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Investigations on the viability as well as on the molecular and serological characterization of *Ornithobacterium rhinotracheale* (ORT) isolates

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I *dedicated this thesis to*

M*y family…*

for their constant supports and unconditional love.

M*y teachers and all the experimental animals*

for their sacrifices.

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Abbreviations

AFLP	amplified fragment length polymorphism
AGP	agar gel precipitation test
APV	avian metapneumovirus
ATCC	American type culture collection
bp	base pair
°C	degree Celsius
CFU	colony forming unit(s)
NCCLS	national committee for clinical laboratory standards
DIA	dot immunobinding assay
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunosorbent assay
IBV	infectious bronchitis virus
kbp	kilobase pair
LPS	lipopolysaccharide
MLEE	multilocus enzyme electrophoresis
NDV	Newcastle disease virus
OMP	outer membrane protein
ORT	*Ornithobacterium rhinotracheale*
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
pH	positive potential of the hydrogen ions
p.i.	post infection/ post inoculation

pmol	picomol
PGNR	pleomorphic gram-negative rod
ppm	parts per million
RAPD	random amplified polymorphic DNA
RAT	rapid agglutination test
rRNA	ribosomal ribonucleic acid
Rep-PCR	repetitive extragenic palindromic-PCR
rpm	rounds per minute
SAT	serum agglutinations test
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophorese
SE-AFLP	single-enzyme amplified fragment length polymorphism
SPAT	serum plate agglutination test
SPF	specific pathogen free
TRT	turkey rhinotracheitis

1. INTRODUCTION

Respiratory disease conditions are continuing to cause heavy economic losses in the poultry industry by increased mortality rates, increased medication costs, increased condemnation rates at slaughter, drops in egg production, reduction of egg shell quality, and decreased hatchability.

The severity of clinical signs, duration of the disease and mortality are extremely variable and are influenced by many factors such as a virulence and pathogenicity of the infectious agent as well as by many environmental factors. Many infectious agents can cause respiratory diseases in poultry such as fungi (Akan et al., 2002; França et al., 2012), viruses (Alexander, 2000; Boroomand et al., 2012; Chansiripornchai et al., 2007; Higgins, 1971; Ignjatovic et al., 2002; Ip et al., 2012; Lee et al., 2012; McFerran and Adair, 1977; Swayne et al., 2001) and bacteria (Blackall, 1999; da Rocha et al., 2002; Noormohammadi et al., 2002; Pruimboom et al., 1996).

Since December 1991 a respiratory disease with different clinical causes has been observed in poultry flocks in different countries (Charlton et al., 1993; Du Preez, 1992; Hafez et al., 1993; van Beek et al., 1994). Bacteriological examinations have resulted in isolation of slowly growing, pleomorphic gram-negative rod (PGNR). Initially, the bacterium was designated as Pasteurella-like, Kingella-like, Taxon 28, or pleomorphic gram-negative rod before the name *Ornithobacterium rhinotracheale* gen. nov. sp. nov. in the rRNA-Superfamily V was suggested (Hafez and Vandamme, 2011; Vandamme et al., 1994).

Currently infections with *Ornithobacterium rhinotracheale* (*O. rhinotracheale,* ORT) occur worldwide and *O. rhinotracheale* is incriminated as a possible causative agent in respiratory disease either alone (mono-causal) or in synergy with different other micro-organisms (multi-causal). Moreover non-infectious factors such as poor management, inadequate ventilation, high stocking density, poor litter conditions, poor hygiene, and high ammonia level are concurrent causes that increase the severity of the disease (Bock et al., 1997; Chin et al., 2003; de Rosa et al., 1996; Hafez, 1998; Odor et al., 1997; van Beek et al., 1994; Vandamme et al., 1994).

O. rhinotracheale has been isolated from chickens, chukar partridges, ducks, geese, guinea fowl, gulls, ostriches, partridges, pheasants, pigeons, quail, rooks, and turkeys (van Empel and Hafez, 1999).

Currently 18 different serotypes designated A to R have been identified (Chin et al., 2008; van Empel and Hafez, 1999). Serotyping can be done with reference antisera using agar gel precipitation test (AGP) or enzyme linked immunosorbent assay (ELISA) (Hafez and Sting, 1999; van Empel et al., 1996; Vandamme et al., 1994). However, AGP is the method of choice for serotyping.

Since clinical signs and post-mortem lesions of *O. rhinotracheale* infections are not sufficiently specific to allow diagnosis, laboratory methods are needed for definite diagnosis. While detection of nucleic acids using polymerase chain reaction (PCR) is reliable and fast (Hassanzadeh et al., 2010), isolation of the bacterium is necessary for serotyping, to determine the antimicrobial susceptibility for an effective therapy, and to produce autogenous vaccines. However, many factors can interfere with isolation of *O. rhinotracheale* such as the time of sampling, presence of secondary infections, and shipment from farm to the diagnostic laboratory, but currently there is no information about optimal conditions for collection and storage conditions for the samples to be transported to laboratory available.

Consequently the **first** objective of this present thesis was to test different transport media and storage temperatures for their ability to allow reisolation of *O. rhinotracheale*. Further the influence of the amount of *O. rhinotracheale* used to inoculate transport swabs and of concurrent inoculation with *Escherichia coli* (*E. coli*) was investigated.

The **second** part of the thesis compiled information about the isolation and diagnosis by PCR at the institute of poultry diseases, Free university Berlin. *O. rhinotracheale* isolates were typed by AGP. Further partial 16S ribosomal RNA (rRNA) genes and the complete Or01 genes of selected field isolates and of reference strains A to H were sequenced and compared in order to investigate if typing by AGP might be replaced by the sequence analysis.

2. LITERATURE

2.1. Definition

O. rhinotracheale infections can cause acute highly contagious diseases in poultry, which can be associated with high economic losses due to an increase in mortality rates, condemnation rates, drop in egg production or due to a decrease of the performance results. Up to now, *O. rhinotracheale* has not been found to be of public health significance. Within this bacterial species several serotypes and isolates with different virulence do exist (Chin et al., 2008).

2.2 History

In 1991 a new respiratory disease in broiler chickens was observed in South Africa by Jan DuPreez (van Beek et al., 1994). The clinical signs observed were relatively mild respiratory symptoms starting with sneezing at an age of 28 days, which lasted up to the end of the fattening period. The respiratory signs were accompanied by increased mortality and poor performances, e.g. daily growth rate and feed conversion ratio. At post mortem investigation foamish, white, “yoghurt like” exudates in the air sacs, predominantly in the abdominal air sac and also pneumonia were observed.

Clinical findings in several turkey as well as broiler flocks in Germany, the Netherlands, and USA included sniveling, sneezing, wet eyes, and swelling of the infraorbital sinus, together with severely decreased growth and slightly increased mortality (Charlton et al., 1993; Hafez et al., 1993; van Beek et al., 1994). From all above mentioned outbreaks a pleomorphic gram-negative rod was isolated and later identified as *Ornithobacterium rhinotracheale* (Vandamme et al., 1994).

A retrospective study showed that antibodies against *O. rhinotracheale* already were present in European poultry since the 1980`s. The early history *of O. rhinotracheale* was described by Hinz and Hafez (1997). Briefly, in September 1981 in northern Germany respiratory signs were observed in 5-weeks-old turkey poults characterized by nasal discharge, facial edema, and fibrino-purulent airsacculitis. A slow growing, pleomorphic, gram-negative rod, phenotypically related to *Pasteurella (Riemerella) anatipestifer* and *E. coli* was isolated from the respiratory tract. This unknown organism was also cultured from the trachea of young

rooks in 1983. Beichel (1986) excluded this strain from the family *Pasteurellacae*, and Mouahid et al. (1992) found significant evidence for a taxonomic relationship to the *Flavobacterium-Cytophaga* complex. Between 1986 and 1988 several outbreaks of diseases in turkey breeding flocks were observed in England. These outbreaks were marked with general depression, drop in egg production, coughing in 1-5% of the birds, and low mortality. At post-mortem investigation lesions such as fibrinous airsacculitis and pneumonia were observed. Bacteriological investigation again showed an unidentifiable bacterium. In 1988 this bacterium was investigated in Denmark by Bisgaard (1992), who found that it did not belong to the family *Pasteurellaceae* and designated it as Taxon 28. In 1988 and later on further strains of this bacterial organism were isolated from turkey and partridges in Belgium (Wyffels and Hommez, 1990). This bacterium also could not be classified into any of the known bacterial species but was found to be identical to bacteria isolated from respiratory diseases in ducks (1987, Hungary). In 1994 *O. rhinotracheale* was named by Vandamme et al. (1994) and it experimentally could be proven that *O. rhinotracheale* was able to cause diseases in poultry (van Empel et al., 1996).

Currently, infection with *O. rhinotracheale* has been recognized in many countries worldwide and is incriminated as a possible additional causative agent in respiratory disease complexes in poultry. Several surveys showed that the majority of chicken and turkey flocks in Europe, Africa, North and South America and some Asian countries have been in contact with *O. rhinotracheale* (Allymehr, 2006; Arns et al., 1998; Canal et al., 2003; Canal et al., 2005; Dudouyt et al., 1995; El-Gohary, 1998; El-Sukhon et al., 2002; Hafez and Friedrich, 1998; Hinz et al., 1994; Koga and Zavaleta, 2005; Misirlioglu et al., 2006; Naeem et al., 2003; Sakai et al., 2000; Soriano et al., 2002; Tanyi et al., 1995; Travers et al., 1996; Turan and Ak, 2002).

