In vitro antibiotic susceptibility profiles, antimicrobial resistance mechanisms and virulence factors of *Ornithobacterium rhinotracheale*: a review

C. D. Gornatti Churria[1,*], G. Vigo[2], M. Machuca[3], M. Píscopo[1], M. Herrero Loyola[1] and M. Petruccelli[1]

[1] Cátedra de Patología de Aves y Pilíferos y Laboratorio de Diagnóstico de Enfermedades de las Aves y los Pilíferos, Facultad de Ciencias Veterinarias, 60 y 118 s/n, Universidad Nacional de La Plata, La Plata, Provincia de Buenos Aires, Argentina
[2] Cátedra de Microbiología y Laboratorio de Diagnóstico e Investigaciones Bacteriológicas, Facultad de Ciencias Veterinarias, 60 y 118 s/n, Universidad Nacional de La Plata, La Plata, Provincia de Buenos Aires, Argentina
[3] Cátedra de Patología Especial y Laboratorio de Patología Especial, Facultad de Ciencias Veterinarias, 60 y 118 s/n, Universidad Nacional de La Plata, La Plata, Provincia de Buenos Aires, Argentina
*Corresponding author. E-mail: danielgornatti@fcv.unlp.edu.ar

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 causing high economic losses throughout the world annually. Ornithobacterium rhinotracheale infection is a contagious disease of avian species, primarily chickens and turkeys, associated with respiratory distress, decreased growth, mortality, and increased condemnation rates at slaughter. 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. European, American, and Asian studies from 1995 to 2012 have described the resistance of worldwide O. rhinotracheale field strains from broiler chickens, turkeys, and layer hens against several antibiotics such as gentamicin, neomycin, danofloxacine, trimethoprim sulfa, colistin, lincomycin, erythromycin, tetracycline, enrofloxacin, clindamycin, mamikacin, cloxacillin, metronidazole, triple sulfa, fosfomycin, sulfamethazine, sulfamerazine, sulfaquinoxaline, sulfachloropyridazine, ampicillin, doxycycline, flumequine, ceftiofur, penicillin, amoxicillin, tilmicosin, tylosin, and norfloxacin. The current literature is still very limited in relation to virulence factors and antimicrobial resistance mechanisms of O. rhinotracheale. Only a few works have described some of the virulence factors of this bacterium such as non-siderophores iron acquisition mechanisms, neurominidase enzymatic activity, and a hemolysin-like protein. Some of the mechanisms associated with antimicrobial resistance of O. rhinotracheale, such as the β-lactamase enzyme, and the resistance-related mutations in gyrA, have also been reported. The purpose of this work is to review and update the current knowledge on antimicrobial susceptibilities, mechanisms of antibiotic resistance, and virulence factors of O. rhinotracheale.

Keywords *Ornithobacterium rhinotracheale*; poultry; antibiotics; resistance; virulence factors

1. *Ornithobacterium rhinotracheale* infection in poultry: introduction

The first report related to the characterization of *O. rhinotracheale* was that of Charlton et al. (1993) [1]. Then, the phylogenetic position and various genotypic, chemotaxonomic, and classical phenotypic characteristics of 21 strains were described, and the name *O. rhinotracheale* assigned [2]. 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 [3].

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 [2]. When cultured on solid media, the bacterium appears as short, plump rods 0.2–0.9 μm in width and 0.6–5 μm in length [3], and less frequently as long filamentous rods or club-shaped rods [4].

The use of 5–10% sheep blood agar plate is recommended for its isolation and optimal growth [3]. The bacterium grows aerobically, microaerobically, and anaerobically, but grows best in air enriched with 7.5–10 % CO_2 at 37°C [4]. 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 [4].

Because of the resistance to gentamicin and polymyxin B observed in 90% of *O. rhinotracheale* field isolates [2], 5 μl/ml of each antibiotic is recommended to be added to blood agar medium for selective isolation of this bacterium [3, 4]. The use of 10 μg of gentamicin per ml of blood agar medium has also been suggested to isolate *O. rhinotracheale* from contaminated samples [3]. Chin and Charlton (2008) [4] 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 [3, 4], but 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 [5].

Ornithobacterium rhinotracheale infection, also known as Ornithobacteriosis, 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 [5]. The most common macroscopic findings in broiler chickens are unilateral pneumonia, pleuritis, and abdominal airsacculitis with foamy, white yogurt-like exudate [3]. Other respiratory lesions, such as catarrhal tracheitis and bilateral exudative pneumonia, have also been found in chickens affected by Ornithobacteriosis [5]. 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 [5]. Unilateral and bilateral consolidations of the lungs due to pneumonic or bronchopneumonic lesions with fibrinous exudate of the pleura [3], mild or severe tracheitis, fibrinosuppurative thoracic and/or abdominal airsacculitis, pericarditis, and peritonitis have also been described in turkeys [3, 4]. 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 [3].

2. Use of antibiotics and antibiotic resistances in commercial poultry

Even though the trend in the poultry industry is to prevent disease and improve management practices, the occurrence of bacterial diseases is not uncommon. Thus, antibiotics have been used in poultry for many years. This practice has played and will continue to play an important role in the commercial production of poultry [6]. Indeed, the development of the modern production of the poultry industry has arisen from genetic selection, improved feeding and health management practices involving the use of antibiotics as growth promoters, coccidiostats, and therapeutic or prophylactic agents [7, 8].

The economic and health advantages of using antibiotics have revolutionized the intensive poultry production [7]. Antibiotics were first used for growth promotion purposes as early as the late 1940's when it was discovered that chickens grew faster when fed by-products of tetracycline fermentation. Subsequently, other antimicrobials have been approved for growth promotion and performance enhancement over the years [8]. Moreover, the intensive production over the past 60 years has been largely due to the introduction of coccidiostats in the feed. These products interfere with various stage(s) of the intestinal development of coccidia [8].

Finally, antimicrobials can also be used as therapeutic agents and can be applied to the target animal by individual injection, oral application or mass application via drinking water (main way of administration) or feed (used on a limited basis). Oral administration via either drinking water or feed has been shown to be the most practical method of application [8].

The key to antibiotic treatment success is related to many principles and includes identification of the pathogen, antibiotic selection based on sensitivity results, presence of effective antibiotic concentrations at the infection site, proper dosing and route of administration, and response to management needs. Antibiotic therapy should be used as a tool to manage disease outbreaks and not as a crutch to deficiencies in management or nutrition [6]. Because many of the diseases that occur in poultry are secondary to other primary infections, identifying the primary cause of infection is paramount in modern poultry production to minimize any overuse of antibiotics [6].

The development of antibiotic resistance in bacteria has been found in both humans and animals [9]. In general, when an antibiotic is applied, the drug eliminates the sensitive bacterial strains, leaving behind or selecting those variants with unusual traits that can resist it. Then, these resistant bacteria multiply, increasing their numbers a million fold a day, becoming the predominant microorganisms in the population [7].

