# *Ornithobacterium rhinotracheale*

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# *Ornithobacterium rhinotracheale*

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**PAULUS CORNELIUS MARIA VAN EMPEL**

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Promotor: Prof. Dr. Bernard A. M. van der Zeijst

Co-promotor: Dr. Johannes F. van den Bosch
Intervet International bv
Afd. Bacteriologische Research

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CHAPTER 1

# General introduction

Respiratory diseases in poultry and
history of *Ornithobacterium rhinotracheale*

## 1. Aim and course of the studies described in this thesis

This thesis deals with an avian disease of the respiratory tract caused by a bacterium named *Ornithobacterium rhinotracheale*. At the start of these studies almost nothing was known about the disease as well as about the bacterium and its effect on poultry. In fact the bacterium was still nameless and only little information about the clinical signs was on hand. The aim of these studies was to get a good picture of the bacterium, the disease and a possible prevention of the disease. During the course of the study several problems had to be solved and questions to be answered such as :

- The pathogenicity of *O. rhinotracheale* for poultry had to be established. The infection route had to be investigated and infection models had to be developed.
- Growth conditions had to be established for the isolation of more field strains. These were needed for further studies.
- Reliable identification methods for *O. rhinotracheale*, also suitable for use in routine laboratories, had to be developed.
- Serological tests had to be developed for screening and identification purposes.
- The proper biochemical and genetic methods to characterize strains had to be selected.
- Searching for the possible reservoirs of the bacterium within the body, the course of the *O. rhinotracheale* infection had to be investigated and the tools to do so had to be developed.
- For the control of the disease, vaccines had to be developed and application routes and vaccination schemes had to be investigated.

In this thesis the research work dealing with these items will be discussed.

## 2. Respiratory diseases in poultry

The globel spread of avian respiratory diseases is well described in the literature. In addition to infectious agents (such as fungi, viruses, and bacteria), also environmental circumstances e.g. high concentrations of ammonia play a role. The microorganisms known to be involved in avian respiratory diseases are : Aspergillus species, laringotracheïtis virus, infectious bronchitis virus, avian paramyxovirus types 1 (Newcastle disease virus), 2, 3 and 6, influenza A virus, adenovirus group 1, reoviruses, pneumovirus, *Escherichia coli, Haemophilus paragallinarum, Pasteurella multocida, Riemerella (Pasteurella) anatipestifer, Bordetella avium, Mycoplasma gallisepticum, Mycoplasma synoviae, Mycoplasma meleagridis* and *Mycoplasma iowae*.

Not all of these agents have been proven to be primary pathogens, but their involvement in respiratory diseases has been established. Most respiratory diseases are caused by

more than one agent or a combination of an infectious agent and poor management practices. Specially sanitation is an important factor in the prevalence of respiratory diseases. These facts are complicating the establishment of pathogenicity of an infectious agent.

### 2.a. Fungal respiratory pathogens

**Aspergillus.** The two major agents causing aspergillosis of poultry are *Aspergillus fumigatus* and *Aspergillus flavus* (13). Symptoms of aspergillosis are dyspnea, gasping, accelerated breathing, dermatitis, ophthalmitis and encephalitis. They occur in almost all bird species. Airsacculitis with caseous nodules were found in turkeys after an aerosol exposure of *A. fumigatus*. The disease resulted in 50% mortality (46). Treatment is difficult because only a few drugs like Nystatin and Amphotericin B are effective. Aspergillus species can be identified by the way the conidia (spores) are formed. *A. fumigatus* forms greenish to gray conidia on uniseriate phialides up on the upper two-thirds of the vesicle while *A. flavus* forms goldish to red brown conidia on uniseriate and biseriate phialides covering the entire vesicle, pointing out in all directions (32).

### 2.b. Viral avian respiratory pathogens

**Adenoviruses of group 1.** These viruses have frequently been isolated out of cases of avian respiratory disease, but their role as primary infectious agents is questionable (38). However, aerosol exposure did induce reactions in the air sacs (38). Quail bronchitis virus is known to be pathogenic for quail, but not for other avian species (56). The viruses have regularly been found together with other pathogenic agents but their role as respiratory pathogen, except for quail bronchitis virus, is not well known. Because of this normally no preventive measures are taken.

**Infectious bronchitis virus (IBV)** causes serous catarrhal or caseous exudates in the trachea, the sinuses, the air sacs and the nasal passages which will result in gasping, coughing, sneezing and nasal discharge. Infections occur in chickens and to a lesser extent in turkeys and pheasants (28). All birds in a flock will become infected. Mortality rates are usually low, only young birds will succumb (28). Mortality also occurs when other agents, like bacteria, are present (15). There is no specific treatment for IBV infections; vaccination is the best way to prevent the disease. Isolation and serotype characterization of the isolates are necessary for a definitive diagnosis. Serology can be performed with ELISA, Vero cell neutralization or haemagglutination inhibition (28).

**Influenza viruses of type A** may cause respiratory, enteric or reproductive infections (19). The severity is dependent on the virulence of the virus strain and the species, age, environment and immune status of the birds. Morbidity and/or mortality can reach up to 100%. Vaccination and eradication programs are used to control the disease. There is no practical specific treatment available (6). Isolation and identification is necessary for a conclusive diagnosis.

**Laringotracheitis virus (LTV)** causes nasal discharge, coughing, gasping, watery eyes and conjunctivitis in chickens but not in turkeys (23). Morbidity rates can be very high, while mortality rates varies (23). No drug has found to be effective against LTV infections; vaccination is the only way to prevent the disease. Diagnosis is done serologically, usually using immunofluorescence (23).

**Newcastle disease virus (NDV)** causes a variety of clinical signs depending on the virulence of the strain involved. In all cases the respiratory tracts are affected. Chickens are highly (3), turkeys are less (1, 9) and ducks and geese are hardly susceptible (24). Morbidity and mortality rates in chickens can be very high. Often other agents, like bacteria, are involved in the disease. The infectivity of the virus is reduced by physical and chemical treatments but no treatment can guarantee the total inactivation of the virus. Vaccination is the most important way to prevent NDV infections, but also eradication programs are used to control the disease (3). Live vaccines may cause reactions in the respiratory tract (8). Virus isolation is the only conclusive method for diagnosis although immunohistological techniques can be helpful (22). NDV is also known under the name paramyxovirus 1.

**Paramyxoviruses other than NDV.** Three other paramyxoviruses (PMV), the types 2, 3 and 6, are able to cause mild respiratory infections and egg production problems in chickens, turkeys and ducks. The mortality is low and prevention by vaccination is only used in turkeys for PMV type 3 (3).

**Pneumoviruses** are the causitive agents of turkey rhinotracheitis (TRT) and swollen head syndrome (SHS) in chickens (4). Also pheasants can be infected but pigeons, ducks and geese are refractory. The signs of TRT in young turkeys are sneezing, sniveling, conjunctivitis, swollen sinuses and foamy nasal discharge (34). In laying birds also a drop in egg production can be seen. Morbidity of TRT is usually very high, while mortality is variable and dependent on the presence of other infectious agents (4). SHS infections cause swelling of the sinuses, depression and torticollis with a low morbidity (39). Young birds appear to be more susceptible than older animals. Protection can be obtained by vaccination using live and inactivated vaccines. Treatment of TRT with antibiotics have been reported to be variably successful; this success is presumably obtained by controlling secondary bacterial infections(16). Virus isolation, although difficult, and serology are the best ways for diagnosis.

**Reoviruses** are associated to arthritis and tenosynovitis. They were also associated with acute and chronical respiratory syndromes in chickens, turkeys and other avian species (49). The primary respiratory pathogenicity of reoviruses is not well known but the role as secundary pathogens has been confirmed (48). Vaccination has been proven to provide protection. Virus isolation is the best way for diagnosis.

### 2.c. Bacterial avian respiratory pathogens

***Bordetella avium*** is the causative agent of turkey coryza (TC), an infection in turkeys which also occurs, but in a less severe form, in chickens (5). TC causes sneezing, coughing, a clear nasal discharge, mucoid exudate in the upper trachea and softening of the trachea. High morbidity together with low mortality is a typical finding of TC. Contact and litter or water contamination are the most common routes of infection (52). Vaccination with bacterins as well as live vaccines is useful for prevention. High doses of penicillin together with oxytetracycline in the drinking water for at least 3 days has been proven to be a good treatment (26). Other antibiotics normally will not cure the disease (5). *B. avium*, formerly *Alcaligenes faecalis* (27), is a gram-negative, motile, small, strictly aerobic rod that grows well on standard media and on some selective media such as McConkey agar (37).

***Escherichia coli.*** *E. coli*, most of the time in combination with viruses or Mycoplasma species, is the causative agent of a disease called air sac disease or chronic respiratory disease (CRD). CRD has been reported in chickens, turkeys and ducks (20). The clinical picture of CRD is characterized by thickened air sacs with caseous exudate, pericarditis usually associated with myocarditis, perihepatitis and occasionally salpingitis (20). Mortality occurs during the first week of the infection. Mortality rates can be very high. Specially when other pathogenic factors, such as infections with IBV, NDV or Mycoplasma, are involved, the disease can cause severe losses. Egg transmission has been found to be important way of for the spread of the infection (20). Usually CRD can be treated effectively with the common antibiotics, but a number of avian isolates of *E. coli* are resistant to one ore more drugs (20). Vaccination has been proven to be an effective way of prevention (20). *E. coli* is a gram-negative, motile, facultative anaerobic, pleomorphic rod from the family *Enterobacteriaceae* that grows rapidly in standard media and on some selective media as McConkey agar (7).

***Haemophilus paragallinarum.*** Infectious coryza (IC), caused by *H. paragallinarum*, is only found in chicken and quails, not in turkey, pigeon or duck (57). The disease is characterized by an acute catarrhal inflammation of the mucous membranes of the nasal passages and sinuses. Also subcutaneous edema of the face and the watt-

les and catarrhal conjunctivitis are common. Pneumonia and/or air sacculitis are rarely present. When not complicated by infections with other agents such as respiratory viruses (e.g. NDV or IBV) or Mycoplasma, IC will hardly result in an increased mortality (57). Airborne transmission is believed to be the most common infection route. Bacterin vaccines do protect, although immunity is serovar-specific. Live vaccines induce better cross-protection (47). Treatment of IC with antibiotics is difficult because most of the therapeutic agents are not bactericidal. Also drug resistance developes rapidly. Only combinations of drugs (e.g. sulfanomides with trimethoprim) are effective (35,36). *H. paragallinarum* is a gram-negative, non-motile, facultative anaerobic, pleomorphic rod from the family *Pasteurellaceae* that needs the reduced form of NAD (NADH) and, sometimes, serum for growth (57). However pathogenic strains of *H. paragallinarum* which were NADH- and serum-independent have been reported, predominantly from South Africa.

**Mycoplasmata.** Avian mycoplasmosis is caused by the four most common avian Mycoplasma species : *M. gallisepticum*, *M. synoviae*, *M. meleagridis* and *M. iowae*. The disease is characterized by high morbidity and low mortality. *M. gallisepticum* causes air sacculitis, severe sinusitis and tenovaginitis in turkeys and chickens (59). *M. synoviae* causes air sacculitis, sinovitis, body-weight loss and lameness in chickens and turkeys (29). The lameness is caused by inflammation and swelling of the joints. *M. meleagridis* causes air sacculitis and skeletal abnormalities in turkeys but not in chickens (58). *M. iowae* causes mild air sacculitis and lesions in the leg in turkeys and chickens (30). Egg-transmission as well as contact and airborne droplets have found to be infection routes. Bacterins and live vaccines are be effective for protection. The succes of treatment of Mycoplasmosis is dependent on the species involved. Not all species are susceptible to the commonly used antibiotics. Egg dipping and injection with Tylosin is effective for infections with *M. gallisepticum*, *M. synoviae* and *M. meleagridis* (29, 58, 59). Mycoplasmas are the smallest free living micro-organisms. They lack a cell wall and need protein-rich media containing at least 10 to 15% animal serum (31). *M. synoviae* also needs NAD for growth (31). Growth is slow and is on solid media marked by typical “fried-egg” colonies. Mycoplasmas are resistant to penicillin and thallium acetate. These compounds are incorporated into media to supress growth of other bacteria (31).

***Pasteurella multocida.*** Fowl cholera (FC) is caused by *P. multocida* and is present in many avian species (45). FC is of historical importance because it is the first bacterial disease investigated for which a vaccine was developed (43). The clinical picture of FC does often, but not always, include respiratory infections. Virulent strains cause acute FC, which is featured by fever, anorexia, strong mucous discharche from the mouth, diarrhea and an increased respiratory rate. These signs occur within a day

before death, which is caused by sepsis (42). Chronic FC, caused by less virulent strains, shows signs generally related to localized infections e.g. in the wattles, foot pads, joints and the respiratory tract (45). Free-flying birds and chronically infected birds are considered to be the source of infection. Vaccination with bacterins induce serovar-dependent protection (unpublished observations). Live vaccines induce better cross-protection but often have residued pathogenicity. Acute FC is very hard to treat. Only high doses of antibiotics e.g. Streptomycin, given intramuscularly just before or at the time of inoculation of *P. multocida* in experimental challenge studies, can prevent death (45). The efficacy of the treatment of chronic FC is dependent on the sensitivity of the strain involved because *P. multocida* strains strongly vary in susceptibility to antibiotics (45). *P. multocida* is a gram-negative, non-motile, small, facultative anaerobic rod from the family *Pasteurellaceae* which grows rapidly on standard media but not on selective media such as McConkey agar (7).

***Riemerella anatipestifer***, formely *Pasteurella anatipestifer* (51), infects ducks, turkeys, geese and pheasants but, not chickens or pigeons (50). The infection causes ocular and nasal discharge, mild coughing and sneezing and ataxia. Tremors of the neck and head are found to be typical for *R. anatipestifer* infections. Mortality does vary from minor to moderate, depending on predisposing factors such as adverse environmental conditions and other diseases. How *R. anatipestifer* infects birds is not clear, but small wounds and air-born droplets are believed to play a role. Experimentally the disease can be reproduced by injection, but not by oral application (50). Treatment with antibiotics is difficult because strains of *R. anatipestifer* differ strongly in their sensitivity patterns. Usually sulphonamides will effective. Vaccination can be an effective way to prevent the disease, but immunity is serovar-specific (50). *R. anatipestifer* is a gram-negative, non-motile, small, facultative anaerobic rod from the family *Pasteurellaceae* which grows rapidly on standard media but not on selective media as McConkey agar (7).

## 3. History of *Ornithobacterium rhinotracheale*

In 1991 a new respiratory disease in broiler chickens was observed in South Africa by Jan DuPreez (53). Relatively mild respiratory symptoms, which started with sneezing at an age of about 28 days and which lasted up to the end of the fattening period, were accompanied by increased mortality (3 % more) and poor performances considering e.g. growth per day and feed conversion. At post mortem investigation foamish, white, “yoghurt like” exudate in the air sacs, predominantly in the abdominal air sac, was the most striking feature; also pneumonia was described. Bacteriological examination revealed a slow growing, pleomorphic, gram negative rod, which could

not be classified as one of the known bacterial species.

A *Pasteurella*-like bacterium, isolated from ducks suffering from a respiratory disease in Hungary in 1987, proved to be identical to the South African isolates in appearance as well as biochemically. Also *R. anatipestifer*-like isolates from turkeys suffering respiratory disease isolated in Germany in 1991 and 1992 (21) looked identical and had the same biochemical reactions as the strains from South Africa and Hungary.

In several turkey flocks in the Netherlands and Germany, clinical findings like sniveling, sneezing, wet eyes and swelling of the sinus infraorbitalis, together with severe growth retardation were detected in 1992 and 1993 (53). On some farms there was a constant increase of mortality despite medication. Also in broilers, minor to moderate respiratory problems and acute deaths were observed. These clinical findings, starting at 2 to 8 weeks of age in turkeys and at 2 to 5 weeks of age in broilers, were characterized in turkeys by a severe purulent pneumonia (53). These features resembled the features of a *P. multocida*-infection, but occurred at a very young age. In the field it was found that even at 2 weeks of age airsacculitis (upper and lower air sacs) and pericarditis could be detected in turkeys as well as chickens (53). Out of tissues from affected animals gram-negative rods, similar to the strains out of South Africa, Germany and Hungary, were be isolated.

In turkeys conventional oral therapeutics gave poor results. Especially when pneumonia was prevalent, the use of e.g. enrofloxacine and trimethoprim + sulphonamide was hardly or not at all effective. Occasionally injections with tetracyclines and synthetic penicillins (usually twice) were effective. In other cases medication failed, resulting in losses of up to 25% within a few weeks (21, 53). Isolated strains out of these cases in the Netherlands were almost all resistant to flumequine, only slightly sensitive for enrofloxacine and trimethoprim + sulphonamide and sensitive for tetracyclin and ampicillin. A German investigation (Hafez, personal communication) showed that 90% of the German strains were resistant to enrofloxacin and all were resistant to trimethoprim + sulphonamide, while all strains were sensitive for tetracyclins and amoxycillin.

The isolation of *Pasteurella*-like bacteria from six meat turkeys flocks with respiratory disease in Germany was reported (21). Also in the U.S.A. these pleomorphic gram-negative rods, associated with avian respiratory disease, were isolated from turkeys and chickens in California (12). Finally, bacteria isolated from a Dutch turkey flock with respiratory problems in 1992, initially defined as *R. anatipestifer* were found to be similar. Not all the strains, mentioned in these reports, did serologically react with antisera against the strains from South Africa, Germany and Hungary but the appearance, the odor and the biochemical reactions of all strains were identical.

Initially the new bacterium was named *Pasteurella*-like or *Kingella*-like. Later on

Bisgaard stated that the organism could be classified within a group of bacteria designated as TAXON 28. In 1994 the name *Ornithobacterium rhinotracheale* gen. nov. sp. nov. was proposed for this new species (54). By nows all the above mentioned strains have been classified as *O. rhinotracheale.*

Investigations of culture collections in Germany, revealed that *O. rhinotracheale* already had been isolated from the respiratory tract of turkeys in 1981 and of rooks in 1983. Also in Belgium, France and Israel *O. rhinotracheale* had been isolated before 1990 (54). Sofar no isolates of *O. rhinotracheale* from before 1981 have been reported. After improving the isolation method of avian respiratory bacteria, the presence of *O. rhinotracheale* was found to be linked to air sacculitis and purulent pneumonia in meat turkeys and broiler chickens (17, 18, 33, 40, 41, 53, 54). In various areas in the U.S.A. *O. rhinotracheale* was associated with acute pneumonia with mortality rates from 2 - 50% in older turkeys (12 weeks or older) in 1995 and 1996 (6). More recently *O. rhinotracheale* was shown to be connected to high losses in broiler chickens of 28 days and older (10). In these cases a subcutaneous oedema over the cranium with a severe bacterial osteitis, but without an infection of the respiratory tract was found.

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CHAPTER 2

# Experimental infection in turkeys and chickens with *Ornithobacterium rhinotracheale*

***Paul van Empel, Han van den Bosch, Danny Goovaerts and Paul Storm***

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**SUMMARY**

*Ornithobacterium rhinotracheale* was found to cause growth retardation in both turkeys and chickens after experimental intra air sac administration and to cause growth retardation together with airsacculitis and pneumonia after aerosol administration. Both turkey and chicken isolates of *O. rhinotracheale* were able to induce the same kind of respiratory inflammations and weight-gain losses in chickens as well as turkeys. Turkey rhinotracheitis virus was found to have a triggering effect on the *O. rhinotracheale* infection in turkeys while Newcastle disease virus and to a lesser extent Infectious Bronchitis virus showed triggering effects on the *O. rhinotracheale* infection in chickens. *O. rhinotracheale* could be reisolated from affected organs of experimentally infected birds.

## INTRODUCTION

Only very recently a pleomorphic gram-negative rod-shaped bacterium was isolated in various countries from turkeys and chickens with respiratory disease (1,4,5). These bacteria could not be classified as belonging to an already known species, and subsequently a new taxon *Ornithobacterium rhinotracheale* gen. nov., sp. nov. was proposed (6). The clinical symptoms associated with the isolation of *O. rhinotracheale* included weakness, gasping, severe dyspnoea, mucus discharge and mortality (1,4). Dissection showed fibrinopurulent pneumonia and fibrinous inflammation of the thoracic air sacs (1,4,5).

In the present paper we describe experimental infection of turkeys and chickens with isolated and pure cultures of *O. rhinotracheale* in order to reproduce the respiratory disease seen in clinical outbreaks. Furthermore, the possible triggering effect of various respiratory viruses on the outcome of experimental *O. rhinotracheale* infection was investigated.

## MATERIALS AND METHODS

**Bacterial strains.** The two strains used for this study were *O. rhinotracheale* strain B3263/91, isolated from a diseased broiler chicken in South Africa by J.H. duPreez and strain GGD-1261, isolated from an affected fattening turkey in Germany by H.M. Hafez. Both strains were maintained lyophilized at -20°C. Bacteria were grown at 37°C on sheep blood agar plates in a 5-10% $CO_2$ atmosphere, or in brain-heart infusion broth (BHI, Oxoid) at 37°C. When grown on bloodagar for at least 48 hr, *O. rhinotracheale* colonies appeared tiny, grey to grey-white, sometimes with a reddish glow, and had a distinct odor related to butyric acid.

