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***In vitro* Antibiotic Susceptibility Patterns of *Ornithobacterium rhinotracheale* from Commercial Chickens in Argentina[#]**

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Abstract: *Ornithobacterium rhinotracheale* is a bacterial pathogen of the poultry industry causing severe economic losses worldwide. Fifteen β -hemolytic and 23 non-hemolytic *O. rhinotracheale* isolates from commercial broiler and broiler breeder chicken flocks were obtained and their sensitivity to ampicillin, trimethoprim-sulfamethoxazole, gentamicin, enrofloxacin, erythromycin, florfenicol, doxycycline and fosfomicin tested by a disk diffusion method. All isolates were non-susceptible to gentamicin. Most were non-susceptible to enrofloxacin, erythromycin, trimethoprim-sulfamethoxazole, doxycycline and fosfomicin, while most were susceptible to ampicillin and florfenicol. Susceptibility to ampicillin and florfenicol was significantly higher compared to all other antibiotics tested. Overall, 34 out of 38 (89.5%) isolates were found to be multiresistant. The presence of these multiresistant *O. rhinotracheale* isolates is suggestive of antibiotic over and misusing in the local poultry industry. To the authors' knowledge, this is the first work in South America reporting *in vitro* antibiotic susceptibility patterns of Argentine β -hemolytic and non-hemolytic *O. rhinotracheale* isolates.

Key words: Antibiotic susceptibilities, commercial chickens, hemolytic activity, *Ornithobacterium rhinotracheale*, South America

INTRODUCTION

Ornithobacterium rhinotracheale is a Gram-negative, non-motile, highly pleomorphic, rod-shaped and non-sporulating bacterium of the rRNA superfamily V within the phylum *Cytophaga-Flavobacterium-Bacteroides*, with inconsistent biochemical profile (Gornatti Churria *et al.*, 2012). Hemolytic activity was recently reported among North American and Argentine *O. rhinotracheale* field isolates and may contribute to the overall virulence of this bacterium (Gornatti Churria *et al.*, 2012). *O. rhinotracheale*, first characterized by Charlton *et al.*

(1993), is a worldwide pathogen causing severe economic losses to the poultry industry (Gornatti Churria *et al.*, 2012). *O. rhinotracheale* infection, also known as ornithobacteriosis, is a contagious disease of avian species, primarily turkeys and chickens, causing respiratory distress, decreased growth and mortality (Chin *et al.*, 2013). The severity of clinical signs, duration of the disease and mortality rates are extremely variable and are influenced by environmental stressors and concurrent infections (Chin *et al.*, 2013). Although several studies performed between 1995 and 2012 have

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described the antibiotic resistance patterns of *O. rhinotracheale* field isolates originating from Europe, Asia, North and Central America (Gornatti Churria *et al.*, 2012), no study has described the antibiotic resistance patterns of isolates from South America. The aim of this study was to describe the *in vitro* antibiotic susceptibility patterns of 38 Argentine *O. rhinotracheale* field isolates and compare the *in vitro* antibiotic susceptibility patterns of β -hemolytic (H) and non-hemolytic (NH) isolates.

MATERIALS AND METHODS

Sixty-one cases, including 57 cases from 21-to 49-day-old broiler chicken flocks with severe respiratory disorders, decreased growth, decreased food consumption, increased mortality rates and increased condemnation rates and 4 cases from 50 to 55-week-old broiler breeder flocks with severe respiratory disorders and increased mortality rates belonging to commercial poultry establishments from several districts of Buenos Aires Province, Argentina, were submitted to the Laboratorio de Diagnóstico de Enfermedades de las Aves y los Píliíferos (Cátedra de Patología de Aves y Píliíferos, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, La Plata, Buenos Aires, Argentina) between September 2010 and April 2012 for the isolation and identification of *O. rhinotracheale* and the study of its antibiotic susceptibility patterns. All animals were subjected to complete necropsies. Samples analyzed were collected from the trachea, lungs and air sacs of necropsied broiler and broiler breeder chickens with mucous tracheal contents, pneumonia or fibrinous airsacculitis, respectively.

Tracheal swabs and lung and air sac tissue samples were inoculated onto 10% goat blood agar (Agar nutritivo, Britania S.A., Argentina) with the addition of 5 μ g/ml gentamicin (Genta G, Laboratorio Vetu , Argentina) and incubated at 37°C in microaerophilia for 24-48 h. After incubation, blood agar plates were placed at room temperature for 48 h and evaluated for the presence of β -hemolytic activity of *O. rhinotracheale* as previously reported (Gornatti Churria *et al.*, 2012). The isolates were analyzed by using Gram's stain (Gram, Britania S.A.), oxidase test (Monodiscos oxidasa, Britania S.A.) and catalase test (Hydrogen peroxide 30%, Merck KGaA, Germany) and identified by using real-time polymerase chain reaction as previously described (Gornatti Churria *et al.*, 2012).