2.3 Taxonomy of the genus Ornithobacterium

O. rhinotracheale belongs to the phylum *Bacteroidetes,* class *Flavobacteria,* order *Flavobacteriales* family Flavobacteriaceae, and the Genus *Ornithobacterium* (Vandamme et al., 1994). Initially *Riemerella columbina, Ornithobacterium rhinotracheale* and *Coenonia anatina* were recognized in the of long-term studies on the etiology of respiratory tract infections in birds as phenotypically unusual isolates (Segers et al., 1993). Moreover, the reclassification of the organism known as *Pasteurella anatipestifer* or *Moraxella anatipestifer* as *Riemerella anatipestifer* (Segers et al., 1993) triggered a series of taxonomic studies

leading to the stepwise characterization and description of *O. rhinotracheale* (Vandamme et al., 1994), as well as *C. anatine* and *R. columbina* (van Empel and Hafez, 1999). The formal description of *O. rhinotracheale* led to a lot of studies and researches on this bacterium because of its acknowledgement as an economically important pathogen in turkey and chicken husbandry. Nowadays *Riemerella, Ornithobacterium* and *Coenonia* are thought to belong to the same major phylogenetic linage, now known as the family Flavobacteriaceae (Bernardet and Bowman, 2006; Bernardet et al., 2002; Bernardet et al., 1996).

2.4 Etiology and Colony Morphology

O. rhinotracheale is a Gram-negative, non-motile, pleomorphic, rod-shaped, non-sporulating bacterium. Its colonies are characterized by a circular, grey to grey- white color, sometimes with a reddish glow (van Empel and Hafez, 1999). They are convex with an entire edge (Devriese et al., 2001; Erganis et al., 2002; Murthy et al., 2008; Roepke et al., 1998; van Empel et al., 1997). Usually it is considered to be not haemolytic, but recently β-hemolytic activity has been revealed in field isolates in North America (Tabatabai et al., 2010) and Latin America (Churria et al., 2011).

No special structures or properties such as pili, fimbriae, or plasmids could be detected (Leroy-Setrin et al., 1998; van Empel and Hafez, 1999). For growth of the organism incubation on 5-10% sheep blood agar for at least 48 hours under micro-aerophilic conditions (5-10% CO_2) at 37°C is required (Chin et al., 2008; Erganis et al., 2002; van Empel et al., 1997; van Empel and Hafez, 1999). No growth occurs on MacConkey agar, Endo agar, Gassner's agar, Drigalski agar, and Simmon's Citrate media (Chin et al., 2008). Moreover, at the first isolation, most *O. rhinotracheale* cultures show a big difference in size of colonies from 1 to 3 mm after 48 hours of incubation; after subcultivation for 2-3 times, the colony size becomes more uniform (van Empel and Hafez, 1999). After several subcultivations, some strains may be adapted to growth under aerobic conditions, although growth is always significantly better under microaerobic conditions (van Empel and Hafez, 1999).

In liquid media, *O. rhinotracheale* are very variable in length (0.6 to 5 µm) and often form clusters, which can hold up to several thousands of organisms, but which can easily be disrupted (van Empel and Hafez, 1999). Not all strains of *O. rhinotracheale* will grow equally in liquid media and a rich medium such as Todd Hewitt broth or Brain Heart Infusion broth supplemented with serum, is required. *O. rhinotracheale* can be suppressed by overgrowth

by less fastidious bacteria in contaminated samples including *E. coli* (van Empel and Hafez, 1999). The G+C content of the genome of *O. rhinotracheale* strains is between 37 and 39 % (Vandamme et al., 1994).

2.4.1 BIOCHEMICAL PROPERTIES

The results of biochemical tests for the identification of *O. rhinotracheale* can be variable (Chin and Charlton, 2008; van Empel and Hafez, 1999). Accordingly, Chin and Charlton (2008) proposed the following tests as those with more consistent reactions to identify *O. rhinotracheale*: oxidase (+), catalase (-), β-galactosidase (+), indole (-), and triple sugar iron agar (no change) (Chin and Charlton, 2008). Most but not all *O. rhinotracheale* isolates react positively in urease test. In addition, Ryll et al. (2002) were able to isolate and identify a cytochrome-oxidase negative strain of *O. rhinotracheale* from turkeys in Germany. *O. rhinotracheale* cannot reduce nitrate to nitrite and does not grow on MacConkey agar.

van Empel et al., (1997) tested 1150 isolates using API 20 NE test-kit (Bio-Mérieux, France). The results revealed for 99.5 % of *O. rhinotracheale* isolates a reaction code of 022 000 4 (61 %) or 002 000 4 (38.5 %), differing only in the urease reaction. The other 0.5% reacted positively in the arginine dihydrolase (ADH) test, corresponding to reaction codes 0320004 or 0120004 (Chin et al., 2003). Another commercial system for the identification of this bacterium is the RapID NF Plus (Remel/Atlanta, USA). Testing 110 isolates resulted in five biocodes: 472264 (41.8%), 476264 (31.8%), 676264 (18.2%), 672264 (7.3%) and 472044 (0.9%), (Chin and Charlton, 2008; Post et al., 1997; van Empel and Hafez, 1999). Further identification could be accomplished by using API ZYM test system or fatty acid profile. Absolute fatty acids detected were 15:0 iso, 16:0, 15:0 iso 3OH, 17:0 iso, 16:0 3OH, 17:0 iso 3OH, and unknown peaks with equivalent chain lengths of 13.566 and 16.580 (Chin et al., 2008; Ryll et al., 2002; Vandamme et al., 1994).

2.4.2 SUSCEPTIBILITY TO CHEMICAL AND PHYSICAL AGENTS

O. rhinotracheale strains have been proven to be highly sensitive to different chemical disinfectants (van Empel and Hafez, 1999). In vitro, preparations based on different organic acids such as formic and glyoxyl acids and products containing different aldehydes at concentrations of 0.5% were able to inactivate *O. rhinotracheale* on bacterial carriers (lime wood blocks) within 15 minutes (Hafez and Schulze, 2003). The ability of *O. rhinotracheale*

to remain viable in poultry litter was studied at different temperatures over time. The results indicated that *O. rhinotracheale* survived for 1 day at 37°C, 6 days at 22°C, 40 days at 4°C, and at least 150 days at -12°C. *O. rhinotracheale* did not survive for 24 hour at 42°C. The survival of *O. rhinotracheale* at lower temperatures may be associated with the higher incidence of *O. rhinotracheale* infection in poultry during winter months (Lopes et al., 2002b).

2.4.3 STRAIN CLASSIFICATION AND CHARACTERISTICS

18 serotypes (A through R) of *O. rhinotracheale* have been determined by using boiled extract antigens (BEAs) and monovalent antisera in AGP or in ELISA (van Empel et al., 1996). Most of the chicken isolates belong to the serotype A, while turkey isolates are more heterogeneous and belonged to serotype A, B and D (Hafez, 2002; van Empel, 1998; van Empel et al., 1996). Van Empel et al. (1997) investigated 443 *O. rhinotracheale* strains which were isolated between 1987 and 1996 from chickens and turkeys in France, Germany, Israel, The Netherlands, United Kingdom, or the United States. 97 % of them belonged to the four major serotypes A, B, D, and E (van Empel et al., 1997). In these, serotype A was found to be the most prevalent serotype among chicken strains (94%), and turkey strains (57%)(van Empel et al., 1997). No relation was found between host specificity and the *O. rhinotracheale* serotype (van Empel et al., 1997). Some geographical differences between the prevalence of serotypes can found. Most of the isolates in the United States and Europe belong to serotype A (Chin and Charlton, 2008; van Empel et al., 1997), while serotype C has been found only in chickens and turkeys of South Africa and the United States (Chin et al., 2008). Serotypes B, D, and E are the predominant ones after serotype A in Europe (Chin and Charlton, 2008; van Empel et al., 1997). In another study 88 German *O. rhinotracheale* isolates, which were collected between 2003 and 2006, were classified by AGP as serotypes A, B, C, D, E, H, I, or J, while 29 tested French isolates were classified as serotypes A, C, I, or J. Within this heterogeneous distribution the serotype A appeared most often (Waldow, 2009). The frequent occurrence of serotype C within the German (14.8%) and the French (10.3%) isolates was surprising. In both groups isolates showing cross reactions occurred (Waldow, 2009). Comparison of the 16s rRNA sequences showed that all Taiwanese pigeon isolates formed a distinct cluster. The chicken isolates from Taiwan were also set apart from isolates from other countries (Tsai and Huang, 2006). Recently, Gutzer et al. (2011) investigated tracheal swabs collected from birds of prey in Germany in aim to determine the presence of *O. rhinotracheale*. The results of investigation using PCR revealed that *O. rhinotracheale*-DNA could be detected in 22 out of 93 examined samples

from birds of the family Accipitridae. From all samples only two *O. rhinotracheale* strains were isolated and serotyped as serotype J and F using AGP. On the other hand, 25 of examined 34 samples from birds of family Falconidae were tested positive by PCR and 5 strains were isolated from which 2 were of serotypes A and 2 of serotypes H and one of serotype J (Gutzer et al., 2011).

van Empel (1998) suggested that *O. rhinotracheale* strains can be divided into more species or subspecies by a special method such as the random-amplification-fragment length-polymorphism (AFLP) method. Popp and Hafez (2003) carried out an investigation to compare *O. rhinotracheale* strains from different countries and species using serological typing as well as pulsed-field gel electrophoresis. In the AGP of the 77 isolates from turkeys, 17 belonged to serotype A and 18 to serotype B. 14 of the serotype A isolates were from Germany, 2 from Hungary and 1 from Austria. The serotype B isolates originated from Germany (16) and Israel (2). The remaining isolates from Germany (42) belonged to other serotypes and showed partial cross-reactions with other serotypes. The majority (21) of the 24 tested isolates from chicken were serotype A. The isolates were analysed by pulsed-field gel electrophorese (PFGE) using the enzyme *SalI*. Each of the 17 investigated standard serotype strains (A - Q) had different pattern. The genomic analysis of the other isolates showed a wide spread of DNA-fingerprints. Comparing the isolates from German turkeys belonging to serotype A, a wide variation of genomic fingerprints was observed. The isolates with serotype B were partially identical. Comparing isolates from different countries high similarity within the isolates of the same serotype, despite of the origin (chicken/turkey) was observed. The result suggests the existence of relationships between the geographic origin, the serotype and the DNA fingerprint pattern.