**Viral strains.** Turkey rhinotracheitis (TRT) virus strains 3B OC21 (turkey challenge strain) and SA-2381-88/OC20 (isolated out of diseased broiler chickens in South Africa) both were obtained as frozen virus suspensions from J. Cook (Intervet UK Ltd, England). The Newcastle Disease (ND) virus strain used was a lentogenic strain LaSota as described by Goldhaft (3). The Infectious Bronchitis (IB) virus strain used was a clone derived from Mild Mass H, belonging to the Massechussetts serotype.

**Experimental animals.** The chickens used were commercial Hybro-broilers (Inter-broed, Gemert, The Netherlands), and fattening turkeys used were commercial British United Turkeys (BUT) animals (Plukon, Ruinen, The Netherlands). Both broilers and turkeys were purchased as embryonated eggs, and housed in isolaters immediately

after hatching. Food (Hendrix' voeders, Heijen, The Netherlands) and water were given *ad libitum*.

**Intra-air sac challenge.** For intra-air sac challenge, the bacterial strains were first passed through eggs. Bacteria were harvested from blood agar plates in 0.04M posphate-buffered saline (pH = 7.2) and inoculated into the yolk sac of 7-9-day-old embryonated eggs. The eggs were incubated until embryo death, which mostly occurred within 2 days. Subsequently, yolks were collected and stored at -20°C. For challenge, 0.2 ml of infected yolk, containing 4.2 x $10^8$ colony-forming units (CFU) of strain B3263/91 or 7.5 x $10^8$ CFU of strain GGD-1261, was injected per bird directly into the right abdominal air sac.

**Aerosol challenge.** For aerosol challenge, the bacterial strains were grown in BHI for 24 h at 37°C with agitation. In each isolator (approximately 1.5 $m^3$), containing the birds to be challenged, 100 ml of *O. rhinotracheale* culture with $10^8$-$10^9$ C.F.U. per ml was administered as a fine spray using a commercial paint sprayer. The developed mist was maintained in the isolators for at least 10 min. with the air circulation closed.

**Virus infection.** In a number of experiments, birds were infected with virus 5-6 days prior to *O. rhinotracheale* aerosol challenge. Turkeys were infected with a culture of TRT virus strain 3B OC21 containing $10^{6.6}$ median ciliostatic doses ($CD_{50}$) per ml, by administering 0.1 ml intranasally and 0.1 ml by eye drop (2). Broiler chickens were infected with either a culture of TRT virus strain SA-2381-88/OC20 containing $10^{4.5}$ $CD_{50}$ per ml by administering 0.1 ml intranasally and 0.1 ml by eye drop, or by a coarse spray of a culture of IB virus containing at least $10^6$ median egg infective doses ($EID_{50}$) per bird, or a coarse spray of a culture of ND virus strain Lasota containing at least $10^6$ $EID_{50}$. The coarse spray method was performed according to the method described by Yadin and Orthel (7) using an Hazelock ASL polysprayer 4075 (Louis Blok B.V., Hoevelaken, The Netherlands).

**Parameters of infection.** During 7 to 14 days after *O. rhinotracheale* challenge, morbidity and mortality were recorded, the daily weight gain after challenge was determined, macroscopical lesions were recorded at postmortem examination of the birds, and attempts to reisolate the challenge bacteria were carried out. At postmortem examination a scoring system for the observed lesions was used as follows : for thoracic air sacs, 0 = no abnormalities, 1 = one air sac seriously affected by fibrinous airsacculitis or limited pin-head sized foci of fibrinous exudate in both air sacs, 2 = both air sacs seriously affected by fibrinous airsacculitis; for abdominal air sacs, 0 = no abnormalities, 1 = pin-head sized foci of fibrinous exudate or slight diffuse fibrinous airsacculitis, 2 = severe fibrinous airsacculitis; for lungs, 0 = no abnormalities, 1 = unilateral pneumonia, 2 = bilateral pneumonia; for trachea, 0 = no abnormalities, 1 =

some exudate in the tracheal lumen, 2 = lumen of the trachea filled with exudate. All data were presented as percentages of the maximal possible lesion scores per group.

**Statistical analysis.** The statistical analyses for the weights and weight gains were performed by using Student's *t*-test (single sided). The statistical analyses for the pathologic lesions were performed by using the Kruskal-Wallis one-way analysis of variance.

## RESULTS

**Intra-air sac challenge.** The intra-air sac challenge of 32 days old turkeys with both *O. rhinotracheale* strains B3263/91 or GGD-1261 resulted in a significant decrease of the daily weight-gain during the 11-day observation period (Table 1).

Table 1. Intra air-sac challenge in turkeys at 32 days of age with *O. rhinotracheale* strains B3263/91 and GGD-1261

| Challenge strain | n | Average weight (g) Age 32 days | Age 43 days[A] | Average daily weight gain (g)[A] |
|---|---|---|---|---|
| Control | 6 | 992 (± 58) | 1889 (± 87)[a] | 82 (± 6)[a] |
| B3263/91 | 8 | 1011 (±141) | 1731 (±217)[b] | 65 (±8)[b] |
| GGD 1261 | 8 | 1044 (±117) | 1657 (±220)[b] | 56 (±11)[b] |

[A] Within columns, averages having different superscripts are significantly different ($P<0.05$)

Table 2. Intra-air sac challenge in broilers at 31 days of age with *O. rhinotracheale* strains B3263/91 and GGD-1261

| Challenge strain | n | Average weight (g) Age 31 days | Age 41 days[A] | Average daily weight gain (g)[A] |
|---|---|---|---|---|
| Control | 10 | 1323 (± 127) | 1988 (± 287)[a] | 60 (± 14)[a] |
| B3263/91 | 10 | 1409 (± 109) | 1735 (± 303)[b] | 30 (± 23)[b] |
| GGD 1261 | 10 | 1356 (± 122) | 1816 (± 178)[b] | 42 (± 9)[c] |

[A] Within columns, averages having different superscripts are significantly different ($P<0.05$)

Mortality or clinical symptoms were not observed after challenge, and macroscopic lesions were absent at autopsy. Furthermore, the challenge bacteria could not be reisolated from the airsacs. The only sites from which *O. rhinotracheale* could be isolated were the brains (occasionally) and the heel joint (when inflamed). Similar results were seen after intra-air sac challenge of 31-day-old broiler chickens. Thus a significant growth retardation was the predominant effect (Table 2).

**Aerosol challenge of turkeys.** Turkeys challenged with an aerosol of *O. rhinotracheale* strain B3263/91 at 14 days of age showed severe airsacculitis at 7 days post challenge, but no growth retardation. When TRT virus was administered to 8-day-old turkeys prior to *O. rhinotracheale* challenge 6 days later, a significant growth retardation was seen, together with the induction of severe airsacculitis (Table 3).

Table 3. Aerosol challenge of turkeys with *Ornithobacterium rhinotracheale* (O.r.) strain B3263/91, using turkey rhinotracheitis (TRT) virus strain 3B/OC21 as a trigger. TRT administration was at 8 days, *O. rhinotracheale* challenge was at 14 days, and post mortem investigation was at 21 days of age.

| Challenge | | | Average daily | Airsacculitis as a % |
|---|---|---|---|---|
| TRT | O.r. | n | weight gain (g)[AB] | of the maximum score[B] |
| NO | NO | 10 | 27 (± 4)[a] | 0[a] |
| YES | YES | 10 | 21 (± 4)[b] | 81[b] |
| NO | YES | 10 | 30 (± 4)[a] | 92[b] |
| YES | NO | 10 | 26 (± 4)[a] | 0[a] |

[A] Daily weight gain measures between day 1 and day 21

[B] Within columns, average and percentages having different superscripts are significantly different ($P<0.05$)

When 31-day-old turkeys were challenged with *O. rhinotracheale* significant growth retardation was induced, but growth retardation was accompanied by a significant increase in the airsacculitis score only when TRT virus was administered prior to *O. rhinotracheale* challenge (Table 4). Administration of TRT virus alone at an earlier or later age induced only very mild airsacculitis and no decrease in growth rate (Tables 3, 4). Using an aerosol challenge of turkeys, with or without the prior administration of TRT virus, neither mortality nor clinical symptoms could be induced and no other pathologic lesions developed apart from airsacculitis. The challenge bacteria could be reisolated from the affected air sacs, but isolation from any other nonaffected organ remained negative.

Table 4. Aerosol challenge of turkeys with *Ornithobacterium rhinotracheale* (O.r.) strain B3263/91, using turkey rhinotracheitis (TRT) virus strain 3B/OC21 as a trigger. TRT administration was at26 days, *O. rhinotracheale* challenge was at 31 days, and post mortem investigation was at 42 days of age.

| Challenge | | | Average daily | Airsacculitis as a % |
|---|---|---|---|---|
| TRT | O.r. | n | weight gain (g)[AB] | of the maximum score[B] |
| NO | NO | 10 | 102 (± 16)[a] | 3[a] |
| YES | YES | 10 | 54 (± 9)[b] | 27[b] |
| NO | YES | 10 | 57 (± 11)[b] | 15[bc] |
| YES | NO | 10 | 100 (± 18)[a] | 11[ac] |

[A] Daily weight gain measured between day 31 and day 42

[B] Within columns, averages and pathologic scores having different superscripts are significantly different ($P<0.05$)

**Aerosol challenge of broilers.** In two separate experiments broiler chickens were challenged at an age of 14 days (Tables 5, 6) or 21 days (Tables 7, 8) with an aerosol of *O. rhinotracheale* strain B3262/91 and observed for 2 wk after challenge. A significant decrease in daily-weight gain was seen, especially in the second week after challenge (Tables 6, 8). Pathologic lesions at postmortem investigation in general were mild, with only significant airsacculitis at 1 wk after challenge and, in one of the experiments, severe exudate in the trachea at 2 wk postchallenge.

As shown in Tables 5 and 6, the administration of IB virus and, to a lesser extent, TRT virus, at 5 days before *O. rhinotracheale* challenge had an aggravating effect, especially on the development of airsacculitis. As shown in Tables 7 and 8, the administration of ND virus at 5 days prior to *O. rhinotracheale* challenge induced a more serious increase of airsacculitis and pneumonia scores, as compared with both *O. rhinotracheale* challenge or ND administration alone. As in turkeys, especially in the right abdominal air sacs, severe fibrinous inflammation was found. The airsacculitis consisted of a foamy exudate with presence of relatively large clots of fibrin (Figure 1). In most cases the pneumonia was unilateral with a clear boundary between the affected and healthy part of the lung (Figure 2). Mortality or clinical symptoms were absent. The challenge bacteria could easily be reisolated from the organs with macroscopical lesions and from tracheal exudate but not from any other non-inflamed organ.

Table 5. Aerosol challenge of broilers with *Ornithobacterium rhinotracheale* strain B3263/91, using an infectious bronchitis (IB) virus Massechusetts serotype strain and turkey rhinotracheitis (TRT) virus strain SA-2381-88/OC20 as triggers. Virus administration was at 9 days, *O. rhinotracheale* challenge was at 14 days and post mortem investigations was at 21 days of age.

| | Average daily weight gain between days 14 and 21 | | Pathologic lesions (as a % of the maximal score) | | | |
|---|---|---|---|---|---|---|
| Challenge | n | weight gain (g)[A] | n | Air sacs[A] | Trachea [A] | Lungs |
| Control | 20 | 52 (± 6)$^{a}$ | 10 | 0$^{a}$ | 0$^{a}$ | 0 |
| O.r. | 20 | 50 (± 8)$^{a}$ | 10 | 18$^{b}$ | 5$^{a}$ | 0 |
| IB and O.r. | 20 | 46 (± 8)$^{b}$ | 10 | 45$^{c}$ | 20$^{b}$ | 10 |
| TRT and O.r. | 19 | 54 (± 6)$^{a}$ | 10 | 28$^{bc}$ | 10$^{ab}$ | 0 |

[A] Within columns, averages and pathologic scores having different superscripts are significantly different ($P$<0.05)

Table 6. Aerosol challenge of broilers with *Ornithobacterium rhinotracheale* strain B3263/91, using an infectious bronchitis (IB) virus Massechusetts serotype strain and turkey rhinotracheitis (TRT) virus strain SA-2381-88/OC20 as triggers. Virus administration was at 9 days, *O. rhinotracheal*e challenge was at 14 days and post mortem investigations was at 28 days of age.

| | | Average daily weight gain between | Pathologic lesions (as a % of the maximal score) | | |
|---|---|---|---|---|---|
| Challenge | n | days 14 and 28[A] | Air sacs[A] | Trachea [A] | Lungs |
| Control | 10 | 56 (± 8)$^{a}$ | 0$^{a}$ | 0$^{a}$ | 0 |
| O.r. | 10 | 43 (± 6)$^{b}$ | 5$^{a}$ | 60$^{b}$ | 0 |
| IB and O.r. | 10 | 44 (± 7)$^{b}$ | 23$^{b}$ | 5$^{a}$ | 0 |
| TRT and O.r. | 9 | 51 (± 7)$^{a}$ | 20$^{b}$ | 0$^{a}$ | 0 |

[A] Within columns, averages and pathologic scores having different superscripts are significantly different ($P$<0.05)

Table 7. Aerosol challenge of broilers with *Ornithobacterium rhinotracheale* (O.r.) strain B3263/91, using Newcastle disease (ND) virus strain LaSota as trigger. ND administration was at 16 days, *O. rhinotracheale* challenge was at 21 days, and postmortem investigation was at 28 days of age.

| | Average daily weight gain between days 14 and 28 | | Pathologic lesions (as a % of the maximal score) | | | |
|---|---|---|---|---|---|---|
| Challenge | n | weight gain (g)[A] | n | Air sacs[A] | Trachea [A] | Lungs |
| Control | 24 | 52 (± 9)[a] | 10 | 3[a] | 0 | 0[a] |
| O.r. | 25 | 41 (± 5)[b] | 10 | 25[b] | 5 | 10[a] |
| ND | 24 | 39 (± 7)[b] | 10 | 15[b] | 0 | 0[a] |
| ND and O.r. | 25 | 43 (± 7)[b] | 10 | 80[c] | 10 | 75[b] |

[A] Within columns, averages and pathologic scores having different superscripts are significantly different ($P<0.05$)

Table 8. Aerosol challenge of broilers with *Ornithobacterium rhinotracheale* (O.r.) strain B3263/91, using Newcastle disease (ND) virus strain LaSota as trigger. ND administration was at 16 days, *O. rhinotracheale* challenge was at 21 days, and post-mortem investigation was at 35 days of age.

| | | Average daily weight gain between days | | Pathologic lesions (as a % of the maximal score) | | |
|---|---|---|---|---|---|---|
| Challenge | n | 21 and35[A] | 28 and 35[A] | Air sacs[A] | Trachea [A] | Lungs |
| Control | 14 | 59 (± 8)[a] | 66 (± 8)[a] | 0[a] | 0[a] | 0 |
| O.r. | 15 | 26 (± 3)[b] | 11 (± 5)[b] | 2[a] | 0[a] | 0 |
| ND | 14 | 46 (± 7)[c] | 54 (± 12)[c] | 14[b] | 21[b] | 0 |
| ND and O.r. | 15 | 43 (± 5[c] | 42 (± 7)[d] | 70[c] | 3[a] | 0 |

[A] Within columns, averages and pathologic scores having different superscripts are significantly different ($P<0.05$)

Figure 1: Airsacculitis of the abdominal air sacs of a 28 days old broiler, showing a foamy exudate with large clots of fibrin (arrows). The bird was challenged with an aerosol of *Ornithobacterium rhinotracheale* at 21 days of age after the administration of Newcastle disease strain LaSota at 16 days of age.

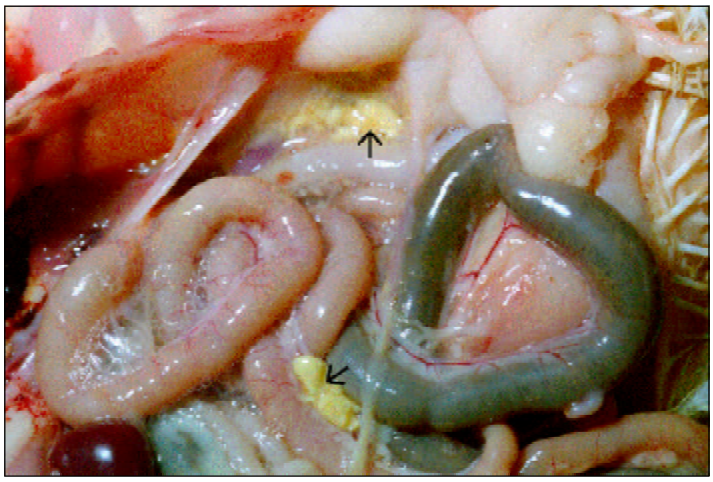
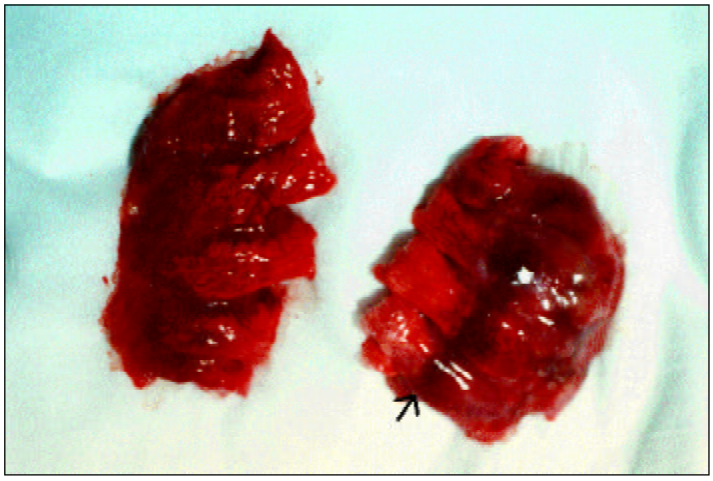


Figure 2: Unilateral pneumonia in a 28 days old broiler, showing a clear boundery between the affected and healthy part of the right lung (arrow) and the not infected left lung. The bird was challenged with an aerosol of *Ornithobacterium rhinotracheale* at 21 days of age after the administration of Newcastle disease strain LaSota at 16 days of age.



## DISCUSSION

In this study we showed that experimental infection of both turkeys and broilers with *O. rhinotracheale* bacteria could evoke respiratory disease, with at least partly the same characteristics as seen in clinical outbreaks. Both a turkey and a broiler *O. rhinotracheale* isolate induced growth retardation in turkeys as well as in broiler chickens after intra-air sac challenge without macroscopical lesions. The *O. rhinotracheale* strain isolated from a diseased broiler was used for aerosol challenge of both turkeys and broilers of various ages, and was capable to induce airsacculitis and/or growth retardation. In turkeys, infection appeared to be aggravated by the prior administration of a turkey TRT virus isolate. In broilers, infection appeared to be aggravated by the prior administration of ND virus, and to a lesser extent by prior administration of IB virus or a chicken TRT virus isolate, in particular with regard to development of airsacculitis and pneu-monia.

The most prominent clinical sign after experimental infection appeared to be the growth retardation. Also, in clinical outbreaks in the field a decreased feed and water intake was associated with the isolation of *O. rhinotracheale* (4, van Empel, pers. obs.). However, other clinical signs seen in outbreaks such as weakness, dyspnoea, mucus discharge and even mortality (4,5), were not observed after experimental infection. This seeming discrepancy between natural and experimental *O. rhinotracheale* infection might be explained by differences in predisposing and aggravating factors. In the present study we showed that infection was aggravated by prior administration of certain avian viruses. But under field conditions also other factors can be of importance such as stress, high stock density, poor ventilation, presence of other bacteria, or high ammonia levels.

With regard to pathology, the most prominent lesion after experimental *O. rhinotracheale* infection was the development of fibrinous airsacculitis, sometimes accompanied by pneumonia or tracheal exudate. These postmortem features were also observed in clinical outbreaks associated with the isolation of *O. rhinotracheale* (1,4,5; van Empel, pers. obs.). Although not all clinical signs and postmortem findings as seen in clinical outbreaks could be reproduced after experimental *O. rhinotracheale* infection, we nevertheless feel that the present study demonstrates that *O. rhinotracheale* is a true infectious agent in turkeys and broiler chickens. All three postulates of Koch are fulfilled : the agent was isolated from diseased birds and not from healthy birds (1,4,5; this study); the agent was grown to purity; and the pure agent was able to reproduce the disease in turkeys and broilers after experimental infection and the agent was recovered from the diseased animal (this study).

In this study a turkey and a broiler isolate of *O. rhinotracheale* were used. Both strains appeared to have the same effects in turkeys as well as broilers after experimental infection. Therefore, we conclude that so far no clonal pathogenicity exists among *O. rhinotracheale* strains for either turkeys or broiler chickens, but that strains are equally able to infect various avian species. But, of course, further research will have to provide more answers to the many remaining questions regarding this new avian respiratory disease.

