Ornithobacterium rhinotracheale Infection in Poultry: an Updated Review

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Abstract Ornithobacterium rhinotracheale is a Gram-negative bacterium of the rRNA superfamily V within the Cytophaga-Flavobacterium-Bacteroides phylum, which has become an emerging pathogen in the poultry industry. The purpose of this work is to review the current literature on O. rhinotracheale infection in domestic poultry. Despite the difficulties to identify the bacteria using biochemical tests, the polymerase chain reaction (PCR) technique has been successfully used to identify suspected isolates. Moreover, pulsed-field gel electrophoresis (PFGE) has allowed the genetic typing of O. rhinotracheale. Recent investigations carried out in the United States and Argentina described the unusual and extensive β-hemolytic activity of field strains isolated from affected chickens and turkeys. According to the latest serological studies, the causing agent has eighteen serotypes (A to R) and serotype A is the most prevalent among chicken and turkey strains. Gross findings typically found in broiler chickens are unilateral pneumonia, pleuritis, and abdominal airsacculitis with foamy, white yogurt-like exudate, which cause increased condemnation rates at slaughter, whereas those found in turkeys include tracheitis, pneumonia or bronchopneumonia, thoracic and/or abdominal airsacculitis, pericarditis, and peritonitis. Despite the worldwide efforts to control O. rhinotracheale infection, the present situation shows several problems not only because antibiotic treatments appear to be less effective due to the resistance acquired in the poultry industry but also because the vaccines developed so far show variable results in commercial poultry.

Keywords Ornithobacterium rhinotracheale; Poultry; Etiology; Pathology; Control

Introduction
Respiratory infections particularly those in which bacterial pathogens are involved (Glisson, 1998), are some of the most serious diseases of poultry (van Empel and Hafez, 1999). Increased mortality, increased costs of medication, increased condemnation rates, drops in egg production, reduction in shell quality, and decreased hatchability directly associated with these respiratory infections in poultry cause heavy economic losses (van Empel and Hafez, 1999). Ornithobacterium 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 and Charlton, 2008; Chin et al., 2008). Although O. rhinotracheale is considered as a primary pathogen in poultry (van Veen et al., 2000b; Pan et al., 2012), the severity of clinical signs, duration of the disease, and mortality are extremely variable and are influenced by housing environmental stressors such as poor management, inadequate ventilation and high ammonia levels, high stocking density, poor litter conditions, poor hygiene, foodborne mycotoxins, suboptimal nutrition, and concomitant infectious diseases (Chin et al., 2008; Hoerr, 2010). After its identification and characterization in 1994 by Charlton et al, O. rhinotracheale has been isolated throughout the world (Hinz et al., 1994; Devriese et al., 1995; van Empel et al., 1997; Goovaerts et al., 1998; Sakai et al., 2000; Sprenger et al., 2000; van Veen et al., 2000a; 2001; Ak and Turan, 2001; Devriese et al., 2001; Hung and Alvarado, 2001; Soriano et al., 2002; Malik...
et al., 2003; Banani et al., 2004; Canal et al., 2005; Türkyilmaz, 2005; Tsai and Huang, 2006; Marien, 2007; Chansiripornchai et al., 2007; Moreno et al., 2008; Murthy et al., 2008b; Uriarte et al., 2009; Walters et al., 2009; Gavrilović et al., 2010; Tabatabai et al., 2010; Chernyshev et al., 2011; Gornatti Churria et al., 2011; 2012).

1 History

The first report related to the characterization of O. rhinotracheale was that of Charlton et al (1993). Then, Vandamme et al (1994) described the phylogenetic position and various genotypic, chemotaxonomic, and classical phenotypic characteristics of 21 strains were then described, and assigned the name O. rhinotracheale (Vandamme et al., 1994). However, this bacterium appears to have been isolated before 1993 (Chin et al., 2008). In 1991 a new respiratory disease in 28-day-old broiler chickens was observed in South Africa. The birds suffered sneezing, associated with increased mortality and poor performance parameters. At postmortem examination, pneumonia and foamy white and “yoghurt-like” exudate were observed in the abdominal air sacs and the bacteriological study revealed a slow growing, pleomorphic, Gram-negative rod unknown among the bacterial species previously reported (van Empel, 1998; van Empel and Hafez, 1999; Bisshop, 2003). A bacterium similar to Pasteurella spp. isolated from 10-week-old Pekin ducks with respiratory disease in Hungary in 1987 (van Empel, 1998; van Empel and Hafez, 1999; Pyzik, 2007; Chin et al., 2008), as well as Riemerella anatipestifer-like strains isolated from turkeys suffering respiratory disease in Germany in 1991 and 1992 showed appearance and biochemical properties similar to those of the South African isolates (van Empel, 1998; van Empel and Hafez, 1999; Pyzik, 2007). This bacterium was named Pasteurella-like, Kingella-like, TAXON 28, or pleomorphic Gram-negative rod before the name Ornithobacterium rhinotracheale gen. nov. sp. nov. was suggested (van Empel and Hafez, 1999; Chin et al., 2008). Investigations of German culture collections revealed that O. rhinotracheale had been already isolated from the respiratory tract of 5-week-old turkeys with nasal discharge, facial edema, and fibrinopurulent airsacculitis in 1981 and from rooks in 1983 (van Empel, 1998). In Belgium, France and Israel, O. rhinotracheale had also been isolated before 1990 (van Empel, 1998). No isolates of O. rhinotracheale had been reported before 1981 (van Empel, 1998).