However, based on the 16s rRNA sequence analysis, all *O. rhinotracheale* isolates from many investigations are close to the isolates from GenBank with identity ranging from 98.3% to 100%, indicating that *O. rhinotracheale* strains from all over the world are closely related (Tsai and Huang, 2006; van Empel and Hafez, 1999). 16s rRNA sequences of 23 Taiwanese *O. rhinotracheale* isolates showed high identities 98-100% to the other sequences in GenBank (Tsai and Huang, 2006).

2.5 Transmission

The infection spreads horizontally by direct and indirect contact through aerosols and/ or drinking water (van Empel and Hafez, 1999). *O. rhinotracheale* infection appears to have become endemic and can affected every new restocking even in previously cleaned and disinfected houses, especially in areas with intensive poultry production as well as in multiple age farms (Hafez, 1996; van Empel and Hafez, 1999). The survival of *O. rhinotracheale* at lower temperatures may be associated with the higher incidence of *O. rhinotracheale* infection in poultry during winter months (Lopes et al., 2002b). Heeder et al. (2001) examined the seroprevalence of *O. rhinotracheale* within a commercial ***layer*** population. Of the pullet flocks examined, 43% and 52% were positive by SPAT and ELISA, respectively.

The prevalence of *O. rhinotracheale* antibody is high in the commercial layer population, suggesting that this respiratory pathogen can easily spread through multiple-age layer farms from older flocks to newly housed pullet flocks. Surveillance of exposure to *O. rhinotracheale* infection in the field has shown that prevalence of the infection is higher during winter months. In addition, Amonsin et al. (1997) raised the hypothesis that *O. rhinotracheale* might be introduced to domesticated poultry flocks from wild bird populations. The results obtained by Gutzer et al. (2011) support the above mentioned hypothesis.

Vertical transmission is suspected, since some reports on the isolation of *O. rhinotracheale* at very low incidence from reproductive organs, hatching eggs, infertile eggs, and dead embryos were published (Back et al., 1998; El-Gohary, 1998; Tanyi et al., 1995). Back et al. (1998) isolated *O. rhinotracheale* from ovaries and oviduct of 56-week-old female breeder turkeys from a commercial farm in Minnesota.

Experimentally *O. rhinotracheale* infection was established by three routes of inoculation: intravenous, intratracheal and intranasal. Tissue samples from several organs (airsacs, brain, intestine, kidney, liver, lung, ovary, oviduct, spleen and trachea) were collected on days 3, 7 and 14, and were examined for the presence of *O. rhinotracheale* by cultural isolation and *in situ* detection by immunofluorescent antibody assay (IFA). *O. rhinotracheale* was recovered from ovaries and oviducts on days 3 and 7 after inoculation and again from the oviduct on day 14 by cultural isolation. By IFA *in situ* detection, *O. rhinotracheale* was recovered from all ovaries and oviducts on day 3 and day 7 after inoculation. This may be due to the ability of the turkey's immune response to clear the infection from most of the tissues by day 14. The isolation of the organism from ovaries and oviducts in this experiment

supports the possibility of the vertical transmission. If this transmission does occur, one would assume that it might happen in the acute stage of the infection (Back et al., 1998). This might be the reason of the world-wide prevalence of the disease (Tanyi et al., 1995). Likewise, van Veen et al. (2003) observed that specific pathogen free (SPF) broiler chickens that were placed in hatching incubators at a commercial turkey hatchery during hatch showed respiratory tract lesions at post-mortem examination that were positive for *O. rhinotracheale* by bacteriological and immunohistological examination. However, Varga et al. (2001) found that at 37°C *O. rhinotracheale* did not survive on egg-shells for more than 24 hours, while upon inoculation into embryonated chicken eggs it killed embryos by the 9th day, and from the 14th day post-inoculation no *O. rhinotracheale* could be cultured from the eggs at all. This suggested that *O. rhinotracheale* was not transmitted via eggs during hatching.

2.6 Clinical signs

The severity of clinical signs, duration of the disease, and mortality are extremely variable and are influenced by many environmental factors such as poor management, inadequate ventilation, high stocking density, poor litter conditions, poor hygiene, high ammonia level, concurrent diseases, and the type of secondary infection.

In ***turkeys*** outbreaks mostly have been observed in male birds over 14 weeks of age, however in many cases young poults up to the 2nd week of age could also found to be affected (Hafez et al., 1993; van Beek et al., 1994). The mortality ranges between 1 and 10 % during the acute phase (8 days). Initial symptoms are coughing, sneezing, and nasal discharge followed in some cases by severe respiratory distress, dyspnoea, prostration, sinusitis, and arthritis. The symptoms are accompanied by a reduction in feed consumption and water intake (Chin et al., 2008; Hafez, 2002). Szalay et al. (2002) observed nervous manifestations in one flock of 5-week-old poults and in three 16- to 20-week-old turkey flocks. The symptom was accompanied by increased mortality and was found to be associated with fibrinopurulent inflammation of the cranial bones and meningitis. The bacterium could be isolated from these lesions.

In ***turkey breeder*** flocks clinical signs are accompanied mostly by slightly increased mortality, drops in egg production (2-5%), and increases in the number of unsettable hatching eggs (Chin et al., 2003; Chin et al., 2008; de Rosa et al., 1996; Hafez et al., 1993; van Beek et al., 1994)

Clinical signs in ***broilers*** generally appear between the 3rd and 4th week of age with a mortality rate of 2-10 %. The clinical signs identified are depression, decrease in food intake, reduced weight gains, transient nasal discharge, and sneezing, followed by facial edema (Cauwerts et al., 2002; Du Preez, 1992; Odor et al., 1997; van Beek et al., 1994).

Sudden deaths of young chickens due to *O. rhinotracheale* infection of the brains and the skull with weakened skull-bones can also found. Moreover, subcutaneous edema over the cranium with a severe bacterial osteitis without respiratory tract infection was also described (van Empel et al., 1999). Furthermore, especially in older turkeys and chickens *O. rhinotracheale* was shown to spread to other body sites, causing arthritis, osteitis, and osteomyelitis that may develop with the formation of a purulent, exudates found in the joints of lame birds (Chin et al., 2008).

In ***broiler breeders*** the disease primarily affects the birds at the peak of production or shortly before entering production, mostly between 24th and 52nd week of age. Before the main symptoms are detected, a slight increase in mortality and decrease in feed intake may be observed. The first signs are mild respiratory distress. The symptoms are generally accompanied by a drop in egg production, decrease in egg size, and poor egg shell quality. Fertility and hatchability are unaffected in many cases (Hafez, 1996). Clinical signs in layer flocks are similar to that found in breeder flocks (Sprenger et al., 2000a).

2.7 Gross lesions

In turkeys lesions generally were localized in the lungs and include edema and uni- or bilateral consolidation of the lungs with fibropurulent exudate. Pericarditis, airsacculitis, peritonitis, and enteritis could be detected. In some cases, swelling of the liver and spleen plus degeneration of heart muscles have been seen (Hafez et al., 1993; Hinz et al., 1994; Roepke et al., 1998; van Beek et al., 1994; van Empel et al., 1996; van Empel and Hafez, 1999).

The lesions in broilers include pneumonic lungs, pleuritis, and airsacculitis. In the air sac accumulation of creamy, “yoghurt-like” exudate could be observed (Charlton et al., 1993; van Empel and Hafez, 1999).

2.8 Pathogenicity

Several *O. rhinotracheale* strains, isolated from turkey, chicken, duck, or partridge, were used for aerosol challenges of both turkeys and broilers of various ages. All the tested strains (8 chicken strains, 7 turkey strains, 2 partridge strains and 1 duck strain) were able to infect turkeys as well as broiler chickens with comparable severity after viral priming (van Empel et al., 1996).

However, differences in the pathogenicity of *O. rhinotracheale* between strains originating from different countries were found after experimental infections without virus primers (Ryll et al., 1997; Travers et al., 1996)

At the beginning, *O. rhinotracheale* was considered to be a secondary pathogen as it could not to cause disease in SPF chickens without priming with avian respiratory viruses, such as Newcastle disease (ND) virus or turkey rhinotracheitis (TRT) virus (van Empel et al., 1996; van Empel and Hafez, 1999).

