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ISOLATION AND IDENTIFICATION OF *Ornithobacteriun rhinotracheale* FROM LAYING HENS IN FARMS OF LA HABANA PROVINCE

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ABSTRACT: **Respiratory infection is the most serious disease affecting poultry and causing great economic losses to the poultry industry worldwide. In avian host, several microorganisms of the genus Pasteurella (*P. multocida*, *P. gallinarum* and *P. anatipestifer*), *Bordetella* (*B. avium*) and *Avibacterium paragallinarum*) were involved in respiratory diseases complex. *Ornithobacterium rhinotracheale* is a recently discovered bacterium, of worldwide distribution in commercial poultry, in which it is associated to respiratory diseases and it is also found in wild birds. Airsacculitis and pneumonia are the most common symptoms of infection with *O. rhinotracheale*. Isolation and identification of pathogenic bacteria associated to respiratory diseases were carried out from a total of 80 samples of animals collected in four different periods from 4 farms in the western region in Cuba. In total 16(20%), 15(18%), 9(11%) and 4(5%), isolates were identified as *P. multocida, E.coli, M.haemolytic* and *O. rhinotracheale*. The *O. rhinotracheale* strains were isolated from infraorbital sinus exudates of animal with clinical symptoms and these strains were identified by biochemical test and by amplification of a fragment of 16S rRNA which was analysed by enzymatic restriction, the fragments with the expected size were obtained. This work is the first report of the presence of *O.rhinotrachelae* by culture and molecular method in layer hens in farms from the Western region in Cuba.**

*(Key words: **Pasteurella multocida**; **Escherichia coli**; **Ornithobacterium rhinotracheale**; layer hens)*

AISLAMIENTO E IDENTIFICACIÓN DE *Ornithobacterium rhinotracheale* EN GALLINAS PONEDORAS DE GRANJAS DE LA PROVINCIA LA HABANA

RESUMEN: **Las enfermedades respiratorias en las aves son de gran importancia por su impacto económico en la conversión alimenticia, disminución en la producción de huevos, incremento en el costo de medicamentos. En hospederos aviares varios microorganismos de los géneros Pasteurella (*P. multocida*, *P. gallinarum* and *P. anatipestifer*), *Bordetella* (*B. avium*) and *Avibacterium paragallinarum*) están asociados al complejo respiratorio. *Ornithobacterium rhinotracheale* es una bacteria de reciente descubrimiento, de amplia distribución mundial, asociada con enfermedades respiratorias, también está presente en aves salvajes. La aerosaculitis y neumonía son los síntomas más comunes de la infección por *O.rhinotracheale*. El objetivo de este trabajo es la identificación de bacterias asociadas a procesos respiratorios en aves ponedoras específicamente en 80 muestras de animales procedentes de 4 granjas de la región occidental. Del total de muestras 16(20%), 15(18%), 9(11%) y 4(5%) correspondieron a *P. multocida, E.coli, M.haemolytic* and *O. rhinotracheale*. Las cepas de *O. rhinotracheale* se identificaron por pruebas bioquímicas, se confirmaron, análisis de restricción por amplificación de un fragmento del RNAr16s. Se obtuvieron los tamaños esperados. Este trabajo constituye el primer reporte de la identificación y presencia de *O.rhinotracheale* por cultivo y métodos moleculares en gallinas ponedoras en granjas de la región occidental.**

*(Palabras clave: **Pasteurella multocida**; **Escherichia coli**; **Ornithobacterium rhinotracheale**; gallinas ponedoras)*

INTRODUCTION

Respiratory infection is the most serious disease affecting poultry causing great economic losses to in the poultry industry worldwide. In avian host, several microorganisms of the genus *Mycoplasma* sp., *Pasteurella multocida, Bordetella avium* and *Avibacterium paragallinarum* are involved in respiratory diseases complex (1, 2). *Escherichia coli* associated to respiratory infection in chickens has also been reported (3).

The etiological agent of ORT is a Gram-negative rod shaped bacterium named *Ornithobacterium rhinotracheale* in 1994 after a detailed phenotypic and genotypic analysis done by Vandamme (4). This disease may affect chickens and turkeys at any age, the birds manifest dyspnoea, sneezing, increased mortality rate and decreased egg production. Currently, 18 serotypes of ORT have been differentiated (from A to R) (5, 6). The infection can be transmitted horizontally by aerosol, as well as vertically through eggs, which probably accounts for its rapid and worldwide spread (6).