2 Etiology

2.1 Morphology, staining, growth requirements, antigenic structure, and colony morphology

Ornithobacterium rhinotracheale is a Gram-negative, non-motile, highly pleomorphic, rod-shaped, and non-sporulating bacterium of the rRNA superfamily V within the Cytophaga-Flavobacterium-Bacteroides phylum. When cultured on solid media, the bacterium appears as short, and plump rods measuring 0.2–0.9 µm in width and 0.6–5 µm in length (van Empel and Hafez, 1999; Chin et al., 2008), and less frequently as long filamentous rods or club-shaped rods (Chin and Charlton, 2008). No structures such as pili, fimbriae, and plasmids or properties such as specific toxic activities have been reported for the species (van Empel and Hafez, 1999; Chin et al., 2008). The use of 5%–10% sheep blood agar plate is recommended for isolation and optimal growth of the causing agent. Ornithobacterium rhinotracheale does not grow on MacConkey agar, Endo agar, Gassner agar, Drigalski agar, or Simmons citrate medium (Chin et al., 2008). The bacterium grows aerobically, microaerobically, and anaerobically, but the best growth occurs in air enriched with 7.5–10 % CO₂ at 37 °C (Chin and Charlton, 2008). Under these conditions and 24 h post-incubation, O. rhinotracheale develops pin-point colonies smaller than 1 mm in diameter. After 48 h, the colonies are approximately 1–2 mm in diameter, gray to gray-white, circular, and convex with an entire edge, and some isolates from chickens have a reddish glow. Cultures of O. rhinotracheale have a distinct smell similar to that of butyric acid (van Empel and Hafez, 1999; Chin and Charlton, 2008). Because of the resistance to gentamicin and polymyxin B observed in 90% of O. rhinotracheale field isolates (Vandamme et al., 1994), 5 µL/mL of each antibiotic is recommended to be added to blood agar media for selective isolation of this bacterium (van Empel and Hafez, 1999; Chin and Charlton, 2008; Chin et al.,
The use of 10 µg of gentamicin per mL of blood agar medium has also been suggested to isolate *O. rhinotracheale* from contaminated samples (Chin et al., 2008). van Empel and Hafez (1999) and Chin and Charlton (2008) also proposed the use of blood agar plates without antibiotic to prevent missing 10% of the antibiotic-susceptible isolates. *Ornithobacterium rhinotracheale* was first identified as a non-hemolytic microorganism (van Empel and Hafez, 1999; Hafez, 2002; Canal et al., 2005; Chin and Charlton, 2008; Chin et al., 2008), such as the ATCC 51463 strain of *O. rhinotracheale* (Tabatabai et al., 2010; Gornatti Churria et al., 2011). However, the presence of extensive and unusual β-hemolytic activity has been recently reported among North American and Argentinean field isolates after the 48-h-period following incubation at room temperature (Walters et al., 2009; Tabatabai et al., 2010; Gornatti Churria et al., 2011). Tabatabai et al (2010) characterized and demonstrated the β-hemolytic activity of *O. rhinotracheale* isolates using *in vitro* kinetic hemolysis assays with sheep red blood cells, western blotting with leukotoxin-specific monoclonal antibodies, and isobaric tagging and quantitative analysis of *O. rhinotracheale* outer membrane protein digest preparation. Moreover, Walters et al (2011) have recently developed an embryo lethality assay to determine potential virulence differences between hemolytic and non-hemolytic *O. rhinotracheale* strains isolated from turkeys in Virginia’s Shenandoah Valley, USA. In search for the optimal dilution to differentiate the pathogenicity of the isolates, these authors compared mortality patterns for 8 days post-inoculation and found the most drastic differences in the $10^3$ dilution groups. The results showed higher mortality numbers in an embryo model caused by hemolytic *O. rhinotracheale* isolates, which appear to be more virulent than non-hemolytic ones.

*Ornithobacterium rhinotracheale* is known for its hemagglutinating activity (Soriano et al., 2003; Tsai and Huang, 2006). Vega et al (2008) tested the hemagglutinating activity of serotypes A to I of *O. rhinotracheale* reference strains by using red blood cells from 15 different species, including avian, mammal, fish, and human erythrocytes, and concluded that rabbit erythrocytes were suitable to test *O. rhinotracheale*. In contrast, Chernyshev et al (2011) have recently reported the hemagglutinating activity of 19 Russian isolates with chicken and sheep erythrocytes.

### 2.2 Biochemical identification

The results of biochemical tests for the identification of *O. rhinotracheale* can be inconsistent (van Empel and Hafez, 1999; Chin and Charlton, 2008; Chin et al., 2008). Therefore, Chin and Charlton (2008) proposed the following tests as those with more consistent reactions to identify *O. rhinotracheale*: oxidase (+), catalase (-), β-galactosidase (+), indole (-), and triple sugar iron agar (no change). Chin et al (2008) also described a cytochrome-oxidase negative strain isolated from turkeys in Germany.

On the other hand, commercial biochemical test kit such as the API-20NE identification strip (bioMérieux, France) have been found useful for the identification of *O. rhinotracheale* (van Empel and Hafez, 1999; Chin and Charlton, 2008; Chin et al., 2008), although this bacterium is not included in the API database (van Empel and Hafez, 1999; Chin and Charlton, 2008). In a study where a total of 1150 isolates were tested, the biocodes found in 99.5% of the strains were 0–2–2–0–0–0–4 (61%) or 0–0–2–0–0–4 (38.5%). In addition, the isolates with positive results for arginine dihydrolase test (0.5%) had the biocodes 0–3–2–0–0–0–4 and 0–1–2–0–0–0–4 (van Empel and Hafez, 1999; Chin and Charlton, 2008). *Ornithobacterium rhinotracheale* showed constant and negative results for five enzymatic activities in the API-ZYM system (bioMérieux, France): lipase, β-glucuronidase, β-glucosidase, α-mannosidase, and α-fucosidase reactions (Chin and Charlton, 2008; Chin et al., 2008). Another commercial system for the identification of this bacterium is the RapID NF Plus (Remel/Atlanta, USA) which was used to test 110 isolates, and found five unique biocodes: 4–7–2–6–4 (41.8%), 4–7–6–2–6–4 (31.8%), 6–7–6–2–6–4 (18.2%), 6–7–2–2–6–4 (7.3%) and 4–7–2–0–4–4 (0.9%), (van Empel and Hafez, 1999; Chin and Charlton, 2008).
2.3 PCR