On the other hand, Ryll et al. (1996) as well as Sprenger et al. (1998) observed after experimental infection of turkeys via aerosol, intratracheal, intravenous, and/or intrathoracal routes without previous priming with virus, airsacculitis, pneumonia, and increased mortality. However in turkeys that were infected intratracheally and intraveniously with *O. rhinotracheale* a mortality rate of 10 to 20 % were observed. A group infected with lung homogenate from *O. rhinotracheale* infected turkeys showed 50% mortality (Sprenger et al., 1998). Also van Veen et al. (2000) tested field strains of *O. rhinotracheale* on their virulence in different chicken breeds (meat and layer types). Aerosol infection was able to induce lesions after challenge without previous priming with virus, and thus O. *rhinotracheale* was proven to be a primary pathogen. In addition, white SPF leghorns were less susceptible than broilers, whereas there was no difference in susceptibility between commercial broilers and SPF broilers (van Veen et al., 2000). They also were able to demonstrate that the tested Dutch isolates and a South African strain were more pathogenic compared with an American strain (van Veen et al., 2000). Also, Travers et al. (1996) inoculated three South African *O. rhinotracheale* field isolates into the caudal abdominal air sacs of 28-day-old broiler chickens and showed a significant difference in incidence of airsacculitis and arthritis. Moreover, the different South African *O. rhinotracheale* isolates were capable of causing primary disease. Even so, they caused significantly more severe respiratory disease in the broilers co-infected with ND virus. Respiratory and arthritis were reproduced, but no sinusitis

was observed. In addition, significant differences in the re-isolation rate of *O. rhinotracheale* were observed.

van Empel et al. (1999) conducted a study about the pathogenesis by immunohistochemistry. Priming of SPF chickens with ND virus was followed after one week by subsequent aerosol challenge with *O. rhinotracheale*. The results demonstrated that the lesions in the air sacs, lungs and in the trachea are caused by *O. rhinotracheale* not by the ND virus. Moreover, two days after *O. rhinotracheale* challenge, *O. rhinotracheale* was found attached to the epithelium on the air sacs followed by thickening of air sacs, edema, and an acute granulomatous airsacculitis (van Empel et al., 1999). Infections with *O. rhinotracheale* appear to be restricted to the respiratory tract, with lesions only evident in birds previously infected with ND virus, even though a strong serological response can be established in the absence of prior viral infection (van Empel et al., 1999). In general, the airsacculitis and pneumonia induced after *O. rhinotracheale* challenge of ND primed birds were fully established at 5 to 7 days after *O. rhinotracheale* challenge (van Empel et al., 1999).

In these studies commercial birds with uncertain microbiologic and/or immunologic status were used. This may have contributed to the infections. Also using SPF – leghorn birds, no pneumonia or airsacculitis could be induced by aerosol, intratracheal or intrathoracal challenge application of *O. rhinotracheale* on its own (van Empel et al., 1999). Only an intravenous challenge was able to induce up to 20 % mortality and clinical reactions such as meningitis, osteitis, and purulent infections of the hock and knee in SPF chickens, but no airsacculitis as seen in the field (Goovaerts et al., 1998).

2.9 Synergism with other avian pathogens

In turkeys, infection was aggravated by the prior administration of TRT virus or ND virus isolates (Marien et al., 2005; van Empel et al., 1996), *Bordetella avium* (Droual and Chin, 1997), *Mycoplasma gallispeticum* and /or *E. coli* (de Rosa et al., 1996; Marien et al., 2007).

In order to clarify the role of other avian pathogens in the course of *O. rhinotracheale* infection, further serological surveillance for antibodies against *O. rhinotracheale*, TRT, *Chlamydophila psittaci* were carried in turkey flocks. Results showed an interaction between *O. rhinotracheale* and other pathogens (Hafez, 1998, 2002; van Loock et al., 2005). On the other hand, a concomitant infection with *Mycoplasma synoviae* did not show an obvious

effect on mortality rates nor on the antibody response against *O. rhinotracheale* in turkeys (Zorman-Rojs et al., 2000).

Marien (2007) used an experimental groups of 15 susceptible 3-week-old turkeys. These animals were inoculated oculonasally with TRT subtype A, *E. coli* O2:K1 and *O. rhinotracheale*, with a 3 days interval between viral and bacterial inoculation and approximately 8 hours between the two bacterial inoculations. Macroscopic findings were comparable between the experimental groups. The lesions of all groups were serous to seromucous exudate in the turbinates and sinuses, as well as hyperaemia of the turbinates and the trachea (Marien et al., 2007). As mentioned before, some bacteria including *Bordetella avium* and *E.coli* have also been suspected to induce the establishment of *O. rhinotracheale* infections, but nevertheless respiratory viral infections are more important, because they lead to more severe respiratory lesions and higher mortality rates than bacterial infection (Marien et al., 2007; Marien et al., 2005; Marien et al., 2006).

In broilers, infection was aggravated by the prior administration of ND virus, and to a lesser extent by prior administration of Infectious Bronchitis (IB) virus or a chicken-TRT virus isolate, in particular with regard to development of airsacculitis and pneumonia. Without the virus no airsacculitis or pneumonia was seen in these studies (van Empel et al., 1996; Odor et al., 1997, Jirjis et al., 2004). Also in field cases viruses had influence on *O. rhinotracheale* infections (Travers, 1996, Vandekerchove et al., 2004).

2.10 Immunity

Immunity against *O. rhinotracheale* induced by inactivated vaccines was serotype specific, while live vaccination induced a degree of cross-protection between some serotypes (Schuijffel et al., 2005a; van Empel and van den Bosch, 1998). After live vaccination cross-reactivity was analyzed by ELISA, using *O. rhinotracheale* serotypes A, B, G, and M as antigens. Cross-reactions between the different serotypes A, B, G, and M could be observed in all birds one week after both aerosol and intravenous challenge, indicating the presence of common antigens and the presence of cross reactive serum IgG antibodies. Highest antibody titres and strongest serological cross-reactivity were found against serotypes A and B (Schuijffel et al., 2005a). In two animal experiments vaccinated chickens were first vaccinated with *O. rhinotracheale* serotype G and then challenged with *O. rhinotracheale* serotype G (homologous challenge) or *O. rhinotracheale* serotype A (heterologous challenge). Vaccination followed by challenge resulted in reduced and significantly different

respiratory pathology scores compared to unvaccinated challenged birds (Schuijffel et al., 2005a). The importance of the humoral immunity was shown by Schuijffel et al. (2005b), who treated broiler chickens with cyclophosphamide (CY) and suppressed B lymphocyte development, as was confirmed by histological and serological analysis. *O. rhinotracheale* challenge to CY-treated birds revealed a significantly higher pathologic score in comparison to immune-competent birds that received the same bacterial and dose of challenge. Serum immunoglobulin levels measurement of immune-competent birds showed a positive correlation between IgA and/or IgG levels and protection against infection. Passive shift of *O. rhinotracheale* specific antiserum to the immune-suppressed birds before the pathogen challenge significantly decreased morbidity (Schuijffel et al., 2005a; Schuijffel et al., 2005b).

2.11 Diagnosis

Diagnosis of poultry diseases basically consists of the farm data history of each case along with management and environmental investigations (Hafez, 2002; Hafez and Friedrich, 1998; Hafez et al., 1993; van Empel et al., 1997). Clinical signs and lesions are of little value in diagnosis, since many other infectious agents such as *E. coli* or *R. anatipestifer* Pasteurellosis, Chlamydiosis, TRT, and also other bacteria causing joint infections such as *E. coli, Staphylococcus aureus,* or *Streptococcus feacalis* bacterial infection can produce similar clinical signs and post mortem lesions (van Empel and Hafez, 1999). Moreover, infections of the brain with *O. rhinotracheale* are often missed, because they are not considered as possible causes of the related clinical symptoms (van Empel et al., 1996). Accurate diagnosis must be substantiated by isolation and identification of the causative bacteria and /or detection of antibodies using serological examination (Fig. 1).

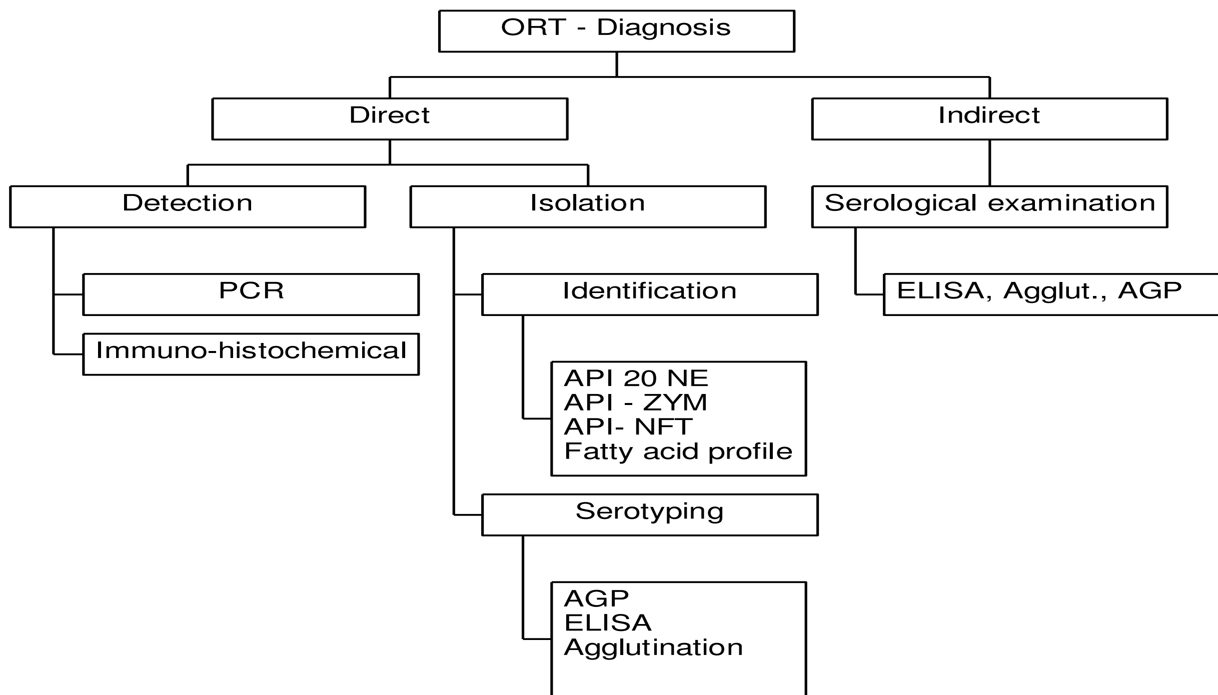

Fig. 1: Laboratory diagnosis of *O. rhinotracheale* (Hafez, 2002)