In vitro antibiotic susceptibility tests were performed by a disk diffusion method as previously described (Gornatti Churria *et al.*, 2012). Briefly, 10% goat blood Mueller-Hinton agar plates (Agar Mueller Hinton, Britania S.A.) were inoculated with *O. rhinotracheale* field isolates and incubated at 37°C in microaerophilia for 24 h. The isolates were tested against the following antimicrobial agents: ampicillin (10 μ g/disk), trimethoprim-sulfamethoxazole (1/24 μ g/disk),

gentamicin (10 μ g/disk), enrofloxacin (5 μ g/disk), erythromycin (15 μ g/disk), florfenicol (30 μ g/disk), doxycycline (30 μ g/disk) and fosfomicin (50 μ g/disk). Because no standardized disk diffusion method for *O. rhinotracheale* has been previously described, the recommendations for zone diameters interpretative criteria of fastidious Gram-negative microorganisms described by the Clinical and Laboratory Standards Institute (CLSI, 2009) for ampicillin, trimethoprim-sulfamethoxazole, gentamicin, enrofloxacin, erythromycin and florfenicol and by the Instituto Nacional de Enfermedades Infecciosas of Argentina "Dr. Carlos G. Malbr n" (INEI, ANLIS "Carlos G. Malbr n, 2003) for doxycycline and fosfomicin were followed.

Since the test of antibiotic disks against reference strains is a key control for accurate results of antimicrobial resistance patterns of field isolates, the ampicillin, enrofloxacin, erythromycin, gentamicin, florfenicol and trimethoprim-sulfamethoxazole disks were tested against the ATCC reference strains of *Escherichia coli* 25922 and *Staphylococcus aureus* 25923 on Mueller-Hinton agar plates following CLSI recommendations (CLSI, 2009).

RESULTS AND DISCUSSION

Ornithobacterium rhinotracheale field isolates were observed as small point colonies, Gram-negative pleomorphic rods, with negative results for catalase tests and positive results for oxidase tests. In addition, several showed β -hemolytic activity. A total of 38 field isolates of *O. rhinotracheale* were obtained from the 61 cases (62.3%) submitted from different locations of Buenos Aires Province. Overall, 36 out of 38 (94.7%) isolates were obtained from broiler chicken flocks and two from broiler breeder flocks (5.3%).

During the 48 h-postincubation period at room temperature, 15 field isolates showed extensive β -hemolytic activity, whereas 23 of them showed no hemolytic activity.

Despite the difficulty in comparing data from several sources related to *O. rhinotracheale* antibiotic resistance patterns because the methods and interpretative criteria have not been defined and the criteria for susceptibility and resistance may differ among reports (Devriese *et al.*, 2001), the results obtained in this study were compared with those available in the limited current literature. The CLSI guidelines for result interpretations of *O. rhinotracheale* antibiotic resistance patterns were previously followed by the studies carried out in Turkey (Ak and Turan, 2001), the USA (Malik *et al.*, 2003) and Taiwan (Tsai and Huang, 2006). Data grouped in contingency tables were analyzed statistically using the chi-square (χ^2) test for comparison of proportions. Statistical significance was set at 0.05.

In our study, all isolates were non-susceptible to gentamicin. Most of the H and NH isolates were non-susceptible to enrofloxacin (12/15 and 17/23),

Table 1: *In vitro* antibiotic susceptibility patterns in 15 β -hemolytic and 23 non-hemolytic field isolates of *Ornithobacterium rhinotracheale* from Argentina

Antimicrobial agent	----- β -hemolytic -----		----- Non-hemolytic -----		----- All isolates -----	
	Non-susceptible	susceptible	Non-susceptible	susceptible	Non-susceptible	susceptible
Ampicillin*	6	9	5	18	11	27
Enrofloxacin*	12	3	17	6	29	9
Erythromycin*	12	3	16	7	28	10
Gentamicin*	15	0	23	0	38	0
Florfenicol*	5	10	6	17	11	27
Trimethoprim-sulfamethoxazole*	12	3	18	5	30	8
Doxycycline [†]	10	5	16	7	26	12
Fosfomycin [†]	8	7	14	9	22	16

*According to the Clinical and Laboratory Standards Institute criteria

[†]According to the National Institute of Infectious Diseases of Argentina "Dr. Carlos G. Malbrán" criteria

erythromycin (12/15 and 16/23), trimethoprim-sulfamethoxazole (12/15 and 18/23), doxycycline (10/15 and 16/23) and fosfomycin (8/15 and 14/23) and susceptible to ampicillin (9/15 and 18/23) and florfenicol (10/15 and 17/23), respectively (Table 1). There was no statistically significant difference between H and NH *O. rhinotracheale* isolates with regard to susceptibility to individual antibiotics tested (data not shown). Susceptibility to florfenicol and ampicillin was significantly higher compared to all other antibiotics tested. Susceptibility to fosfomycin was significantly higher compared to trimethoprim-sulfamethoxazole (data not shown).