## ACKNOWLEDGMENTS

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CHAPTER 3

# Identification

CHAPTER 3A

## Identification and serotyping of *Ornithobacterium rhinotracheale*

***Paul van Empel, Han van den Bosch, Peter Loeffen and Paul Storm.***

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**SUMMARY**

In the present study 443 strains of *Ornithobacterium rhinotracheale*, a causative agent of respiratory disease in fowl, were investigated biochemically and serologically. In both ways *O. rhinotracheale* could be differentiated from other gram-negative rods and, more particularly, from the *Pasteurella*-like bacteria potentially pathogenic for fowl. For the biochemical characterisation of *O. rhinotracheale* the API-20NE identification strip proved to be useful, although *O. rhinotracheale* is not included in the API system.

Serologically, by using monovalent antisera in agar gel precipitation (AGP) tests and enzyme-linked immunosorbent assays (ELISAs), seven serotypes (serotypes A to G) of *O. rhinotracheale* could be discriminated. The AGP test was chosen as the preferred method to be used for serotyping. Isolates of serotype A was found to be the most prevalent, especially in chickens. Isolates from turkey were more heterogeneous devided over the serotypes. Some strains showed cross-reactivity between the serotypes A, B and E. Five *O. rhinotracheale* strains could not be serotyped with the available antisera. Relationships between the geographic origin and the serotypes were found. By the ELISA the presence of antibodies against *O. rhinotracheale* could be detected in 1-day-old birds as well as in birds with clinical signs, and therefore, it might be useful for diagnostic purposes.

## INTRODUCTION

Respiratory problems, together with purulent pneumonia, airsacculitis, severe growth retardation and rapidly increasing mortality were reported in meat turkeys and broilers in South Africa, Germany, the United States, France and the Netherlands (1, 2, 4 and 6). A gram-negative, pleomorphic rod, could repeatedly be isolated from affected organs. This *Pasteurella*-like organism has recently been referred to as *Ornithobacterium rhinotracheale* gen. nov. sp. nov. (7). In experimental infections, it was possible to evoke severe growth retardation and airsacculitis in turkeys and chickens with this bacterium (8). *O. rhinotracheale* strains from different countries reporting fowl infected with the organism were investigated for their bacteriological, biochemical and serological relationships and their differences from other gram-negative rods.

## MATERIALS AND METHODS

**Bacterial strains.** The 443 *O. rhinotracheale* strains, involved in this study, were isolated between 1987 and 1996 from the respiratory tract, liver, joints or brain of diseased birds in various countries. All except three strains were isolated from chickens or turkeys; the other three strains were isolated from a duck (from Hungary), and from a guinea fowl and a partridge (both from France). Immediately after arrival the strains were freeze-dried and stored at -20°C. The strains were cultivated on sheep blood agars at 37° C, in a 5 to 10 % $CO_2$ atmosphere for at least 48 hours.

For comparative investigations, the 16 serotype-specific strains of *Pasteurella multocida* (3), the 17 serotype-specific strains of *Riemerella anatipestifer* (5) and the 3 serotype-specific strains of *Haemophilus paragallinarum* (9) together with 2 NADH-independent *H. paragallinarum* strains from South Africa (J. du Preez) and a fieldstrain of *Pasteurella gallinarum* (our own collection) were used. Because in the beginning we found *O. rhinotracheale* to react biochemically as a *Kingella*-like bacterium, the strains *Kingella kingae* ATCC 23330, *Kingella denitrificans* ATCC 33394 and *Kingella (Suttonella) indologenes* ATCC 25869 were included in the comparative studies.

**Biochemical and enzymatic reactions.** The biochemical and enzymatic reactions were tested using the A.P.I. system (BioMérieux SA, La Balmes-Les Grottes, France). All tests were performed under the prescribed conditions. As in the identification method used by the API system, *O. rhinotracheale* strains, which are oxidase-positive, gram-negative, facultatively anaerobic rods, were characterised with use of the API-20NE strip.

**Serological investigations.** Monovalent antisera were prepared by injecting specific-pathogen-free chickens subcutaneously twice, with a bacterin containing between $10^8$ and $10^9$ formalin-killed bacteria per dose in an oil adjuvant at an interval of 3 to 4 weeks. At 3 to 4 weeks after the second injection, serum was collected.

Boiled extract antigens (BEAs) were prepared as described by Heddleston (5) by washing well-grown cultures from sheep blood agar with 0.02 M Phosphate buffer-8.5 % NaCl-0.3 % formaldehyde (pH 7.2). The same buffer was used to adjust the suspensions to an optical density at 660 nm of 0.5 to 0.6 when the suspensions were diluted 1:20. Subsequently, the suspensions were boiled for 60 min at 100° C. After centrifugation the supernatants were sterilized by filtration through a 0.22-ìm-pore-size filter and were then used as antigen in agar gel precipitation (AGP) tests as well as in enzyme-linked immunosorbent assays (ELISAs).

For the AGP test, glass slides were flooded with ± 1 ml of preheated sterile 1.5 % Noble agar-8.5 % NaCl-0.1 % thimerosal per 5 $cm^2$. Patterns consisting of six or seven wells (2 mm in diameter) located around a central well at a distance of ± 5 mm were punched out of the agar. At 1-h intervals the wells were filled twice with ± 15 ìl of undiluted BEAs or serum. The slides were incubated for at least 48 h in a moist chamber at 37° C and were then observed for precipitation lines under UV light.

For the ELISA, polystyrene microtitre plates were coated with a 1/100 dilution of BEAs and were incubated overnight at 37°C. The coated plates were incubated for 60 min at 37°C with serial dilutions of the test sera. Subsequently, the bound antibodies were quantified using rabbit anti-chicken Immunoglobulin peroxidase conjugate (Nordic) and tetramethylbenzidine (Fluka) as the substrate.

## RESULTS

**Bacteriological identification.** Optimal growth of *O. rhinotracheale* was obtained when the organism was incubated on 5% sheep blood agar for at least 48 hours under microaërobic conditions (5 to 10% $CO_2$) at 37° C. Under these circumstances *O. rhinotracheale* developed small, grey to grey-white colonies, sometimes with a reddish glow and always with a distinct odor, similar to the odor of butyric acid. Upon primary isolation, most *O. rhinotracheale* cultures showed great differences in the colony size from 1 to 3 mm after 48 h of incubation. When the primary cultures were subcultured, the colony size became more uniform.

The most important biochemical and enzymatic reactions of *O. rhinotracheale* in comparison to those of *Kingella* spp. and the other gram-negative rods related to the family *Pasteurellaceae* potentially pathogenic for fowl are listed in Table 1. By using the same tests and references, *O. rhinotracheale* can also be differentiated from other

potential pathogenic gram-negative rods in the families *Enterobacteriaceae* and *Neisseriaceae*. Within the species *O. rhinotracheale*, the enzymatic reactions (performed with API ZYM strips) showed uniform results.

All 443 *O. rhinotracheale* strains reacted positively in the oxidase test. When tested in the API 20NE strip at the recommended temperature of 30°C, the *p*-nitrophenyl-ß-D-galactopyranoside test (to observe the presence of ß-Galactosidase) became positive within 3 h for all strains. With the API 20NE strip, 65 % of the strains also reacted positively in the urease test and four strains (1 %) reacted positively in the arginine dihydrolase (ADH) test. For all other tests, used in the API 20NE strip, no reactions were seen even after 72 hours of incubation at 30°C. When strains were tested at 37°C in the API 20E strip, the urease, ADH and gelatine (for the presence of protease) tests sometimes reacted positively within 48 hours, even if they were negative at 30°C by the API 20-NE test.

**AGP test.** By using BEAs in the AGP test, seven different serotypes (serotypes A to G) could be distinguished (Table 2 and Fig. 1). Within serotypes A, B, D and E, different precipitation reactions were seen. The reactions of 88% of the strains belonging to serotypes A,B,D or E were visualized as sharp precipitation lines. When BEAs of different strains within a serotype were tested against the corresponding antiserum the precipitation pattern of the sharp lines showed that the precipitating antigens were identical. The BEAs of the remaining 12% of the strains produced faint precipitation lines that also appeared to represent identical precipitating antigens within each serotype, as indicated by the precipitation pattern found. The BEAs of eight strains were found to react with more than one antiserum. In addition to a sharp precipitation line against serotype A or B antiserum with these strains several faint precipitation lines against serotype A, B or E antiserum were seen. Five strains (1%) could not be typed by the AGP test with the presently available antisera. All four strains which reacted positively in the ADH test with the API 20NE identification strip at 30°C were positive in the AGP test, indicating they do belong to the species *O. rhinotracheale*. Information about the geographic origin in relation to the serotype of 440 *O. rhinotracheale* strains isolated from chickens and turkeys is listed in the Tables 3 and 4, respectively.

No reactions were seen in the AGP test between the antigens (BEAs) of the 45 tested *O. rhinotracheale* strains and the antisera against the *Kingella* strains or the *P. gallinarum* strain. Also, no reactions were seen between the antigens of the 45 tested *O. rhinotracheale* strains and all the serotype-specific antisera of 3 *Pasteurella*-like rods (*P. multocida, R. anatipestifer* and *H. paragallinarum*) known to be pathogenic for fowl.

Table 1. Differentiation of *Ornithobacterium rhinotracheale* from *Kingella* and gram-negative rods related to *Pasteurellaceae* potentially pathogenic for fowl[a]

| Reaction | *Haemophilus paragallinarum*[b] | *Riemerella anatipestifer*[b] | *Pasteurella multocida*[b] | *Pasteurella haemolytica*[b] | *Pasteurella gallinarum*[b] | *(Pasteurella) pseudotuberculosis* | *Actinobacillus spp.*[b] | *Kingella spp.*[b] | *Ornithobact. rhinotracheale* |
|---|---|---|---|---|---|---|---|---|---|
| Nitrate reduction | +[c] | - | + | + | + | +/- | | +/- | - |
| Catalase | - | + | + | + | + | + | + | - | - |
| Oxidase (cytochrome) | - | + | + | + | + | - | + | + | + |
| Urease | - | +/- | - | - | - | + | +/- | - | +/- |
| ß-Galactosidase (ONPG [d]) | + | - | +/- | - | +/- | + | +/- | - | + |
| Arginine dihydrolase | - | + | - | | - | | - | - | -/+ |
| Indole | - | +/- | + | - | - | - | - | +/- | - |
| Growth on McConkey agar | - | - | - | - | - | +/- | + | | - |
| Lysine decarboxylase | - | | - | | - | - | - | - | - |
| Ornithine decarboxylase | - | | - | | - | - | - | - | - |
| Fermentation/Oxidation of : | | | | | | | | | |
| Fructose | + | - | | | + | + | | -/+ | + |
| Lactose | - | - | - | +/- | - | - | +/- | - | + |
| Maltose | + | - | +/- | + | + | + | + | +/- | + |
| Galactose | - | - | + | | + | + | +/- | - | + |

[a] Data are from *Diseases of Poultry*, 9th ed. (9).
[b] Data on biochemical reactions are from *Bergeys Manual of Determinative Bacteriology* 9th ed. (3a), and the analytical profile index of the API identification system.
[c] +, positive; +/-, majority of strains positive; -/+, majority of strains negative; -, negative.
[d] ONPG, *o-nitrophenyl-ß-D-galactopyranoside.*

Table 2. Serotyping of *O. rhinotracheale* by AGP and differentiation of *O. rhinotracheale* from other relevant gram-negative rods by ELISA.

| Strain no. | Monovalent antiserum Species | Strain | Serotype | ELISA titer ($^2$log) against antigens of strain no.: 1 | 2 | 3 | 4 | 5 | 6 | 7 | homologous |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *O. rhinotracheale* | B 3263/91 | A | 20 | 15 | 8 | 12 | 12 | 11 | 13 | 20 |
| 2 | *O. rhinotracheale* | GGD 1261 | B | 13 | 19 | 8 | 11 | 13 | 11 | 11 | 19 |
| 3 | *O. rhinotracheale* | ORV K91-201 | C | 9 | 10 | 17 | 7 | 9 | 11 | 11 | 17 |
| 4 | *O. rhinotracheale* | ORV 94108 no. 2 | D | 10 | 11 | 10 | 19 | 11 | 11 | 12 | 19 |
| 5 | *O. rhinotracheale* | O-95029 no. 12229 | E | 13 | 16 | 11 | 12 | 20 | 11 | 13 | 20 |
| 6 | *O. rhinotracheale* | ORV 94084 K858 | F | 10 | 11 | 11 | 8 | 8 | 20 | 9 | 20 |
| 7 | *O. rhinotracheale* | O-95029 no. 16279 | G | 11 | 12 | 12 | 9 | 10 | 11 | 20 | 20 |
| 8 | *P. multocida* | X-73 | 1 | <6 | <6 | <6 | -[a] | - | - | - | 22 |
| 9 | *P. multocida* | P-1059 | 2 | <6 | <6 | <6 | - | - | - | - | 21 |
| 10 | *P. multocida* | P-1662 | 3 | <6 | <6 | <6 | - | - | - | - | 20 |
| 11 | *P. multocida* | P-1702 | 4 | <6 | <6 | <6 | - | - | - | - | 19 |
| 12 | *R. anatipestifer* | PAA CV | 1A | 7 | <6 | <6 | - | - | - | - | 18 |
| 13 | *R. anatipestifer* | PAB BRD | 6B | <6 | 7 | 7 | - | - | - | - | 20 |
| 14 | *R. anatipestifer* | PAD CV | 10D | <6 | <6 | <6 | - | - | - | - | 21 |
| 15 | *H. paragallinarum* | 0083 | A | 8 | 8 | 8 | - | - | - | - | 16 |
| 16 | *H. paragallinarum* | Spross | B | 8 | 8 | 7 | - | - | - | - | 18 |
| 17 | *H. paragallinarum* | H-18 | C | 9 | 8 | 8 | - | - | - | - | 16 |
| 18 | *H. paragallinarum* [b] | 281/91 | A | 10 | 8 | 7 | - | - | - | - | 16 |
| 19 | *H. paragallinarum* [b] | 4620/91 | A | 8 | 8 | 7 | - | - | - | - | 12 |
| 20 | *P. gallinarum* | Fieldstrain | | 8 | 6 | 7 | - | - | - | - | 17 |
| 21 | *K. kingae* | ATCC 23330 | | 9 | 7 | 8 | - | - | - | - | 18 |
| 22 | *K. denitrificans* | ATCC 33394 | | 7 | 7 | 7 | - | - | - | - | 19 |
| 23 | *K. indologenes* | ATCC 25869 | | 8 | 6 | 7 | - | - | - | - | 17 |

a -, not determined
b NADH-independent strain

**ELISA.** By using the ELISA and monovalent antisera (Table 2), *O. rhinotracheale* could be serotyped as described above for the AGP test. Also with use of this ELISA, *O. rhinotracheale* could be distinguished from other relevant, gram-negative rods potentially pathogenic for fowl and with which *O. rhinotracheale* could be confused. The monovalent antisera contained large amounts of homologous antibodies ($^2$log titers up to 22), meaning that background reactions can easily occur. So, with these sera, we regard ELISA titers up to 10 ($^2$log) to be a negative reaction, which is an arbitrary cut-off. In addition to the data presented in Table 2, antisera against *O. rhinotracheale* serotypes did not react in ELISAs with antigens prepared from the other species listed. Within each species cross-reactions occurred between the serotypes, but few cross-reaction between the species were seen. All antisera showed the highest titer against the homologous antigen. Within the species *O. rhinotracheale* the cross-reactions were mainly between the serotypes A, B, D and E.

In day-old turkeys as well as day-old broilers, antibodies against *O. rhinotracheale* could be detected. Antibody titers between 8 and 12 ($^2$log) were found at that time. At 3 weeks of age, the same birds were negative in the ELISA (titers, < 5), suggesting that these antibodies were maternally derived. Broiler and turkey flocks from The Netherlands, Germany, France, the United States, and South Africa presenting with clinical signs of an *O. rhinotracheale* infection were investigated for the presence of antibodies by the ELISA. In all cases, antibodies, especially against serotypes A and B, could be found (titers, between 8 and 16).

Figure 1. Differentiation of the seven serotypes of *O. rhinotracheale* (serotypes A to G) by the AGP test. Capital letters, antigens; lowercase letters, monovalent antiserum.

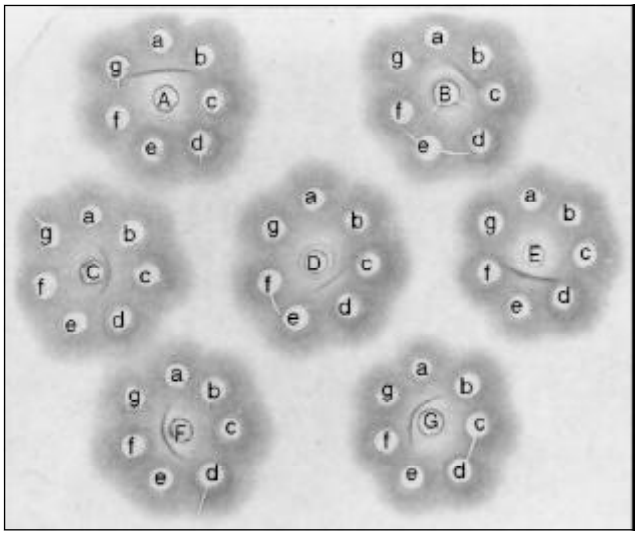

Table 3. Serotypes and geographic origin of the isolates of *O. rhinotracheale* from chickens.

| Country | No. of strains of serotype: | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | NT[a] | |
| France | 3 | | | | 1 | | 1 | 1 | 6 |
| Germany | 4 | | | | | | | | 4 |
| Italy | 1 | | | | | | | | 1 |
| South-Africa | 57 | | | | | | | | 57 |
| The Netherlands | 148 | 6 | | | 3 | | | 2 | 159 |
| USA | 19 | | 2 | | | | | | 21 |
| Total | 232 | 6 | 2 | 0 | 4 | 0 | 1 | 3 | 248 |
| Percentage | 94 | 2 | 1 | 0 | 2 | 0 | 0 | 1 | 100 |

[a] NT, Not typable with the presently available antisera

Table 4. Serotypes and geographic origin of the isolates of *O. rhinotracheale* from turkeys.

| Country | No. of strains of serotype: | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | NT[a] | |
| France | 35 | 8 | | 7 | 12 | | | | 62 |
| Germany | 1 | 9 | | | 1 | | | | 11 |
| Israel | | | 2 | | | | | | 2 |
| The Netherlands | 47 | 22 | | 9 | 4 | 1 | | 1 | 84 |
| United Kingdom | 12 | | | | | | | 1 | 13 |
| USA | 14 | 3 | 3 | | | | | | 20 |
| Total | 109 | 44 | 3 | 16 | 17 | 1 | 0 | 2 | 192 |
| Percentage | 57 | 23 | 2 | 8 | 9 | 1 | 0 | 1 | 100 |

[a] NT, Not typable with the presently available antisera

## DISCUSSION

*O. rhinotracheale* could be distinguished from other gram-negative rods potential pathogenic for fowl biochemically as well as serologically. Within the API system *O.rhinotracheale* could easily be identified by the API 20NE strip at 30°C. Almost all strains (99 ) showed an API 20NE code of 0-2-2-0-0-0-4 (65%) or 0-0-2-0-0-0-4 (34%). If the possibility of a positive ADH test is included (code 0-3-2-0-0-0-4 or 0-1-2-0-0-0-4), a 100% identification score with the API 20NE strip was found. The enzymes urease, protease, and ADH of *O. rhinotracheale* showed temperature-dependent reactions in the API 20E and the API 20NE strips.

By the AGP as well as the ELISA, seven serotypes of *O. rhinotracheale* could be distinguished. Some cross-reactions were found between the serotypes A, B, D and E in both tests. In the AGP test it was found that two different precipitating antigens can occur within the serotypes A, B and E, which probably explains why these serotypes also show cross reactions in the ELISA. These reactions, as well as the 5 strains (1%) with for *O. rhinotracheale* positive API 20NE codes which did not react by the AGP test, need to be investigated more thoroughly.

For identification of *O. rhinotracheale*, we propose the use of the API 20NE strip at the recommended temperature of 30°C. Strains with the four possible API 20NE result codes should be further investigated in the AGP test. We recommend the AGP test for serotyping, whereas the ELISA might be used for diagnosting infections in infected birds. For optimal results the antigens or antisera of all seven serotypes should be used. Therefore we plan to deposit the serotype-specific *O. rhinotracheale* strains, as listed in Table 2, in the American Type Culture Collection as reference strains for general use. The serotype specificity can be seen as a disadvantage for the use of the ELISA for diagnostic screening purposes because seven tests must be performed with each serum to exclude an infection. We also found that not all the birds within an infected flock were serologically positive and that serological responses in field infections sometimes were low and could disappear after several weeks. These findings should be kept in mind to prevent incorrect interpretations of ELISA results. More serological from experimentally and naturally infected birds are necessary to judge the true value of ELISA for diagnostic purposes. Such studies are in progress.