The acquired resistance to an antimicrobial drug is the result of an alteration in the genome of those microorganisms. The primary pathway is the mutation of a gene into a resistance gene (*de novo* resistance). The secondary pathway is the incorporation of such resistance genes by donor bacteria into acceptor ones via conjugation, transformation, or transduction. These three processes constitute the three different forms of horizontal gene transfer [10]. As a consequence, the clinical effect of acquired resistance can be divided into two different types according to the change in the bacterial genome. The first type is gradual, and in general the consequence of stepwise mutations (*de novo* or primary resistance pathway). While lower concentrations of antimicrobial drug have no effect, higher concentrations are needed to inhibit bacterial growth. This resistance mechanism has been described in *Mycobacterium tuberculosis* for rifampicin, in *Streptococcus* species for β-lactam antibiotics mediated by altered penicillin-binding proteins, and in different bacterial species for quinolones (*gyr* and *par* genes). The second type of clinical effect is responsible for complete insensitivity of the microorganism to the antimicrobial drug. Even higher concentrations of the antimicrobial drug do not inhibit the growth of the bacterial population. This second resistance pathway usually involves horizontal gene transfer and is the most common type of antimicrobial resistance [10].

3. *In vitro* antibiotic susceptibility profiles

The control of *O. rhinotracheale* infections is important to minimize economic losses in poultry flocks [11]. *Ornithobacterium rhinotracheale* infections can be successfully treated with antibiotics, but the bacterium rapidly develops antibiotic resistance [12]. The antibiotic treatment against this bacterium shows several difficulties because of the variable susceptibility of the strains associated with the regime used in the poultry industry of each geographical area [3]. The susceptibility of *O. rhinotracheale* to antibiotics is very inconsistent and appears to depend on the source of the strain analyzed [13]. Studies on the antimicrobial susceptibility of *O. rhinotracheale* have been reported in several American, European, and Asian countries.

Devriese et al. (2001) [14] explained the difficulty in comparing data from different investigation sources related to *O. rhinotracheale* antibiotic susceptibility because the methods and interpretative criteria have not been defined and the criteria for susceptibility and resistance may differ 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 [5].

Following both the disc diffusion and broth microdilution methods, different authors have tested *O. rhinotracheale* isolates using a wide range of antibiotics such as amikacin, tiamulin, clindamycin, chloramphenicol, spectinomycin, tylosin, tilmicosin, spiramycin, cephalexin, cloxacillin, ceftiofur, penicillin G, amoxicillin, ampicillin, bacitracin, furazolidone, doxycycline, polymyxin B, novobiocin, flumequine, florfenicol, fosfomycin, enrofloxacin, norfloxacin, danofloxacine, chlortetracycline, oxytetracycline, streptomycin, erythromycin, lincomycin, metronidazole, pefloxacin, gentamicin, neomycin, colistin, triple-sulfa, sulfadimethoxine, sulfachloropyridazine, sulfamethazine, sulfamerazine, sulfaquinoxaline, trimethoprim sulfa, cefoperazone, and sarafloxacin [5].

In Turkey, Ak and Turan (2001) [15] tested eight antibiotics using the broth microdilution method against 11 strains of *O. rhinotracheale* isolated from tracheal samples of broiler chickens between 1999 and 2000. The interpretation criteria of antibiotic resistance and susceptibility patterns followed the guidelines of CLSI. The antibacterial agents used in that study had been initially diluted from 128 mg/ml to 0.008 mg/ml in doubling dilution in peptone water. Oxytetracycline was the most effective antibiotic with minimum inhibitory concentrations (MICs) ≤ 0.125–1 μg/ml, and all isolates were resistant to gentamicin and neomycin. All the field isolates were found to be sensitive to doxycycline. In addition, all the isolates except for one were resistant to danofloxacine. Tilmicosin (MICs ranging from ≤ 0.5–4 μg/ml), erythromycin (MICs ranging from ≤ 0.5–8 μg/ml), and penicillin G (MICs ranging from ≤ 4–16 μg/ml) significantly inhibited the isolates but with higher antibiotic concentrations. These results suggest that acquired antibiotic resistance is common in *O. rhinotracheale* and that the development of antibiotic-resistant strains could be associated with serious economic problems in the local poultry industry.

In Iran, Banani et al. (2004) [13] isolated 105 strains from tracheal swabs of 187 chicken flocks with respiratory diseases and increased mortalities between early-2001 and late-2002. The strains were tested 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. In addition, all were resistant to trimethoprim sulfa, colistin, and neomycin, and most were completely resistant to gentamicin, lincomycin, erythromycin, tetracycline, and enrofloxacin. Since this antibiotic was the one commonly used in the Iranian poultry industry during the study period, results showed that *O. rhinotracheale* rapidly acquired antibiotic resistance.

In Taiwan, Tsai and Huang (2006) [18] obtained a total of 40 *O. rhinotracheale* field isolates from tracheal swabs of chickens (28 isolates) and pigeons (12 isolates) with or without respiratory signs, and used the disc diffusion method to study antimicrobial susceptibility patterns according to CLSI guidelines. The antibiotics tested were amoxicillin, ampicillin, ceftiofur, clindamycin, enrofloxacin, erythromycin, gentamicin, oxytetracycline, penicillin, polymyxin B, trimethoprim sulfa, and tetracycline. Most of the chicken isolates (> 80%) were sensitive to amoxicillin, ampicillin, penicillin, and oxytetracycline, and resistant to clindamycin, erythromycin, and trimethoprim sulfa. In general, the resistance rate to amoxicillin, ampicillin, ceftiofur, enrofloxacin, oxytetracycline, penicillin, polymyxin B, and trimethoprim sulfa was similar, but lower in the pigeon isolates. In contrast, the resistant rates to clindamycin, erythromycin, gentamicin, and tetracycline were significantly different between chicken and pigeon isolates.

In Malaysia, Mohd-Zain et al. (2008) [16] studied 18 field isolates obtained from swabs of the nasal cavity, trachea, and air sacs of chickens and tested them against amoxicillin, ampicillin, chloramphenicol, doxycycline, enrofloxacin, trimethoprim sulfa, and tetracycline by using the disc diffusion method. They also tested the isolates against tylosin and tilmicosin using the broth microdilution method. They found that all isolates were susceptible against chloramphenicol, but resistant against ampicillin, enrofloxacin and trimethoprim sulfa. Most of the isolates (77.8%) were resistant to amoxicillin. The broth microdilution method showed that tylosin had a MIC ≥ 64 μg/ml and tilmicosin a MIC of >128 μg/ml in all the isolates. In agreement with previous works, these authors concluded that acquired antibiotic resistances are common characteristics of *O. rhinotracheale* strains. The *O. rhinotracheale* field strains analyzed in that study were shown to be resistant to many of the main antibiotics normally used for the treatment of respiratory diseases of poultry and for the implementation of preventive measures against *O. rhinotracheale* infections.