In Cuba, serological evidence of the ORT infection has been observed (date no yet published) through antibodies against the bacteria which have been detected using commercial ELISA test. This study was aimed at isolation and identification of bacteria associated to sinusitis and pneumonia with special reference to *O. rhinotracheale* in four layer hens' farms in the province La Habana.

MATERIALS AND METHODS

Samples such as exudates from infraorbital sinus, tracheas and lungs fragments were collected from 80 layer hens from *White leghorn* line L33 belonging to four different farms in the Occidental region and in four different months: July and October 2008; February and December 2009. The animals showed respiratory disease symptoms. The collected samples were streaked onto 5 % sheep blood agar media(Columbia Blood Agar base; Oxoid) and Chocolate agar. The culture plates were incubated for at least 48 hour at 37°C in anaerobic or aerobic conditions with or without CO_2 (Anaerobic Oxoid).

Cultures with pure growth were analyzed for identification by subculture in Columbia blood agar, Chocolate agar and Mac Conkey agar. Pure culture were stained by Gram's Method, identified biochemically by oxidase and catalase test and api systems api, Biomerieux, France to confirm the main phenotypic traits. Suspected *P.multocida* and *O.rhinotracheale* were confirmed genetically by PCR with specific primers to region of 16S rRNA.

DNA extraction: A few colonies from suspicious ORT cultures were transferred into tubes containing 3 ml of Brain Heart Broth. The tubes were incubated at 37°C for 18 hours. The cultures were then centrifuged at 13000 g for 5 min and the cell pellet was resuspended with 0.5 ml of TE buffer and the supernatant was removes and resuspended in 375 µl of fresch cell wall disruption buffer (10mg/ml lysozyme) and incubated al 37°C for 30 minutes. The cells were lysed by addition of 20 µl Lysis buffer (SDS 20%) and 3 µl of proteinase K and this mix was incubated at 37°C for 1 hour and 200 µl saturated NaCl (approx. 6,0M) was added. Genomic DNA was precipitated with absolute ethanol and 0.3 M sodium acetate at –20°C overnight. The mixture was then centrifuged at 120000 g for 10 min and the pellet was washed with 300 µl of 70% ethanol. The pellet was dried and resuspended in 50 µl of TE buffer containing RNAase A and used as a target DNA in PCR (7). Bacterial DNA of *P.multocida* and ORT to be amplified were also released from whole organisms by boiling from single colony (8).

PCR was carried out in a total volume of 25 µl containing 5 µl of template DNA, 20 pmol of each primer, 200 µM of dNTPs, 1x PCR buffer, 3 mM $MgCl_2$ and 1.5 U of amplicen. The amplified DNA was visualized in 0.8% agarose gels stained with ethidium bromide. The 1Kb ladder (Promega) was used as standard. The oligonucleotide primers were custom synthesized (CIGB, Cuba).

Amplification of *P.multocida* was obtained with initial denaturation step at 95°C for 4 min followed by 45 cycles at 95°C for 60s, 55°C for 60s and at 72°C for 60s, with a final extension at 72°C for 9 min. The pair of primers has the fallowed sequence: KMT1SP6 5'GCTGTAAACGAACTCGCCAC-3' and KMT1T75'-ATCCGCTATTTACCCAGTGG-3' were used for amplification of a fragment of DNA *P.multocida* (8).

Amplifications for ORT were carried out using conditions modified from Van Empel and Hafez (9). The sequence of primers to the ribosomal 16S rRNA gene of ORT: OR16S-F1 (5'-GAGAATTAATTTACGGATTAAG) and OR16S-R1 (5'-TTCGCTTGGTCTCCGAAGAT). Amplification was obtained with initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 60s and extension at 72°C for 90s, with a final extension at 72°C for 7 min. The amplified products were visualized by ethidium bromide (0.5µg/mL) staining after electrophoresis at 70 volts for 1 hour in 0.8% agarose gels. Chromosomal and amplified DNA of ORT was

digested for 3 h with the restriction endonucleases *Cla*I according to the recommendations of the manufacturer (Promega). The 20 µl digestion mixture consisted of DNA, buffer (10X), enzyme (10U) and water. Following restriction digestion, reactions were stopped by heating solutions for 10 min at 65ºC, then cooling for 5 min on ice. The digested DNA was separated in a horizontal gel containing 1% (w/v) agarose. Electrophoresis was done at room temperature (RT) at 100 V for 30 minute.