As shown in Tables 3 and 4, serotype A was the predominant serotype among the isolates of *O. rhinotracheale* from chickens and the most frequently among the isolates from turkeys. All strains of the serotypes D and F and most strains of serotypes B (88%) and E (77%) in this study were of turkey origin. It is obvious that in this study the distribution of isolates of *O. rhinotracheale* from turkeys among the seven

serotypes was more heterogeneous than was the case for the isolates from chickens. Also, relationships between the geographic origins of the strains and the serotypes seem to exist; e.g. all strains of serotype C originated from California, and all strains received from South Africa and the United Kingdom (except for one nontypable strain) belong to serotype A. By testing sera of chicken and turkey flocks suspected of being infected with *O. rhinotracheale* by the ELISA, serotypes A and B were found to react the strongest, which is in agreement with the prevalence of these serotypes among the isolated strains. However, it should be kept in mind that the majority of the strains and the sera tested originated from only three areas (France, The Netherlands and South Africa) which could bias the results. It is not yet possible to explain the observed differences in distribution of serotypes among isolates of *O. rhinotracheale* from chickens and turkeys. Previously, we have shown that a serotype A strain from a chicken and a serotype B strain from a turkey have similar virulences for both chickens and turkeys (8). Strains of serotypes C, D and E are also pathogenic for chickens as well as turkeys (unpublished data). So there is no indication of any host specificity of the serotypes. A possible explanation may perhaps be found in different breeding practices in the chicken and turkey industries, but more thorough epidemiological and pathogenicity studies are needed to obtain final answers to the remaining questions about the emerging *O. rhinotracheale* infection in poultry.

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CHAPTER 3

# Identification

CHAPTER 3 B

## Molecular characterization of *Ornithobacterium rhinotracheale*

*Paul van Empel[1*], Paul Savelkoul[2], Ruud Segers[1], Jeroen Stoof[2], Peter Loeffen[1] and Han van den Bosch[1].*

*[1] Intervet International B.V., Boxmeer, The Netherlands*
*[2] Department of Clinical Microbiology and Infection Control, University Hospital, Amsterdam, The Netherlands.*

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## SUMMARY

*Ornithobacterium rhinotracheale* is a recently described bacterium associated with respiratory diseases in fowl. In this study we characterized 56 strains belonging to different serotypes, independently isolated from bird species from various countries, by amplified fragment length polymorphism (AFLP), PCR, and polyacrylamide-gel-electrophoresis (PAGE) of the outer-membrane-proteins (OMP).

With use of the AFLP assay we were able to group *O. rhinotracheale* strains into 5 minor or 3 major clusters which showed some association with serotyping. Because the linkage levels between the AFLP banding patterns of these *O. rhinotracheale* clusters is comparable to the linkage levels of AFLP banding patterns between (sub)species of other bacteria, there may be several subspecies of *O. rhinotracheale* or even more species of *Ornithobacterium*. However, this is in contradiction to the PAGE profiles of the OMP's of the same 56 strains which show high similarity rates. Also the fact that the developed PCR proved to be specific for all *O. rhinotracheale* strains tested, indicating that the strains are closely related. Further investigation will elucidate whether the actual AFLP clustering represent other (sub)species.

## INTRODUCTION

During the period 1991 - 1997, respiratory diseases with mild to moderate symptoms in different bird species were observed in several places in the world (4, 5, 9, 12, 13). Bacteriological examination revealed a slowly growing, pleomorphic, gram negative rod, for which in 1994 the name *Ornithobacterium rhinotracheale* was proposed (13). Since the pathogenicity of *O. rhinotracheale* was proven in experimental infection (14), a worldwide collection of strains was set up. Up to now 1096 strains of *O. rhinotracheale* have been investigated biochemically and serologically. For the biochemical characterization of *O. rhinotracheale* the API-20NE identification strip (Bio Merieux, France), incubated at 30°C, was shown to give a 99.5 % identification score in combination with the agar gel precipitation (AGP) test (15). Serologically, using monovalent antisera in AGP tests, so far nine serotypes of *O. rhinotracheale* could be discriminated, designated A - G (15) and H and I (our own unpublished observation).

To study the bacterium and the epidemiology of this disease, 56 strains of different serotypes were selected. The strains, isolated from different bird species out of different area's of the world, were investigated by PCR, DNA fingerprinting and by outer-membrane-protein analysis.

## MATERIALS AND METHODS

**Bacterial strains.** The geographic origin, the serotype and the origin of the *O. rhinotracheale* strains are listed in Table 1. The strains were grown on 5% sheep-blood agar for at least 48 hours under microaerobic conditions (5-10 % $CO_2$) at 37° C or in Todd Hewitt (TH) medium (Difco, Detroit, MI, USA) for 24 hours at 37°C. All strains were identified as *O. rhinotracheale* with API identification strips and were serotyped with the agar gel precipitation (AGP) test as described (15).

For comparison, 4 strains of *Pasteurella multocida* (X-73, P-1059, P-1662 and P-1702), 3 strains of *Riemerella anatipestifer* (PAA CV, PAB BRD and PAD CV), 1 strain of *Pasteurella gallinarum* (fieldstrain), 3 strains of *Haemophilus paragallinarum* (0083, Spross and H-18) and 2 strains of NADH-independent *Haemophilus paragallinarum* (281/91 and 4620/91), *Kingella kingae* (ATCC 23330), *Kingella denitrificans* (ATCC 33394) and *Kingella (Suttonella) indologenes* (ATCC 25869) were used. These strains were previously used and described when identifying *O. rhinotracheale* (15). Vandamme *et al.* (13) found that based on 16S rRNA sequences, *O. rhinotracheale* showed most similarity towards the genus *Flavobacterium*. Therefore we also included *F. breve* (ATCC 14234) and *F. meningosepticum* (ATCC 13253) in this study.

**PCR.** PCR was performed using the primer combination OR16S-F1 (5'-GAGAATTAATTTACGGATTAAG) and OR16S-R1 (5'-TTCGCTTGGTC-TCCGA-AGAT). This combination amplifies a 784 bp fragment on the 16S rRNA gene of *O. rhinotracheale*, and was derived after comparing the sequence of this gene (Genbank nucleotide sequence accession number L19156) with 16S rRNA gene sequences from related species (10). The final PCR volume was 50 µl containing 1µl of a strength of 1 McFarland suspension of fresh culture in TH medium as template, 2µl dNTP's (2mM), 4pmol of both primers, 0.5 U Supertaq polymerase (HT-Biotechology, Cambridge, UK) and 5 µl 10X Supertaq buffer (HT-Biotechnology) in water. PCR conditions were as follows : denaturation at 94°C for 5 min, followed by 45 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 60 sec and elongation at 72°C for 90 sec followed by a final extension at 72°C for 7 min. Storage for longer periods was performed at -20°C. PCR products were separated by electrophoresis in a 1%, ethidium bromide-stained, agarose gel and were visualized by viewing the gel with an ultraviolet transilluminator.

**Amplified fragment length polymorphism (AFLP).** DNA was isolated according to Boom (3), using fresh cells. AFLP was performed essentially as described by Janssen *et al.* and Vos *et al.* (6, 16). Briefly, 50 ng purified chromosomal DNA was digested with 1 U of *Eco*RI (Pharmacia Biotech, Uppsala, Sweden) and 1 U of *Mse*I (New England Biolabs Inc., Beverly,MA, USA). Subsequently, ligation of adapters to the restriction fragments was performed overnight at 20°C in a final volume of 30 µl. The ligation mixture consisted of 50 ng of chromosomal DNA, 50 pMol of each *Eco*RI- and *Mse*I adapter, 1.2 U T4 DNA ligase (Pharmacia Biotech), 1mM ATP and ligase buffer (10mM Tris acetate, pH 7.5, 10mM magnesium acetate, 50 mM dithiothreitol and 50 ng/µl BSA). The structure of the *Eco*RI adapter is :

5' - C T C G T A G A C T G C G T A C C - 3'
3' - C T G A C G C A T G G T T A A - 5'

The structure of the *Mse*I adapter is :

5' - G A C G A T G A G T C C T G A G - 3'
3' - C T A C T C A G G A C T C A T - 5'

After ligation the DNA was diluted with water to a final volume of 500 µl and stored at -20°C until use. The ligated restriction fragments were amplified according to Koeleman *et al.* (7) with the fluorescent labeled *Eco*-A primer (5'-GACTGCGTACCAATTCA-3') and *Mse*-C primer (5'-GATGAGTCCTGAGTAA C-3'), both having an one nucleotide 3'-overhang in the unknown chromosomal DNA. The final PCR volume was 10 µl containing 0.5 ng template DNA, 20ng Texas Red labeled *Eco*-A primer (Isogen Bioscience, Maarssen, The Netherlands), 1µl dNTP's (2mM), 60 ng unlabeled *Mse*-C primer, 1U Taq polymerase (Perkin Elmer) in 10

mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$. Amplification was carried out in a GeneAmp PCR System 9600 thermal cycler (Perkin Elmer) during 35 cycles: denaturation (30 sec at 94°C), annealing (30 sec at 65°C-56°C) and extension (60 sec at 72°C). In the first 12 cycles the annealing temperature was lowered 0.7°C per cycle. After the completion of the cycle program, 3 µl loadingsbuffer (Amersham Life Science, Cleveland, USA) was added to the reaction mixtures. Prior to gel loading the amplicons were denatured by heating for 1 min at 95°C and rapidly cooling on ice. Amplified fragments were separated on a denaturing polyacrylamide gel (6 M Urea, RapidGel-XL-6%, Amersham Life Science) in 1x TBE buffer (100 mM Tris, 100 mM boric acid, 2 mM EDTA, pH = 8.0) in a Vistra 725 automated DNA sequencer (Amersham Life Science). Two µl sample of each reaction mixture was loaded on the gel and gels were run at 1500 V for 6 h.

Clustering analysis of the results of the gelelectrophoresis (saved as TIFF files) was performed with use of GelCompar 4.0 software (Applied Maths, Kortrijk, Belgium). The similarity between all possible pairs of traces was expressed by the Pearson product moment correlation coefficient (r) and grouping was obtained by the unweighted pair-group clustering algorithm (UPGMA).

**Outer membrane proteins (OMP)**. The OMP's were prepared according to Barenkamp *et al*. (2, 11). Briefly : The cells were suspended in 10 mM HEPES buffer (pH 7.4) and sonicated 10 times, 30 seconds on ice, using a Branson, model 250 sonifier with standard flat tip at a maximum output with a minimum of aeration. The cell-debris was removed by centrifugation (25 min; 23,000 g at room temperature). The supernatant was mixed with 1/4 volume of 5% (w/v) sodium-N-laurylsarcosine in HEPES buffer and stirred for 2-18 hours at room temperature. Subsequently the insoluble outer membranes were centrifugated (90 min; 183,000 g at room temperature) and the pellet was resuspended in a minimal volume of HEPES buffer with 0,1% sodium azide and stirred until the suspension was completely homogeneous. The OMP's were separated through sodium dodecyl sulphate (SDS)-polyacrylamide-gel-electrophoresis (PAGE) according to Laemmli (7), using a 12% T-gel and a 2.6% C-gel. The interpolation of the protein profiles was performed by using the model GS-700 imaging densitometer and the algorithm of Ward in the Molecular analyst software package (Bio-Rad, Hercules, CA, USA).

## RESULTS

**PCR.** A 784 bp amplicon was observed in the PCR with all the 56, API-20NE and AGP confirmed, *O. rhinotracheale* strains. None of the tested strains of the species *P. multocida, R. anatipestifer, H. paragallinarum,* NADH-independent *H.*

*paragallinarum, P. gallinarum, K. kingae, K. denitrificans, K. indologenes, F. breve* and *F. meningosepticum* amplified the 784 kb amplicon product. In the collection of 1096 *O. rhinotracheale* strains, 6 strains were found to react biochemically but not serologically as *O. rhinotracheale*. These 6 strains did react as *O. rhinotracheale* in the PCR and are currently under investigation to identify possible new serotypes. The sensitivity limit of the PCR was between 180 and 4500 bacteria. It was found to be critical to use fresh cultures for PCR. If cultures were allowed to grow for longer than 48 hours, the PCR failed in most cases.

**AFLP.** Using a similarity coefficient (Sd) of 70% as a distinctive linkage level, 5 different clusters of *O. rhinotracheale* strains could be discriminated (Figure 1 and Table 1). The clusters 1 and 2 showed an Sd of 67%, cluster 3 showed an Sd of 51% with clusters 1 and 2, cluster 4 showed an Sd of 46% with the clusters 1, 2 and 3 and cluster 5 showed an Sd of 28% with the other clusters. In cluster 1 we found 9 out of the 10 tested serotype A strains, 6 out of 10 serotype B strains, 4 out of 5 serotype C strains, 7 out of 10 serotype E strains, 1 out of 3 serotype F strains and 1 out of the 3 tested serotype H strains. In cluster 2 we found 1 out of the 10 tested serotype A strains, 4 out of 10 serotype B strains, 1 out of 10 serotype E strains and 1 out of 2 serotype G strains. The serotype-C-strain ORV K91-201 (strain no. 21) was the only strain that was placed in cluster III. All the serotypes D and I strains were placed in cluster IV together with 2 out of 10 serotype E strains, 1 out of 3 serotype F strains, 1 out of 2 serotype G strains and 2 out of the 3 tested serotype H strains. Cluster IV contained only strains isolated from turkeys. One strain, serotype-F-strain ORV 94084 K858 ORT (strain no 46), showed a totally different profile and was placed in cluster V.

If an Sd of 50% was used as distinctive linkage level 3 clusters ($\alpha$,ß and $\gamma$) could be distinguished. Cluster $\alpha$ contained the clusters I, II and III, in which all of the serotype A, B and C strains were placed. The clusters ß and $\gamma$ were identical to the clusters IV and V respectivily.

**OMP.** Using a Sd of 90% as a distinctive linkage level, 5 different OMP-profile clusters could be discriminated (Figure 2 and Table 1). The lowest Sd was found to be 84%, comparing the clusters 1, and 2 with the clusters 3, 4 and 5. Clusters 1 and 2 had an Sd of 85%, cluster 3 had an Sd of 87% with the clusters 4 and 5 and cluster 4 had an Sd of 89% with cluster 5. No correlations could be found between the OMP clusters and AFLP, serotyping, bird species and origin.

## DISCUSSION

All strains that tested biochemically and serologically as *O. rhinotracheale*, were identified as such by AGP or the PCR. The PCR was found to be specific and thus useful for identification purposes. The primer combination OR16S-F1 and OR16S-

R1 amplifies a 784 bp fragment of the *rnn* gene of *O. rhinotracheale* but not of other bacteria with which *O. rhinotracheale* may be confused. This PCR should also be suitable for the demonstration of *O. rhinotracheale* in e.g. eggs, faeces, dust- or tissue samples. But the sensitivity of the assay needs to be improved.

The results of the AFLP indicate that, within the species *O. rhinotracheale*, subgroups do exist. The choice of the primer-set affects the number of fragments in the AFLP banding patterns and therefore is of importance for the interpretation of similarities, Janssen *et al.* found that a decrease in the number of bands resulted in significantly lower linkage levels between clusters (6). The primer-set we used, produced between 18 and 28 bands in the AFLP banding pattern of *O. rhinotracheale* strains. Janssen *et al.* (6) found that for the genera *Xanthomonas* and *Aeromonas*, using between 15 and 25 bands in the AFLP banding patterns, species could be distinguished at Sd-values of about 60% and 64% respectivily. In the same study most of the subspecies of *Xanthomonas axonopodis* and *Xanthomonas vasicula* could be distinguished at Sd-levels higher than 70%. Although we do not know anything about the differences between these genera and *Ornithobacterium* with regard to e.g. the ability to exchange genetic material, we propose the arbitrary Sd-values of 70% and 50% as levels at which subspecies and species should be separated, respectivily. Based on this assumption *O. rhinotracheale* should be divided into at least 5 subspecies (I to V) or even that the genus *Ornithobacterium* should be divided into 3 "species" α, ß and γ, of which α can be divided into 3 subspecies). However, we did not find other arguments in this study that convincingly could confirm the division of *Ornithobacterium* into 3 species. Although all serotype A, B and C strains were found in "species" α including the AFLP clusters I, II and III, and all serotype D strains were found in "species" ß, several other serotypes were found to be represented in both these "species" (Table 1). Furthermore, the 3 serotype F strains than would belong to the 3 different "species".

Except for the studies by Amonsin *et al.* (1) and Vandamme *et al.* (13), no other genetic studies of *O. rhinotracheale* have been reported. Amonsin *et al.* found 6 different rep-PCR types of *O. rhinotracheale*, with only minor differences, together with >99% 16S rRNA sequence-identity among the tested strains. Vandamme *et al.* investigated the 16S rRNA sequence, DNA-DNA binding values and G+C contents; they found high similarity between strains. Only 2 strains used in both studies, B3263/91 (AFLP cluster I, serotype A) and GGD-1261 (AFLP cluster I, serotype B), were included in our study, so no comparison between the studies can be made. In conclusion, we believe that the clusters of *O. rhinotracheale* strains, found with the genomic fingerprinting AFLP-assay, might be regarded as (sub)species, but more research is needed before changes should be proposed in the current nomenclature.

The OMP profiles showed high similarity levels (Sd > 84%) similar to the data

from Vandamme *et al.* (13) with total protein profiles (Sd > 89%). Using an Sd of 90% as an arbitrary distinctive level, no correlation of OMP profiles with either the serotyping or AFLP profile as tested in this study was found. These data do not support the idea that (sub)species of *O. rhinotracheale* do exist. However, the Sd-levels of the total-protein profiles of the 5 species of the genus *Capnocytophaga*, found to be closely related to *O. rhinotracheale*, showed the same narrow range of Sd-levels of the total-protein profiles (13) and the OMP-profiles of *O. rhinotracheale* (this study).

In conclusion, the high similarity of OMP profiles among the tested *O. rhinotracheale* strains without any correlation with the different serotypes found, suggesting that the tested isolates, originating from all over the world and from several bird-species, are represented by a small group of closely related clones. But the AFLP results in this study suggest a larger distance between subspecies of *O. rhinotracheale* or even species of Ornithobacterium. This should be investigated more thoroughly e.g. by looking at markers covering the whole genomen.

*Footnote table 1:*

[a] Strain no 34 was at the beginning of these studies misinterpreted as serotype D.
[b] Number as used in the tests.
[c] AFLP cluster as determined with an Sd of 70% as distinctive linkage level.
[d] OMP cluster as determined with an Sd of 90% as distinctive linkage level.
[e] Not tested

Table 1: Designations, sources and test results of *Ornithobacterium rhinotracheale* strains used, listed according to serotype[a].