In India, Murthy et al. (2008) [17] tested eighteen isolates obtained from the trachea, lungs, air sacs, swabs of infraorbital sinus exudates, heart blood, and liver samples of 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 cephalexin, norfloxacin, pefloxacin, streptomycin, and furazolidone was variable. The authors suggested that the continuous use of drugs may have resulted in the development of acquired antibiotic resistance in the *O. rhinotracheale* isolates studied. They thus proposed a monitoring program design for antibiotic susceptibilities for the control of *O. rhinotracheale* infections.

In Mexico, Soriano et al. (2003) [19] studied the MICs of ten Mexican field isolates obtained from broiler chickens and layer hens together with reference strains from South Africa, Europe and the United States. Amoxicillin, enrofloxacin, gentamicin, oxytetracycline, fosfomycin, sulfamethazine, sulfamerazine, sulfaquinoxaline, sulfachloropyridazine, and trimethoprim were used following the microdilution broth method. Amoxicillin, enrofloxacin, and oxytetracycline inhibited all the reference strains at 2–64 mg/ml, 4–64 mg/ml, and 4–32 mg/ml, respectively, whereas gentamicin, fosfomycin, trimethoprim, sulfamethazine, sulfamerazine, sulfaquinoxaline, and sulfachloropyridazine inhibited them at values of ≥ 128 mg/ml. 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, whereas gentamicin, fosfomycin, trimethoprim, sulfamethazine, sulfamerazine, sulfaquinoxaline, and sulfachloropyridazine inhibited them at values of ≥ 128 mg/ml. The authors observed no improvement in suspected *O. rhinotracheale* outbreaks in broiler chicken flocks when fosfomycin or gentamicin was used, in accordance with the marked resistance (≥ 128 mg/ml) found *in vitro* of the Mexican isolates tested against these two antibiotics. In contrast, amoxicillin, enrofloxacin, and oxytetracycline provided clinical improvement of suspected *O. rhinotracheale* infection outbreaks. In agreement with that previously reported, the authors concluded that *O. rhinotracheale* strains had acquired antibiotic resistance and suggested that antimicrobial susceptibility tests should be used to guide treatment of *O. rhinotracheale* infections.

In the United States, Malik et al. (2003) [11] 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. Field strains were tested against 12 antibiotics: clindamycin, erythromycin, spectinomycin, ampicillin, sulfadimethoxine, trimethoprim sulfa, gentamicin, ceftiofur, sulfachloropyridazine, enrofloxacin, penicillin, and tetracycline 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 sulfachloropyridazine decreased from 1996 to 2002, whereas that against 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. Based on their results, Malik et al. (2003) [11] suggested the need of continued antibiotic resistance monitoring of *O. rhinotracheale* and the establishment of baseline resistance patterns. Because indiscriminate use of antibiotics may lead to antibiotic resistance in pathogens and resident microflora, these results should be eventually helpful in planning strategies for the control of *O. rhinotracheale* infections.

In Belgium, the work conducted by Devriese et al. (1995) [20] was the earliest one describing the antimicrobial resistance of *O. rhinotracheale* field isolates. Fourteen isolates were obtained from the respiratory tract with lesions of gallinaceous birds (eight from chickens, four from turkeys, one from a guinea fowl, and one from a partridge) from Belgian farms. These authors used the microdilution broth method, and tested the following antibiotics: doxycycline, spectinomycin, tylosin, lincomycin, lincomycin-spectinomycin, ampicillin, penicillin G, ceftiofur, enrofloxacin, trimethoprim sulfa, and flumequine. The results showed susceptibility to enrofloxacin, and acquired resistance to lincomycin, tylosin, doxycycline, and flumequine. The authors concluded that acquired antibiotic resistance is exceptionally frequent in *O. rhinotracheale* strains. They determined the antibiotic susceptibilities of poultry strains only when strains of wild birds were included in the study, and highlighted that the *O. rhinotracheale* type strain was erroneously considered to be penicillin-sensitive in the species studied based on the disc diffusion method. 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. Less than 10% of the strains were susceptible to tylosin and spiramycin, and a few strains were susceptible to enrofloxacin and doxycycline [14].

In the Netherlands, van Veen et al. (2001) [21] obtained 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 of the 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 of the multiresistant isolates of *O. rhinotracheale* obtained. 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. The results showed that the *O. rhinotracheale* field isolates obtained in the Netherlands between 1996 and 1999 had become

less sensitive to antibiotics *in vitro*. The authors proposed that one of the contributing factors might be the doubling of the number of broiler chicken flocks treated with antibiotics during the study period.

In summary, European, American, and Asian studies from 1995 to 2012 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, doxycycline, flumequine, ceftiofur, penicillin, amoxicillin, tilmicosin, tylosin, and norfloxacin [5].

4. Mechanisms of antibiotic resistance

4.1. Reduced enrofloxacin susceptibility and resistance-related mutations in *gyr*A

In order to investigate the mechanism responsible for *in vivo* reduced enrofloxacin susceptibility in *O. rhinotracheale*, Marien et al. (2006) [22] investigated the antimicrobial resistance to enrofloxacin of Belgian and French *O. rhinotracheale* isolates, particularly its association with the amino acid changes at position 87 of the GyrA subunit. To this end, these authors used *O. rhinotracheale* strains with increased MIC values to enrofloxacin, isolated either from field cases or from turkeys treated with enrofloxacin under experimental conditions. Four groups of specific-pathogen-free turkeys received continuous treatment with enrofloxacin (10 mg/kg, 3 or 5 days), florfenicol (20 mg/kg, 5 days), and amoxicillin (20 mg/kg, 5 days) in the drinking water after oculonasal inoculation of avian pneumovirus and *O. rhinotracheale*. In the experimental group, which received 5 days of enrofloxacin, *O. rhinotracheale* strains with a higher enrofloxacin MIC (0.25 μg/ml) than that of the inoculated *O. rhinotracheale* strains (≤ 0.03 μg/ml), were isolated initially from the trachea of one turkey and subsequently from the other turkeys within that group. In addition, for comparative purposes, the authors included other *O. rhinotracheale* field strains with different *in vitro* sensitivity levels for enrofloxacin. These included one *O. rhinotracheale* strain with lower enrofloxacin sensitivity (MIC 0.5 μg/ml), five enrofloxacin-sensitive strains (MIC ≤ 0.03 μg/ml), and 17 strains with unusually high enrofloxacin MIC values (MIC 1–2 μg/ml), close or equal to the clinical breakpoint of 2 μg/ml, all isolated from turkeys in France and broiler chickens in Belgium.