The PCR procedure is considered a useful laboratory tool for the identification of suspected *O. rhinotracheale* isolates (van Empel and Hafez, 1999; Chansiripornchai et al., 2007; Hung and Alvarado, 2001; Chin et al., 2008; Gornatti Churria et al., 2011; 2012), and also for diagnostic or investigation purposes (van Empel and Hafez, 1999; Canal et al., 2003b; Eroksuz et al., 2006; Tsai and Huang, 2006; Pyzik et al., 2007). The primers OR16S-F1 (5’-GAGAATTAATTTACGGATTAAG-3´), and OR16S-R1 (5´-TTCGCTTGGTCTCCGAAGAT-3´) have allowed the amplification of a 784 bp fragment on the 16S rRNA gene of *O. rhinotracheale* (van Empel and Hafez, 1999; Hafez, 2002; Hung and Alvarado, 2001; Canal et al., 2003; Eroksuz et al., 2006; Tsai and Huang, 2006; Pyzik et al., 2007; Uriarte et al., 2009; Gornatti Churria et al., 2011; 2012). According to van Empel and Hafez (1999) and Hafez (2002) no other bacteria closely-related to *O. rhinotracheale* could be confused when those primers are used.

On the other hand, Thachil et al (2007) studied the enterobacterial repetitive intergenic consensus (ERIC)-PCR and the random amplified polymorphic DNA (RAPD) assay with Universal M13 primer-based fingerprinting techniques to differentiate *O. rhinotracheale* isolates. To this end, they evaluated A total of 50 field strains and 8 reference strains for genetic differences using the primers ERIC 1R (5´-AIGTAAGCTCCTGGGGATTAC-3´) and Universal M13 (5´-TTATGAAAAACGACGGCCAGT-3´). M13 fingerprinting revealed different patterns for six reference serotypes of *O. rhinotracheale* (van Empel and Hafez, 1999; Hafez, 2002; Hung and Alvarado, 2001; Canal et al., 2003; Eroksuz et al., 2006; Tsai and Huang, 2006; Chansiripornchai et al., 2007; Pyzik et al., 2007; Uriarte et al., 2009; Gornatti Churria et al., 2011; 2012). According to van Empel and Hafez (1999) and Hafez (2002) no other bacteria closely-related to *O. rhinotracheale* could be confused when those primers are used.

The authors concluded that M13 fingerprinting technique was more discriminative in differentiating *O. rhinotracheale* isolates than the ERIC 1R fingerprinting technique.

2.4 PFGE

Moreno et al (2009) genotyped *O. rhinotracheale* strains obtained from Spanish red-legged partridges with neurological signs, otitis and cranial osteomyelitis, following adaptations of previously reported PFGE assays. The authors incubated isolates on Columbia agar in aerobic conditions at 37°C for 24 h and prepared DNA plugs from fresh colonies of bacteria suspended into 1 mL of TE1× buffer with a transmittance of 20%. The suspension was incubated at 37°C for 10 min, and 60 mL of lysozyme was added. The suspension was mixed with an equal volume of 2% molten agarose, 10% sodium dodecyl sulphate, and recombinant proteinase K. The DNA plugs were incubated at 56°C for 2 h in TE 1× buffer with 30 mL of recombinant proteinase K, and lysis buffer, and washed six times. The restriction enzymes Apal and Smal were used according to the manufacturer’s recommendations. DNA fragments were resolved in a 1% gel on a PFGE equipment, CHEF-DR III, at 6 V/cm for 21 h, with switching times ramped from 0.1 to 25 sec at 14°C, with an angle of 120°. A *Salmonella enterica* serovar Braendorup H9812 restricted with Xba I was included as the internal molecular ladder, and Mid Range PFG Marker II was included as a molecular weight ladder for size determinations. The gel was stained with ethidium bromide, and destained in distilled water. The PFGE patterns were examined and the genetic relationship among isolates was evaluated. An epidemiologically unrelated French strain of *O. rhinotracheale* from a pheasant was added. The study revealed indistinguishable macrorestriction patterns of the *O. rhinotracheale* Spanish strains with the enzymes Apal and Smal and no relation was noted with the restriction pattern of the French isolate.

2.5 Serological typing

Eighteen serotypes (A through R) have been differentiated because of the results observed in enzyme-linked immunosorbent assays (ELISAs) and
agar gel precipitation tests using boiled extract antigens and monovalent antisera (van Empel et al., 1997; van Empel and Hafez, 1999; Türkyilmaz, 2005; Chin and Charlton, 2008; Chin et al., 2008). Some geographical differences exist in the serotypes found throughout the world. Most of the isolates in the United States and Europe belong to serotype A (Chin and Charlton, 2008), whereas serotype C has been found only in chickens and turkeys of South Africa and the United States (Chin et al., 2008). Serotypes B, D, and E are the predominant ones after serotype A in Europe (Chin and Charlton, 2008). Also, serotype A has been found to be the most prevalent serotype among chicken strains (97%), and turkey strains (61%), (van Empel et al., 1997; Chin et al., 2008). No relation has been found between host specificity and the O. rhinotracheale serotype (Chin et al., 2008).

Nowadays, the Institute of Poultry Diseases (Free University Berlin, Germany) directed by Prof. Dr. Hafez Mohamed Hafez is the worldwide reference to identify the serotypes of O. rhinotracheale strains (Hafez Mohamed Hafez, 2011, private communications).

3 Transmission, incubation period, hosts, and clinical signs

Ornithobacterium rhinotracheale spreads horizontally by direct and indirect contact through aerosol or drinking water (Chin et al., 2008). Besides its isolation from ovaries, oviduct, hatching eggs, infertile eggs, dead embryos, and dead-in-shell chickens and turkeys (van Empel, 1998; Chin et al., 2008), there are circumstantial evidences of vertical transmission in birds affected by ornithobacteriosis (van Empel, 1998). According to experimental reproductions of the disease, the clinical signs are seen 24–48 h post-inoculation (Chin et al., 2008). Ornithobacterium rhinotracheale infection has been reported mostly in broiler chickens and turkeys, and less frequently in other avian species such as pheasants, quails, gray partridges, chukar partridges, red-legged partridges, guinea fowls, ostriches, rooks, pigeons, ducks, geese, and gulls (van Empel and Hafez, 1999; Chin et al., 2008; Moreno et al., 2009).

