Journal of Animal and Veterinary Advances 10 (12): 1623-1626, 2011
ISSN: 1680-5593


# Reference Strains of *Ornithobacterium rhinotracheale* Raise Detectable Hemagglutination-Inhibition Antibodies in Immunized Specific-Pathogen-Free Chickens

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**Abstract:** The ability of nine reference strains of *Ornithobacterium rhinotracheale* (serovars A-I) to raise detectable, hemagglutination-inhibition antibodies in immunized specific-pathogen-free chickens was investigated. Six of the nine *O. rhinotracheale* serovar (A-H) reference strains raised detectable hemagglutination-inhibition antibodies 14 and 21 days after vaccination. Detectable serum hemagglutination-inhibition antibody titers ranged from 512-16348. Furthermore, transmission electron microscopic examination of a hemagglutinating reference strain (B 3263/91, serovar A) demonstrated a close interaction between the bacterial and chicken erythrocyte surfaces. The results indicate that most of the hemagglutinating *O. rhinotracheale* reference strains included in this study are able to raised detectable hemagglutination-inhibition antibodies in immunized specific-pathogen-free chickens. The findings have the potential to become an alternative laboratory approach for detecting serum antibodies from chickens immunized with *O. rhinotracheale*.

**Key words:** *Ornithobacterium rhinotracheale*, hemagglutination-inhibiton test, antibodies, chickens, serum, vaccination

## INTRODUCTION

The bacterium *Ornithobacterium rhinotracheale* of the family Flavobacteriaceae (order Eubacteria, genus *Ornithobacterium*) has been isolated from both domestic and wild birds (Vandamme *et al*., 1994). It is associated with respiratory disease, decreased growth and mortality in chickens and turkeys. Lesions such as pneumonia and airsacculitis are observed in diseased birds. Economic loss can be considerable when breeders are involved (Chin *et al*., 2008).

Currently, at least 18 Agar Gel Precipitin (AGP) serovars in *O. rhinotracheale* have been recognized (Chin *et al*., 2008). The hemagglutinating activity of nine AGP reference strains (A-I) of *O. rhinotracheale* has been elsewhere reported (Vega *et al*., 2008). However, the bacterial structure involved in the hemagglutinating activity of this bacterium has yet to be identified. Furthermore, the ability of reference strains of *O. rhinotracheale* to raise serum hemagglutination-inhibition antibodies in chickens is unknown.

The aim of the present study was to investigate the ability of nine well-characterized, hemagglutinating reference strains of *O. rhinotracheale* to raise hemagglutination-inhibition antibodies in immunized chickens. Furthermore, the interaction of *O. rhinotracheale* with chicken erythrocytes was visualized by electron transmission microscopy.

## MATERIALS AND METHODS

**Bacterial strains:** The reference strains of *O. rhinotracheale* used were B 3263/91, GGD 1261, ORV K91-201, ORV 94108 nr. 2, O-95029 no. 12229, ORV 94084

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Table 1: Origin of *Ornithobacterium rhinotracheale* serovar reference strains included in the present study

| Strain | AGP serovar | Isolated from | Origin | Year of isolation |
|---|---|---|---|---|
| B 3263/91 | A | Broiler | South Africa | 1991 |
| GGD 1261 | B | Turkey | Germany | 1991 |
| ORV K91-201 | C | Broiler | United States | 1991 |
| ORV 94108 no. 2 | D | Turkey | France | 1994 |
| O-95029 no. 12229 | E | Broiler | France | 1995 |
| ORV 94084 K858 | F | Turkey | The Netherlands | 1994 |
| O-95029 no. 16279 | G | Broiler | France | 1995 |
| E-94063 4.2 | H | Turkey | The Netherlands | 1994 |
| BAC 96-0334 #MINN18 | I | Turkey | United States | 1996 |

K858, O-95029 no. 16279, E-94063 4.2 and BAC 96-0334 #MINN 18 for serovars A-I, respectively. The reference strains have been studied extensively (Van Empel *et al*., 1997; Soriano *et al*., 2003) and were all sourced from the culture collection held at the Poultry CRC, Australia. The origin and source of strains used are shown in Table 1.

**Media:** Bacteria were cultivated on 10% sheep blood agar at 37°C and incubated overnight in a candle jar. Brain-heart infusion broth was used for propagation and maintenance of bacterial cultures. For improved growth this medium was supplemented with 1% (v/v) filter-sterilized, heat-inactivated horse serum (Soriano *et al*., 2002).