2.11.1 SELECTION OF SAMPLES FOR ISOLATION

Samples for bacterial culture should be collected at an early stage of the disease. Collection of suitable samples is very important. *O. rhinotracheale* can usually be isolated from several organs. The trachea, lungs and air sacs are the best tissues for the isolation (Vandamme et al., 2006). The infraorbital sinus and nasal cavity are also suitable sites for culture, but *O. rhinotracheale* can be masked easily by the overgrowth of other bacteria (El-Sukhon et al., 2002; Vandamme et al., 2006). Hafez et al (1993) revealed that isolation from heart blood and liver tissue under field condition mostly brought negative results. In 2005 Lüschow and Hafez (2005) investigated heart blood, liver, and muscle from broiler flocks with a history of respiratory diseases at the time of slaughter. From none of the samples *O. rhinotracheale* could be isolated. Nevertheless, using PCR, *O. rhinotracheale* specific DNA was detected in some samples from heart and from muscle. Moreover, some experiments show that *O. rhinotracheale* can be isolated from those organs, as well as joints, brain, ovary, and oviduct after experimental infections (Back et al., 1997; van Beek et al., 1994).

2.11.2 ISOLATION

The most frequently used method to isolate *O. rhinotracheale* in the laboratory is isolation on Columbia agar plates supplemented with 5-10 % sheep blood. For an optimal growth, the

plates should be placed at 37°C in a 5-10 % CO_2 atmosphere (Chin et al., 2008; Erganis et al., 2002; van Empel et al., 1997; van Empel and Hafez, 1999). *O. rhinotracheale* colonies are catalase negative and mostly oxidase-positive. However, some isolates revealed negative oxidase reaction (Ryll et al., 2002). *O. rhinotracheale* can be isolated on blood or chocolate agar and colonies grow in 24 hours, but it is best to hold inoculated plates for 48-72 hours under microaerophilic condition (Chin et al., 2008; Erganis et al., 2002; van Empel et al., 1997; van Empel and Hafez, 1999). Due to secondary infections caused by other infectious agents that grow faster and are less fastidious than *O. rhinotracheale*, recovery of *O. rhinotracheale* at a late stage of infection usually fails (van Empel, 2002). In contaminated samples with fast-growing bacteria including *E. coli, Proteus sp., or Pseudomonas sp., O. rhinotracheale* colonies may be overgrown and are difficult to detect in routine diagnostic (Hassanzadeh et al., 2010; Vandamme et al., 2006). To prevent the overgrowth of other bacteria, Back et al. (1997) recommended the use of 10 µg of gentamycin per ml of blood agar medium in an effort to isolate *O. rhinotracheale* from contaminated samples. Blood agar containing 5 µg/ml gentamicin and polymyxin B was also effective (Back et al., 1997; van Empel et al., 1997). However, as only about 90 % of *O. rhinotracheale* strains are resistant to both these antimicrobials, so sheep blood agar without these additives should always be included (van Empel and Hafez, 1999).

2.11.3 IDENTIFICATION

2.11.3.1 BIOCHEMICAL TESTS

Characterization of *O. rhinotracheale* can be done by biochemical tests (Charlton et al., 1993; van Empel and Hafez, 1999; Vandamme et al., 1994). The biochemical properties can be determined using API-20NE system (BioMÈrieux, France). Moreover, the API-ZYM system (BioMÈrieux, France) can be used to determine the enzymatic activity (Hafez, 2002; van Empel and Hafez, 1999; Vandamme et al., 1994).

2.11.3.2 SEROLOGICAL TYPING

By using monovalent antisera in AGP tests, serotypes of *O. rhinotracheale* could be discriminated (Hafez and Sting, 1999; van Empel, 1998). However, cross reactions between serotypes were found (Hafez et al., 2000; Hafez and Sting, 1999; Schuijffel et al., 2005a). As

mentioned above currently 18 serotypes, which are discriminated by AGP and designated A to R, have been reported and most chicken isolates belong to serotype A, whereas turkey isolates are more heterogeneous and belong to serotype A, B, and D. Relationships between the geographic origin and the serotypes were found (van Empel et al., 1997). Cross reactions between serotype A and B as well as between B and E were reported by Hafez and Sting (1999).

Turkyilmaz (2005) tried isolation of *O. rhinotracheale* from 257 chickens and 214 turkeys and further tested serum of 333 chickens and 250 turkeys by AGP. *O. rhinotracheale* was isolated from the sinus discharge of three broiler breeders at 37, 42, and 46 weeks of age. It was determined that all three isolates were *O. rhinotracheale* serotype i having cross reactions with serotype L. Of 333 chicken sera examined 86 were found seropositive in chickens by AGP. Thirty sera were against serotype A, 25 sera were against serotype B, nine sera were against serotype i and six sera were against serotype L, while 13 sera were against serotype A with cross reactions against serotype B, and three sera were serotype i with cross reactions with serotype L. Of the 250 turkey sera 64 were seropositive and of those 17 were against serotype A, 29 were against serotype B, five were against serotype i, and two were against serotype L, while eleven sera were against serotype A with cross reactions against serotype B (Turkyilmaz, 2005).

An ELISA using sodium dodecyl sulphate extracted antigen was proven to be less serotype-specific than using boiled extract as antigen (Hafez and Sting, 1999) and less sensitive as well (van Empel et al., 1997)

Furthermore, when analyzed by ELISA after using live vaccination, cross-reactions between the serotypes A, B, G, and M could be observed in all birds one week after both aerosol and intravenous challenge. The strongest serological cross-reactivity was found against serotypes A and B (Schuijffel et al., 2005a).

2.11.3.3 MOLECULAR BIOLOGY

Because the current method of serotyping of *O. rhinotracheale* by the AGP can be subjective and since cross-reactions among serotypes can occur, DNA fingerprinting techniques have been used for characterizing *O. rhinotracheale* isolates (Hafez and Beyer, 1997). Using the primers M13 (5'-TAT GTA AAA CGA CGG CCA GT- 3) and enterobacterial repetitive intergenic consensus (ERIC) 1R (5'- ATG TAA GCT CCT GGG GAT TCA C -3'), variations were found between all tested serotypes, so PCR fingerprints with M13 and ERIC 1R primers are a useful tool for typing and epidemiological investigation of *O. rhinotracheale*

isolates (Hafez and Beyer, 1997). Also, Thachil et al (2007), investigated *O. rhinotracheale* isolates by the ERIC polymerase chain reaction and random amplified polymorphic DNA assay with Universal M13 primer–based fingerprinting techniques. The fingerprint patterns were compared with serotyping results of *O. rhinotracheale* by the AGP. The results revealed that the 58 isolates of *O. rhinotracheale* that were fingerprinted belonged to 8 *O. rhinotracheale* serotypes. 10 different fingerprints were found with M13 fingerprinting and 6 different fingerprints were found with ERIC 1R fingerprinting. M13 fingerprinting technique was found to be more useful in differentiating *O. rhinotracheale* isolates than the ERIC 1R fingerprinting technique. Moreover, the investigation suggests that fingerprinting techniques may be a more certain means for characterizing *O. rhinotracheale* isolates than the serological test using the AGP (Thachil et al., 2007). In addition, Waldow (2009) evaluated the suitability of primers ERIC 1R and ERIC 2 as well as of primer M 13 and the corresponding PCRs (ERIC-PCR or RAPD-PCR). RAPD-PCR by using primer M 13 was able to distinguish reference strains of serotypes A, B, D, E, F, G, I, K, M, and O based on fragment patterns. In contrast fragment patterns of reference serotypes C, H and Q, J and L as well as N and P were very similar so that a differentiation between these serotypes was not possible. Based on these results selected German isolates of serotype A, B, C, and E were investigated by RAPD-PCR. Of all serotypes the patterns of the field isolates of serotype A showed the highest genetic similarity to each other as well as to the pattern of reference serotype A (92% and 79%, respectively). The field isolates characterized as serotype B showed two different patterns which could hardly be differentiated from patterns of serotype A field isolates and had only a slight similarity to the reference serotype B (10%). For the remaining isolates of serotypes C and E a large number of fragment patterns were produced. The genetic similarity between the patterns of the field isolates and their reference serotypes was very low (13%, serotype C; 27%, serotype E). A reliable fingerprinting by RAPD-PCR seems to be possible only for isolates of serotype A.