Despite the successful experimental reproduction of O. rhinotracheale infection in broiler chickens without the presence of any concurrent infectious agents or environmental stressors (van Veen et al., 2000b; Pan et al., 2012), the role of O. rhinotracheale as the primary pathogen is still uncertain (Chin and Charlton, 2008). In many reported cases of affected broiler chickens and turkeys, O. rhinotracheale infection played an associated role with other respiratory pathogens such as Escherichia coli, Bordetella avium, Streptococcus zoopneumoniae, Mycoplasma gallisepticum, Mycoplasma synoviae, Chlamydophila psittaci, Newcastle disease virus, avian metapneumovirus, infectious bronchitis virus, and Cryptosporidium spp. (van Empel et al., 1999; Sakai et al., 2000; Marien et al., 2005; 2007; Chin et al., 2008; Thachil et al., 2009; Gornatti Churria et al., 2012; Pan et al., 2012). The severity of clinical signs, the duration of the disease and the mortality caused by O. rhinotracheale outbreaks are extremely variable, and can be influenced by a wide range of environmental factors (Chin et al., 2008).

Clinical signs of broiler chickens include depression, decreased food intake, reduced weight gains, nasal discharge, sneezing, and facial edema, and appear at 3–6 weeks of age. Also, sudden death with or without respiratory signs has been reported in chickens with nervous signs (Gooovaerts et al., 1998; Chin and Charlton, 2008; Chin et al., 2008).

Broiler breeders aged 20–50 weeks have been seen to be affected by O. rhinotracheale infection in the peak of the laying period or soon before entering the egg production period suffering mild respiratory signs, decreased food intake, variable to low mortality, decreased egg production, decreased egg size, and poor quality of eggshell (Chin and Charlton, 2008; Chin et al., 2008).

Commercial 20-to-50-week-old layers have been found to show increased mortality, decreased egg production, and increased numbers of misshapen eggs (Sprenger et al., 2000; Chin and Charlton, 2008).

In turkeys, O. rhinotracheale infections have been detected as early as at two-weeks of age, but, the severity of clinical signs and mortality has been found
to be higher in older flocks (Chin and Charlton, 2008; Chin et al., 2008). The normal mortality is approximately 1%~15%, but can be up to 50%. Coughing, sneezing, nasal discharge, followed by severe respiratory disease, sinusitis, dyspnea, reduction in food and water intake, and prostration are the signs involved (Chin et al., 2008). In turkey breeder flocks, ornithobacteriosis is associated with a decreased egg production, and an increased number of unsuitable hatching eggs (van Empel and Hafez, 1999). *Ornithobacterium rhinotracheale* infection has also been found to be the cause of neurological signs or paralysis due to arthritis, meningitis, encephalitis, otitis, and cranial osteitis and osteomyelitis in turkeys, chickens, and red-legged partridges (Goovaerts et al., 1998; van Empel and Hafez, 1999; Chin et al., 2008; Moreno et al., 2009).

4 Experimental reproductions

In order to investigate the role and pathogenesis of *O. rhinotracheale* in poultry, several works of experimental reproductions by using turkey embryo, chicken, turkey, layer hen, and quail models with or without the involvement of other bacterial and/or viral respiratory pathogens have been reported (Goovaerts et al., 1998; van Empel et al., 1999; van Empel and Hafez, 1999; van Veen et al., 2000b; Marien et al., 2005; Eroksuz et al., 2006; Thachil et al., 2009; Pan et al., 2012). Intra-tracheal, intra-thoracic, intranasal, intravenous, and oculonasal administration routes were used to inoculate birds with the causing agent. Results show that neither the origin nor the serotypes of the strains appear to have an effect on the pathogenicity in experimental trials (van Empel and Hafez, 1999). Apart from the turkey embryo model described by Walters et al (2011), no experimental poultry trials have been used to study possible pathogenicity differences between hemolytic and non-hemolytic *O. rhinotracheale* field strains (Louisa Tabatabai, 2010, private communications). Previous viral infection with Newcastle disease virus, infectious bronchitis virus, or avian metapneumovirus was necessary for the presence of gross and/or microscopic lesions associated with *O. rhinotracheale* during experimental infections (van Empel et al., 1999; van Empel and Hafez, 1999; Marien et al., 2005; Eroksuz et al., 2006). Although some bacteria such as *E. coli* and *B. avium* are considered less effective triggers than viruses for *O. rhinotracheale* infections (van Empel and Hafez, 1999; Thachil et al., 2009), Pan et al (2012) have recently reported that the association between *O. rhinotracheale* and *S. zooepidemicus* causes higher mortality and severe bronchial lesions than that observed in chickens experimentally infected with *O. rhinotracheale* or *S. zooepidemicus* alone. However, *O. rhinotracheale* has been found to be able to cause lesions in chickens and quails, without previous viral priming (Goovaerts et al., 1998; van Empel and Hafez, 1999; van Veen et al., 2000b; Eroksuz et al., 2006; Pan et al., 2012).

5 Pathology

5.1 Gross pathology

The most common macroscopic findings in broiler chickens are unilateral pneumonia, pleuritis, and abdominal airsacculitis with foamy, white yogurt-like exudate (Chin et al., 2008). Other respiratory lesions, such as catarrhal tracheitis (Gavrilović et al., 2010) and bilateral exudative pneumonia (Gornatti Churria et al., 2011), has also been found in chickens affected by ornithobacteriosis. Condemnation rates of 60% in broilers at slaughter due to airsacculitis in 84% of the birds examined, and due to pericarditis and pneumonia in a few birds have been reported to be associated with *O. rhinotracheale* infection (van Veen et al., 2000a). In addition, more than one third of the respiratory lesions in broiler chickens at slaughter age have been reported to be caused by *O. rhinotracheale* infection, indicating the wide distribution of this bacterium in the broiler industry of Europe (van Veen et al., 2005).