| No.[b] | Strain | Bird species | Origin | Year of isolation | Serotype | AFLP[c] | OMP[d] |
|---|---|---|---|---|---|---|---|
| 1 | B 3263/91 | Chicken | South Africa | 1991 | A | I | 1 |
| 2 | O-96218 GEE 5 | Turkey | The Netherlands | 1996 | A | I | 1 |
| 3 | O-96214 SP 4542 | Chicken | The Netherlands | 1996 | A | I | 1 |
| 4 | O-96148 BAC.96.0108 | Chicken | USA, Virginia | 1996 | A | I | NT[e] |
| 5 | O-96069 BAC.960043 | Chicken | USA Delaware | 1996 | A | NT | 2 |
| 6 | O-96100 1530 | Turkey | France | 1996 | A | I | 2 |
| 7 | O-95264 95.2932 | Guinee fowl | France | 1996 | A | I | 2 |
| 8 | OND.NR.2141(5) EXP 2030 | Chicken | South Africa | 1990 | A | I | 3 |
| 9 | ORV PAST.HONG 87121 | Duck | Hungary | 1987 | A | I | 1 |
| 10 | ORV 94080 STRAIN 5 | Chicken | Italy | 1994 | A | I | 1 |
| 11 | GGD 1261 | Turkey | Germany | 1991 | B | I | 2 |
| 12 | O-96177 SP 426 TRA 1 | Turkey | The Netherlands | 1996 | B | I | 1 |
| 13 | O-96121 SP 254 PE 1 | Turkey | The Netherlands | 1996 | B | I | 3 |
| 14 | O-96148 BAC.96.0107 | Chicken | USA, Virginia | 1996 | B | I | 3 |
| 15 | ORV 448 GELATINE 95-0134 | Turkey | USA West Verginia | 1995 | B | I | 2 |
| 16 | O-96111 DDX 125 | Turkey | France | 1996 | B | II | 2 |
| 17 | O-95159 7442 TR | Turkey | France | 1995 | B | II | 1 |
| 18 | ORV 94099 NR:1648 | Turkey | Germany | 1994 | B | I | 1 |
| 19 | ORV 94099 NR:2009 | Turkey | Israel | 1994 | B | II | NT |
| 20 | ORV 94099 NR:2010 | Turkey | Israel | 1994 | B | II | NT |
| 21 | ORV K91-201 | Chicken | USA California | 1991 | C | III | 1 |
| 22 | O.R K90-118 | Turkey | USA California | 1990 | C | I | NT |
| 23 | O.R K89-1234 | Turkey | USA California | 1989 | C | I | 1 |
| 24 | O.R.K90-1455 | Turkey | USA California | 1990 | C | I | 1 |
| 25 | O.R K91-524 | Chicken | USA California | 1991 | C | I | 1 |
| 26 | ORV 94108 nr.2 | Turkey | France | 1994 | D | IV | 1 |
| 27 | O-96156 SP 401 OOG | Turkey | The Netherlands | 1996 | D | IV | NT |
| 28 | ORV 94081 494 | Turkey | The Netherlands | 1994 | D | IV | 3 |
| 29 | O-95156 SP 401 LZ | Turkey | The Netherlands | 1995 | D | IV | 3 |
| 30 | O-095156 SP 401 TRA 4 | Turkey | The Netherlands | 1995 | D | IV | 1 |
| 31 | O-095156 SP 401 TRA 5 | Turkey | The Netherlands | 1995 | D | IV | 1 |
| 32 | O-96100 SP 3471 | Turkey | France | 1996 | D | IV | 3 |
| 33 | O-95231 95-2498 | Turkey | France | 1995 | D | IV | 1 |
| 34 | O-96051 60201 | Turkey | France | 1996 | A | II | 4 |
| 35 | O-95246 25791 | Turkey | France | 1995 | D | IV | 1 |
| 36 | 0-95029 NR. 12229 | Chicken | France | 1995 | E | II | 1 |
| 37 | ORV 94067 14200-283 | Chicken | The Netherlands | 1994 | E | I | 4 |
| 38 | ORV 94077 SP 758 COR | Turkey | The Netherlands | 1994 | E | IV | 5 |
| 39 | O-96117 SP 254 | Turkey | The Netherlands | 1996 | E | I | 2 |
| 40 | O-96117 SP 198 | Turkey | The Netherlands | 1996 | E | I | 4 |
| 41 | O-95069 SL-95-105NR.1 | Turkey | France | 1995 | E | I | 5 |
| 42 | O-95029 NR. 13778 | Partridge | France | 1995 | E | I | 4 |
| 43 | O-95246 52787-6 | Turkey | France | 1995 | E | I | 4 |
| 44 | ORV 94087 SL-94-141 NR.1 | Turkey | France | 1994 | E | I | 5 |
| 45 | O-93075 | Turkey | Germany | 1993 | E | IV | 5 |
| 46 | ORV 94084 K858 ORT | Turkey | The Netherlands | 1994 | F | V | 5 |
| 47 | O-96222 SP 704 LONG 1 | Turkey | The Netherlands | 1996 | F | IV | 5 |
| 48 | O-96222 SP 704 LONG 2 | Turkey | The Netherlands | 1996 | F | I | 1 |
| 49 | O-95029 NR. 16279 | Chicken | France | 1995 | G | II | 1 |
| 50 | O-94038 K334 | Turkey | The Netherlands | 1994 | G | IV | 1 |
| 51 | E-94063 4.2 | Turkey | The Netherlands | 1994 | H | IV | 1 |
| 52 | O-97041 VRE 9 | Turkey | The Netherlands | 1997 | H | IV | 1 |
| 53 | O-97041 EVE 9 | Turkey | The Netherlands | 1997 | H | NT | 1 |
| 54 | O-97071 BUT 2266/3 | Turkey | United Kingdom | 1997 | H | I | 1 |
| 55 | O-95265 sp 1071 | Turkey | The Netherlands | 1996 | H | NT | 3 |
| 56 | BAC 96-0334 #MINN 18 | Turkey | USA Minnesota | 1996 | I | IV | 1 |

Figure 1: Digitized AFLP patterns obtained from DNA of 53 *O. rhinotracheale* strains. Clusters I - V were separated by using an Sd of 70% as a distinctive linkage level. Clusters α, ß and γ were separated by using an Sd of 50% as a distinctive linkage level. bp = base pairs. Numbers correspond with the strain numbers listed in Table 1.

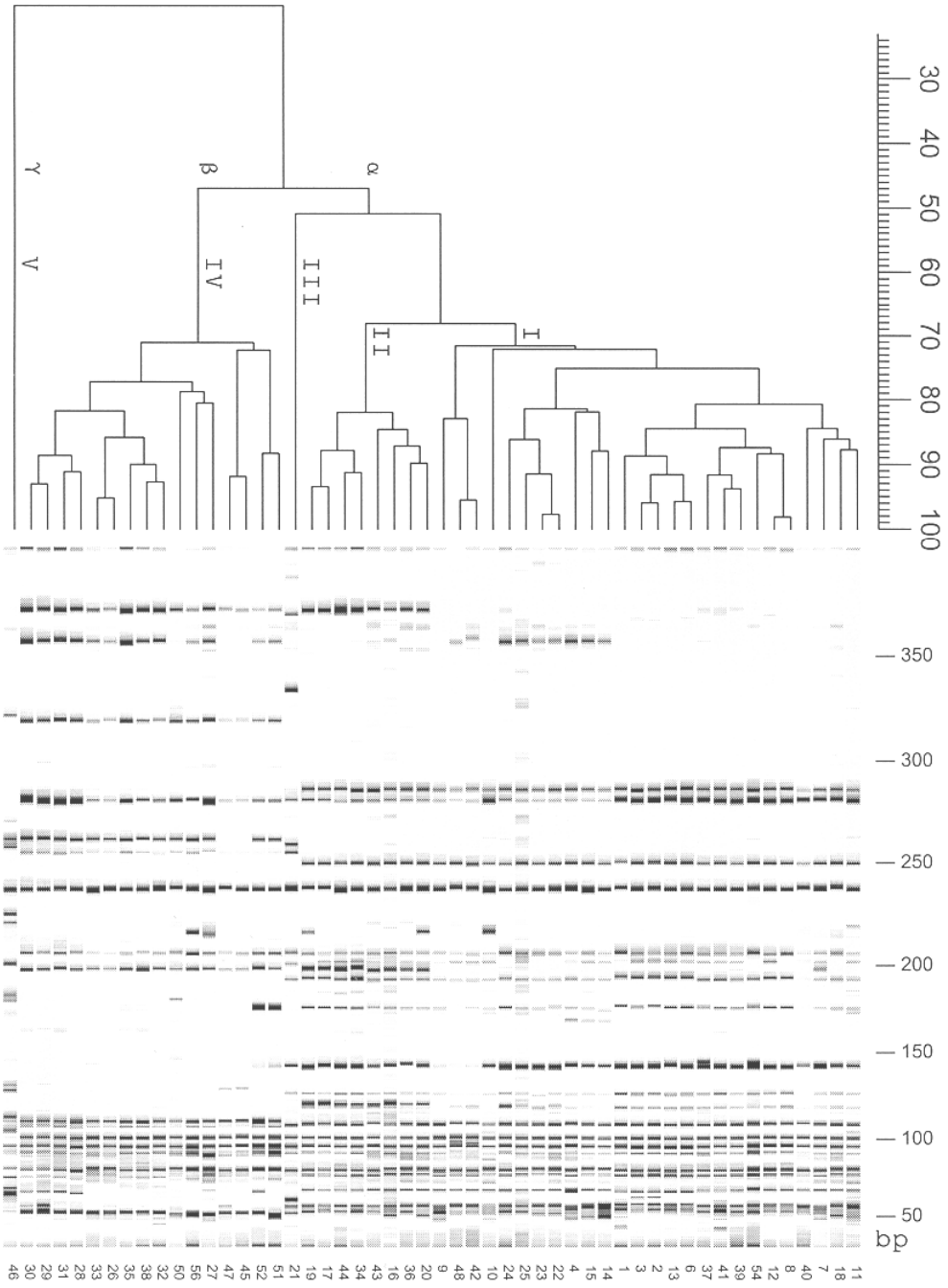
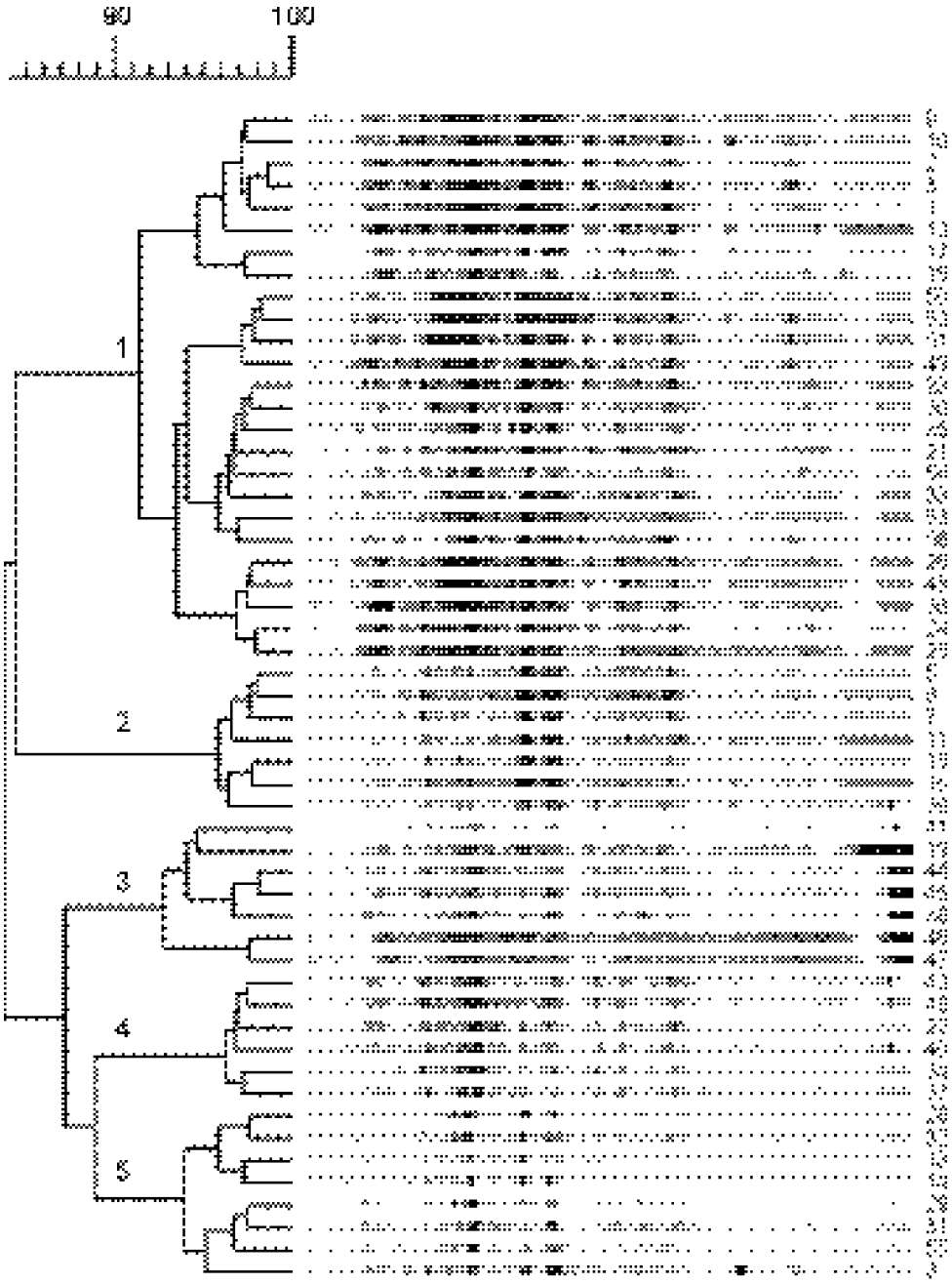


Figure 2: SDS-PAGE patterns obtained from OMP's of 51 *O. rhinotracheale* strains. Clusters 1 - 5 were separated by using an Sd of 90% as a distinctive linkage level. Numbers correspond with the strain numbers listed in Table 1.



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CHAPTER 4

# Pathogenesis

CHAPTER 4A

## *Ornithobacterium rhinotracheale* infection by egg transmission

***Paul van Empel, Han van den Bosch and Paul Storm.***

## SUMMARY

*Ornithobacterium rhinotracheale*, the causitive agent of a respiratory disease in poultry, was cultivated from egg-shells and from yolk sacs of one day old birds. Treatment of broiler chickens and turkeys, apparently carrying the bacterium, with known triggering respiratory viruses induced the the same kind of lesions in the air sacs of birds as elicited by *O. rhinotracheale* in experimental infection experiments. From these affected air sacs *O. rhinotracheale* was isolated. These experiments were carried out on conventionally kept animals. Additional experiments were carried out in which animals were housed under conditions used for specific pathogen free animals. Broiler chickens and turkeys hatched from breeder flocks with a known *O. rhinotracheale* history were used. *O. rhinotracheale* also was cultivated from inflamed air sacs after an infection with Newcastle disease or turkey rhinotracheitis virus. In conclusion, evidence was found that *O. rhinotracheale* can be transmitted by eggs. To determine whether cloacal vertical transmission occurs, additional experiments would be requested.

## INTRODUCTION

*Ornithobacterium rhinotracheale* has been shown to be the causative agent of a respiratory disease in poultry with clinical signs such as weakness, gasping, severe dyspnoea and mucus discharge. Dissection of diseased birds showed fibrinopurulent pneumonia and fibrinous inflammation of the air sacs (1, 5, 6, 7). It was proven that various respiratory viruses, such as turkey rhinotracheitis virus (TRT) and Newcastle disease virus (ND), have a triggering effect on the outcome of *O. rhinotracheale* infections (3).

Up to now the infection route of *O. rhinotracheale* is not known. The occurrence of *O. rhinotracheale* infections in various countries at an early age of the birds (1, 7), and the finding that maternally derived antibodies can be found in broiler and turkey flocks (4) indicates that the breeders of these flocks are in contact with *O. rhinotracheale* and that transmission by eggs may occur.

In this study we present the results of challenge tests in turkeys, using TRT virus as trigger, and in broiler chickens, using ND virus as trigger. In these tests we repeatedly found that eggs transmitted *O. rhinotracheale*. To investigate this transmission further, tests under specific pathogen free (SPF) conditions were carried out in broiler chickens and turkeys, using the same viral triggers. During these tests we also investigated whether *O. rhinotracheale* could be cultivated from eggs and from yolk-sacs of one-day-old birds.

## MATERIALS AND METHODS

**Isolation of bacteria.** For isolation of *O. rhinotracheale* swabs from egg-yolk, yolk sacs or affected organs (air sacs, lungs and tracheae) were taken and inoculated onto sheep blood agar. Samples of eggshells, egg-white and egg membranes from infertile eggs, as well as the remainders of the eggs and samples from yolk sacs of one-day-old birds, were inoculated into 10 ml of brain heart infusion (BHI) broth, incubated for 3 days at 37°C and subcultured on sheep blood agar and on sheep blood agar with 5 µg/ml gentamicin and 5 µg/ml polymyxin (to inhibit growth of other bacteria). The agar plates were incubated at 37°C in a atmosphere containing 5-10% $CO_2$ for at least 3 days. Each day the agar plates were checked for suspected colonies (small, grey and not haemolytic). Such colonies were subcultured under the same conditions.

**Identification of bacterial isolates.** Bacterial isolates were identified using the API identification system (BioMérieux, La Balme les Grottes, France).
*O. rhinotracheale*, which is not included in the API system, was identified with the

API-20NE identification strip and the agar gel precipitation (AGP) test as previously described (4). Briefly, boiled extract antigens of oxidase positive, gram negative rods with an API-20NE code 0-2-2-0-0-0-4 or 0-0-2-0-0-0-4 were tested in an AGP, using monovalent anti-sera against the seven known serotypes of *O. rhinotracheale* (4).

**Animals and eggs.** In preliminary tests, fattening turkeys were commercial British United Turkeys (Plukon, Ruinen, The Netherlands) and chickens were commercial Hybro-broilers (Interbroed, Gemert, The Netherlands). Both turkeys and chickens were purchased as 18 to 20 days embryonated eggs and housed in isolators immediately after hatching. Food (Hendrix' voeders, Heijen, The Netherlands) and water were given *ad libitum*.

In the experiments under SPF conditions the same breeds of birds were used. The eggs were purchased from breeder flocks with a known *O. rhinotracheale* history and with ELISA antibodies against *O. rhinotracheale*. Embryonated eggs were cleaned with a dry cloth but not dipped in a desinfectant or antibiotic. The eggs were transported immediately after lay to separated, thoroughly cleaned and previously formalinized incubators. Immediately after hatching, the birds were transported in closed, sterile sacs to sterilized isolators. Food, sterilized by γ-radiation (GS 4700, Hope Farms, Woerden, The Netherlands), and heat sterilized water were given ad libitum. The 36 turkey eggs and 65 chicken eggs, used for direct cultivation, as well as the 40 one-day-old turkeys and 50 one-day-old broiler-chickens, used for yolk-sac cultivation, were purchased from breeder flocks with a known *O. rhinotracheale* history.

**Virus infection.** Virus infection was performed as described (3). A culture of TRT virus strain 3B OC21 was administered to 25 turkeys by eye drop and nose drop containing at least $10^{6.6}$ median ciliostatic doses per bird, at 8 days of age in the initial test. In the test under "SPF" conditions, TRT was administered at 7 days of age to 25 turkeys.

A lentogenic ND virus strain LaSota was administered to two groups of broiler chickens (group A contained 20 and group B contained 24 birds), at an age of 21 days, by spray containing at least $10^6$ median egg infectious doses per bird. In the initial tests virus was administered to the birds at 21 days of age and in the "SPF test" at 7 (23 birds) or at 21 (24 birds) days of age.

**Post mortem investigation.** In the initial tests, post mortem examinations were performed on turkeys 12 days post TRT administration and on chickens 12 and 19 days post ND administration. The birds in the "SPF tests" were examined 10 days post TRT administration and 9 days post ND administration. The macroscopical lesions were recorded as described previously (3) and affected organs were cultured for *O. rhinotracheale*.

**Serological investigation.** Serum and yolk-sac samples were tested in an enzyme linked immunosorbent assay (ELISA) against boiled extract antigens of seven strains of *O. rhinotracheale* with different serotypes as described previously (4).

## RESULTS

**TRT administration to turkeys.** Twelve days after TRT administration, at 20 days of age, 2 out of 25 turkeys showed severe airsacculitis. This group of turkeys was meant as a TRT-control group in a challenge experiment with *O. rhinotracheale* and was supposed to be kept free of *O. rhinotracheale*. Still from the abdominal air sacs of one turkey an almost pure culture of *O. rhinotracheale* could be isolated. In the negative control group of this challenge experiment, which did not recieve TRT, no airsacculitis was found.

**ND administration to chickens.** In 2 separate experiments, ND strain LaSota was given to a group of chickens (groups A and B). One chicken out of group A died 7 days post ND administration. This bird, and 3 further birds of this group showed severe airsacculitis at post mortem investigation 12 days post ND administration. From the air sacs of 2 of these birds *O. rhinotracheale* was isolated. The remaining 16 birds out of group A showed no or minor airsacculitis.

The 10 chickens from group B, examined 12 days post ND administration, all showed severe airsacculitis, mostly in the abdominal air sacs (typical for an *O. rhinotracheale* infection) with a total group score of 50% of the maximal possible score. The remaining 14 animals were examined 19 days post ND administration and 13 of them showed severe airsacculitis with a total group score of 54 % of the maximal possible score. *O. rhinotracheale* could be isolated from the affected air sacs of 14 of the 23 chickens of group B that showed airsacculitis.

Both groups of chickens served as a ND control group in challenge experiments with *O. rhinotracheale*. They were housed in isolators and supposed to be kept free of *O. rhinotracheale*. In the negative control groups of these challenge experiments, which did not receive ND, no airsacculitis was found and *O. rhinotracheale* could not be isolated.

**Tests on animals housed under SPF conditions.** In these tests, the chickens which were given ND at 7 days of age, all showed an inflammation of the thoracic air sacs but not of the abdominal air sacs at 9 days post ND administration. *E. coli*, *Aerococcus viridans* and *Enterobacter cloacae* but no *O. rhinotracheale* could be cultured from the affected air sacs. Also all chickens, which were treated with ND at 21 days of age, showed airsacculitis in the thoracic but hardly any in the abdominal air sacs 9 days post ND administration. From the thoracic air sacs of 1 and the abdomi-

nal air sacs of 2 of these birds, *O. rhinotracheale* could be isolated. From the air sacs of all birds *E. coli* and *Aerococcus viridans* and of some birds *Staphylococcus aureus* could be cultured.

In the conditioned test in turkeys, 21 of the 25 turkeys which were given TRT at 7 days of age, showed inflammation of the thoracic air sacs and 4 turkeys showed minor inflammation of the abdominal air sacs 10 days post TRT administration. *E. coli*, *Aerococcus viridans* and *Proteus mirabilis* could be cultured from all the affected air sacs. From the thoracic air sacs of one turkey and the abdominal airsacs of another bird, *O. rhinotracheale* could be isolated.

**Cultivation of eggs and yolk sac material.** From the inner surface of the shell of only one chicken egg (out of the in total 101 eggs sampled) *O. rhinotracheale* could be isolated. All other samples remained negative. The remainders of the eggs and swabs from yolk-sacs of 40 one-day-old turkeys were investigated for the presence of *O. rhinotracheale*. All samples were overgrown by *E. coli* and/or *Enterobacter cloacae*. Even after subculturing, these samples remained negative for *O. rhinotracheale*. The remainders of the eggs and swabs from yolk-sacs of 50, one-day-old broiler chickens were also investigated for the presence of *O. rhinotracheale*. In most samples *E. coli* and/or *Enterobacter cloacae* was found. But from 2 yolk-sac samples *O. rhinotracheale* could be cultured.