RESULTS AND DISCUSSION

According to growth characteristics, colony morphology, biochemical reactions and carbohydrate fermentation, the bacterial isolates were identified as *P. multocida* 16(20%), *E.coli* 15(18%), *Mainhelia haemolytic* 9(11%) and 4(5%) *O. rhinotracheale*.

Bright pink colonies observed as a result of lactose fermentation on MacConkey's agar were identified as *E. coli* and confirmed by negative oxidase test and Api 20 E test. Colibacillosis caused by *E.coli* infections account for significant morbidity and mortality in poultry industry (3, 10). Yet, despite the importance of colibacillosis, much about the virulence mechanisms employed by avian *E.coli* remains unknown (11, 12).

Smooth, circular, convex colonies were observed on the Columbia blood agar, staining by Gram method revealed gram negative, coccobacillary organisms and no growth on MacConkey's agar was identified as possible *P.multocida. P.multocida* isolates were confirmed using api 20NE and PCR assay, a band of 460 pb was amplified from all isolates identified. In poultry, infection with *P. multocida* may result in fowl cholera, a disease of economic importance in commercial production that may occur in different forms, such as per acute, acute and chronic infections (13). It is probably that *P.multocida* recovered in this study is associated with chronic respiratory process.

Ewers *et al.* in 2003 (14), Songserm *et al.* in 2003 (15) and Nasrin *et al.* 2007 (16) isolated *E. coli* and *Pasteurella* spp. from layers. The presence of these bacteria could be alarming for industry poultry since the bacteria may produce disease especially when birds are immunologically suppressed due to severe stress conditions (16).

Circular and small colonies (1–3 mm in diameter), opaque to greyish and non-haemolytic, negative in Mac Conkey, positive oxidase and negative for catalase were then selected as possibly to belonging to ORT using the criteria of Vandamme *et al*., 1994 (4). Gram-negative pleomorphic bacteria were visualised as described by Van Empel and Hafez en 1999 (9) and Chin and Droual en 1997(17) .

Two isolates were obtained from one flock and the remaining two isolates were from another flock. The four isolates possibly belonging to ORT identified in this study showed the fallowing code: 0-0-2-0-0-0-4 when the api 20 NE was used, similar to 30.55% from the strains tested by Van Empel and Hafez en 1999 (9). The isolates were β galactosidasa positive similar to the ORT isolation detected by Ozbey *et al*. en 2004 (18). API-20NE identification strip (Bio Merieux, France) has prove to be useful, although ORT is not included in the API data files;in studies where a total of approximately 1150 strains were tested, the strains showed different code by API-20NE. However to Canal *et al*. in 2005 (19) the identification of ORT through the API 20NE system was not possible since the reactions were negative.

The results of biochemical test were confirmed by PCR. All ORT suspicious isolates were positive in PCR. A 784 bp amplification product was obtained, corresponding to the expected size (Figure 1) when the DNA was extracted using chemical lyses from broth cultures. No amplified products were obtained from the negative control.