Uncommon lesions such as subcutaneous edema of the skull with severe osteitis and osteomyelitis together with encephalitis without the involvement of the respiratory tract have been described in 28-day-old broiler chickens (Goovaerts et al., 1998). In turkeys, unilateral and bilateral consolidations of lungs due to pneumatic or bronchopneumonic lesions with fibrinous exudate of the pleura have been found (Hinz et al., 1994; Chin et al., 2008; Thachil et al.,
Mild or severe tracheitis, fibrinosuppurative thoracic and/or abdominal airsacculitis, pericarditis, and peritonitis have also been described in turkeys (Hinz et al., 1994; Chin and Charlton, 2008; Chin et al., 2008; Thachil et al., 2009). Swelling of the liver and spleen, degeneration of the heart muscle, and infection of vertebrae and joints have been observed in some cases of *O. rhinotracheale* infection in turkeys (Chin et al., 2008).

5.2 Histopathology

Most histological lesions can be found in lungs, pleura, and air sacs (Chin et al., 2008). Lung lesions caused by *O. rhinotracheale* are similar to those produced by *Pasteurella multocida* (Fletcher et al., 2008). They are characterized by large and coalescing areas of necrosis centered in the lumen of parabronchi, filled with degenerated and necrotic heterophilic infiltrate or fibrinous exudate. Collections of fibrin with macrophages and heterophils occupying the interstitial tissues and air passages are also found (Chin et al., 2008; Fletcher et al., 2008). According to Fletcher (2010), fibrinoheterophilic diffuse pneumonia in turkeys is suspected to be caused by *O. rhinotracheale* infection. Pleura and air sacs can be thickened with interstitial fibrin, diffuse heterophilic infiltrate, necrotic foci, and fibrosis (Chin et al., 2008).

5.3 Immunohistochemistry (IHC)

In spite of the low immunogenicity capacity of *O. rhinotracheale* (Paul van Empel, 2011, private communications), the immunohistochemical staining is a valuable and sensitive method to demonstrate the presence of this bacterium among the tissues of broiler chickens with respiratory signs in field trials (Hafez, 2000). Immunohistochemistry has been reported with the use of a monovalent rabbit antiserum against the *O. rhinotracheale* strain named B3263/91 belonging to serotype A. The antiserum was prepared by injecting specific-pathogen-free New Zealand white rabbits twice in a 3-week interval, using an oil adjuvant bacterin. Serum was collected four weeks after the second injection (van Empel et al., 1999).

A second option of antiserum elaboration recently suggested is to make a polyclonal antiserum. The use of three oil adjuvant bacterin doses of 0.5 mL each, administered subcutaneously in New Zealand white rabbits with 5–6 weeks of interval, has been recommended (Paul van Empel, 2011, private communications).

Detection of *O. rhinotracheale* by IHC has been carried out following the peroxidase anti-peroxidase staining method. Results of experimental trials have shown minimal, acute and short-lived microscopic air sac lesions associated with focal bacterial attachment to the epithelium of the respiratory tract of chickens exposed to *O. rhinotracheale* aerosol without prior viral infection (van Empel et al., 1999).

6 Serology

Serological tests are useful for flock monitoring and/or the diagnosis of *O. rhinotracheale* infection (Chin et al., 2008). The advantage of serology over bacteriological examination is that antibodies persist for several weeks after infection and bacterial shedding. However, *O. rhinotracheale* excretion and its antibody response may also be affected by a number of factors such as antibiotic therapy and vaccination (Hafez, 2002). ELISAs, serum plate agglutination tests, and a dot-immunobinding assay have been reported for the detection of *O. rhinotracheale* antibodies (Back et al., 1998; van Empel and Hafez, 1999; Sakai et al., 2000; Erganiş et al., 2002; Hafez, 2002; Canal et al., 2003a; Allymehr, 2006; Eroksuz et al., 2006; Chansiripornchai et al., 2007; Ghanbarpour and Salehi, 2009; Uriarte et al., 2010; Walters et al., 2010).

6.1 ELISA

ELISA allows detecting the presence of antibodies against *O. rhinotracheale* in one-day-old chicks and egg-yolk as well as in birds with clinical signs (van Empel and Hafez, 1999). ELISAs have been developed using different serotypes and extracted antigens of *O. rhinotracheale* (Chin et al., 2008), and both self-made and commercially available ELISAs (Biochek, Inc., USA, and IDEXX Laboratories, Inc., USA) are used to detect antibodies against most *O. rhinotracheale* serotypes (Hafez, 2002).
Studies of seroprevalence and serological evidences of *O. rhinotracheale* field or experimental infections among broiler chickens and broiler breeders have been reported throughout the world (Sakai et al., 2000; Canal et al., 2003a; Allymehr, 2006; Eroksuz et al., 2006; Chansiripornchai et al., 2007; Ghanbarpour and Salehi, 2009; Uriarte et al., 2010; Walters et al., 2010).

Both the unvaccinated status against *O. rhinotracheale* and the ELISA seropositivity results are indicative of field infections in developing countries (Canal et al., 2003a; Allymehr, 2006; Chansiripornchai et al., 2007; Ghanbarpour and Salehi, 2009; Uriarte et al., 2010).

In Iran, Allymehr (2006) carried out serological surveys and described 44.2% of positive serum samples from chickens against *O. rhinotracheale* in West Azerbaijan Province. Some years later, Ghanbarpour and Salehi (2009) found lower percentages of positivity among serum samples of broiler chickens (31.9%) in south-eastern Iran.

In southern Brazil, Canal et al (2003a) reported the true prevalence of positive flocks of broiler chickens (63.83%) and broiler breeders (100%) and suggested a positive correlation between the presence of respiratory signs and *O. rhinotracheale* antibodies.

In Argentina, Uriarte et al (2010) analyzed a total of 739 serum samples from broiler chicken and broiler breeder flocks located in Buenos Aires and Entre Ríos provinces and found 345 positive serum samples. The statistical analysis demonstrated higher possibilities of seropositivity among breeders (Uriarte et al., 2010).