To determine the *gyr*A sequences, Marien et al. (2006) [22] extracted the DNA using alkaline lysis following a previously described method, and the QRDR of the GyrA subunit of *O. rhinotracheale* was first amplified using a consensus degenerate universal primer pair, gyrAF and gyrAR. On the basis of the determined sequences of 14 *O. rhinotracheale* isolates and a type strain, amplified with the universal primers, these authors designed specific primers for the *O. rhinotracheale gyr*A gene, namely ORTgyrAF (5'-CACAGAAGGGTGCTCTATGGG-3') and ORTgyrAR (5'-TTCCAGCGGCACCATTTACC-3'). PCR reactions were performed in a volume of 40 μl containing a final primer concentration of 1 μM for each of the oligonucleotides, 40 μM of each deoxynucleoside triphosphate, 3 mM $MgCl_2$, 0.03 U/μl Polymerase Taq , and 1 X PCR buffer. Template DNA (2 μl) was added. The conditions used for the amplifications were the following: initial denaturation at 95°C for 5 min, 35 cycles of 1 min denaturation at 95°C, 1 min of annealing at 48°C for the PCR with the universal primer pair and at 58°C with the specific primer pair, 1 min of elongation at 72°C, and a final extension of 5 min at 72°C. The amplified fragments were analyzed by agarose gel electrophoresis. The amino acid sequences obtained were numbered and aligned with the *Escherichia coli gyr*A amino acid sequence for DNA gyrase A subunit, and the amino acid sequences of the QRDR of *gyr*A of the *O. rhinotracheale* strains with different enrofloxacin susceptibilities were compared. Molecular analysis of the *O. rhinotracheale* strains with increased MIC value (0.25 μg/ml), as reisolated during the *in vivo* experiment, consistently revealed a point mutation (G →T) at nucleic acid position 646 (*E. coli* numbering) of *gyr*A resulting in an amino acid change from aspartic acid to tyrosine at position 87 of GyrA, a known hot spot for fluoroquinolone resistance. Resistance to fluoroquinolones primarily occurs either altering the two target enzymes, DNA gyrase and topoisomerase IV, or by inhibiting the drug from reaching its intracellular target through modifications in the outer membrane proteins or by active efflux systems. Indeed, these authors decided to focus on mutations in *gyr*A, the gene most frequently mutated in Gram-negative organisms. They concluded that a single course of enrofloxacin treatment may contribute to the selection of the first mutant with reduced fluoroquinolone susceptibility, which could eventually contribute to the emergence of fluoroquinolone resistance in *O. rhinotracheale*.

4.2. β-lactamase in *O. rhinotracheale*

In order to investigate the presence of β-lactamase, the enzyme that provides resistance to β-lactams, in *O. rhinotracheale*, Devriese et al. (1995) [20] carried out tests with β-lactamase (Nitrocefin) strips and revealed that all resistant strains tested by, including the type strain, produced this enzyme. Only the strains obtained from rooks were negative. β-lactamase was not detectable with the acidometric method. The nitrocefin β-lactamase tests gave reliable results only with some bacterial genera, notably *Staphylococcus*, *Neisseria*, *Haemophilus*, and *Bacteroides*. Because of the results, *O. rhinotracheale* could be added to this list [20].

Devriese et al. (2001) [14] indicated that the resistance mechanisms against antibiotics in *O. rhinotracheale* are unknown except in the case of β-lactams because the activity of β-lactamase has been previously reported in this bacterium [20]. This enzyme was absent only in the three normally sensitive strains studied at that time, all of which originated from rooks. The β-lactamase involved appears to affect a wide range of β-lactam antibiotics. Devriese et al. (2001) [14] also showed that penicillin G was less active on strains carrying the enzyme, in a manner similar to the broad-spectrum penicillin ampicillin and the cefalosporin, ceftiofur.

Because of the common occurrence of cross-resistance, it is probable that other members of these antibiotic groups are less active on the less sensitive strains, as are other macrolides, quinolones and tetracyclines on strains showing resistance to the representatives of these antibiotic families. The detection of β-lactamase activity in the strains with decreased sensitivity to ampicillin and ceftiofur confirms the interpretation of the MIC values in terms of sensitivity and resistance. This finding is remarkable because none of the poultry strains, including the type strain isolated from a turkey, can be considered normally sensitive [14].

5. Virulence factors

5.1. Introduction

Virulence, or pathogenicity, is defined as the ability of a bacterium to cause infection. Virulence factors denote a bacterial product or strategy that contributes to virulence or pathogenicity [23].

Briefly, virulence factors can be loosely classified into two categories: those that promote bacterial colonization and invasion of the host such as pili, non-fimbrial adhesins, bacterial triggering of actin rearrangement in host cells, binding to and entry of M cells, motility and chemotaxis, sIgA proteases, siderophores, surface proteins that bind transferrin, lactoferrin, or hemin, capsules, C5a peptidase, toxic proteins, and variation in surface antigens; and those that cause damage to the host, mainly, endotoxins, exotoxins, hydrolytic enzymes, and bacterial products associated with autoimmune responses [23].

5.2. Virulence factors in *O. rhinotracheale*

5.2.1. Neuraminidase enzymatic activity of *O. rhinotracheale*

Kastelic et al. (2013) [24] have recently described for the first time the presence of neuraminidase enzymatic activity (NEAC) in 47 field strains of *O. rhinotracheale* isolated from broiler chickens and turkeys with pneumonia, tracheitis, arthritis, and encephalitis, from Croatia, Denmark, England, Germany, Hungary, Israel, Slovenia, and South Africa.

The authors measured the NEAC of *O. rhinotracheale* by using the chromogenic substrate 5-bromo-4-chloro-3-indol-α-D-N-acetylneuraminic acid sodium salt (BIN). The pellets of the strains were suspended in PBS pH 7.2 and 1–2% cell suspensions were used. Then, 10 μl of the each sample with cells was mixed with 10 μl of PBS, pH 7.2, containing 5 μg of BIN in small tubes with caps, and then incubated at room temperature. The time at which the positive reaction occurred, i.e. the colorless sample became indigo blue, was recorded following the results of previous works. The positive control of NEAC was neuraminidase of *Clostridium perfringens* (type V) considering 0.5 mg per sample. Negative controls contained BIN and PBS alone. Supernatants were assayed for NEAC as previously described by the authors. Briefly, 10 ml of supernatant was mixed with 10 ml of PBS containing BIN (5 mg) and incubated at room temperature. The time at which the positive reaction occurred was recorded. Relative NEAC, probably released from *O. rhinotracheale* cells during harvest, was estimated considering the volume of supernatant and that of its pelleted cells and their NEAC.