O. rhinotracheale isolates have also been typed by multilocus enzyme electrophoresis (MLEE), repetitive sequence based-PCR (rep-PCR), and 16S rRNA gene sequencing. (Amonsin et al., 1997). They investigated 55 *O. rhinotracheale* isolates from eight countries of four continents. The *O. rhinotracheale* isolates could be discriminated into six electrophoretic types (ETs), of which only three were isolated from domesticated poultry. 50 out of 55 examined isolates (90.9%) examined were assigned to one of two closely related clones (ET 1 and ET 2) that comprise the ET 1 complex. The results suggested host specificity among clones of *O. rhinotracheale*. The *O. rhinotracheale* isolates recovered from domesticated poultry were assigned to the ET 1 complex. In contrast, none of the four isolates from rooks or guinea fowl were assigned to the common ET 1 complex. In addition,

the clustering obtained by analysis of rep-PCR predicts that *O. rhinotracheale* clones infecting passeriform birds (rooks) are genetically distinct from clones infecting galliform birds (chickens, turkeys, and guinea fowl). In a further investigation, Leroy-Sétrin et al. (1998) compared 23 strains of *O. rhinotracheale,* which were isolated in France between 1994 -1995 from 17 geographical regions, using plasmid profiles, ribotyping, and random amplified polymorphic DNA (RAPD) analysis. All isolates were poorly discriminated by ribotyping although different enzymes were used.

Popp and Hafez (2009) analyzed several *O. rhinotracheale* isolates by pulsed-field gel electrophoresis (PFGE). The restriction digestion of genomic DNA of each isolate was accomplished by using the enzyme *Sal*I. The result of the genetic analysis showed that there was a noticeable diversity of DNA fingerprint patterns and each of the 17 tested reference strains (A–Q) revealed a specific pattern. In addition, the serotype A isolates, which originated from German turkeys, showed a wide variation, while serotype B isolates were identical. Comparison of isolates from various countries showed a high similarity within the isolates of the same serotype regardless of the host species. A relationship between the geographic origin, the serotype, and the DNA fingerprint pattern was suggested by this investigation (Waldow, 2009). Koga and Zavaleta (2005) investigated *O. rhinotracheale* isolates from broilers, breeders, and layers from several geographic zones of Peru using PCR and repetitive extragenic palindromic PCR (rep-PCR) techniques. All 25 isolates tested had a genetic profile similar to that of the *O. rhinotracheale* type strain which was isolated from a turkey in the United Kingdom (American Type Culture Collection, ATCC-Number: 51463).

Or01 gene

Protein Or01 is found in the cytoplasmic-inner membrane fraction. The sequence of Or01 shows similarity to dihydrolipoamide acetyltransferase (E2p), a membrane-associated component of the pyruvate dehydrogenase (PDH) complex (Schuijffel, 2005) The PDH complex is involved in energy conversion and production (de Kok et al., 1998). This E2p component is usually very immunogenic and therefore was repeatedly identified by serological screenings (Ala'Aldeen et al., 1996; Hemila et al., 1990; Jan et al., 2001). The length of the Or01 gene is 1614 base pair (bp), while the size of the protein is 59.8 kDa. Sequence analysis showed the highest similarity to gram-negative bacteria *Bradyrhizobium japonicum* (35% identity, 51% similarity) and *Brucella suis* and *Brucella melitensis* (both 35% identity, 52% similarity). The protein contains 2 conserved lipoyl-binding sites and strongly

hydrophobic regions. The N-terminus of the protein showed a highly hydrophilic region (Schuijffel et al., 2005a).

2.11.3.4 HEMAGGLUTINATING ACTIVITY

Fitzgerald et al. (1998) tested 25 *O. rhinotracheale* isolates for their ability to agglutinate chicken red blood cells. Ten of the 25 isolates, which were sensitive to fosfomycin (MIC values below 128ug/ml), were able to agglutinate red blood cells. The remaining 15 isolates were resistant to fosfomycin (MIC values above 128ug/ml). Only five of these isolates were found to have the ability to agglutinate red blood cells. Other results were obtained by testing some isolates from Mexico by Soriano et al. (2002). They found that all isolates tested showed hemagglutination activity with glutaraldehyde-fixed erythrocytes. On the other hand, Vega et al. (2008) investigated the hemagglutinating activity of nine reference strains (serovars A-I) using fresh erythrocytes from 15 different species: chicken (broiler, rooster, and hen), turkey, pigeon, quail, duck, Harris hawk (*Parabuteo unicinctus*), house finch (*Carpodacus mexicanus*), cow, sheep, horse, dog, rabbit, pig, human (groups A, B, AB, and O), and rainbow trout (*Oncorhynchus mykiss*). All nine strains agglutinated rabbit erythrocytes. None of the strains was able to agglutinate hen, cow, horse, or rainbow trout erythrocytes. The number of positive reactions among the remaining species varied. Results indicate that the use of rabbit erythrocytes is better suited for testing the hemagglutinating activity of *O. rhinotracheale* (Vega et al., 2008). Vega-Sanchez et al. (2011) demonstrated the alternative laboratory approach of the haemagglutination-inhibition test. This test showed that most of the hemagglutinating *O. rhinotracheale* are able to raise detectable hemagglutination-inhibition antibodies in immunized specific-pathogen-free chickens (Vega-Sanchez et al., 2011).

2.11.3.5 DETECTION OF O. RHINOTRACHEALE BY PCR

A specific PCR can be performed using the primer combination OR16S-F1 (5'-GAG AAT TAA TTT ACG GAT TAA G) and OR16S-R1 (5'-TTC GCT TGG TCT CCG AAG AT). This combination amplifies a 784 bp fragment on the 16S rRNA gene of *O. rhinotracheale*, but not of other closely related bacteria with which *O. rhinotracheale* could be confused (Hung and Alvarado, 2001; van Empel, 1998). This method was currently widely used in the routine diagnosis to detect *O. rhinotracheale* -DNA in tracheal swabs, organs, eggs and environmental samples.

2.11.3.6 IMMUNO-HISTOCHEMICAL STAINING

In field trials, using a sensitive immuno-histochemical staining, it was found that *O. rhinotracheale* was detected in 70% of samples collected from broiler suffering from respiratory infection, while using culture and/or serology only 30% of the examined cases could be associated with *O. rhinotracheale* (van Empel et al., 1999; van Veen et al., 2000).

2.11.3.7 SEROLOGICAL EXAMINATION FOR ANTIBODY DETECTION

Serology is useful for flock monitoring and helpful for the diagnosis of *O. rhinotracheale* infection (Asadpour et al., 2008). For serological diagnosis, antibodies against *O. rhinotracheale* can be detected by many tests such as rapid agglutination test (Back et al., 1998), ELISA tests (Hafez, 1996; Lopes et al., 2002b) or DOT- Immunobinding assay (Erganis et al., 2002).

2.11.3.7.1 ELISA

The serotype specificity of the ELISA depends on the method of antigen extraction used for coating the ELISA plates. Boiled extract antigens are serotype-specific (van Empel et al., 1997). Conversely, antigen extraction with sodium dodecyl sulphate (SDS-antigen) (Hafez et al., 2000; Hafez and Sting, 1999) or extracted outer membrane proteins of *O. rhinotracheale* (Lopes et al., 2000) will result in more cross-reactions allowing detection of antibodies against different serotypes with one test (Hafez et al., 2000; Hafez and Sting, 1999). Self-made ELISA (SDS-extraction) as well as two commercial available ELISA-kits (Biocheck and IDEXX) were compared and showed that all allowed detecting antibodies against all tested *O. rhinotracheale* serovars (Hafez et al., 2000).

Field surveys using self-made ELISA or commercial ELISA kits were shown to be a useful tool for monitoring flocks (Allymehr, 2006; Ballagi et al., 2000; Chansiripornchai et al., 2007; Hafez et al., 2000; Ryll et al., 1997; Sakai et al., 2000; Turkyilmaz and Kaya, 2005; van Empel et al., 1997; van Veen et al., 2005). ELISA has been successfully used for screening and to detect maternal antibodies in day-old chickens and turkeys (van Empel and Hafez, 1999). Moreover, using ELISA antibodies against *O. rhinotracheale* can be detected in serum and egg yolk shortly after infection. Titers will peak between 1 to 4 weeks post infection (van Empel et al., 1996). Lopes et al. (2000) revealed that by this method antibodies could be detected in the early period of infection up to 8 weeks post infection, while titers also usually peak between 1 to 4 weeks post infection and will decline rapidly afterwards, what suggests that serum samples for flock screening should be taken frequently (Lopes et al., 2000).

2.11.3.7.2 THE SERUM PLATE AGGLUTINATION TEST (SPAT)

The SPAT was reported as a rapid test for the detection of antibodies against *O rhinotracheale* (Bock et al., 1995). Additionally, a SPAT was developed using a non-serotyped Minnesota isolated of *O. rhinotracheale* and reported to have good sensitivity (Bock et al., 1997). However, in another study (Lopes et al., 2000) the SPAT detected only 65% of infected birds during the first 2 weeks of infection and that number declined significantly thereafter. This suggests that the SPAT detects IgM antibodies, which are efficient in agglutination with specific antigens. In addition, most SPAT-reactions are serotype-specific, although cross-reactions do occur (Lopes et al., 2000).