In Thailand, 68% of broiler flocks were found to be positive. This value was higher than the 60% and 26% previously observed in Brazil and Germany, respectively (Chansiripornchai et al., 2007). The 32.5% prevalence of the individual broiler sera analyzed in Thailand was greater than the 6.52% found in Brazilian chickens and the 9.4% found in German chickens (Chansiripornchai et al., 2007). The serum analysis of the individual broiler breeder sera showed 87.8% of positivity in Thailand, which was lower than the 94.6% found in broiler breeders in Brazil and higher than the 13.9% found in broiler breeders in Japan (Chansiripornchai et al., 2007).

The differentiation between serological responses caused by hemolytic and non-hemolytic *O. rhinotracheale* infections was studied by Walters et al (2010). These authors tested a total of 1 200 serum samples from market hens with 12–14 weeks of age by the use of a specially designed ELISA test. Although 41% of the flocks were positive for both hemolytic and non-hemolytic *O. rhinotracheale* infections, 49.5% were positive for hemolytic *O. rhinotracheale* isolates and 64.6% were positive for non-hemolytic *O. rhinotracheale* isolates.

### 6.2 Serum plate agglutination test (SPAT)

Back et al (1998) developed and standardized the SPAT for rapid detection of antibodies against *O. rhinotracheale* infection. The antigen was prepared by growing *O. rhinotracheale* on 5% sheep blood agar at 37°C for 24 h in 5% CO₂, considering maximum growth of the bacterium at 24 h. The cells were harvested by scraping the bacterial growth, and resuspended in distilled water. The bacterium was inactivated with 0.8% formalin, and incubated at 37°C for 3 h. The cells were harvested by centrifugation at 3 000 × g for 10 min, and the pellet was resuspended in distilled water. Serum samples from chickens and turkeys experimentally infected were tested, and antibodies against *O. rhinotracheale* were detected by SPAT in both avian species, whereas the serum samples from not exposed birds remained negative (Back et al., 1998).

### 6.3 Dot-Immunobinding assay (DIA)

Erganiş et al (2002) developed a DIA and compared it with agglutination assays by testing serum samples from turkeys with respiratory signs. For DIA antigen, *O. rhinotracheale* was cultured on 5% sheep blood agar base at 37°C for 48 h in 10% CO₂, and harvested with phosphate buffer solution (PBS) containing 0.3% formaldehyde. Then, 1 mL of *O. rhinotracheale* suspension of 0.2 g wet weight/mL PBS was boiled for 60 min at 100°C. After centrifugation (2 500 × g for 45 min), the supernatant was passed through a 0.22 µm pore-size filter and used as antigen. Following standardization of the optimal dilutions of antigen, antiserum and conjugate, these were used in DIA tests. The authors concluded that the sensitivity of the DIA appeared to be lower than the agglutination assays studied (Erganiş et al., 2002).
7 Control
7.1 Antimicrobial susceptibility
Antimicrobial resistance profiles of *O. rhinotracheale* isolates from broiler chickens, turkeys, and layer hens have been reported worldwide. However, the antibiotic treatment against this bacterium shows several difficulties, because of the variable susceptibility of the strains associated with the regime used by the poultry industry of the geographical area studied (Chin et al., 2008). *Ornithobacterium rhinotracheale* infections have become more common in the poultry industry and the treatment with antibiotics has become less effective due to an increased pathogenicity, an increased burden of infection, and/or an increased level of acquired antibiotic resistance (van Veen et al., 2001; Malik et al., 2003).

The susceptibility of *O. rhinotracheale* to antibiotics is very inconsistent and appears to depend on the source of the strains analyzed (van Empel and Hafez, 1999; Banani et al., 2004).

The development of resistance to commonly used antimicrobials may result in increased costs of care associated with the use of alternative antimicrobials, which are often more costly (Malik et al., 2003). Following both disc diffusion and broth microdilution methods, different authors tested *O. rhinotracheale* isolates using a wide range of antibiotics such as amikacin, tiamulin, clindamycin, chloramphenicol, spectinomycin, tylosin, tilmicosin, spiramycin, cephalxin, cloxacillin, ceftiofur, penicillin G, amoxicillin, ampicillin, bacitracin, furazolidone, doxycycline, oxytetracycline, polymyxin B, novobiocin, flumequine, florfenicol, fosfomycin, enrofloxacain, norfloxacain, danofloxacain, chlorotetracycline, oxytetracycline, streptomycin, erythromycin, lincomycin, metronidazole, pefloxacin, gentamicin, neomycin, colistin, triple-sulfa, sulfadimethoxine, sulfachloropyridazine, sulfamethazine, sulfamerazine, sulfaquinoxaline, trimethoprim sulfa, cefoperazone, and sarafloxacin (Devriese et al., 1995; 2001; van Empel and Hafez, 1999; van Veen et al., 2001; Malik et al., 2003; Soriano et al., 2003; Banani et al., 2004; Tsai and Huang, 2006; Murthy et al., 2008a; Chernyshev et al., 2011; Gornatti Churria et al., 2011; 2012). Devriese et al (2001) explained the difficulty in comparing data from different investigation sources related to *O. rhinotracheale* antibiotic susceptibility because the methods and interpretative criteria had not been defined and the criteria for susceptibility and resistance may have differed among reports.

Although no standard methods are known for *in vitro* antibiotic susceptibility tests of *O. rhinotracheale*, several reports have followed the suggestions of the Clinical and Laboratory Standard Institute (CLSI) previously known as National Committee for Clinical Laboratory Standards (NCCLS) for fastidious Gram-negative microorganisms (Ak and Turan, 2001; Malik et al., 2003; Tsai and Huang, 2006; Murthy et al., 2008a; Gornatti Churria et al., 2011; 2012).

Works of antimicrobial susceptibility of *O. rhinotracheale* have been reported in the Middle-East (Ak and Turan, 2001; Banani et al., 2004) and Asian countries (Tsai and Huang, 2006; Murthy et al., 2008a).