**Chickens:** A total of 30, 7 weeks old, Specific Pathogen Free (SPF) Leghorn chickens were used in the study. All chicken were placed in isolator units at the Universidad Nacional Autonoma de Mexico (UNAM), individually identified and received antibiotic-free food and water *ad libitum*. To confirm no serum antibodies against *O. rhinotracheale*, all chickens were bled and serum samples tested by the Slide Plate Agglutination (SPA) test as elsewhere described (Back *et al*., 1998).

**Immunization:** Vaccines for each reference strain of *O. rhinotracheale* were produced. Briefly, bacteria were grown overnight in brain-heart infusion broth and supplemented with 1% (v/v) filter-sterilized, heat-inactivated horse serum. A viable count was performed and the culture inactivated with 0.01% (w/v) thimerosal. Once the viable counts results were available, the cells suspensions were adjusted to $5\times10^8$ colony-forming units $mL^{-1}$ and aluminum hydroxide (SIGMA; St. Louis, MO, USA) added to a final concentration of 10%. Purity tests were carried out on final suspensions. Groups of three chickens were inoculated subcutaneously at 9 and 11 weeks of age with 1 mL of the relevant vaccine.

**Hemagglutination-inhibition tests:** Before immunization and 1 week after each immunization, all chickens were bled and sera of all chickens were examined in hemagglutination-inhibition test as previously described (Soriano *et al*., 2002). Hemagglutinin antigens the bacterial cells grown overnight in brain-heart infusion broth were harvested, washed 3 times in Phosphate-Buffered Saline (PBS), pH 7.0 and resuspended in PBS solution with 0.01% (w/v) thimerosal. Glutaraldehyde-fixed rabbit erythrocytes used were prepared as previously described (Soriano *et al*., 2002). Briefly, rabbit blood was collected into Alsever solution and the cellular packages were harvested by centrifugation and washed 3 times in 0.15 M NaCl. A 1% glutaraldehyde solution was prepared by dilution with a solution containing $Na_3PO_4$, pH 8.2 (1 volume) and 0.15 M NaCl (9 volumes) in distilled water (5 volumes). A 1-2% erythrocyte suspension was prepared in the 1% glutaraldehyde solution and held at 4°C for 30 min. Fixed erythrocytes were centrifuged, washed five times with 0.15 M NaCl and suspended in distilled water to 30% suspension. A 0.01% (w/v) thimerosal was added and the suspension was stored at 4°C. Hemagglutination titers of antigens were determined with 50 μL volumes of reagent and 1% glutaraldehyde-fixed erythrocytes with a diluent of PBS that contained 0.01% (w/v) thimerosal in a microdilution method. The hemagglutination titer was the reciprocal of the highest dilution of antigen causing complete agglutination of the glutaraldehyde-fixed erythrocytes.

The hemagglutination-inhibition tests were performed with four hemagglutinin units, the serum hemagglutination-inhibition antibody titers were expressed as the reciprocal of the highest dilution of serum sample that showed complete inhibition of the hemagglutinating activity (Soriano *et al*., 2002).

**Transmission Electron Microscopy (TEM):** Transmission electron microscopy study was performed as previously described (Zepeda *et al*., 2009). A mixed suspension of strain O-95029 no. 16279 and fresh chicken erythrocytes was harvested by centrifugation and fixed in a 3% glutaraldehyde solution. Thin sections (approximately 70-100 nm) for transmission electron microscopy analysis were visualized with a JEOL-1200EXII microscope with a field emission gun at an accelerating voltage of 65 kV at the Instituto de Fisiologia Celular, Universidad Nacional Autonoma de Mexico.

## RESULTS AND DISCUSSION

Hemagglutination-inhibition antibody titers values of serum samples of experimental immunized chickens with different *O. rhinotracheale* vaccines are shown

Table 2: Hemagglutination-inhibition antibody titers against *Ornithobacterium rhinotracheale* detected in immunized chickens included in the study

| Serovar | Chicken | Reciprocal of the serum hemagglutination-inhibition antibody titer | |
|---|---|---|---|
| | | 14 | 21 |
| A | 1 | 1024 | 2048 |
| | 2 | 1024 | 2048 |
| | 3 | 512 | 2048 |
| B | 1 | 8192 | 16384 |
| | 2 | 4096 | 8192 |
| | 3 | 2048 | 2048 |
| C | 3/3 | 0 | 0 |
| D | 1 | 2048 | 4096 |
| | 2 | 2048 | 4096 |
| | 3 | 2048 | 2048 |
| E | 1 | 8192 | 8192 |
| | 2 | 2048 | 4096 |
| | 3 | 4096 | 4096 |
| F | 3/3 | 0 | 0 |
| G | 1 | 16348 | 16348 |
| | 2 | 8192 | 16348 |
| | 3 | 16348 | 16348 |
| H | 1 | 8192 | 8192 |
| | 2 | 4096 | 8192 |
| | 3 | 8192 | 8192 |
| I | 3/3 | 0 | 0 |
| Control | 3/3 | 0 | 0 |

