	Lopburi	42	10	0	1	9
Vet-28.1	Lopburi	42	10	1	2	7
Vet-28.2	Lopburi	41	10	0	4	6
Vet-29	Lopburi	42	10	3	4	3
Vet-30	Chainart	45	10	10	0	0
Vet-31	Lopburi	42	10	10	0	0
Vet-32	Chaiyaphum	42	10	10	0	0
Vet-33	Lopburi	42	10	9	1	0
Vet-34	Lopburi	42	10	9	0	1
Vet-35	Lopburi	42	10	8	1	1
Vet-36	Chonburi	42	30	18	8	4
Vet-37.1 <sup>A</sup>	Chonburi	39	25	21	1	3
Vet-37.2	Chonburi	40	25	24	1	0
Vet-38 <sup>A</sup>	Chonburi	39	25	25	0	0
Vet-39 <sup>A</sup>	Chonburi	39	23	22	1	0
Vet-40	Chonburi	30	20	18	0	2
Total			280	189	36	55
%			100%	67.5	12.9	19.6

<sup>A</sup>The isolates from these farms revealed negative results, which were tested by PCR.

farms (28 flocks) of the major producing companies of Thailand (Tables 1, 2) in the period between October 2004 and September 2005 and kept at  $-20\text{ C}$  until tested.

**Enzyme-linked immunosorbent assay (ELISA).** Sera were analyzed by indirect ELISA with an ORT antibody test kit (Biochek, Gauda, the Netherlands) that detects antibodies against serotypes A to M. Optical densities were determined in an ELISA reader and processed with software from Biochek. According to the test, three categories of the results had been established. The negative, suspect, and positive results are in the titer range  $\leq 424$ ,  $425-1431$  and  $\geq 1432$ , respectively.

**Isolation of bacteria.** The bacteria were collected from chickens in the seropositive flocks. In each flock, the tracheas of 20 mild clinically ill birds (coughing or sneezing) had been swabbed from a flock of approximately 25,000 birds, and five samples had been pooled to one sample. Additionally, the severe clinically ill birds (nasal discharge, severe coughing, no appetite, or severe depress) were necropsied and the trachea or air sacs had been swabbed and pooled as described previously. To accomplish this, the sterile swabs were inserted approximately 2.5 cm into the trachea and carefully withdrawn to avoid contamination from the pharyngeal region. The swabs were placed in sterile tube with tryptone soya agar (Oxoid, Hampshire, England), capped, and cooled transported within 2 days to the laboratory. The bacteria were cultured in a Columbia agar base with 5% sheep blood and 5  $\mu\text{g/ml}$  gentamicin under microaerophilic conditions, for 48–72 hr. After incubation, colonies that were pinpoint, circular, opaque to grayish, and nonhemolytic were considered to be suspect (31). Gram-negative, catalase-negative, and oxidase-positive colonies were tested before identification by polymerase chain reaction (PCR).

**PCR analysis.** Primers used in this study were OR16S-F1 (5'-GAGAATTAA TTTACGGATTAAG-3') and OR16 S-R1 (5'-TTCGCTTGGTCTCCGAAGAT-3'), which were very specific in amplifying a 784-base pair fragment of the 16S rRNA gene of ORT, but not of other closely related bacteria (30). Bacterial DNA was extracted and purified by DNA trap (Biotec, Bangkok, Thailand). PCR was conducted in a Thermal Cycler (MyCycler<sup>™</sup>; Bio-Rad). A reaction volume of 20  $\mu\text{l}$  contained  $10\times$  PCR buffer with  $(\text{NH}_4)_2\text{SO}_4$ , 2.5 mM  $\text{MgCl}_2$ , 0.2 mM dNTP, 1.0  $\mu\text{M}$  each primer, 50–100 ng of DNA template, and 1.5 U of *Taq* DNA polymerase. Samples were subjected to 94 C, 90 sec followed by 35 cycles at 58 C, 60 sec and 72 C, 90 sec. PCR products were examined in 1.8% agarose gels with a  $0.5\times$  Tris

borate-EDTA buffer, stained with 10  $\mu\text{l}$  of ethidium bromide (20 mg/ml), and exposed to ultraviolet light.

## RESULTS

**ELISA.** The ELISA results for the chickens from 40 farms (47 flocks) were revealed. The results showed that 12 of the 19 (63%) broiler flocks were ORT antibody positive. All broiler breeder flocks were ORT antibody positive. The ELISA results from 17 broiler farms for those ages between 30 and 45 days were 67.5% negative, 12.9% suspected, and 19.6% positive (Table 1). The ELISA results for 23 broiler breeder farms for those ages between 17 and 62 weeks were 12.2% negative, 38.0% suspected, and 49.8% positive (Table 2). ORT vaccines have never been used in Thailand; so, the suspected results may be regarded as positive results. The suspected + positive results in the broiler farms were 32.5%. The suspected + positive ELISA results in the broiler breeder farms were 87.8%, and older birds were more positive than younger birds.

**Isolation of ORT.** In total, 165 pooled samples were obtained from 51 broiler flocks and 114 pooled samples from broiler breeder flocks. Thirty-seven suspected bacterial samples (22.4% of the total submitted pooled samples) could be grown and isolated. Twelve (three and nine isolates from broiler and broiler breeder flocks, respectively) (32.4% of the cultured isolates) of the 37 isolates, revealed gram-negative stain, positive to catalase, and negative to oxidase. The rest of bacteria (25 strains) were kept for further identification.