Ak and Turan (2001) tested eight antibiotics using the broth microdilution method against 11 strains of *O. rhinotracheale* isolated from broiler chickens in Turkey and found that oxytetracycline was the most effective antibiotic, with minimum inhibitory concentrations (MICs) $\leq 0.125$–$1 \mu$g/mL, and that all isolates were resistant to gentamicin and neomycin. All field isolates were found to be sensitive to doxycycline. Except for one field isolate, the remaining ones were resistant to danofloxacine. Tilmicosin (MICs ranging from $\leq 0.5$–$4 \mu$g/mL), erythromycin (MICs ranging from $\leq 0.5$–$8 \mu$g/mL), and penicillin G (MICs ranging from $\leq 4$–$16 \mu$g/mL) caused effective inhibition of Turkey field isolates but with higher antibiotic concentrations.

Banani et al (2004) isolated 105 strains from chicken flocks and tested them against 19 antibiotics using the disc diffusion method. All the isolates were susceptible to tiamulin, and most of them were completely susceptible to chloramphenicol and the combination of lincomycin and spectinomycin. All the isolates were resistant to trimethoprim sulfa, colistin, and neomycin. Most of the field isolates were completely resistant to gentamicin, lincomycin, erythromycin,
tetracycline, and enrofloxacin. This antibiotic was the antibiotic commonly used in the Iranian poultry industry during the period of time studied.

Tsai and Huang (2006) obtained a total of 40 *O. rhinotracheale* Taiwanese field isolates from chickens (28 isolates), and pigeons (12 isolates), and used the disc diffusion method to study antimicrobial susceptibility patterns. Most of the chicken isolates (> 80%) were sensitive to amoxicillin, ampicillin, penicillin, and oxytetracycline, and resistant to clindamycin, erythromycin, and trimethoprim sulfa. There were significant differences in the resistance rates to clindamycin, erythromycin, gentamicin, and tetracycline between the pigeon and chicken isolates.

In India, Murthy et al (2008a) tested eighteen isolates from laying hens against twenty antibiotics and antibacterial agents by the disc diffusion method. These authors found that all the *O. rhinotracheale* field isolates were resistant to amikacin, cloxacillin, trimethoprim sulfa, gentamicin, metronidazole, and triple sulfa, and sensitive to amoxicillin, ampicillin, chloramphenicol, ciprofloxacin, doxycycline, enrofloxacin, erythromycin, oxytetracycline, and penicillin G. Susceptibility against cephalixin, norfloxacin, pefloxacin, streptomycin, and furazolidone was variable.

In Mexico, Soriano et al (2003) studied the MICs of ten Mexican field isolates together with reference strains from South Africa, Europe and the United States. Ten antibiotics were used following the microdilution broth method. Amoxicillin, enrofloxacin, and oxytetracycline inhibited all reference strains at 2–64 mg/mL, 4–64 mg/mL, and 4–32 mg/mL, respectively. For all the reference strains, MIC values of ≥ 128 mg/mL were recorded for gentamicin, fosfomycin, trimethoprim, sulfamethazine, sulfamerazine, sulfadiazine, and sulfachloropyridazine. Amoxicillin, enrofloxacin, and oxytetracycline inhibited the Mexican isolates at 32 mg/mL to 128 mg/mL, 4 to > 128 mg/mL, and 8 to > 128 mg/mL, respectively. For all the Mexican isolates, MIC values of ≥ 128 mg/mL were recorded for gentamicin, fosfomycin, trimethoprim, sulfamethazine, sulfamerazine, sulfadiazine, and sulfachloropyridazine. Soriano et al (2003) observed no improvement in suspected *O. rhinotracheale* outbreaks in broiler chicken flocks when fosfomycin or gentamicin was used, in accordance with the marked (≥ 128 mg/mL) resistance found in vitro of the Mexican isolates tested against these two antibiotics.

Malik et al (2003), on the other hand, obtained 125 field strains of *O. rhinotracheale* from lung tissues, tracheal swabs and sinus exudate swabs from Minnesota turkeys with respiratory disease during 1996–2002 in the United States. Field strains were tested by using the disc diffusion method following the CLSI guidelines. Most of the field isolates were sensitive to clindamycin, erythromycin, spectinomycin, and ampicillin. Resistance against sulfachloropyridiazine decreased from 1996 to 2002, whereas that gentamicin, ampicillin, trimethoprim sulfa, and tetracycline increased. The annual trend slopes for these antibiotics were 7.36%, 3.02%, 2.43%, and 1.95%, respectively. Resistance against penicillin remained constant, with a trend slope of only 0.54% per year. Therefore, based on their results, Malik et al (2003) suggested the need of continued antibiotic resistance monitoring of *O. rhinotracheale* and the establishment of baseline resistance patterns.

The European work conducted by Devriese et al (1995) was the earliest worldwide report describing the antimicrobial resistance of *O. rhinotracheale* field isolates. Fourteen isolates were obtained from gallinaceous birds (chickens, turkeys, a guinea fowl, and a partridge) from Belgian farms. The microdilution broth method was performed, and the results showed susceptibility to enrofloxacin, and acquired resistance to lincomycin, tylosin, doxycycline, and flumequine. Some years later, these authors described the MICs of 10 antibiotics for 45 strains of *O. rhinotracheale* isolated from Belgian broiler chickens from 1995 to 1998. All the strains were susceptible to tiamulin and resistant to lincomycin, ampicillin, and ceftiofur as well. Less than 10% of the strains were susceptible to tylosin and spiramycin, and a few strains were susceptible to enrofloxacin and doxycycline (Devriese et al., 2001).

In the Netherlands, van Veen et al (2001) obtained a
total of 395 Dutch field isolates from affected broiler chickens from 1996 to 1999 and initially tested them against amoxicillin, tetracycline, enrofloxacin, and trimethoprim sulfa by the agar gel diffusion test. The susceptibility to amoxicillin and tetracycline decreased in successive years from 62% to 14%, and four strains were sensitive to enrofloxacin or trimethoprim sulfa. Seven alternative antibiotics (erythromycin, penicillin, gentamicin, tilmicosin, tylosin, ceftiofur, and clavulanic acid-potentiated amoxicillin) were later used to test 12 multiresistant isolates of O. rhinotracheale. Except for the susceptibility against clavulanic acid-potentiated amoxicillin observed in the isolates studied, all of them were resistant to the rest of antibiotics used.