**ELISA.** When serological responses against *O. rhinotracheale* were found, they were predominantly against serotype A. In all turkeys and chicken sera antibodies against *O. rhinotracheale* were found at one day of age. The antibody levels in the yolk-sacs at one day of age were similar to those in the sera ($^2$log titres between 7 and 12). No antibodies were found at 14 days of age anymore (titre $\leq 4$).

When measured 12 days post ND administration, 5 of 10 chickens out group B showed weak serological reactions against *O. rhinotracheale* (titres between 5 and 8). At 19 days post ND administration 5 of 14 chickens out of group B showed slightly increased serum titres against *O. rhinotracheale* (titres between 8 and 12). The negative control group (no ND administration) remained serologically negative against *O. rhinotracheale*.

In the “SPF tests”, at one day of age, the chickens as well as the turkeys had serum antibodies against *O. rhinotracheale* (titres between 8 and 11). At 7 and 21 days of age, when viruses were administered, all serum samples were negative (titres $\leq 4$). Nine days post ND administration and 10 days post TRT administration, weak serological reactions against *O. rhinotracheale* were seen for 10 of the 47 chickens and 7 of the 25 turkeys (titres between 5 and 9). Birds not treated with viruses remained serologically negative.

## DISCUSSION

In 3 separate challenge tests, one using turkeys and two using chickens, it was found that birds, that were supposed to be kept free of *O. rhinotracheale*, showed airsacculitis after administration of the respiratory viruses which can be used as trigger for *O. rhinotracheale* infections. The airsacculitis was similar to that encountered in challenge tests with *O. rhinotracheale* (3) and from the affected air sacs *O. rhinotracheale* could be isolated. So the probable cause of the airsacculitis was an infection with *O. rhinotracheale*. In the control groups of these tests, which did not receive respiratory viruses, no airsacculitis was found and *O. rhinotracheale* could not be isolated. Because the animals used in these tests were purchased as embryonated, commercial eggs and placed in separated, clean isolaters immediately after hatching, there is hardly any chance that the *O. rhinotracheale* infections were caused by contact, unless the incubator, food or water was contaminated with *O. rhinotracheale*. This indicated that *O. rhinotracheale* might have been transmitted through the eggs.

To exclude any external contaminations, the broiler chickens and turkeys in the next tests were purchased immediately after lay and then hatched and housed under SPF conditions. Also sterile food and water was given. Knowing that it is very difficult to cultivate *O. rhinotracheale* from diseased birds, even in experimentally infected birds (4), it is important to use highly susceptible birds. So to make the birds more susceptible for the triggering viruses and by this way giving an *O. rhinotracheale* infection a good chance to develop, the birds did not receive any vaccinations prior to the ND or TRT administration. As a result of the virus administrations, almost all birds showed airsacculitis in the thoracic air sacs probably caused by the virus administration as reported before (2, 3). From affected air sacs *O. rhinotracheale* together with bacteria commonly found on eggs, like *E. coli*, *Enterobacter cloacae*, *Aerococcus viridans* or *Staphylococcus aureus*, could be isolated. The incidence of the *O. rhinotracheale* isolations was low, which is in agreement with the findings in the field (1, 3, 7). The isolation of *O. rhinotracheale* from air sacs in these tests proves that *O. rhinotracheale* infections in broilers can be caused by the presence of the bacterium in the breeder flock. We also found that treatment of birds with respiratory viruses evoked weak antibody reactions against *O. rhinotracheale* while untreated birds remained negative. *O. rhinotracheale* could be isolated from yolk-sacs of one-day-old birds and from the the eggshell but not the inner parts of the egg. These results provide evidence that some kind of vertical transmission of *O. rhinotracheale* from infected breeders to progeny takes place. This is of importance to control the disease by hygienic measures or vaccination. Whether cloacal or ovarial

transmission is involved still remains to be established.

## ACKNOWLEDGMENTS

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CHAPTER 4

# Pathogenesis

CHAPTER 4B

## Immuno-histochemical and serological investigation of experimental *Ornithobacterium rhinotracheale* infection in chickens

***Paul van Empel, Mieke Vrijenhoek, Danny Goovaerts and Han van den Bosch.***

*Submitted for publication*

## SUMMARY

With use of an immuno-histochemical technique, *Ornithobacterium rhinotracheale* was proven to be the causitive agent of lesions in the air sacs and lungs in chickens, but only when *O. rhinotracheale* was exposed to the bird after priming with Newcastle Disease (ND) virus. At first the bacteria attached to the epithelium of the air sacs, lateron they infiltrated the air sacs and caused thickening, the formation of oedematous and granulomatous tissue and a strong accumulation of macrophages. The infection peaked at 5 to 9 days post *O. rhinotracheale* exposure after which recovery was seen. In the lungs some areas with bronchial associated lymphoid tissue were affected. Further no other organs were proven to be affected.

Without ND priming, aerosol exposure of *O. rhinotracheale* to chickens only resulted into minimal and temporary microscopic air sac lesions. No *O. rhinotracheale* cells or fragments could be detected at any time point later than 2 days post exposure. However, without showing visible lesions, chickens exposed to *O. rhinotracheale* without ND priming reacted serologically equally as compared to chickens exposed after ND priming, with the same duration and strength of response.

## INTRODUCTION

*Ornithobacterium rhinotracheale* has been repeatedly found to be involved in cases of respiratory diseases in broiler-chickens and meat turkeys (1, 3, 4, 5, 6, 7, 9, 10, 11, 12). Clinically, relatively mild respiratory symptoms were accompanied by increased mortality and growth retardation. Post mortem investigations showed a foamy, yellow-white, "yogurt like" exudate in the air sacs, predominantly in the abdominal air sacs and sometimes accompanied with a purulent pneumonia, often unilateral, and/or exsudate in the trachea. Experimental infection demonstrated that aerosol exposure with an *O. rhinotracheale* culture was only capable of reproducing the clinical features of the disease, if the birds were primed previously by the administration of respiratory viruses (13).
In this study, bacteriological, serological and immuno-histochemical examinations were performed to study the course of disease after experimental infection of chickens.

## MATERIALS AND METHODS

**Animals.** Chickens were SPF leghorns (Intervet, Boxmeer, The Netherlands). All animals were placed in isolaters at one day of age. The birds did not receive any vaccination or treatment and only sterilized food and water was given ad libitum.

**Challenge.** The first group, containing 30 birds, was infected at an age of 26 days as previously described (13), using an aerosol, made of 100 ml of a culture of *O. rhinotracheale* strain B3263/91 (serotype A) containing 3.8 x $10^8$ CFU per ml. The birds were infected 5 days post administration of a spray of Newcastle Disease (ND) virus strain LaSota as viral primer. The second group of 30 birds was treated with an aerosol of the same culture of *O. rhinotracheale* strain B3263/91 at 26 days of age, but without the ND priming. The third group of 30 birds received a spray of ND strain LaSota at 21 days of age but no *O. rhinotracheale* exposure. The fourth group of 30 birds was kept as a negative control group.

**Serology.** Bloodsamples were taken every week up to 14 weeks post *O. rhinotracheale* exposure and at post-mortem investigation. Antibodies against *O. rhinotracheale* serotype A were quantified with use of an enzyme linked immunosorbent assay (ELISA) as previously described (14).

**Post-mortem examination.** Post-mortem examination was performed at 1, 2, 3, 4, 5, 7, 13 and 15 days after *O. rhinotracheale* exposure on 2 birds of each group and on the remaining birds 14 weeks after exposure. The birds were bled, lesions were scored as described (13) and samples for histological investigation were taken from the thoracic airsacs, abdominal airsacs, lung, trachea, brains, pericardium, heart,

duodenum, pancreas, spleen, liver, proventriculus, gizzard, bursa, thymus, skin of the head and flexor tendons of the hock. Samples were fixed in 10 % neutral buffered formalin, routinely processed to paraffin sections and stained with haematoxylin and eosin.

**Bacteriology.** During autopsy, the air sacs, lungs, trachea and any abnormal organs were sampled with cotton swabs for isolation of *O. rhinotracheale*. The swabs were inoculated on sheepblood agars and on sheepblood agars + polymyxin + gentamicin (both 5 µg per ml, used to suppress growth of other bacteria). The agars were incubated at 37°C under 5 - 10 % $CO_2$ atmosphere for 72 hours. Colonies, suspected to be *O. rhinotracheale*, were subcultured and identified with use of a Gram-stain, API-20NE identification strips and agargel precipitation (AGP) test as previously described (14).

**Peroxidase anti peroxidase (PAP) staining.** PAP tests were performed on paraffin sections. The primary antiserum against *O. rhinotracheale*, used in the PAP method, was a monovalent rabbit antiserum against strain B3263/91 (serotype A). The serum was prepared by injecting SPF New Zealand white rabbits (van Broekhoven, Someren, The Netherlands) with a bacterin in oil-adjuvant twice with 3 weeks interval. Serum was collected 4 weeks after the second injection.

The PAP method was performed as described by Bourne (1983). Briefly, sections were treated with $H_2O_2$ to remove endogeneous peroxidase activity, then blocked with swine serum and incubated with the monovalent rabbit antiserum against *O. rhinotracheale*. Subsequently, the sections were incubated with a linking swine-anti-rabbit serum. After washing they were incubated with a horseradish peroxidase-rabbit-antiperoxidase complex (Dako, Santa Barbara, USA) and treated with diaminobenzidin as substrate. Finally the slides were counterstained with haematoxylin. (8) and examined microscopically (magnifications 200 x and 600 x).

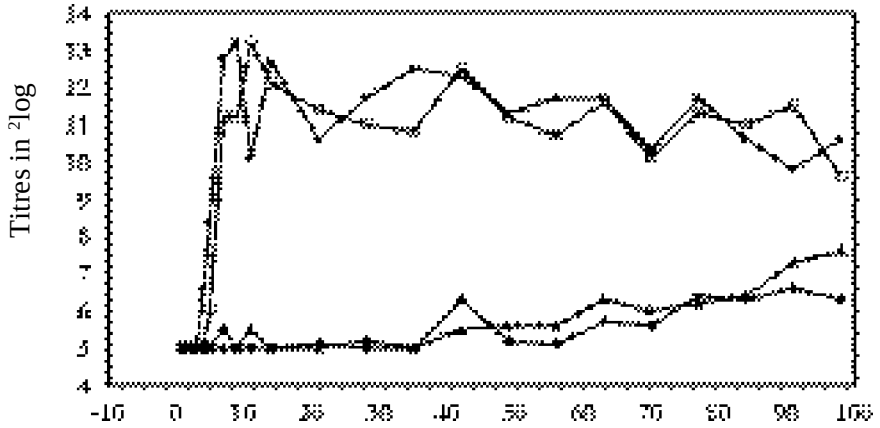
## RESULTS

**Serology**. The serological responses after challenge are visualized in Figure 1. Responses were detectable at 5 days and peaked at 7 days post *O. rhinotracheale* exposure with individual $^2$log titres from 9 up to 16. No differences were found between chickens exposed to *O. rhinotracheale* after ND priming and chickens exposed to *O. rhinotracheale* without ND priming. Hardly any decrease in antibody titres was observed within the 100 days observation period.

**Bacteriology.** During the first 10 days post exposure, *O. rhinotracheale* could be isolated from 34% of the samples taken from the air sacs and the lungs of birds exposed to *O. rhinotracheale* after ND priming. From the trachea samples *O. rhino-*

*tracheale* could only be isolated during the first two days post challenge. *O. rhinotracheale* could not be isolated anymore at time points later than 10 days post challenge.

Figure 1. Average serological response against *O. rhinotracheale* serotype A. ND virus was administered at 21 days of age and *O. rhinotracheale* was exposed at 26 days of age. □ = ND + *O. rhinotracheale*; ■ = *O. rhinotracheale* only; ▒ = ND only; ● = Control.




After exposure of chickens to *O. rhinotracheale* without ND priming, isolation of *O. rhinotracheale* was possible out of 18% of the air sacs and trachea samples, only during the first two days post exposure.

**Post-mortem investigation.** Airsacculitis was found in the chickens exposed to ND alone or ND and *O. rhinotracheale*. The airsacculitis in the group chickens exposed to ND alone scored between 0% and 20% of the maximal possible score while the airsacculitis in the group chickens exposed to ND and *O. rhinotracheale* scored between 60 and 100% with the strongest reaction at 5 - 7 days post *O. rhinotracheale* exposure. Pneumonia was found in chickens exposed to *O. rhinotracheale* after ND priming only. Tracheitis was found in 10 - 20 % of both groups of chickens treated with ND but only at the beginning of the experiment.

**Histology.** Throughout the test no consistent abnormalities were detected microscopically in the heart, pericard, cerebrum, cerebellum, brain stem, thymus, nose, eyelid, head-skin, kidney, spleen, bursa, ceacum, ileum, duodenum, pancreas, proventriculus, gizzard or flexor tendons at the hock.

In the control-group, no consistent abnormalities were detected. In the group that

was treated with ND alone, moderate to severe tracheitis with epithelial hyperplasia and lymphocytic infiltration in the epithelium and lamina propria, as well as minimal airsacculitis, characterised by mononuclear inflammatory cell infiltration and lymphoid follicular hyperplasia of the air sacs, were found up to 10 days post ND treatment.

In the group that was treated with *O. rhinotracheale* alone minimal infiltration of the air sacs by polymorphonuclear granulocytes (PMN) was seen at one day post exposure. Immuno-histochemistry of these air sacs could demonstrate bacteria on the epithelial surface of the air sac at the site of inflammation. Mild infiltration of the epithelium of the respiratory side by PMN's was found and *O. rhinotracheale* bacteria or bacterial fragments were attached to the cilia of the epithelium (Figure 2). Immuno-histochemistry could not demonstrate any *O. rhinotracheale* bacteria or bacterial fragments at time points later than one day post *O. rhinotracheale* exposure in the group treated with *O. rhinotracheale* alone.

In the group exposed to *O. rhinotracheale* after ND treatment, several lesions, related to *O. rhinotracheale*, could be seen in the air sacs, the lungs and to a lesser extent in the trachea. One day post *O. rhinotracheale* exposure the reactions were similar to the reactions seen in the group that was exposed to *O. rhinotracheale* alone. Two days post exposure several *O. rhinotracheale* cells were seen, attached to the epithelium of the thoracal air sacs, especially at the cuboidal epithelium at the site of transition of the air sacs to the lungs (Figure 3). Infiltration of the air sac epithelium and lamina propria by accumulation of macrophages and PMN’s was seen with presence of cell debris and inflammatory cells in the air sac lumen (Figure 4). The air sacs were thickened, oedema was present and acute granulomatous airsacculitis related to the presence of *O. rhinotracheale* antigen, as demonstrated by immunohistochemistry (Figure 5), was developing. Four and five days post exposure, the air sacs were clearly thickened and *O. rhinotracheale* cells or fragments were abundantly found in the epithelium, in the lamina propria and on the surface of the air sacs in the lumen. Most of the positive staining in immunohistochemistry was associated with single and accumulated macrophages (figure 6). At 5 days post exposure, infiltration and necrosis of the bronchial associated lymphoid tissue (BALT) was seen in some areas of the lungs (Figure 7) while other parts of the same lung were not affected at all. Within the affected BALT-areas strong staining was found for *O. rhinotracheale* by immuno-histochemistry (Figure 8). No staining for *O. rhinotracheale* cells or fragments could be detected in any tissue other than lungs or air sacs. Air sac lesions were still present at 13 days post exposure in the group exposed to *O. rhinotracheale* after ND treatment. At that time point they consisted of multifocal hyperplasia of the epithelium with thickening of the air sac wall and

infiltration of the lamina propria by mononuclear inflammatory cell accumulations and few granulomas. Immuno-histochemistry could demonstrate *O. rhinotracheale* cells or fragments in the chronically inflamed areas. After 15 days recovery was seen and only focal hyperplasia of the epithelium was seen in some air sacs and *O. rhinotracheale* cells or fragments could be found sporadically.

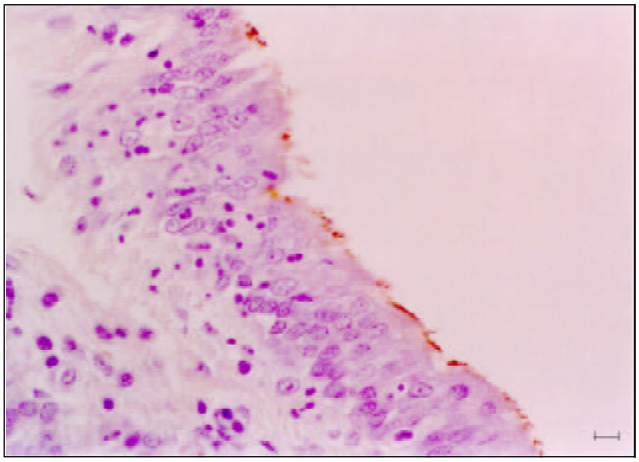


Figure 2. Immuno-histochemistry on the air sac of a chicken, 1 day post *O. rhinotracheale* exposure without ND treatment. The brown spots indicate *O. rhinotracheale* cells or fragments attached to cilia of the epithelium on the respiratory side. A mild infiltration of the epithelium by PMN's can be seen. Bar = 10 µm.

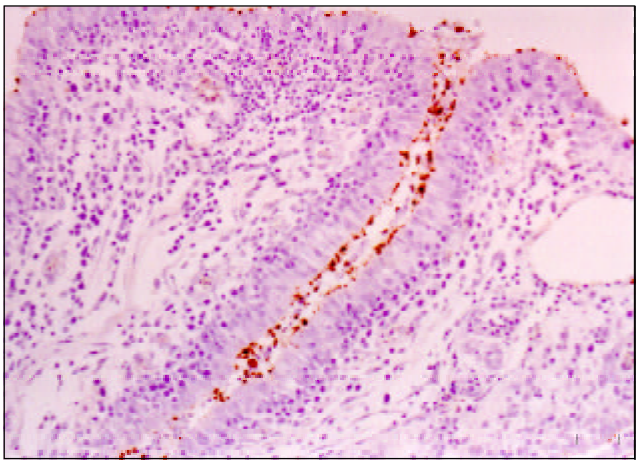


Figure 3. Immuno-histochemistry on the air sac of a chicken, 2 days post *O. rhinotracheale* exposure with ND treatment. The brown spots indicate *O. rhinotracheale* cells or fragments attached to the respiratory epithelium and associated to inflammatory cells in the lumen of the air sac. Also infiltration of the epithelium by PMN's and macrophages can be seen. Bar = 50 µm.

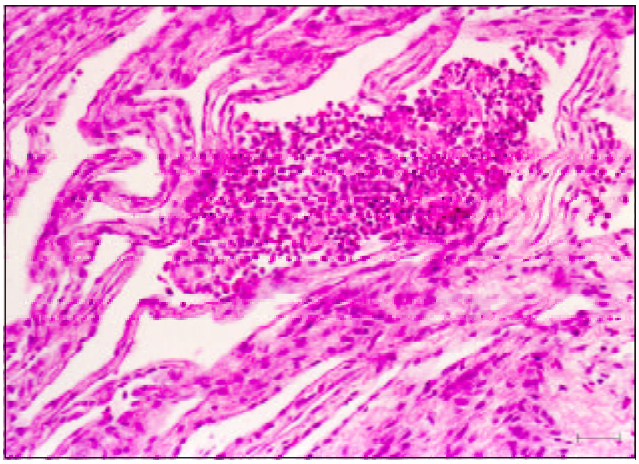


Figure 4. The air sac of a chicken, 2 days post *O. rhinotracheale* exposure with ND treatment. Haematoxylineosin stain. Infiltration of the air sac epithelium by PMN's and macrophages can be seen with presence of inflammatory cells in the air sac lumen. Bar = 50 µm.

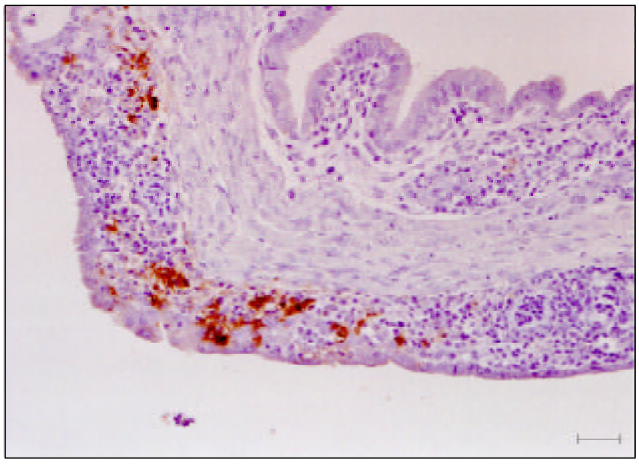


Figure 5. Immuno-histochemistry on the air sac of a chicken, 4 days post *O. rhinotracheale* exposure with ND treatment. The brown spots indicate *O. rhinotracheale* cells or fragments located within the air sac underneath the epithelium causing acute granulomatous airsaccilitis. Bar = 50 µm.