The advantage of the serological tests over bacteriological examination is that antibodies persist for several weeks after infection, while the bacterial shedding is short. However, *O. rhinotracheale* excretion and antibody response may also be affected by a number of factors such as antibiotic therapy and vaccination. The influence of antibiotic therapy on the serological response to *O. rhinotracheale* remains unclear. Popp and Hafez (2002) carried out an investigation in aim to determine the effect of drug therapy using amoxicillin on the antibody kinetics after experimental infection. Amoxicillin was confirmed to be very effective against most isolates tested in vitro (Hafez et al., 1993). Three groups of SPF layers, each of 10 birds, were experimentally intravenously infected with an *O. rhinotracheale* strain at 36 weeks of age. Each bird received $5x10^8$ CFU. Group 1 was kept as infected non-treated control. Group 2 was infected and treated immediately with amoxicillin at a dose level of 250 ppm via drinking water for 5 days. Group 3 was infected as mentioned above and received amoxicillin for 5 days started at 7th day post infection. An additional group (Group 4) was kept as non-infected non-treated control. Blood samples were collected in five day intervals till the 50th day post infection and tested for antibodies against *O. rhinotracheale* using ELISA. The results showed that prompt treatment did not influence the antibody response, while the treatment started at 7th day post infection resulted in a lower antibody response compared to the non-treated control.

2.12 Intervention Strategies

2.12.1 TREATMENT

The treatment of *O. rhinotracheale* infections with antibiotics is not easy due to the variation of resistance and susceptibility of strains. *O. rhinotracheale* infections in poultry can be successfully treated and/or controlled by many antimicrobial drugs in combination together with hygienic measurement (Devriese et al., 2001; Devriese et al., 1995). The treatment of infections with antibiotics is very difficult because of the inconstant sensitivity of the strains and regional variations with regard to the sensitivity of *O. rhinotracheale* to antibiotics. It has also been proven that *O. rhinotracheale* strains are able to acquire resistance easily against several antibiotics (Canal et al., 2003; Devriese et al., 1995; Dudouyt et al., 1995; Hafez et al., 1993; Nagaraja et al., 1998).

Moreover, the antimicrobial drug sensitivity of *O. rhinotracheale* was found to be dependent on the type of bird (Devriese et al., 2001). When *O. rhinotracheale* strains isolated from gallinaceous birds were compared with isolates from rooks using the agar dilution method, the minimal inhibitory concentrations for penicillin and cephalosporin antibiotics differed 5- to 20-fold and were higher in isolates from gallinaceous birds than in isolates from rooks (Devriese et al., 2001).

O. rhinotracheale isolates from Germany originating from the first outbreaks showed a high susceptibility to amoxicillin, chloramphenicol and chlortetracycline in the disc diffusion tests (Hafez et al., 1993). Ninety per cent and 36 % of the isolates were found to be susceptible to erythromycin and furazolidone, respectively. In addition, only 6 % of tested isolates were found to be susceptible to enrofloxacin. None of isolates were susceptible to apramycin, neomycin, gentamicin and sulphonamide/trimethoprine (Hafez et al., 1993). The susceptibility to enrofloxacin seems to be geography-related, since most turkey isolates from Germany and the Netherlands are resistant, while 98 % of isolates from France (Dudouyt et al., 1995) and 71 % of isolates from Belgium (Devriese et al., 1995) are susceptible to enrofloxacin. In Canada, *O. rhinotracheale* could be isolated from enrofloxacin-treated birds in mono-cultures (Joubert et al., 1999). Likewise, Nagaraja *et al.* (1998) investigated 68 *O. rhinotracheale* isolates from the United States and found that they were susceptible to ampicillin, erythromycin, penicillin, spectinomycin, and tylosin. Further 54 of the 68 isolates were susceptible to neomycin, sarafloxacin, and tetracycline. These isolates differed

significantly from German isolates in their pattern of susceptibility for at least two antibiotics, erythromycin and sarafloxacin (Nagaraja et al., 1998)

In contrast *O. rhinotracheale* isolated in Minnesota between 1996 and 2002 showed an increase of the resistance to gentamicin, ampicillin, tetracycline, and trimethoprim sulfa, while the resistance against penicillin remained constant from year to year (Malik et al., 2003). Similar results were obtained by van Veen et al. (2001) who tested *O. rhinotracheale* isolates collected in the Netherlands between 1996 and 1999 in the agar gel diffusion test. The percentages of strains susceptible to amoxicillin and tetracycline decreased in successive years from approximately 62 % to 14 %, and four strains were resistant to enrofloxacin or sulphonamide/trimethoprine. Twelve multiresistant strains were tested against seven alternative antibiotics; they were resistant to all of them except clavulanic acid-potentiated amoxycillin.

Several investigations were published on the susceptibility of *O. rhinotracheale* using Minimum Inhibitory Concentration (MIC) test. Varga et al. (2001) examined *O. rhinotracheale* isolates from Hungary by MIC test. Among the 16 drugs examined, penicillin G, ampicillin, ceftazidim (with MICs from < or = 0.06 microgram/ml to 0.12 microgram/ml), erythromycin, tylosin, tilmicosin (with some exceptions MICs ranged from < or = 0.06 microgram/ml to 1 microgram/ml), and tiamulin (MICs varied from < or = 0.06 microgram/ml to 2 micrograms/ml) were the most effective. Lincomycin, oxytetracycline, and enrofloxacin also gave good inhibitions, but for most strains only in a higher concentration (MICs ranged in most cases from 2 micrograms/ml to 8 micrograms/ml). The other antibiotics inhibited the growth of *O. rhinotracheale* only in very high concentrations (colistin) or not at all (apramycin, spectinomycin, polymyxin B).

Using also the MIC method Popp and Hafez (2002) investigated the susceptibility profiles of *O. rhinotracheale* isolates from several countries; namely 78 isolates from Germany, 12 isolates from Hungary, 2 from Israel, 5 from Spain, and 3 from Turkey. The obtained results showed that 84 to 88 % of the isolates were sensitive to amoxicillin, ceftiofur and tiamulin. 45 % of the isolates were found to be sensitive to chlortetracycline and 30 % to tetracycline and penicillin, while 25 % were sensitive to tilmicosin. In addition, only 6 % - 12 % of tested isolates were found to be sensitive to enrofloxacin, lincomycin/spectinomycin, cotrimoxazol (sulfonamide) and lincomycin. Comparing the sensitivity of isolates corresponding to their origin showed that nearly all isolates are sensitive for amoxicillin except those from Spain, which showed an intermediate sensitivity. Also 94 % of isolates were resistant to enrofloxacin regardless their origin. Against penicillin G all isolates from Hungary and Turkey

were sensitive. Concerning the sensitivity to tetracycline origin related differences were determined. The most isolates from Hungary and Turkey were sensitive.

Also Waldow and Hafez (2007) tested 117 isolates collected from Germany (88 isolates) and from France (29 isolates) collected between 2003-2006 using the MIC method. The results showed that 87.5 % of German isolates and all French isolates were sensitive to tiamulin (MIC ≤ 1 µg/ml). Regarding the antibiotics erythromycin, ceftiofur, and ampicillin, over 72 % of the German isolates showed MIC values ≥ 4 µg/ml. A similar result was observed in the French isolates. MIC values ≥ 1 µg/ml for penicillin, lincomycine and tetracycline. A MIC value of 2 µg/ml for enrofloxacin was observed with nearly 45 % of all German strains. A very similar result was observed with the French isolates. However, approximately 38 % showed a MIC of ≤ 0.06 µg/ml.

For the combination trimethoprim/sulfamethoxazole over 76 % of German isolates exhibited a MIC value of ≥ 1/19 µg/ml. Only 51 % of the French isolates also showed this value. In general the German strains exhibited higher MICs values to all of the antibiotics tested compared to the French strains.

Soriano et al. (2002) tested Mexican isolates and found that the susceptibility of *O. rhinotracheale* to amoxicillin, enrofloxacin, and oxytetracycline was variable. However, consistently higher MIC values were obtained for gentamicin, fosfomycin, trimethoprim, sulfamethazine, sulfamerazine, sulfaquinoxaline, and sulfachloropyridazine.

Under field conditions administration of chlortetracycline, amoxicillin, or ampicillin via drinking water for three to seven days is often used to treat *O. rhinotracheale* infections (Marien et al., 2007). In some case, injection with tetracycline and synthetic penicillin show an effect, but often medication fails resulting in a loss of up to 25 % of the animal within a few weeks (van Beek et al., 1994).

The results of administering enrofloxacin via drinking water to control *O. rhinotracheale* infections in turkeys were investigated by Garmyn et al. (2009a; 2009b). In different experiments four-week-old turkeys were first infected with avian metapneumovirus (APV) and three days later with *O. rhinotracheale*. In the first trial enrofloxacin treatments in the drinking water 5 successive days was started 24 h after *O. rhinotracheale* challenge using several doses ranged between 5 to 20 mg/kg/ body weight. In addition, further trials were carried out in aim reduce the duration. Medication mostly started with a high initial dose at the first day and was reduced at 2nd or 4th days. This treatment compared the effect of with 10 mg/kg body weight for five successive days. In all trials enrofloxacin treatments were

equally efficacious. However, none of the alternative enrofloxacin treatment regimens yielded better results than 10 mg/kg of BW for 5 successive days via drinking water to combat *O. rhinotracheale* infections in turkeys (Garmyn et al., 2009a). Likewise in further experiments Garmyn et al. (2009a; 2009b) compared the efficacy a single-day treatment regimen using 50 mg/kg to a multiple-day treatment regimen with 10 mg/kg body weight for five days. They infected 22 days old poults with APV and *O. rhinotracheale.* Subsequently the birds were treated via the drinking water with enrofloxacin, using either a single-day treatment regimen at 50 mg/kg body weight during a 5-h, 10-h or 20-h period or a standard five-day treatment regimen at 10 mg/kg body weight/day for 20 hours. All dosage regimens cleared *O. rhinotracheale* from the trachea, four days after onset of treatment. *O. rhinotracheale* bacteria were re-excreted in the single-day regimens but without worsening of the clinical symptoms. The five-day treatment with 10 mg enrofloxacin/kg in turkeys provided the best results for the treatment of an *O. rhinotracheale* infection in turkeys by shortening the course and reducing the severity of clinical disease and by eliminating *O. rhinotracheale* from the respiratory tract without re-emergence and also found that none of the used treatment regimens promoted the selection of bacterial clones with reduced susceptibility or resistance (Garmyn et al., 2009a; Garmyn et al., 2009b).