In the our study, the susceptibility to ampicillin in most of the Argentine H and NH *O. rhinotracheale* field isolates is in accordance to the results previously observed in the studies carried out in India (Murthy *et al.*, 2008) and Taiwan (Tsai and Huang, 2006) and in contrast to the resistance against ampicillin observed in Belgium (Devriese *et al.*, 2001), the USA (Malik *et al.*, 2003) and Malaysia (Mohd-Zain *et al.*, 2008). The non-susceptibility to trimethoprim-sulfamethoxazole observed in most of the Argentine H and NH *O. rhinotracheale* field isolates of the present study is in accordance to previous results observed in the studies carried out in Iran (Banani *et al.*, 2004), the USA (Malik *et al.*, 2003), Malaysia (Mohd-Zain *et al.*, 2008), India (Murthy *et al.*, 2008), Taiwan (Tsai and Huang, 2006) and the Netherlands (van Veen *et al.*, 2001). The non-susceptibility to enrofloxacin observed in most of the Argentine H and NH *O. rhinotracheale* field isolates of the present study is in accordance to the results observed in the studies carried out in Iran (Banani *et al.*, 2004) and Malaysia (Mohd-Zain *et al.*, 2008) and in contrast to the susceptibility to enrofloxacin previously observed in the studies carried out in Belgium (Devriese *et al.*, 1995), India (Murthy *et al.*, 2008) and Mexico (Soriano *et al.*, 2003). The non-susceptibility to erythromycin observed in most of the Argentine H and NH *O. rhinotracheale* field isolates of the present study is in accordance to the results observed in the studies carried out in Iran (Banani *et al.*, 2004), Taiwan (Tsai and Huang, 2006) and the Netherlands (van Veen *et al.*,

2001) and in contrast to the susceptibility to erythromycin previously observed in the studies carried out in Turkey (Ak and Turan, 2001), the USA (Malik *et al.*, 2003) and India (Murthy *et al.*, 2008).

The non-susceptibility to gentamicin observed in all *O. rhinotracheale* isolates of the present study is in accordance to the natural resistance of this bacterium against gentamicin (Gornatti Churria *et al.*, 2012) and to results previously observed in studies carried out in Turkey (Ak and Turan, 2001), Iran (Banani *et al.*, 2004), the USA (Malik *et al.*, 2003), Mexico (Soriano *et al.*, 2003) and the Netherlands (van Veen *et al.*, 2001). The non-susceptibility of most of the Argentine H and NH *O. rhinotracheale* field isolates to doxycycline is in accordance to the results observed in Belgian isolates (Devriese *et al.*, 1995; Devriese *et al.*, 2001) and in contrast to the susceptibility previously reported in studies carried out in Turkey (Ak and Turan, 2001) and India (Murthy *et al.*, 2008). The non-susceptibility of most of the Argentine H and NH *O. rhinotracheale* field isolates of the present study to fosfomycin is in accordance to the results observed in Mexican isolates (Soriano *et al.*, 2003).

The present study describes for the first time the antibiotic susceptibility patterns of *O. rhinotracheale* field isolates in South America and the comparison of antibiotic susceptibility patterns of H and NH *O. rhinotracheale* field isolates.

Antibiotics are used to treat and prevent infectious diseases in the poultry industry throughout the world (Malik *et al.*, 2003). The antibiotic treatment of respiratory diseases in commercial poultry has always been problematic because of the poor absorption from the gut of several antibiotics or because of their high economic costs (Devriese *et al.*, 2001). Their indiscriminate use may lead to antibiotic resistance in both pathogenic and resident microflora (Malik *et al.*, 2003). Nowadays, the emergence of multidrug resistance in *O. rhinotracheale* is becoming one of the major veterinary threats in the poultry industry throughout the world (Mohd-Zain *et al.*, 2008).

Antibiotic multiresistance within *O. rhinotracheale* field isolates was considered when non-susceptibility against three or more antibiotic families was observed. In the present study, 34 out of 38 (89.5%) *O. rhinotracheale* field isolates, both H (n = 14) and NH (n = 20), obtained from several commercial poultry establishments from Buenos Aires Province and tested against antimicrobial agents of eight different antibiotic families were found to be multiresistant (data not shown).

Conclusions and suggestions: The wide range of antibiotic non-susceptibility among the multiresistant H and NH isolates obtained in our study is probably a consequence of over- and misuse of antimicrobial agents in the local poultry industry (Mohd-Zain *et al.*, 2008) and may be associated with the absence of restrictions on antimicrobial use among local poultry producers.

The antibiotic susceptibility patterns observed in the present study could be helpful to plan and carry out possible control strategies against *O. rhinotracheale* infections in commercial poultry in Argentina through the more refined and informed selection of antibiotics.

The implementation of preventive measures such as autogenous and/or commercial bacterins in commercial broiler breeders could also be helpful in controlling *O. rhinotracheale* infections in broiler chicken cases with no clinical improvements in which those selected antibiotics were previously used with negative results.

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[¶]We would like to dedicate this article to the memory of Dr. Germán B. Vigo who encouraged the publication of this work

[§]Deceased