The authors then inhibited NEAC by using neuraminidase inhibitor 2-deoxy-2,3-didehydro- N-acetylneuraminic acid (DANA) to confirm the specificity of NEAC observed in *O. rhinotracheale* samples (cells and supernatants). They included DANA in appropriate samples, and compared their reactions with those without DANA. DANA was also used in assays in which the capacity of *O. rhinotracheale* to desialylate chicken and turkey glycoproteins was investigated. Serum glycoproteins containing SAα(2-6)gal moieties were desialylated. The DIG Glycan Differentiation commercial Kit was used to determine the sialylation of glycoproteins, as well as the levels of their desialylation with *O. rhinotracheale* isolates. Sialylation with the SAa(2-6)galactose linkage was detected by *Sambucus nigra* agglutinin (SNA) as recommended. The desialylation of chicken serum glycoproteins was assayed as previously described. Serum samples were taken from turkeys without *O. rhinotracheale* infection. Samples with glycoproteins strongly binding SNA were used in the desialylation assay. Samples of diluted serum samples were mixed with the *O. rhinotracheale* strain tested, and incubated at 37–38 °C overnight. Following incubation, microcentrifuges with samples were centrifuged (30,000 x *g*, 20 min), supernatants collected, and their proteins subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the PhastSystem and blotting (transfer) to Immobilon P, as previously described. Levels of the SAα(2-6)gal moiety or serum glycoproteins were estimated by visual comparison of staining intensities of bands to which SNA bound. Levels of desialylation by *O. rhinotracheale* were estimated with reference to control serum samples to which 0.2 mg of neuraminidase of *C. perfringens* was added. DANA (10–30

mg/sample) was used to confirm that NEAC of *O. rhinotracheale* desialylated serum glycoproteins. Desialylation of glycoproteins from tracheas of chickens and turkey was carried out. Desialylation of glycoproteins from tracheal mucus was studied as previously described by the authors. Freeze-dried samples were reconstituted with distilled water and then analyzed by the MAA probe for glycoproteins containing SAα(2-3)gal moiety. Their desialylation by *O. rhinotracheale* strains was analyzed as described above for desialylation of serum glycoproteins by this bacterium. DANA (30 mg/sample) was used to confirm that NEAC of *O. rhinotracheale* was involved in desialylation. PCR and sequencing analysis of the neuraminidase gene were performed. DNA of *O. rhinotracheale* strains was isolated with the RTP Spin Bacteria DNA commercially available mini kit. The annotated *O. rhinotracheale* DSM 15997 incomplete genome sequence was used to design primers able to amplify and sequence the region from the 5'-end of the α-amylase gene, the *nanO* (neuraminidase) gene to the 3'-end of the hypothetical protein. PCRs were performed in a total volume of 30 ml, consisting of 1 ml bacterial DNA, 10 pmol each primer, 0.2 mM each dNTP, 3 ml 10-fold concentrated Taq DNA polymerase synthesis buffer, 2.4 ml 25 mM $MgCl_2$ stock solution, and 1.5 units of Taq DNA polymerase. The program consisted of initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation for 1 min at 95 °C, annealing for 30 s, extension for 90 s at 72 °C, and final extension for 10 min at 72 °C. The PCR products were analyzed by horizontal agarose gel electrophoresis in 1% (w/v) agarose gels in Tris/Borate/EDTA buffer. Gels were stained for 15 min in a solution of 1 mg ethidium bromide ml^{-1}, washed for 15 min in deionized water and analyzed and photographed under UV light. The sequence data were aligned and analyzed with Sequencher 4.8 (Gene Codes Corporation).

In this study, Kastelic et al. (2013) [24] demonstrated for the first time that *O. rhinotracheale* strains have potent NEAC. Their genomes contain a *nanO* gene (1071 bp) encoding a protein with motifs that are characteristic of bacterial neuraminidases/sialidases. All 47 *O. rhinotracheale* strains tested revealed relatively strong NEAC. They showed five types of the *nanO* sequence. It seems that the NanO polymorphisms observed do not cause considerable differences in NEAC. This can be expected because all amino acid substitutions were found outside NanO regions/motifs considered important for the function of neuraminidase.

At present, it is not known whether antibodies to NanO potentially produced by *O. rhinotracheale*-infected poultry can have any influence on the NanO sequence diversification. Poultry generate antibodies to several *O. rhinotracheale* proteins. Kastelic et al. (2013) [24] concluded that further studies using recombinant NanO are needed to confirm its immunogenicity in poultry. This investigation demonstrated that its NanO can desialylate host proteins. Like the neuraminidase NanH of the poultry pathogen, *Mycoplasma synoviae*, NanO cleaves SAα(2-6)gal moiety from serum glycoproteins.

Kastelic et al. (2013) [24] described that *O. rhinotracheale* cleaves SAα(2-3)gal moiety from tracheal mucus glycoproteins of chickens and turkeys. Generally, such sialic acid-rich glycoproteins represent host defense barriers, especially against pathogens which target sialic acid moieties of receptors on epithelial cells. They are false receptors able to inhibit binding of pathogens to receptors of target host cells. Receptors for *O. rhinotracheale* remain to be identified, so it is not clear if NanO plays any role in colonization of the respiratory tract. NanO neuraminidase can liberate sialic acid from poultry glycoproteins, but it is not known whether *O. rhinotracheale* utilizes such free sialic acid [24].

Kastelic et al. (2013) [24] evidenced for the first time that *O. rhinotracheale* has neuraminidase and that NEAC is common in strains of this poultry pathogen. *Ornithobacterium rhinotracheale* desialylates host glycoproteins, including IgG and also cleaves sialic acid from SAα(2-3)gal moiety of turkey and chicken sialylated glycoproteins present in tracheas which are frequently colonized by this bacterium.

5.2.2. Non-siderophores iron acquisition mechanisms of *O. rhinotracheale*

Iron acquisition is one of the mechanisms of bacterial pathogens to overcome the host capacity to limit available iron during the infection process [25]. Iron acquisition mechanisms such as Fur-independent, constitutive Ton-B dependent mechanism, Fur-dependent low-molecular-weights iron chelators or siderophores, and Fur-dependent receptor/soluble receptor systems have been previously described [25].

In order to investigate the iron acquisition mechanism of *O. rhinotracheale*, Tabatabai et al. (2008) [25] cultured reference and North American field isolates under iron deprivation by using brain heart infusion (BHI) broth and agar with the addition of 200 μM of 2, 2'-dipyridyl (DP). To examine the tolerance of *O. rhinotracheale* strains to DP, they carried out growth studies in microtiter plate format. Wells containing 150 ml of sterile BHI broth containing from 0 to 400 mM final concentrations of DP were inoculated with 50 ml of a 24 h culture adjusted to provide a final A_{600} of 0.1. Cultures were incubated for 24 h at 37 °C and 7.5% humidified CO_2 on a microplate shaker.

The authors then performed the filter disk assay for iron uptake. A volume of 0.1 ml of a 10-fold dilution of a 16 h culture adjusted to 0.5 absorbance units at 600 nm was spread on pre-warmed plates of BHI agar containing 200 mM DP. Sterile 10 mm Whatman N° 1 filter disks were placed on the surface of the agar. A volume of 30 ml of a filter-sterilized 200 mM iron source prepared in Chelex-100-treated deionized water was deposited on the filter disk. Hemin was first dissolved in 0.01 N NaOH as a 10 mM solution, and then diluted to provide a solution containing 200 mM in iron. Plates were incubated, agar surface facing up, at 37 °C in 7.5% humidified CO_2. After 1 h of incubation, plates were inverted, and then incubated up to 72 h. Growth around the filter disks was recorded as positive or negative as

follows: + (< 3-mm zone of growth), ++ (3-to-5-mm zone of growth), +++ (<5-mm zone of confluent growth), and – (no growth).