**PCR analysis.** The PCR analysis revealed nine positive samples from 12 ORT suspected samples of the seroconversion flocks. All PCR-positive samples were isolated from the broiler breeder flocks (Table 2).

## DISCUSSION

Our work is the first report of the seroprevalence and the presence of ORT in Thailand within the commercial broiler and broiler

Table 2. ELISA results of ORT from broiler breeder farms and the PCR results of ORT tested from the gram-negative, catalase positive and oxidase negative bacterial colonies.

Farm code	Province	Age (wk)	No. of samples	ELISA results		
				Negative	Suspect	Positive
Vet-1.1	Prachinburi	21	10	0	0	10
Vet-1.2	Prachinburi	21	10	0	0	10
Vet-2	Phetchabun	26	12	2	5	5
Vet-3	Phetchabun	36	10	4	5	1
Vet-4	Lopburi	27	10	1	4	5
Vet-5	Lopburi	29	27	11	13	3
Vet-6	Lopburi	31	25	8	12	5
Vet-7	Lopburi	30	38	3	16	19
Vet-8	Lopburi	17	30	14	13	3
Vet-9	Lopburi	46	24	1	13	10
Vet-10 <sup>A</sup>	Chonburi	36	30	1	11	18
Vet-11	Chonburi	22	12	0	6	6
Vet-12	Chonburi	53	12	2	4	6
Vet-13	Chonburi	58	11	2	5	4
Vet-14	Chonburi	43	12	0	1	11
Vet-15	Chonburi	32	12	0	3	9
Vet-16	Chonburi	60	12	1	2	9
Vet-17	Chonburi	38	11	1	5	5
Vet-18	Chonburi	44	12	2	2	8
Vet-19.1 <sup>A</sup>	Chonburi	61	25	0	9	16
Vet-19.2 <sup>A</sup>	Chonburi	48	25	4	12	9
Vet-19.3 <sup>A</sup>	Chonburi	47	22	3	14	5
Vet-20.1 <sup>A</sup>	Chonburi	60	15	1	5	9
Vet-20.2 <sup>A</sup>	Chonburi	59	15	0	4	11
Vet-21.1 <sup>A</sup>	Chonburi	61	20	1	9	10
Vet-21.2	Chonburi	62	19	0	6	13
Vet-22 <sup>A*</sup>	Chonburi	61	25	0	11	14
Vet-23 <sup>A</sup>	Chonburi	61	24	0	4	20
Total			510	62	194	254
%			100	12.2	38.0	49.8

<sup>A</sup>Isolates from these farms revealed positive results, which were tested by PCR.

breeder population. We found that the antibody against ORT was common even though ORT vaccines have never been applied in Thailand. Herein, the ELISA test revealed that broiler breeder flocks had a greater serologic prevalence for ORT than did broilers. All broiler breeder flocks tested were positive for the ORT antibody, and this was similar to the 100% positive flocks found in southern Brazil (8) and higher than the 79% found in broiler breeder flocks in Germany (15). In broiler flocks, 68% (13/19) had been found positive, and this was higher than the 60% positive flock found in southern Brazil (8) and the 26% found in broiler flocks in Germany (20). The high prevalence of positive broiler flocks may be because of the continuous introduction of the agent from their parent flocks (6,30). The results revealed that age might influence the seroprevalence of ORT in Thailand. The broiler chickens, raised over a short period, had been found to display seroconversion to a lesser degree than those of the broiler breeder raised over a longer period. The sera analysis revealed that in the individual 510 broiler breeder sera, 87.8% were positive, which was lower than the 94.6% found in broiler breeders in southern Brazil (8) and higher than the 13.9% found in broiler breeders in Japan (21). Overall, these numbers show a high prevalence of ORT antibodies in broiler breeders in Thailand. The prevalence of the individual 280 broiler sera, 32.5%, that were positive was much greater than the 6.52% found in broilers in southern Brazil (8) and the 9.4% found in broilers in Germany (20). The higher prevalence of serum-positive broiler breeders may be the result of a higher challenge and longer life span of breeders. The presence of antibodies and ORT organisms identified in the broiler breeders may lead to the transmission of the

bacteria to broilers through the eggs (5). In contrast, serum-positive breeders may passively protect their progeny at the beginning of their life (30), although broilers may become susceptible to clinical disease sometime later (19). The lower prevalence of ORT antibodies in broilers in Thailand, compared with broiler breeders, may be influenced by differences in age, environmental factors, and local strains of the bacteria that spread slowly. All these results might suggest that the rolling infection had been occurring in the broiler and broiler breeder farms. Bacterial isolation and identification revealed that nine isolates of the 12 PCR analysis samples revealed positive results to PCR analysis. All the positive PCR samples that were collected from the broiler breeder farms suggested that age might be an influence on the positive ORT isolation. Our study shows that the prevalence of ORT antibodies and ORT organisms is present in the commercial broiler and broiler breeder population, even though no ORT vaccine had been used in Thailand, suggesting that rolling infection may occur and that the organism can spread through parent flocks to newly housed pullet flocks.

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