In Russia, Chernyshev et al (2011) have recently identified and characterized 19 Russian isolates from chickens and turkeys and found resistance to amikacin, penicillin, gentamicin, clindamycin, norfloxacin, erythromycin, and trimethoprim sulfa.

In summary, European, American, and Asian studies from 1995 to 2012 have described the resistance of worldwide O. rhinotracheale field strains against the following antibiotics: gentamicin, neomycin, danofloxacine, trimethoprim sulfa, colistin, lincomycin, erythromycin, tetracycline, enrofloxacin, clindamycin, amikacin, cloxacillin, metronidazole, triple sulfa, fosfomycin, sulfamethazine, sulfamerazine, sulfaquinoxaline, sulfachloropyridazine, ampicillin, tylosin, doxycycline, fluencequine, ceftiofur, penicillin, amoxicillin, tilmicosin, tylosin, and norfloxacin (Devriese et al., 1995; 2001; Ak and Turan, 2001; van Veen et al., 2001; Malik et al., 2003; Soriani et al., 2003; Banani et al., 2004; Tsai and Huang, 2006; Murthy et al., 2008a; Chernyshev et al., 2011; Gornatti Churria et al., 2011, 2012).

Only a few works have described some of the responsible mechanisms associated with antimicrobial resistance of O. rhinotracheale (Devriese et al., 1995; 2001; Marien et al., 2006). Devriese et al (1995) demonstrated the presence of the enzyme β-lactamase among Belgian field strains. In a later study, β-lactam antibiotics such as penicillin G, ampicillin, and ceftiofur were reported to be less effective when they were exposed against those selected strains (Devriese et al., 2001).

Marien et al (2006) investigated the antimicrobial resistance of O. rhinotracheale isolates to enrofloxacin, particularly its association with the amino acid changes at position 87 of the GyrA subunit. To this end, they used O. rhinotracheale strains with increased MIC values to enrofloxacin, and isolated either from field cases or from turkeys treated with enrofloxacin under experimental conditions. They observed point mutations in amino acids at positions 83 or 87 of the GyrA subunit, in the field strains less susceptible or resistant to enrofloxacin. The isolates with reduced susceptibility following experimental enrofloxacin treatment showed a constant point mutation at nucleic acid position 646 of gyrA resulting in an amino acid change at position 87 of the GyrA subunit (Marien et al., 2006).

7.2 Vaccination

The best strategy for the control or prevention of O. rhinotracheale infection is probably vaccination, because most worldwide O. rhinotracheale isolates have acquired resistance against the antibiotics regularly used in poultry (Schuijffel et al., 2006b). However, in spite of the availability of autogenous vaccines, economic losses related to O. rhinotracheale infections in the poultry industry are estimated in hundreds of millions of dollars annually in the United States (Tabatabai et al., 2010). Bacterins, live vaccines, and subunit recombinant vaccines have been developed and reported, with variable results for the control of experimental and natural infections associated with O. rhinotracheale (van Empel and van den Bosch, 1998; Cauwerts et al., 2002; Lopes et al., 2002; Bisshop, 2003; Chansiripornchai, 2004; Schuijffel et al., 2006a; 2006b; Murthy et al., 2007).

Injectable and inactivated vaccines were found to be impractical for commercial broiler flocks (van Empel and van den Bosch, 1998), whereas, autogenous bacterins were successfully used for the control of O. rhinotracheale outbreaks in turkeys in Israel (Chin et al., 2008).

Some authors obtained high maternal antibodies titers by using inactivated vaccines in broiler breeders,
causing enough protection in the progeny against experimental challenges (van Empel and van den Bosch, 1998; Cauwerts et al., 2002; Bisshop, 2003). In addition, Cauwerts et al (2002) found lower condemnation rates, lower mean mortality and higher mean production index in the progeny from vaccinated broiler breeders. Indeed, De Herdt et al (2012) have recently reported the improved performance of broiler chickens derived from broiler breeders vaccinated with Nobilis® OR inac (Merck Animal Health, USA and Canada). These authors demonstrated a significant 22.3% lower broiler loss and a significant 3.9% higher production index in the broiler progeny of the vaccinated broiler breeder flocks.

Murthy et al (2007) then developed inactivated vaccines in order to find the bacterin candidate in pullets. To this end, they produced eighteen different vaccines with different inactivating substances (formalin and thiomersal) and with or without adjuvant (mineral oil, alum, and aluminum hydroxide gel). The experimental trials showed that bacterin in mineral oil adjuvant induced the highest serological response and a significant decrease in macroscopic lesions such as pneumonia and airsacculitis in the vaccinated group of birds (Murthy et al., 2007). Other authors also developed subunit recombinant vaccines against \textit{O. rhinotracheale} infection (Chansiripornchai, 2004; Schuijffel et al., 2006a; 2006b). The genes encoding eight cross-reactive antigens were amplified, cloned in an expression vector, and expressed in \textit{E. coli} and then used as subunit vaccine. The protection against an experimental challenge composed of homologous and heterologous serotypes of \textit{O. rhinotracheale} was also evaluated. As a result, the production of reactive antibodies against the recombinant proteins on Western Blot assays provided homologous and heterologous protection in the challenged group of chickens (Schuijffel et al., 2005). Although the eight previously reported antigens were not expressed by all \textit{O. rhinotracheale} serotypes, a four component subunit recombinant vaccine was able to protect against \textit{O. rhinotracheale} heterologous infection challenge (Schuijffel et al., 2006b).

Another candidate vaccine was the temperature-sensitive mutant strain of \textit{O. rhinotracheale} considered by Lopes et al (2002). Turkeys aged one-day-old were vaccinated using drinking water and via oculonasal instillation. The trial showed successful results not only because the strain showed the ability to colonize the upper respiratory tract and was recovered for 13 days after administration, but also because the humoral response was observed only in the vaccinated group of turkeys compared with the unvaccinated control group (Lopes et al., 2002).

* The first author would like to dedicate this article to the memory of his mother

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