In order to evaluate the siderophore production, Tabatabai et al. (2008) [25] then cultured the isolates carried in BHI broth in the presence or absence of 200 mM DP for 24 h or 48 h and centrifuged them at 10,000 x *g*. A volume of 0.5 ml of supernatant was transferred to a cuvette, and 0.5 ml of CAS assay solution was added. Change in absorbance at 680 nm was determined after 48 h of incubation at room temperature. In addition, the assay solutions were scanned from 500 nm to 700 nm to confirm reduction in absorbance at 600 nm signifying iron removal from the CAS complex. Culture supernatant from *E. coli* strain ATCC 35218 and *E. coli* strain DH5α served as positive and negative controls, respectively. In addition, BHI broth with and without DP served as medium controls. Pre-warmed CAS-BHI agar plates were streaked with a loop of culture obtained from one 2-mm colony grown on trypticase soy agar-blood agar plates. Plates were incubated for 48 h at 37 °C in a humidified 7.5% CO_2 atmosphere for *O. rhinotracheale* strains, and then observed for the appearance of an orange-yellow zone around the streaked culture. *Escherichia coli* strain ATCC 35218 and *E. coli* DH5α were streaked on the same plate 24 h after streaking the *O. rhinotracheale* cultures. These strains served as positive and negative controls, respectively. Growing *E. coli* under CO_2 had no effect on the outcome of the results. *Escherichia coli* DH5α served as a negative control because this phage T1 deletion mutant is reportedly negative for the ferric siderophore receptor and siderophore production.

These authors also performed 2-dimensional (2-D) sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) mass spectrometry. The samples for 2-D PAGE were then prepared. Cultures of 100 ml grown in BHI broth for 48 h at 37 °C with agitation were centrifuged at 7500 x *g*. The cell pellet was washed with 0.1 M PBS, pH 7.2, and re-suspended in 0.5 ml of 2.5% NaCl in 20 mM *N*-(2 hydroxyethyl)piperazine-N9-(butanesulfonic acid) (HEPES), pH 7.4, and heated for 1 h at 56 °C to extract outer membrane proteins. The extracted protein was dialyzed against 20 mM HEPES, pH 7.4. Protein concentration was determined using the bicinchoninic acid assay. Then, 100 µg of protein in 50 ml was mixed with 100 ml of rehydration buffer and applied to the Zoom IEF strip. Isoelectric focusing was performed using Zoom IPGRunner according to the manufacturer's instructions. The IEF strip was removed, equilibrated in SDS-reducing buffer, and applied to the NuPAGE Zoom Bis-Tris 4–12% gradient gel. After 2-D PAGE for 183 V h, the gel was stained. The electrotransfer to polyvinylidene difluoride (PVDF) was performed in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, pH 11.0, at 4 °C to 60 V h. After transfer, the PVDF was submerged in methanol, allowed to air dry, and incubated with hyperimmune chicken serum to *O. rhinotracheale* diluted 1:5000 in
0.1 M PBS, pH 7.4, and 0.05% Tween 80 containing 0.25% fish gelatin. The blot was subjected to rapid immunodetection according to the manufacturer's instructions. The conjugate, horseradish peroxidase labeled goat-anti-chicken IgG, was diluted 1:5000 in the same buffer. Blots were developed using the TMB substrate system. For MALDI-TOF analysis, gel plugs were removed from the stained 2-D gel and digested with trypsin as described previously. Briefly, gel plugs were removed, digested on an automated digester, and subjected to mass spectrometric analysis using a QStar XL mass spectrometer. The peptide mass spectra were analyzed using the Prospector software utility MS-FIT. *De novo* sequences were obtained using Analyst QS and Bioanalyst v1.1. The iron chelator DP was added to the growth medium to render the medium iron restricted and to mimic the host's environment for the invading pathogen.

Since very little is known about the virulence factors and virulence mechanisms of this bacterium, Tabatabai et al. (2008) [25] examined the mechanism of iron acquisition with the end to provide insights into the pathogenesis of this microorganism. To this end, they examined several *O. rhinotracheale* field strains of known serotype, an ATCC type strain, and 72 field strains of unknown serotype for their ability to grow in BHI broth rendered iron restricted by using the iron chelator DP. First, they examined the effect of iron-restricted media on the proliferation of *O. rhinotracheale*.

Because of the observed differences in sensitivity of the strains to DP, these authors extended the DP sensitivity studies and included several field strains. Interestingly, a wide range in sensitivity was observed among the field strains. Twenty-two out of the 72 strains grew well in 50 mM and 100 mM DP. The remaining strains did not grow well in the BHI broth with or without DP. The ability of these 22 field strains of *O. rhinotracheale* to grow in the presence of DP suggests that this attribute may be related to the disease-producing potential of these strains. To determine whether *O. rhinotracheale* produces siderophores as a mechanism to acquire iron, the authors used previously reported protocols. Their results support the conclusion that *O. rhinotracheale* strains do not produce siderophores. The alternative mechanism for iron acquisition is through the expression of one or more iron-protein receptor-mediated uptake systems.

Tabatabai et al. (2008) [25] demonstrated that the mechanism of iron acquisition for *O. rhinotracheale* under iron-restricted conditions occurs via the iron-bound protein pathway rather than through the siderophore secretion pathway. They also showed that many of the field strains had different sensitivities to the iron chelator DP. Only about one third of the isolates tested were resistant to DP, suggesting that these strains may potentially be more virulent.

5.2.3. Hemolysin-like protein with pore-former ability of *O. rhinotracheale*

Ornithobacterium rhinotracheale was first identified as a non-hemolytic microorganism [3, 4]. 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 [5, 26]. In order to compare and characterize the

hemolysin-like activity of North American *O. rhinotracheale* field isolates obtained from diseased turkeys biochemically and kinetically, Tabatabai et al. (2010) [26] used *in vitro* kinetic hemolysis assays with sheep red blood cells (RBCs), western blotting with leukotoxin-specific monoclonal antibodies, and isobaric tagging and quantitative analysis of *O. rhinotracheale* outer membrane protein digest preparation.

Tabatabai et al. (2010) [26] carried out an *in vitro* hemolysis assays with sheep and turkey RBCs. To this end, they collected blood aseptically and centrifuged it at 400 x *g* for 10 min at 20 °C. Erythrocytes were washed three times with PBS, pH 7.4 until the supernatant was colorless. A suspension of 1% RBCs was made in 50 mM PBS containing 0.1% bovine serum albumin. The hemolysis assay was performed as described previously. Briefly, proteins in 0.5-ml volumes containing 0.5, 1.5, and 2.25 mg protein/ml in 50 mM PBS were added to microfuge tubes containing 0.5 ml of RBC suspension. The tubes were gently inverted and incubated in a 37 °C water bath. RBC lysis control (100%) was prepared by adding 0.5 ml of a 0.4% saponin solution in water to 0.5 ml of RBC suspension. At specified time intervals, 0.2-ml aliquots were removed and centrifuged at 5000 x *g* for 1 min at room temperature. The absorbance was read at 540 nm.