in Table 2. The serum samples collected from all the experimental groups before the immunization with any type of vaccine showed no positivity in both SPA and hemagglutination-inhibition tests. Similarly, serum samples collected from the unimmunized control group showed no positive reactions in both SPA and hemagglutination-inhibition tests. All immunized chickens raised detectable, agglutinating antibodies 12 and 21 days after vaccination.

Transmission electron micrographs showed *O. rhinotracheale* strain B 3263/91 in close association with the surface of a fresh chicken erythrocyte (Fig. 1).

The present study appears to be the first published investigation on the hemagglutination-inhibition antibodies raised by *O. rhinotracheale* in immunized chickens. A previous study showed the ability of an isolate to raise hemagglutination-inhibition antibodies in immunized rabbits (Soriano *et al*., 2002).

The results confirmed that *O. rhinotracheale* reference strains possess hemagglutinating antigens as elsewhere reported (Vega *et al*., 2008). Furthermore, most of the *O. rhinotracheale* reference strains included in the study raised detectable, serum hemagglutination-inhibition antibodies. It is unknown why the remaining three *O. rhinotracheale* strains with hemagglutinating activity did not raised detectable, serum hemagglutination-inhibition antibodies. A similar result

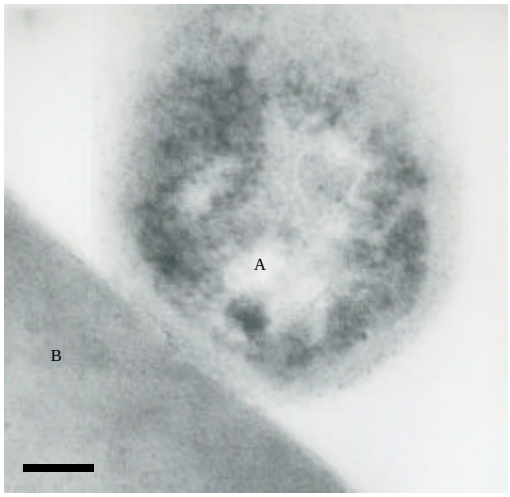


Fig. 1: Transmission electron micrograph of a cross-section of *Ornithobacterium rhinotracheale* B 3263/91 reference strain (A) adhered to the surface of a fresh chicken erythrocyte (B). Bar = 50 nm

was observed when glutaraldehyde-fixed chicken erythrocytes were used in the test. However, all reference strains raised detectable antibodies in immunized chickens when tested by the SPA test. Future studies focused on immunogenicity of hemagglutinating *O. rhinotracheale* strains need to be performed.

The TEM examination showed a close interaction between bacterial and fresh chicken erythrocytes surfaces, yet structural adhesions could not be visualized. Hemagglutination mainly depends of the expression of hemagglutinins (adhesins) and the interaction with erythrocyte receptors (Goldhar, 1995). Specific bacterial hemagglutinins or erythrocyte surface receptors involved in *O. rhinotracheale* hemagglutination have yet to be identified. Also, the effect of sera raised in this study, on the close interaction between the bacterium and erythrocytes, need to be studied.

In this study, a reduced number of chickens per *O. rhinotracheale* reference strain were immunized. However, we were able to detect serum hemagglutination-inhibition antibody titers raised by most of the *O. rhinotracheale* reference strains. The findings have the potential to become an alternative laboratory approach for detecting serum antibodies from chickens immunized with *O. rhinotracheale*.

## CONCLUSION

In this study, the results confirmed the hemag glutinating ability of a reference strain of *O.*

*rhinotracheale* and suggest that most of the *O. rhinotracheale* reference strains included in this study have the ability to raise hemagglutination-inhibition antibodies in immunized SPF chickens.

## ACKNOWLEDGEMENTS

Dr. Pat Blackall (Poultry CRC, Australia) is greatly acknowledged for providing *Ornithobacterium rhinotracheale* reference strains. This research was supported by Consejo Nacional de Ciencia y Tecnología (CONACYT), project 52244-Z (UAEM 1774/2007C).

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