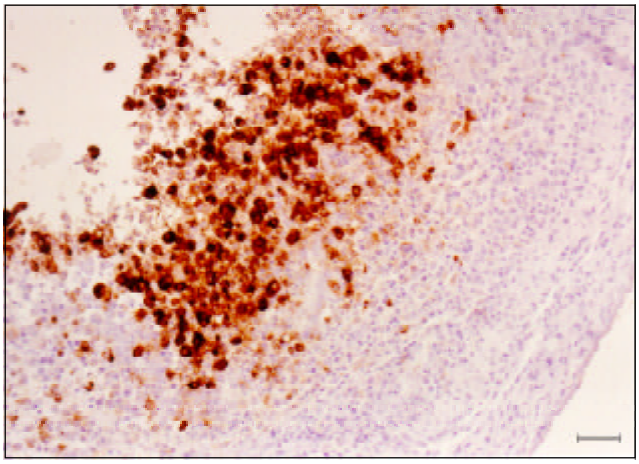


Figure 6. Immuno-histochemistry on the air sac of a chicken, 5 days post *O. rhinotracheale* exposure with ND treatment. The brown spots indicate *O. rhinotracheale* cells or fragments located deep in the air sac tissue, associated to single and accumulated macrophages. Bar = 50 µm.

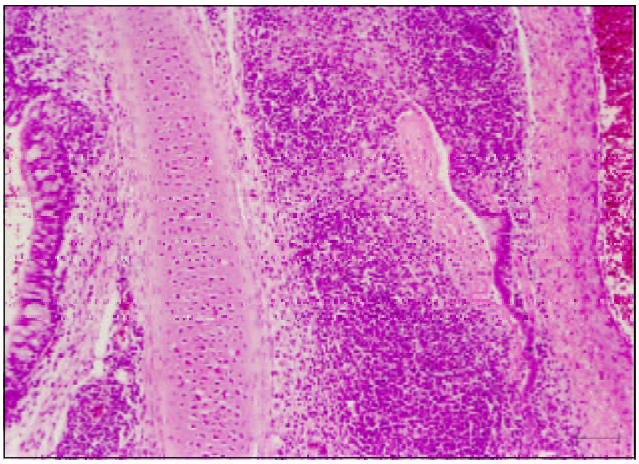


Figure 7. The lung of a chicken, 5 days post *O. rhinotracheale* exposure with ND treatment. Haematoxylin-eosin stain. In the center an infected BALT-area with infiltration by bacteria and necrosis of the tissue can be seen. On the left side a negative BALT-area is shown. Bar = 50 µm.

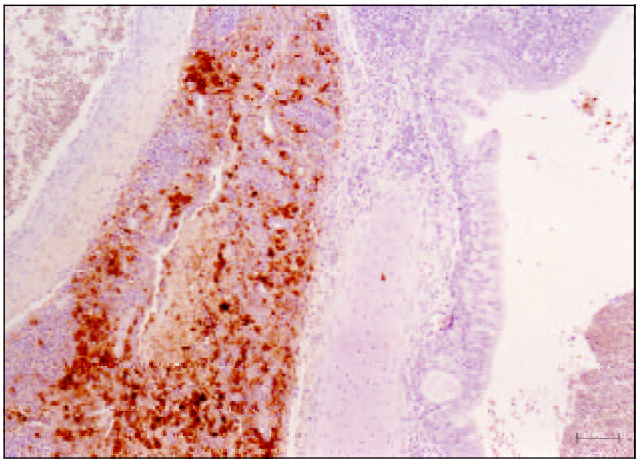


Figure 8. Immuno-histochemistry on the lung of a chicken, 5 days post *O. rhinotracheale* exposure with ND treatment. The brown spots indicate *O. rhinotracheale* cells or fragments located in an infected BALT-area. Bar = 50 µm.

## DISCUSSION

When given to susceptible birds post ND priming, *O. rhinotracheale* was shown to be invasive in the air sacs and lungs. By immunohistochemistry we could clearly prove that *O. rhinotracheale* was the cause of the induced inflammations. At 2 days post *O. rhinotracheale* exposure, the air sacs showed infiltration of the epithelium by macrophages and PMN's. Then the air sacs thickened, oedematous and granulomatous tissue was formed and a strong accumulation of macrophages at the site of infection was seen. *O. rhinotracheale* cells or fragments were demonstrated all over the airsacs but predominantly associated with macrophages. The airsacculitis peaked between 5 and 9 days post exposure and lateron recovery was seen. Some of the BALT-areas within the lungs showed lesions attributed to *O. rhinotracheale*

approx. 4 to 5 days post exposure and *O. rhinotracheale* antigen could be demonstrated within these lesions. No other organs became affected and *O. rhinotracheale* cells or fragments could not be found in any other investigated organ.

This immuno-histochemical investigation confirms the finding in a previous clinical study (13), that aerosol exposure of chickens with *O. rhinotracheale* without (viral) priming will not result in significant lesions of the respiratory tract. In non primed birds only minimal and temporary microscopic air sac lesions could be induced with focal attachment of the bacteria to the epithelium. Both immuno-histochemically and bacteriologically, *O. rhinotracheale* could not be recovered anymore at time points later than two days post aerosol exposure. This seems in contradiction with the fact that serologically the *O. rhinotracheale* exposure without ND-treatment resulted in a longstanding and high response, equal to the response found in the heavily infected birds out of the group which was exposed to *O. rhinotracheale* after ND priming. In contrast with serological results of fieldsera from birds with known *O. rhinotracheale* infections whose titres decreased substantially within 6 - 10 weeks (personal observations, not published), the serological responses remained at the same level up to at least 14 weeks post *O. rhinotracheale* exposure. In the field we regularly found that both the serological response and the bacteriological recovery of the bacterium decreased rapidly after an *O. rhinotracheale* infection. An explanation for this difference could be that the way of exposure used in this challenge test, very fine aerosol containing high amounts of viable bacteria, allows the bacterium to enter the respiratory tract of the bird far better compared to exposure in the field. But even then the high and longstanding serological responses indicate that the bacterium should be invasive somewhere else in the body or capable to induce the immune-system in another way. We were only able to detect *O. rhinotracheale* bacteria or bacterial fragments in the respiratory tract but not in any other part of the infected birds but the method used might not be sensitive enough. If the bacterium is hiding itself somewhere in the body more sensitive detection methods, such as PCR, should be developed to find the place of concealment.

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CHAPTER 5

# Prevention

## Vaccination of chickens against *Ornithobacterium rhinotracheale* infection

***Paul van Empel and Han van den Bosch.***

*Submitted for publication*

## SUMMARY

Vaccination of young broilers against experimental *O. rhinotracheale* challenge was found to be effective but the results of vaccination were influenced in a negative way by the presence of maternal antibodies. The use of a strong adjuvant, such as mineral-oil, in a bacterin was necessary to obtain good protection when maternal antibodies were present.

Vaccination of broiler-breeders resulted into high serological responses and protection of their progeny against experimental *O. rhinotracheale* challenge up to an age of 4 weeks.

Vaccination of broilers with a live vaccine was found to be effective when the maternal antibody-levels were low. A combination of vaccinating the breeders with a bacterin and their progeny with a live vaccine at approx. 3 weeks of age seems to be the best way to protect broilers against *O. rhinotracheale* infection.

## INTRODUCTION

*Ornithobacterium rhinotracheale* is found to be able to cause respiratory disease in turkeys and chickens (1,2,3,6,7,8,9,10,11,12). Clinically the isolation of *O. rhinotracheale* could be connected to weakness, gasping, severe dyspnoea, mucus discharge, weight-gain losses and increased mortality rates. Dissection of diseased birds showed fibrinopurulent, often unilateral, pneumonia and fibrinous inflammation of the air sacs (11). Up to now 7 different serotypes, designated A - G, were reported (14) but recently 2 more serotypes were found, serotypes H and I (own observation, not published). The most predominant serotype is type A. In chickens, more than 95 % of the isolates are found to be of serotype A (14).

Experimentally the disease could be evoked by aerosol administration of *O. rhinotracheale,* but only as a secondary infection after the administration of primers. As viral primers Turkey rhinotracheitis virus and Newcastle Disease virus (ND) can be used for turkeys while ND and Infectious Bronchitis virus can be used for chickens (13). Also *Escherichia coli* (5) and *Bordetella avium* (4) were found to have a triggering effect on *O. rhinotracheale* infections.

In this study we investigated the influence of vaccination on experimental *O. rhinotracheale* infections in broiler chickens, using serotype A vaccines. Normally broilers only will come in hand at one day of age so vaccinations with inactivated vaccine were performed on one-day-old birds. Because maternal antibodies regularly were found in the field (14), the influence of these antibodies on vaccine efficacy was investigated by comparing vaccination results in one-day-old commercial broilers (with maternal antibodies) with the vaccination results in older SPF (= special pathogen free) birds (without antibodies at time of vaccination). Also it was investigated if vaccination of broiler-breeders will induce protection in their progeny against experimental *O. rhinotracheale* challenge. Finally, the feasibility of live vaccination was investigated in young broilers.

## MATERIALS AND METHODS

**Bacterial strains.** The *O. rhinotracheale* serotype A strains used for this study were strain B3263/91 (isolated out of a diseased broiler chicken in South Africa by J.H. duPreez) and strain BAC 96-0034 (isolated out of a broiler chicken in Delaware, USA by E. Odor; originally designated as 95-1339). For the live vaccine, strain B3263/91 was passed in vitro 35 times (B3263/91/35). The strains were maintained lyophilized at -20°C. Bacteria were grown at 37°C on sheepbloodagar plates in a 5-10% $CO_2$ atmosphere or in Todd Hewitt broth (THB) at 37°C.

**Vaccines.** Inactivated vaccines were bacterins, produced from strain B3263/91, with 3 different adjuvants (mineral-oil, corn-oil or saponin), containing ± 1 x $10^9$ cells per dose. Vaccinations were performed by injecting 0.25 ml subcutaneously in the neck.
For live vaccinations, 100 ml of THB-cultures of *O. rhinotracheale* strain B3263/91/35, containing between 1 x $10^8$ and 1 x $10^9$ colony forming units (CFU) per ml, were administered as a fine spray to the birds in an isolater of ± 1.5 $m^3$, using a commercial paint sprayer. The developed mist in the isolaters was maintained for at least 10 min. with the air circulation closed.

**Experimental animals**. Chickens used were commercial broiler-breeders and broilers or special pathogen free (SPF) white leghorns. The broilers and SPF leghorns did arrive as embryonated eggs and were housed in isolaters immediately after hatch. The broiler-breeders were purchased at an age of 11 weeks. Food (Hendrix, Boxmeer, the Netherlands) and water were given ad libitum to the broilers and SPF leghorns but on ratio to the broiler-breeders. The broilers and SPF leghorns did not receive any other vaccination or treatment during the experiments. The broiler-breeders were vaccinated according to the schedule of the supplier.

**Challenge.** *O. rhinotracheale* challenge was performed as described before (13). Briefly : Five or six days before the *O.rhinotracheale* challenge the chickens were treated with a coarse spray of a culture of a lentogenic ND virus, strain LaSota, containing at least $10^6$ median egg infective doses per bird. Aerosol *O. rhinotracheale* challenges were performed with THB cultures of strain BAC 96-0034, containing between 1 x $10^8$ and 1 x $10^9$ CFU per ml, which were given as an aerosol in the same way as the live vaccine was applied.

**Parameters of infection.** Post-mortem investigations were performed 7 days post challenge and lesions were scored. Airsacculitis was scored for the thoracic and the abdominal airsacs separately. The thoracic airsacs were scored : 0 = no abnormalities, 1 = one airsac seriously affected by fibrinous airsacculitis or limited pinhead-sized foci of fibrinous exudate in both air sacs, 2 = both air sacs seriously affected by fibrinous airsacculitis. The abdominal air sacs were scored : 0 = no abnormalities, 1 = pinhead-sized foci of fibrinous exudate or slight diffuse airsacculitis, 2 = severe fibrinous airsacculitis. The airsacculitis score is given as the sum of both scores. Pneumonia was scored : 0 = no abnormalities, 1 = unilateral pneumonia, 2 = bilateral pneumonia. The average group-scores are given as a percentage of the maximal possible score. Statistical analysis was performed using the Kruskal-Wallis test.

**Serology.** Bloodsamples were tested in an enzyme-linked immunosorbent assay (ELISA) against antigens of *O. rhinotracheale* strain B3263/91 as previously described (14). All sera were prediluted 1:16. Statistical analysis was performed using the Students-t test.

## EXPERIMENTAL DESIGN

**Experiment 1.** Ten one-day-old broilers per group were vaccinated with one of the 3 different bacterins. ND was administered at 21 days of age, *O.rhinotracheale*- challenge was performed at 26 days of age and post-mortem investigation was performed at 33 days of age. Ten, not vaccinated, birds per group were used as negative controls, controls for ND administration or challenge controls.

**Experiment 2.** Ten SPF leghorns per group were vaccinated at an age of 21 days with one of the 3 different bacterins. In this experiment it was chosen to use 3-weeks-old SPF birds to minimilize the chance of the presence of antibodies at time of vaccination. ND was administered at 42 days of age, *O.rhinotracheale* challenge was performed at 48 days of age and post-mortem investigation was performed at 56 days of age. Ten, not vaccinated, birds per group were used as negative controls, controls for ND administration or challenge controls.

**Experiment 3.** At 12 and 18 weeks of age, 25 broiler-breeders were vaccinated with the bacterin in mineral-oil-adjuvant and 25 broiler-breeders were kept as not vaccinated controls. At the age of 32 and 37 weeks from both groups of broiler-breeders embryonated eggs were collected and broilers were hatched. From all groups 10 broilers were treated with ND at 9 days of age, challenged with *O. rhinotracheale* at 15 days of age and post-mortem investigations were performed at 22 days of age. From the not vaccinated breeder group, 10 broilers were used as negative controls and 10 broilers were used as controls for ND administration. From both breeder groups 10 broilers, hatched out of eggs layed at a breeder-age of 37 weeks, were treated with ND LaSota at 24 days of age, challenged with *O. rhinotracheale* at 30 days of age and post-mortem investigations were performed at 37 days of age. From the not vaccinated breeder group, 10 broilers were used as negative controls and 10 broilers were used as controls for ND administration.

**Experiment 4.** Seventeen one-day-old, 19 seven-days-old and 20 fourteen-days-old broilers were vaccinated with live vaccine. Seventeen one-day-old, 21 seven-days-old and 15 fourteen-days-old broilers from the same hatches were kept as not vaccinated controls. Seven days post vaccination, 5 birds from each group were sacrificed and a post-mortem investigation was performed to see if any pathology was induced by the vaccination. To the remaining birds ND was administered 7 days post vaccination, *O. rhinotracheale* challenge was performed 14 days post vaccination. Post-mortem investigation was performed 21 days post vaccination.

## RESULTS

**Experiment 1.** The results of this experiment are listed in Table 1. The average ELISA titre (in $^2$log) against *O. rhinotracheale* serotype A in the blood of the one-day-old broilers, used in this experiment, was 8.9 (± 1.0). Good protection and a moderate serological response was measured in the birds vaccinated with the bacterin in mineral-oil-adjuvant. The bacterin in saponin-adjuvant induced a low protection and a minimal serological response, whereas the bacterin in corn-oil-adjuvant hardly showed any response or protection.

Table 1: Post-mortem results of experiment 1 (Inactivated *O. rhinotracheale* vaccines in broilers). Vaccination at day 1, ND administration at day 21, challenge at day 26, post-mortem at day 33.

| Group Treatment | N | avg. ELISA titre (in $^2$log) at age of challenge | lesion-scores in % of the maximal possible score Airsacculitis | Pneumonia |
|---|---|---|---|---|
| Mineral-oil-adjuvant | 10 | 8.4 (±1.8)$^a$ | 15%$^a$ | 0%$^a$ |
| Corn-oil-adjuvant | 10 | 5.4 (±0.9)$^{bc}$ | 85%$^c$ | 5%$^a$ |
| Saponin-adjuvant | 10 | 6.2 (±1.1)$^b$ | 65%$^b$ | 15%$^{ab}$ |
| Challenge controls | 10 | 4.1 (±0.5)$^c$ | 95%$^c$ | 35%$^{bc}$ |
| ND controls | 10 | 4.3 (±0.7)$^c$ | 10%$^a$ | 0%$^a$ |
| Negative controls | 10 | 4.1 (±0.3)$^c$ | 5%$^a$ | 0%$^a$ |

Within columns, values having different superscripts are significantly different ($p<0.05$)

**Experiment 2.** The results of this experiment are listed in Table 2. No antibodies were detected in the blood of the 21 days old SPF leghorns, used in this experiment. Good protection and a very high serological response was measured in the birds vaccinated with the bacterin in mineral-oil-adjuvant. The bacterin in saponin-adjuvant induced a significant decrease of lesions and a moderate serological response, whereas the bacterin in corn-oil-adjuvant showed the lowest response or protection.

**Experiment 3.** The average serological responses after vaccination of the broiler-breeders against *O. rhinotracheale* serotype A, which declined slightly but remained rather stable during the course of this experiment, are shown in Figure 1. The kinetics of antibody titres in the hatched broilers are shown in Figure 2. The challenge results of the broilers are listed in Table 3. Moderate levels of antibodies against *O.*

*rhinotracheale* serotype A were measured in the breeders at time of vaccination (average titre of 9.7 ± 1.2). The first vaccination already boosted the serological responses to very high levels, which did not increase after the second vaccination (Fig. 1).

Table 2 : Post-mortem results of experiment 2 (inactivated *O. rhinotracheale* vaccines in SPF leghorns). Vaccination at day 21, ND administration at day 42, challenge at day 48, post-mortem at day 56.

| Group Treatment | N | avg. ELISA titre (in $^2$log) at age of challenge | lesion-scores in % of the maximal possible score Airsacculitis | Pneumonia |
|---|---|---|---|---|
| Mineral-oil-adjuvant | 10 | 18.0 (±2.4)[a] | 22.5%[a] | 5%[a] |
| Corn-oil-adjuvant | 10 | 7.1 (±2.0)[b] | 45%[b] | 5%[a] |
| Saponin-adjuvant | 10 | 9.1 (±1.7)[b] | 37.5% [b] | 0%[a] |
| Challenge controls | 10 | Not tested | 65%[c] | 30%[b] |
| ND controls | 10 | Not tested | 20%[a] | 0%[a] |
| Negative controls | 10 | ≤ 4.0[c] | 0%[d] | 0%[a] |

Within columns, values having different superscripts are significantly different ($p<0.05$)

Figure 1. Average serological response of broiler-breeders against *O. rhinotracheale* serotype A. Breeders were vaccinated with *O. rhinotracheale* strain B3263/91 bacterin in mineral-oil-adjuvant at 12 and 18 weeks of age. ■ = vaccinated group; □ = control group.

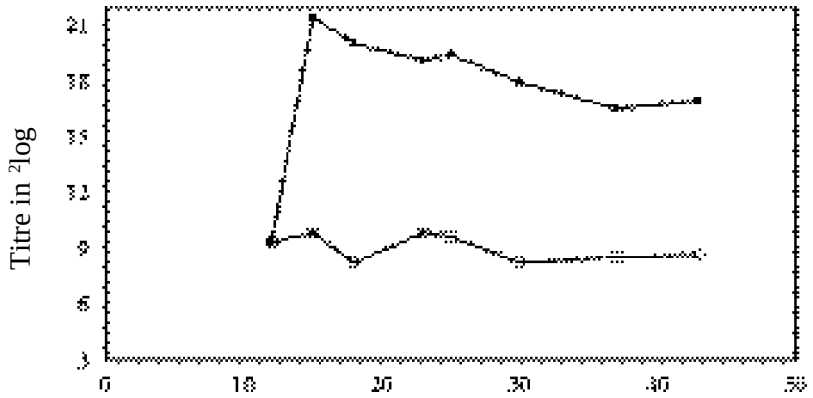
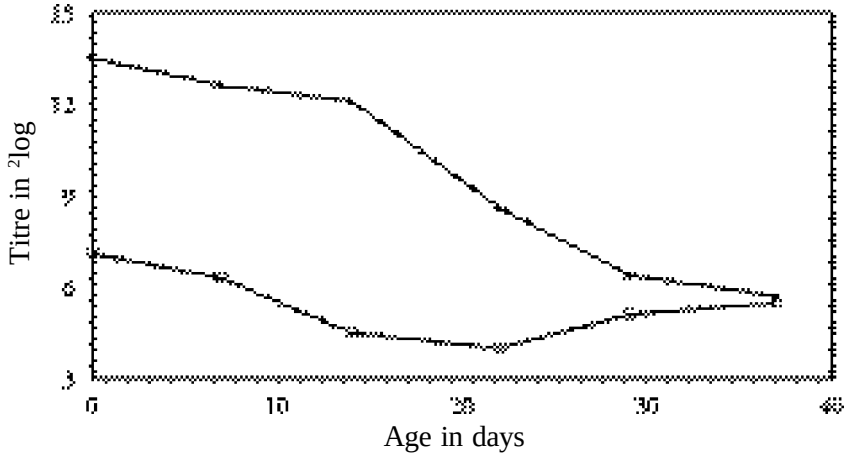


Figure 2. Average *O. rhinotracheale* serotype A titres of broilers, hatched from eggs layed by 37 weeks old breeders. Breeders were vaccinated with *O. rhinotracheale* strain B3263/91 bacterin in mineral-oil-adjuvant at 12 and 18 weeks of age.
■ = broilers out of eggs layed by the vaccinated group; □ = broilers out of eggs layed by the control group.