Then, the authors carried out SDS-PAGE and western blotting. They denatured and chemically reduced the protein samples using sample buffer containing 1 mM dithiothreitol and loaded onto 10-or-15-well 4–20% gradient gels. Samples were run according to the manufacturer's instructions, stained with Coomassie blue R250, and destained. Western blotting was performed using nitrocellulose membranes. For leukotoxin visualization, membranes were probed with a 1:10,000 dilution of anti-leukotoxin IgG monoclonal antibodies 2C9-1E8 (neutralizing) and 6A7-2E7 and prepared from *Mannheimia haemolytica* strain D153 serotype 1 using preparative SDS-PAGE. Unrelated IgG2b monoclonal antibody at 1:10,000 served as a control. Blots were incubated with the appropriate conjugates, alkaline phosphatase-conjugated anti-mouse IgG at 1:1,000 or alkaline phosphatase-conjugated anti-turkey IgG at 1:1000. Color was developed for 10 min at room temperature using one 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablet.

In order to perform the isobaric tagging and relative and absolute quantitative (iTRAQ) analysis of *O. rhinotracheale* outer membrane proteins for leukotoxin peptides, the authors analyzed four of the strains of *O. rhinotracheale*. The strains were grown in 100 ml of BHI broth for 48 h at 37 °C with agitation, and centrifuged at 7500 x *g*. The cell pellet was washed with PBS, pH 7.2, and resuspended in a small volume of 2.5% NaCl in 20 mM HEPES, pH 7.4, and heated for 1 h at 56 °C to extract the outer membrane proteins. The extracted protein was dialyzed against 20 mM HEPES, pH 7.4. Protein concentration was determined using a bicinchoninic acid assay. Protein preparations were precipitated with ice-cold acetone, and 100 mg of each protein preparation was treated with one of four isobaric amino-modification reagents labeled with biotin according to the manufacturer's recommendations. The protein preparations were digested with trypsin and mixed, and the peptides were separated by affinity chromatography on an avidin column following the manufacturer's instructions. Samples were analyzed by liquid chromatography/mass spectrometry/mass spectrometry using the Q-Star XL-TOF quadrupole tandem mass spectrometer.

Tabatabai et al. (2010) [26] observed that most of the field strains showed hemolytic activity after 48 h at room temperature aerobically following 48-h incubation under 7.5% CO_2. Some of the strains evaluated and the additional field strains subsequently tested often showed α-hemolysis (greening of the blood agar) after 48 h of incubation and upon removal of the plates from the CO_2 environment. Upon further incubation at room temperature, partial or complete hemolysis was observed, depending on the isolate tested. Because sheep blood agar plates gave more consistent results, all experiments were subsequently performed using sheep blood agar plates. Because of the initial unexpected observations of hemolysis on blood agar, comparative proteomics using iTRAQ were used to specifically compare labeled peptide fragments derived from the same protein or proteins expressed by different strains of *O. rhinotracheale*.

In order to confirm the observation of β-hemolytic activity observed on sheep blood agar plates and the iTRAQ results, the authors then performed *in vitro* hemolysis assays with both sheep and turkey RBCs. The highest hemolysin-like activity was observed from protein extracts prepared from one of the strains evaluated. They observed that hemolysis took place within the first minute of incubation at 37 °C. Therefore, for subsequent assays, the first sample was removed at 30 s and at 2-to-3-min intervals thereafter. Protein extracts from this particular isolate were used in subsequent assays for concentration-dependent and kinetic analyses. Initial rates of hemolysis measured at the 30-s time point were directly proportional to the protein concentration of the cell-free sonicates. The RBC concentration in the assay suggests that the hemolytic activity was due to a pore-forming ability of the protein and not to an enzyme action on the RBC membrane, as the percentage of hemolysis decreased with increasing RBC concentration.

Because iTRAQ comparative experiments for global analysis of the *O. rhinotracheale* proteome identified proteins with sequence identity toward four peptide sequences of lktA of *M. haemolytica*, Tabatabai et al. (2010) [26] then performed western blotting by using highly specific IgG monoclonal antibodies prepared to lktA. Numerous proteins appeared on the Coomassie-stained gel, but only three proteins were visualized on the western blot using the neutralizing monoclonal antibody, 2C9, and the non-neutralizing antibody, 6A7. The weakly stained bands corresponded to proteins at 181 and 92 kDa and the strongly stained band corresponded to a protein or proteins at 56 kDa.

Most of the North American field isolates of *O. rhinotracheale* studied by Tabatabai et al. (2010) [26] showed β-hemolytic reactions on sheep blood agar when the incubation period was extended beyond 48 h under CO_2 with further

incubation at ambient temperature for an additional 48 h. One explanation may be the acquisition of the hemolysin/cytolysin gene by horizontal transfer. It is unknown if the hemolytic-like activity is related to virulence of *O. rhinotracheale*. Furthermore, Tabatabai et al. (2010) [26] did not directly associate the hemolytic activity with any of the three proteins identified by western blotting with the lktA-specific monoclonal antibody. The authors concluded that it would be of interest to compare the relative virulence of the hemolytic and non-hemolytic phenotypes in turkey models. In order to identify the hemolytic activity of *O. rhinotracheale* strains, they examined secreted proteins, outer membrane proteins, and whole-cell lysates by iTRAQ mass spectrometry. The analysis of iTRAQ experiments allowed them to identify peptides from *O. rhinotracheale* protein extracts that were identical to peptides of lktA and peptides of *Actinobacillus actinoacetemcomitans*. Kinetically, three different types of hemolytic (cytolytic) mechanisms have been previously described based on the analysis of concentration-dependent *in vitro* assays, such as porins, enzymes, and surfactants [26]. Based on kinetic analysis of the hemolytic activity, the authors determined that the hemolytic activity was a pore-forming molecule rather than an enzyme molecule. However, because the kinetic analysis cannot distinguish between a pore-former and a molecule with surfactant properties, the authors suggested that complete DNA and protein sequence information is needed to make this determination. These authors concluded that North American field isolates of *O. rhinotracheale* express a hemolysin-like protein present in the whole cell lysate, and that this hemolytic activity may contribute to the overall virulence of *O. rhinotracheale*.

5.2.4. Plasmid pOR1 from *O. rhinotracheale*

Finally, regarding virulence factors of *O. rhinotracheale*, Jansen et al. (2004) [12] reported the presence of plasmid pOR1 in two strains of the 56 strains tested in their study. The panel consisted of two isolates of the rare serotype K, 34 isolates of non-serotype K from Minnesota, and 20 isolates obtained from different European and African bird species. The authors analyzed the isolates by dot blot DNA hybridization with the full-length pOR1 clone in lambda EMBL3 phage as a probe. They detected plasmid pOR1 only in the two isolates from serotype K. The authors concluded that the apparently rare occurrence of pOR1 suggests that the plasmid was introduced into *O. rhinotracheale* on a single and recent occasion and has not spread in the population. The selective advantage of carrying pOR1 thus remains unclear.