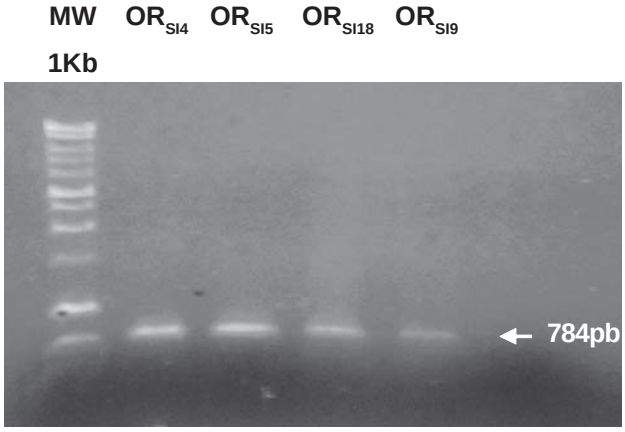

FIGURE 1. Electrophoreses of PCR products on a 0.8% agarose gel stained with ethidium bromide: 1 Kb Promega molecular weight marker (lane 1), amplification products from four isolates from flocks OR_{SI4} (lane 2), OR_{SI5} (lane 3), OR_{SI8} (lane 4), OR_{SI19} (lane 5) and *Pasteurella multocida* as the negative control (lane 6)./ *Electroforesis de productos de PCR en geles de agarosa al 0.8% teñidos con bromuro de ethidium: línea 1: Marcador de peso molecular de 1 Kb Promega, línea 2-5: productos de amplificación de 4 aislados de ORT: OR_{SI4} (línea 2), OR_{SI5} (línea 3), OR_{SI8} (línea 4), OR_{SI19} (línea 5) y línea 6 P. multocida como control negativo.*

The ORT fragment of 784 bp was also amplified when the ORT PCR was also performed directly from a single colony grown on agar plates using DNA extraction by boiling, similar to *P. multocida*, this technique is useful for the rapid identification of *ORT* directly from bacterial cultures without culture, extraction and purification of genomic DNA even in mixed culture.

To confirm that the amplified PCR fragment corresponding to 16S rRNA it was cleaved with the restriction endonucleases and the products were resolved by agarose gel electrophoresis showed the fragments with the expected size 671 and 124 pb when the *Cla*I restriction enzyme was used (date not shown). The primers described by Van Empel and Hafez en 1999 (9) are highly specific only for a 784bp fragment on the 16S rRNA gene of ORT and at the same time are not consistent with any other closely related bacteria occurring in a genome.

In the present study *O.rhinotracheale* was isolated only from the infraorbital sinus exudates from four layer hens but not from other tissues. ORT can usually be isolated from the lungs, trachea and air sacs in broilers (1, 20, 21), although there are some reports about the isolation of ORT from infraorbital sinus from layer hens (21, 22, 23). In the experimental infection with ORT aerosol in broiler chickens, the histological lesion in sinuses were predominant but the culture and PCR was negative, however ORT was detected by culture and PCR in trachea, lungs and air sacs only (24).

Because of the difficulties of ORT isolation, it can be incorrectly diagnosed as viral infection or bacterial infection due to the overgrowing of different genus of bacterial, particularly *E. coli, Proteus* sp., *Pseudomonas* sp. thereby making it difficult to identify by the routine methods used in most diagnostic laboratories. This can be avoiding by isolation at early infections stage or by the use of antibiotic in the culture media. Since it has been shown that the most of ORT isolates are resistant to gentamicin and recommended the use of 10 ug of gentamicin per ml of blood agar medium (25).

ORT was isolated only in two months corresponding to winter November and January however in July and October there not was any isolation of ORT in the studied samples. It is well known that respiratory infections are significantly affected by environmental factors, and that disease severity is increased during the winter months. Temperature, ventilation, humidity, atmospheric ammonia, and dust have important interactions with infectious agents in producing respiratory disease (1).

The study revealed that the percentage of isolation of *P.multocida* and *E.coli* was higher (20% and 18% respectively) than the ones of *O.rhinotracheale* (5%). These results were consistent with the ones reported by Ozbey *et al.* 2004 (18) where ORT was isolated from trachea only (1.5%) from chickens while the presence of antibodies against ORT was detected by ELISA in 33 (10.2%) of the samples.

Ornithobacterium rhinotracheale has been isolated from chickens, turkeys, quails, ducks, geese, ostriches, guinea fowls, pheasants, rooks and pigeons (4, 26). ORT was also reported producing infections in the United States, Germany, South Africa, The Netherlands, France, Israel, Belgium, Hungary, Japan, the United Kingdom, Turkey, Canada, Jordan and Brazil (18, 26, 27, 28, 29, 30). The isolates obtained in our study were compatible with ORT and for the first time it is recognized in Cuba by the culture and genetic identification from infrorbital sinus of layer hens with respiratory disorders. The results will allow the development of future study with the aim at generating information about the serotyping of ORT, characterization of antimicrobial susceptibility and pathogenicity specifically in layer chickens.

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