Vaccination of broiler-breeders with a bacterin in mineral-oil-adjuvant did induce enough maternal antibodies in their progeny to give good protection against challenge with *O. rhinotracheale* at 14 days of age and still a significant protection at 30 days of age (Table 3).

**Experiment 4.** The live vaccine strain did not induce any disease as judged at the post-mortem investigation of vaccinated birds seven days post vaccination. The results with regard to antibody responses and protection are shown in Table 4. Maternal antibodies were measured at one day of age. Live vaccination of one-day-old broilers did not induce a significant protection against experimental *O. rhinotracheale* infection and the serological response was low and not significant. Vaccination at 7 days of age induced a significant protection and serological response. A very good protection was obtained by the vaccination at 14 days of age.

Table 3 : Post-mortem results of experiment 3, using an inactivated *O. rhinotracheale* vaccine in oil-adjuvant in broiler-breeders at 12 and 18 weeks of age and challenges of hatched broilers at various ages.

| Broiler-breeders | | Broilers | | | Lesion-scores in % of the maximal possible score. | |
|---|---|---|---|---|---|---|
| Treatment | Age lay | Challenge | avg. ELISA titre (in $^2$log) at age of challenge | Age at time of challenge | Airsacculitis | Pneumonia |
| Control | 32 weeks | None | 5.1 (±1.1[a] | 15 days | 0%[a] | 0%[a] |
| Control | 32 weeks | ND | Not tested | 15 days | 25%[b] | 0%[a] |
| Control | 32 weeks | ND +*O. rhinotracheale* | Not tested | 15 days | 100%[d] | 50%[b] |
| Vaccinated | 32 weeks | ND + *O. rhinotracheale* | 13.3 (±1.6)[b] | 15 days | 40%[c] | 0%[a] |
| Control | 37 weeks | None | 4.7 (±0.8)[a] | 15 days | 0%[a] | 0%[a] |
| Control | 37 weeks | ND | Not tested | 15 days | 17.5%[b] | 0%[a] |
| Control | 37 weeks | ND +*O. rhinotracheale* | Not tested | 15 days | 72.5%[c] | 35%[b] |
| Vaccinated | 37 weeks | ND + *O. rhinotracheale* | 12.3 (± 1.7)[b] | 15 days | 17.5 %[b] | 0%[a] |
| Control | 37 weeks | None | 5.3 (± 1.0)[a] | 30 days | 0%[a] | 0%[a] |
| Control | 37 weeks | ND | Not tested | 30 days | 28%[b] | 0%[a] |
| Control | 37 weeks | ND + *O. rhinotracheale* | Not tested | 30 days | 97.5%[d] | 50%[b] |
| Vaccinated | 37 weeks | ND + *O. rhinotracheale* | 6.9 (± 1.0)[b] | 30 days | 57.5%[c] | 2.5 %[a] |

Within columns of the 3 groups, values having different superscripts are significantly different ($p<0.05$)

Table 4 : Post-mortem results of experiment 4 (live *O. rhinotracheale* vaccine in broilers given at various ages). ND administration 7 days post vaccination, challenge 14 days post vaccination, post-mortem 21 days post vaccination.

| Age at vaccination | avg. ELISA titre (in $^2$log) at age of vaccination | Treatment | avg. ELISA titre (in $^2$log) at age of challenge | Lesion-scores in % of the maximal possible score. Airsacculitis | Pneumonia |
|---|---|---|---|---|---|
| 1 day | 7.6 (±1.8) | Vaccinated | 6.2 (±1.3) | 50% | 16.7%[a] |
| 1 day | | Control | 5.3 (±0.5) | 62.5% | 31% |
| 7 days | 5.8 (±1.4) | Vaccinated | 8.1 (±1.1)[a] | 46%[a] | 4%[a] |
| 7 days | | Control | 5.2 (±0.3) | 93% | 43% |
| 14 days | 5.1 (±0.4) | Vaccinated | 9.5 (±1.6)[a] | 11%[a] | 0%[a] |
| 14 days | | Control | 4.6 (±0.5) | 95% | 40% |

[a] Value significantly different from the value of the control group ($p<0.05$).

## DISCUSSION

In the experiment in which one-day-old broilers were vaccinated and where moderate antibody titres were found at time of vaccination, the vaccine in mineral-oil-adjuvant was the only vaccine able to induce good protection. This in spite of the low to moderate serological response at time of challenge. The same vaccine was able to induce good protection and very high antibody titres, when used in birds whithout antibodies at time of vaccination, as proven in the experiment with 3-weeks-old SPF leghorns. As compared with the mineral-oil vaccine, poor protection was induced by the corn-oil and saponin based vaccines but it appeared that these vaccines performed relatively better in leghorns without antibodies at time of vaccination than in broilers in which antibodies were present at time of vaccination. Furthermore, the results with the live vaccine showed that serological and protective responses increased with age at vaccination and with decrease of maternal antibody levels at time of vaccination. These results might indicate that maternal antibodies have a negative influence on vaccine efficacy of non-mineral-oil vaccines, although at present also a difference in immunocompetence between the chickens used in the various experiments can not be excluded.

Only the mineral-oil-adjuvant was found to give a good protection in young birds even when maternal antibodies were present. However, a disadvantage of vaccinating one-day-old birds with bacterins in mineral-oil-adjuvants is that the vaccination on its own can have a negative effect on the performance of the birds and induce local tissue reactions. Out of these findings one can conclude that vaccination of one-day-old broilers in the field, where maternal antibodies are widespread and therefore a mineral-oil-adjuvant will be needed, only will be considered in case of emergency. This means that another strategy should be followed to prevent *O. rhinotracheale* infections in broilers.

In the vaccination experiment of broiler-breeders it was found that the bacterin in mineral-oil-adjuvant induced high and longstanding responses in the broiler-breeders. The serological response at the start of the experiment, the strong serological response after the first vaccination and the lack of booster effect after the second vaccination support the idea that most probably the flock already had been in contact with *O. rhinotracheale*. If so, it means that the first vaccination already reacted as a booster on the former infection, indicating that two vaccinations will be needed in breeders that had not been in contact with *O. rhinotracheale* before the vaccination. The vaccinations induced high levels of maternal antibodies in the progeny resulting in good protection against experimental challenge of the hatched broilers at an age up to 30 days of age. However, protection decreased with increasing age of the broilers,

since protection at 15 days was better than protection at 30 days of age. As mentioned before, the efficacy of live vaccination of broilers increased with the age at time of vaccination. Therefore, the most practical approach in combating *O. rhinotracheale* infections in broilers seems to be breeder vaccination with an inactivated vaccine combined with live vaccination of broilers at 2-3 weeks of age. However, the efficacy of live vaccination of broilers hatched from vaccinated breeders still needs to be determined.

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CHAPTER 6

# General discussion and conclusions

## GENERAL DISCUSSION AND CONCLUSIONS

The objective of the research discribed in this thesis was to investigate the bacterium *Ornithobacterium rhinotracheale*, the disease it is causing in fowl, the epidemiology and the possible ways of prevention of the disease.

*O. rhinotracheale* has been isolated from several bird-species with respiratory infections in different parts of the world. The cause of the disease was hard to determine because the isolation of *O. rhinotracheale* is problematic, especially since *O. rhinotracheale* is often overgrown by other bacteria. Therapeutic treatment of the disease was found to be difficult and sometimes even impossible because many *O. rhinotracheale* strains are resistant to the regularly used antibiotics. Economic losses are hard to estimate because the disease is often just slumbering with increased mortality rates of 2 - 10% and growth retardation which is difficult to determine. Only in older turkeys losses are dramatic with mortality rates up to 50% in a few days.

The first goal of this study was to fulfil the three postulates of Koch and thus prove that *O. rhinotracheale* is capable to cause the disease seen in the field. Experiments with *O. rhinotracheale* alone, in which several challenge-routes were investigated, revealed only growth retardation but no visible respiratory infections. However, when birds were treated with avian respiratory viruses prior to an aerosol infection with *O. rhinotracheale*, the bacterium was found to be able to induce growth retardation as well as airsacculitis and pneumonia in both chickens and turkeys (Chapter 2). Clinically the features of this infection were identical to those observed in the field. Later on, with use of the immuno-histochemical peroxidase-anti-peroxidase (PAP) staining, we proved beyond any doubt that *O. rhinotracheale*, and not the virus, was the cause of the lesions in the air sacs (Chapter 4B). With the same method it could be proven that a (viral) trigger is needed to induce lesions in air sacs and/or lungs caused by *O. rhinotracheale*. Without the trigger, using the same challenge dose, *O. rhinotracheale* was not infectious and could not been demonstrated at time points later than 2 days post challenge (Chapter 4B).

Not only viruses but also bacteria such as *Escherichia coli* (3) and *Bordetella avium* (2) are able to trigger an *O. rhinotracheale* infection. Up to now only one report is known in which *O. rhinotracheale* was found to be pathogenic after intratracheal inoculation in 22 weeks old turkeys without a second infection in the birds (4). Because the immune status of the birds in this study was not known, nor the possible presence of agents able to trigger *O. rhinotracheale* infections, we can not conclude from these studies that *O. rhinotracheale* is a primary pathogen.

The second goal was to prove that *O. rhinotracheale*, which had not yet been named by that time, is a new bacterial species that is pathogenic for fowl. Therefore growth conditions had to be optimized, a reasonable number of field strains had to be collected and the necessary tests had to be performed to distinghuish *O. rhinotracheale* from other bacterial species with which it could be confused. It could be proven biochemically and serologically that *O. rhinotracheale* was a new bacterial species and a new fowl pathogen (Chapter 3A). Vandamme *et al.* (5) provided genetic *evidence for this* new species and proposed the name of the bacterium. In total about 1100 strains were investigated biochemically and serologically. As result of these studies an identification method could be developed which appeared to be highly specific and practical (Chapter 3A). Another result was that, up to now, 9 different serotypes of *O. rhinotracheale* can be distinghuished (Chapters 3A and 3B).

Investigation of the genomic DNA of selected strains by amplified fragment length polymorphism gave an indication for the existence of more subspecies within *O. rhinotracheale* or even new species within the genus *Ornithobacterium* (Chapter 3B). In contrast to these findings, results of other studies, such as PCR (1, Chapter 3B), cellular-protein profiles (5) and outer-membrane-protein profiles (Chapter 3B), show that the reactions of the *O. rhinotracheale* strains investigated were very homogeneous. More study will be required to clarify these contradictions. Nevertheless it seems that *O. rhinotracheale* was only recently introduced into domestic poultry and that the isolates recovered throughout the world belong to a small group of closely related clones.

The third goal was to learn more about the disease and the infection route. During the infection studies indications were found that *O. rhinotracheale* can be transmitted via eggs. This could be the explanation for the global spread of *O. rhinotracheale* within a few years, since eggs are distributed worldwide. Egg-transmission was demonstrated by hatching eggs from suspected flocks. The hatched birds were kept in isolation and treated with viruses that had been proven to be able to trigger *O. rhinotracheale* infections. *O. rhinotracheale* was isolated from air sacs of such birds (Chapter 4A). *O. rhinotracheale* could also be isolated, although sporadically, from egg-shells and yolk-sacs of one-day-old birds (Chapter 4A).

The possibility of egg-transmission together with the fact that almost all *O. rhinotracheale* can be placed in a small group of closely related clones and the fast global spread of the disease, supports the idea that the bacterium is a new mutant or that the bacterium was only recently introduced into the domestic poultry population e.g. from wild birds.

The course of the respiratory disease could be followed with the histo-immunochemical PAP-staining (Chapter 4B). After a viral priming, *O. rhinotracheale* was found to infiltrate the epithelium of the air sacs and to cause an acute granulomatous airsacculitis. Also an infiltration of some, but not all, parts of the bronchial associated lymphoid tissue in the lungs by *O. rhinotracheale* could be demonstrated. *O. rhinotracheale* cells or cell-fragments were often associated with macrophages. The disease peaked 5 - 9 days post challenge, after which birds recovered fast. The bacterium was not demonstrated in other tissues than those of the respiratory tract.

Without viral priming, *O. rhinotracheale* disappeared within two days after challenge but the serological reactions in these birds were as strong and longlasting as those found in heavily infected birds (Chapter 4B). This means that *O. rhinotracheale* may hide somewhere else in the body or that it stimulates the immune-system in another way. If *O. rhinotracheale* can invade cells (e.g. macrophages, where most of the reaction was found with the immuno-histochemical staining), even without viral priming than there is a good chance that *O. rhinotracheale* is able to survive intracellularly. In this way the bacterium could hide itself somewhere in the body. This may also explain how *O. rhinotracheale* causes infections, as soon as the immune-system is suppressed, or tissues are damaged e.g. by a virus.

The fourth goal was to prevent infections caused by *O. rhinotracheale*. The result of vaccination of one-day-old broiler-chickens with bacterins was found to depend on the kind of adjuvant used (Chapter 5). Only vaccines in mineral-oil-adjuvant showed good protection. The other adjuvants tested were not able to induce a reasonable protection, most probably due to the maternal antibodies which are detected in almost all tested commercial birds. This poses a problem since the use of mineral-oil-adjuvants is known to have negative effects on the broilers.

Vaccination of broiler-breeders with a bacterin of *O. rhinotracheale* in mineral-oil-adjuvant resulted in high and long lasting protection and serological responses (Chapter 5). A good protection of the progeny of these animals against experimental challenge with *O. rhinotracheale* was also observed. Protection of broilers, hatched from eggs layed by vaccinated broiler-breeders, lasted up to about 4 weeks of age, then it decreased.

Live vaccination of young broilers with *O. rhinotracheale* was found to be feasible (Chapter 5). The protection against experimental challenge with *O. rhinotracheale* increased with the age at time of vaccination and was negatively related to the amount of maternal antibodies.

The conclusion of the vaccination trials sofar is that vaccination of one-day-old broilers will not be feasible, exept for emergency cases where mineral-oil-adjuvant bacterins can be of use in spite of the possible local tissue reactions. The best approach

to prevent infections of *O. rhinotracheale* in broilers is vaccination of broiler-breeders with an inactivated vaccine in combination with live vaccination of broilers at 2 to 3 weeks of age. The same strategy can be used for turkeys, although, because turkeys are handled at older ages, it may also be possible to use inactivated vaccines with other adjuvants at 2 to 3 weeks of age.

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# Samenvatting

Het doel van de in dit proefschrift beschreven studie was het onderzoeken van de bacterie *Ornithobacterium rhinotracheale*, de ziekte die deze bacterie veroorzaakt, de epidemiologie van de ziekte en de preventie van de ziekte. Bij de start van het onderzoek was er nog niets bekend over de bacterie en het ziektebeeld. De eerste berichten over luchtwegaandoeningen in pluimvee, veroorzaakt door een nog niet geïdentificeerde *Pasteurella*-achtige bacterie kwamen uit Zuid Afrika in 1991, waar de bacterie uit ontstoken luchtzakken van mestkuikens geïsoleerd kon worden. Nader onderzoek leerde dat deze bacterie, biochemisch zowel als uiterlijk, grote overeenkomst vertoonde met een bacterie die in 1987 geïsoleerd was uit een eend met longontsteking in Hongarije en een bacterie die begin 1992 geïsoleerd was uit een ontstoken luchtzak van een kalkoen.

Het leveren van het bewijs dat *O. rhinotracheale* de veroorzaker van de luchtweg-aandoeningen in pluimvee was werd de eerste doelstelling. Hiervoor werden besmettings-proeven in kippen en kalkoenen uitgevoerd, waaruit bleek dat de bacterie op zichzelf het ziektebeeld niet kon opwekken. Pas nadat een dier behandeld was met virussen, die in staat zijn lichte luchtwegaandoeningen in pluimvee op te wekken, kon met een aerosol toediening van *O. rhinotracheale* het ziektebeeld geinduceerd worden. Later kon met behulp van een immuno-histochemische kleuring onomstotelijk worden bewezen dat *O. rhinotracheale* de veroorzaker van de luchtweg infectie was en dat de virale behandeling vooraf noodzakelijk was voor het opwekken van het ziektebeeld.

Als tweede doel werd gesteld dat bewezen moest worden dat *O. rhinotracheale* een nieuw bacterie-species en/of een nieuwe pathogeen voor pluimvee was. Nadat de groei condities geoptimaliseerd waren kon, serologisch zowel als biochemisch, worden bewezen dat *O. rhinotracheale* niet behoorde tot een bekend bacterie-species of pluimvee-pathogeen. Door anderen is dit ook genetisch bewezen en werd de naam *Ornithobacterium rhinotracheale* aan de bacterie gegeven. Er werd een stammen collectie opgezet en met behulp daarvan werd een identificatie methode ontwikkeld en konden 9 serotypes onderscheiden worden.

Onderzoek naar het DNA van relevante stammen wees uit dat er de mogelijkheid bestaat dat *O. rhinotracheale* onderverdeeld kan worden in subspecies of zelfs dat het genus *Ornithobacterium* onderverdeeld dient te worden in verschillende species. Echter andere testen, zoals PCR en het bepalen van het eiwit-patroon van de buiten

membranen, gaven een grote homogeniteit binnen de onderzochte stammen aan. Dit laatste wijst erop dat *O. rhinotracheale* een recente mutant is of dat de bacterie slechts kortgeleden, via bijvoorbeeld contact met wild gevogelte, geïntroduceerd is in de commerciele pluimvee wereld.

De derde doelstelling was het in kaart brengen van de infectie route en een beter inzicht krijgen over de manier hoe *O. rhinotracheale* de infectie veroorzaakt. Er kon worden aangetoond dat, naast de route via de lucht, *O. rhinotracheale*, en daarmee ook de ziekte, ook via het ei kan worden overgebracht. Dit verklaart waarschijnlijk de snelle verspreiding van de ziekte (binnen enkele jaren werd de bacterie in 4 continenten aangetroffen) omdat bevruchte eieren over de hele wereld verstuurd worden.

Een experimenteel opgewekte infectie werd bacteriologisch en serologisch gevolgd en het ontstaan van het ziektebeeld werd bekeken met behulp van een immunohistochemische methode. Hieruit kwam naar voren dat *O. rhinotracheale* waarschijnlijk in staat is op een schuilplaats binnen het lichaam van het dier te overleven van waaruit hij, na bijvoorbeeld een virale infectie, het ziektebeeld kan op wekken.

De vierde doelstelling was het voorkomen van de ziekte, veroorzaakt door *O. rhinotracheale,* door middel van vaccinatie. Het succesvol vaccineren met bacterins bleek afhankelijk van het soort adjuvant dat gebruikt werd. Ook bleek de aanwezigheid van (maternale) antistoffen te interfereren met de vaccinatie, hoe meer antistoffen er aanwezig waren op het tijstip van vaccinatie hoe minder bescherming er geinduceerd werd. Het vaccineren van eendags mestkuikens was alleen succesvol als er een minerale olie als adjuvant gebruikt werd. Maar, omdat vaccinatie met vaccins in minerale olie op zichzelf al schadelijke gevolgen voor het dier kan hebben, zoals lokale entreacties en groeiachterstand, zal vaccinatie met dit vaccin alleen in noodgevallen in eendags kuikens gebruikt kunnen worden.

Vaccinatie van mestkuiken-moederdieren was succesvol m.b.t. de bescherming van de nakomelingen van gevaccineerde dieren in de eerste weken van hun leven. Het reduceren van de spreiding van *O. rhinotracheale*, bijvoorbeeld d.m.v. eieren, kon niet bewezen worden omdat de incidentie van *O. rhinotracheale* laag en de isolatie zeer moeilijk is.

Vaccinatie met levende *O. rhinotracheale* cellen induceerde een goede bescherming in jonge mestkuikens. Ook hier bleek dat het resultaat van de vaccinatie afhankelijk was van de hoeveelheid (maternale) antistoffen op het tijdstip van vaccinatie.

De combinatie van het enten van mestkuiken-moederdieren met een bacterin en een levende vaccinatie van de mestkuikens op 2 - 3 weken leeftijd lijkt de beste methode ter voorkoming van de ziekte in mestkuikens.

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# Curriculum vitae

De schrijver van dit proefschrift werd geboren op 23 juli 1954 te Helvoirt (N-Br.). In 1973 werd het H.A.V.O. diploma behaald aan het St. Janslyceum te ‘s Hertogenbosch. Na een tweejarige opleiding tot derdegraads leraar Natuurkunde-Scheikunde aan het Moller instituut te Tilburg, werd in 1978 het diploma HBO-A Medisch Microbiologisch analist behaald aan de Hogeschool te Eindhoven. Hetzelfde jaar trad hij in dienst als bacteriologisch analist bij de afdeling Controle en Standaardisatie van het Centraal Diegeneeskundig Instituut te Rotterdam. In 1981 werd het diploma HBO-B Biologisch analist behaald aan het van ‘t Hoff instituut te Rotterdam. Vanaf 1982 werkt de auteur op de afdeling Bacteriologische Research bij Intervet International B.V. te Boxmeer, eerst als bacteriologisch analist, later als wetenschappelijk laboratorium assistent en momenteel als assistent projectleider.

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