References

[1] Charlton BR, Channing-Santiago SE, Bickford AA, Cardona CJ, Chin RP, Cooper GL, Droual R, Jeffrey JS, Meteyer CU, Shivaprasad HL, Walker RL. Preliminary characterization of a pleomorphic Gram-negative rod associated with avian respiratory disease. *J. Vet. Diagn. Invest*. 1993;5:47–51.

[2] Vandamme P, Segers P, Vancanneyt M, van Hove K, Mutters R, Hommez J, Dewhirst F, Paster B, Kersters K, Falsen E, Devriese LA, Bisgaard M, Hinz KH, Mannheim W. *Ornithobacterium rhinotracheale* gen. nov., sp. nov., isolated from avian respiratory tract. *Int. J. Syst. Bacteriol*. 1994;44:24–37.

[3] Chin RP, van Empel PCM, Hafez HM. *Ornithobacterium rhinotracheale* infection. In: Saif YM, Fadly AM, Glisson JR, McDougald LR, Nolan LK, Swayne DE, eds. *Diseases of Poultry*. 12th ed. Oxford, UK: Blackwell Publishing Ltd; 2008:765–774.

[4] Chin RP, Charlton BR. Ornithobacteriosis. In: Dufour-Zavala L, Swayne DE, Glisson JR, Pearson JE, Reed WM, Jackwood MW, Woolcock PR, eds. *A Laboratory Manual for the Isolation, Identification, and Characterization of Avian Pathogens*. 5th ed. Madison, WI: American Association of Avian Pathologists; 2008:75–76.

[5] Gornatti Churria CD, Machuca M, Vigo G, Petruccelli M. *Ornithobacterium rhinotracheale* infection in poultry: an updated review. *Int. J. Mol. Zool*. 2012;2: 23–38.

[6] Wages DP. Antimicrobial therapy. In: Saif YM, Fadly AM, Glisson JR, McDougald LR, Nolan LK, Swayne DE, eds. *Diseases of Poultry*. 12th ed. Oxford, UK: Blackwell Publishing Ltd; 2008:42–46.

[7] Apata DF. Antimicrobial resistance in poultry. *Int.J. Poult. Sci*. 2009;8:404−408.

[8] Löhren U, Ricci A, Cummings TS. Guidelines for antibiotic use in poultry. In: Guardabassi L, Jensen LB, Kruse H, eds. Guide to Antimicrobial Use in Animals. Oxford, UK: Blackwell Publishing Ltd; 2008:126–142.

[9] Teuber M. Veterinary use and antibiotic resistance. *Curr. Opin. Microbiol*. 2001;4:493–499.

[10] Catry B, Laevens H, Devriese LA, Opsomer G, de Kruif A. Antimicrobial resistance in livestock. *J. Vet. Pharmacol.Therap*. 2003;26:81–93.

[11] Malik YS, Olsen K, Kumar K, Goyal SM. *In vitro* antibiotic resistance profiles of *Ornithobacterium rhinotracheale* strains isolated from Minnesota turkeys during 1996–2002. *Avian Dis*. 2003;47:588–593.

[12] Jansen R, Chansiripornchai N, Gaastra W, van Putten JPM. Characterization of pOR1from Ornithobacterium rhinotracheale and construction of a shuttle plasmid. Appl. Environ. Microbiol. 2004;70:5853–5858.

[13] Banani M, Pourbakhsh SA, Deihim AH. Antibiotic sensitivity of Ornithobacterium rhinotracheale isolates associated with respiratory diseases. Arch. Razi Ins. 2004;58:111–117.

[14] Devriese LA, De Herdt P, Haesebrouck F. Antibiotic sensitivity and resistance in Ornithobacterium rhinotracheale strains from Belgian broiler chickens. Avian Pathol. 2001;30:197–200

[15] Ak S, Turan N. Antimicrobial susceptibility of Ornithobacterium rhinotracheale isolated from broiler chickens in Turkey. Vet.Arh.2001;71:121–127.

[16] Mohd-Zain Z, Jee TL, Jusoff K. Phenotypic characteristics, antibiotic susceptibility, and pathogenicity of Ornithobacterium rhinotracheale. WSEAS Trans. Biol. Med. 2008;7:133–142.

[17] Murthy TRGK, Dorairajan N, Balasubramaniam GA, Dinakaran AM, Saravanabava K. *In vitro* antibiotic sensitivity of *Ornithobacterium rhinotracheale* strains isolated from laying hens in India. *Vet. Arh*. 2008;78:49–56.
[18] Tsai HJ, Huang CW. Phenotypic and molecular characterization of isolates of *Ornithobacterium rhinotracheale* from chickens and pigeons in Taiwan. *Avian Dis*. 2006;50:502–507.
[19] Soriano VE, Vera NA, Salado CR, Fernández RP, Blackall PJ. *In vitro* susceptibility of *Ornithobacterium rhinotracheale* to several antimicrobial drugs. *Avian Dis*. 2003;47:476–480.
[20] Devriese LA, Hommez J, Vandamme P, Kersters K, Haesebrouck F. *In vitro* antibiotic sensitivity of *Ornithobacterium rhinotracheale* strains from poultry and wild birds. *Vet. Rec*. 1995;137:435–436.
[21] van Veen L, Hartman E, Fabri T. *In vitro* antibiotic susceptibility of strains of *Ornithobacterium rhinotracheale* isolated in the Netherlands between 1996 and 1999. *Vet. Rec*., 2001;49:611–613.
[22] Marien M, Decostere A, Nauwynck H, Froyman R, Devriese L, Haesebrouck F. *In vivo* selection of reduced enrofloxacin susceptibility in *Ornithobacterium rhinotracheale* and its resistance-related mutations in *gyr*A. *Microb. Drug Resist*. 2006;12:140–144.
[23] Salyers AA, Whitt DD. *Bacterial Pathogenesis: a Molecular Approach*. Washington DC: American Society for Microbiology; 1994.
[24] Kastelic S, Berčič RL, Cizelj I, Benčina M, Makrai L, Zorman-Rojs O, Narat M, Bisgaard M, Christensen H, Benčina D. Ornithobacterium rhinotracheale has neurominidase activity causing desialylation of turkey and chicken serum and tracheal mucus glycoproteins. Vet. Microbiol. 2013;162:707–712.
[25] Tabatabai LB, Zehr ES, Zimmerli MK, Nagaraja KV. Iron acquisition by Ornithobacterium rhinotracheale. Avian Dis.2008;52:419–425.
[26] Tabatabai LB, Zimmerli MK, Zehr ES, Briggs RE, Tatum FM. Ornithobacterium rhinotracheale North American field isolates express a hemolysin-like protein. Avian Dis., 2010;54:994–1001.