Also Marien et al. (2007; 2005) infected three-week-old turkey poults oculonasally with APV subtype A followed by infection with *E. coli* and *O. rhinotracheale*, with a three days interval between viral and bacterial inoculation and approximately eight hours between the two bacterial inoculations to assess the efficacy of drinking-water administration of enrofloxacin for three and five days, amoxicillin for five days and florfenicol for five days for the treatment. Antimicrobial treatment started one day after the dual bacterial inoculation. After infection, the birds were daily examined and scored for clinical signs, weighed at different times, and their tracheae were swabbed daily. Five birds were euthanized and examined for macroscopic lesions at necropsy five days post bacterial inoculation and the remainder 15 days post bacterial inoculation. Samples of the turbinates, trachea, lungs, sinuses, air sacs, heart, pericardium, and liver were collected for bacteriological examination. Recovery from respiratory disease caused by an APV/ *E. coli O. rhinotracheale* triple infection in three-week-old turkey poults was overall most successful after enrofloxacin treatment, irrespective of treatment duration, followed by florfenicol treatment. Compared with the untreated group, clinical signs as well as *O. rhinotracheale* and *E. coli* multiplication in the respiratory tract were significantly reduced by both enrofloxacin treatments and the florfenicol treatment, with the enrofloxacin treatments showing significantly better reductions than the florfenicol treatment. Five day treatment with amoxicillin, compared with the untreated group, did not

cause a significant reduction in any of the prior parameters (Marien et al., 2007; Marien et al., 2005).

Warner, et al. (2009) found that a protocol of metaphylactic tilmicosin at a rate of 15 mg/kg/day for a period of five days immediately, as well as a vaccination programme against APV in a turkey farm with a history of severe respiratory problems caused by *O. rhinotracheale* and TRT was very effective in mitigating the clinical signs and reducing abattoir condemnations.

2.12.2 VACCINATION

Several attempts to combat the infection using live or inactivated vaccines in broiler, broiler breeder, as well as in turkey and layer flocks were carried and revealed different results (Cauwerts et al., 2002; Schuijffel et al., 2005b; van Empel and van den Bosch, 1998). A major problem with *O. rhinotracheale* inactivated vaccines is that those vaccines do not provide broad cross-protection against many serotypes of *O. rhinotracheale.* For this reason including different serotypes in the vaccines should be considered (Bock et al., 1997; Schuijffel et al., 2005a; Schuijffel et al., 2006; van Empel and van den Bosch, 1998).

2.12.2.1 INACTIVATED VACCINES

In the field, vaccinations with autogenic inactivated oil-adjuvant vaccines were proven to be successful in reducing the outbreaks of *O. rhinotracheale* (Bock et al., 1997; van Empel and van den Bosch, 1998). Vaccination of 1-day-old broilers with one of three different bacterins (in mineral oil adjuvant, corn oil adjuvant, or saponin adjuvant) 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 (van Empel and van den Bosch, 1998).

Also, vaccination of broiler breeders with the inactivated vaccine at 12th and 18th week of age induce high level of antibodies and allowed them to pass to their offspring and supply the offspring with a good protection against lesions and clinical disease after *O. rhinotracheale* challenge at day 14 and day 30 (Bisschop et al., 2004; van Empel and van den Bosch, 1998). The protection, however, decreased with increasing age of the progenies (van Empel

and van den Bosch, 1998). Antibody titers in unvaccinated flocks increased during the study period, suggesting that there was circulation of the bacterium among broiler breeders (van Empel and van den Bosch, 1998).

Cauwerts et al. (2002) investigated the effect of *O. rhinotracheale* vaccination of broiler breeders on antibody titres and performance of breeders and their offspring. Statistical analyses revealed no differences in performance between vaccinated and unvaccinated breeder flocks. On the other hand, a significantly lower mean mortality rate and higher mean production index in the broilers derived from vaccinated breeders could be observed. Recently, De Herdt et al. (2012) published a report on the effect of *O. rhinotracheale* inactivated vaccines applied at week 8 and week 18 in breeder flocks on several economic parameter of their offspring under field condition. They evaluated the parameters from 100 broiler flocks derived from four vaccinated breeder farms and four unvaccinated breeder farms of the same organization in Belgium. The obtained results showed a significant lower broiler loss and a significant higher production index in the broiler flocks derived from vaccinated breeders.

To determine the efficacy of vaccination of pullets against *O. rhinotracheale* infection investigation were carried out by Murthy et al. (2007) using eight different vaccines, with different inactivating substances (formalin and thiomersal) and with or without adjuvant (mineral oil, alum, and aluminium hydroxide gel). The birds were vaccinated twice, namely at week 8 and at week 12 of age and challenged in week 15, 6 days after priming with Newcastle disease virus (NDV). The results showed that bacterin in mineral oil adjuvant induced the highest serologic response and a significant decrease of lesions such as air sacculitis and pneumonia in vaccinated birds compared with the unvaccinated challenge control birds.

Vaccination of young turkeys with an autogenous bacterins successfully reduced the number of outbreaks of *O. rhinotracheale* infections in the field of turkeys in Israel (Bock et al., 1997). However, repeated infections caused by other serotypes commonly occur during the long rearing period of turkeys (van Empel, 2002).

In meat turkey flocks Hafez et al. (1999) carried out field trials using monovalent or trivalent inactivated vaccines. Both trials were carried out on farms with a known history of *O. rhinotracheale* infections. In trial 1 male and female birds (house 1) were vaccinated subcutaneously at seven and ten weeks of age with a monovalent bacterin of *O. rhinotracheale* serotype A in watery adjuvant, while the birds in house 2 were kept as non-vaccinated control. In the second trial male and females birds were kept in three houses

(houses 1-3) and vaccinated twice at 1st day and at 3rd week of age with a trivalent bacterin, containing the *O. rhinotracheale* serotypes A, B, and D, while the birds in the house 4-6 were kept as non- vaccinated control. Vaccination resulted in induction of antibodies for a short duration. In trial 1, the mortality rate and the total quantity of condemned meat were higher in the non-vaccinated control birds. No differences were found with regard to the body weight. In the 2nd trial, the mortality rate was comparable for both groups. The total quantity of condemned meat was higher in non-vaccinated groups. No differences were found with regard to the body weight.

Also Sprenger et al. (2000b) vaccinated 6-week-old turkeys subcutaneously with a killed *O. rhinotracheale* vaccine and challenged them intratracheally with live *O. rhinotracheale* at 14 or 21 weeks of age. Lesion such as air sacculitis and pneumonia occurred less frequently in vaccinated birds than unvaccinated birds after challenge, and *O. rhinotracheale* was recovered from unvaccinated, challenged birds but not from vaccinated, challenged or from unchallenged birds. Furthermore, serum samples from turkeys vaccinated with killed *O. rhinotracheale* vaccines contained antibodies to the organism within 1 week after vaccination. These antibodies were detected for eight weeks after vaccination (Sprenger et al., 2000b).

A cross-protective immunity against different *O. rhinotracheale* serotypes can be induced by live vaccination, and sera from live vaccinated and cross-protected birds were used for immunoscreening of an *O. rhinotracheale* serotype G expression library (Schuijffel et al., 2005a). Based on earlier results, they identified several candidate proteins for the development of a cross-protective vaccine against *O. rhinotracheale* infections (Schuijffel et al., 2005b). Eight genes encoding cross-reactive antigens were amplified, cloned in an expression vector, and expressed in *Escherichia coli*. The purified recombinant proteins with a molecular mass ranging from 35.9 kDa to 62.9 kDa were mixed and tested as a subunit vaccine for (cross-) protection against challenge with homologous and heterologous *O. rhinotracheale* serotypes in chickens. This vaccination resulted in the production of antibodies against the recombinant proteins. It further provided homologous as well as heterologous protection against *O. rhinotracheale* challenge in chickens. In a further study a four component subunit vaccine was able to protect the birds against challenge with a heterologous *O. rhinotracheale* serotype (Schuijffel et al., 2006).

2.12.2.2 LIVE VACCINES

Vaccination of broilers with a live vaccine per spray was found to be effective, when the maternal antibody levels were low. Comparing vaccination at day 1, 7, or 14 followed by challenge 14 days post vaccination brought good results and the lowest (11 %) incidence of airsacculitis and pneumonia in birds, which had been vaccinated at 14 days of age (van Empel and van den Bosch, 1998).

Roepke (1998) administered an autogenous live vaccine via the oral route in 6-week-old turkeys, that resulted in a reduction of pathologic lesions and mortality when the turkeys were older. Turkeys were simultaneously spray vaccinated with a live Newcastle disease vaccine without any problems (Roepke et al., 1998).

Lopes et al. (Lopes et al., 2002b) developed a temperature-sensitive (Ts) mutant of *O. rhinotracheale* for use as a live vaccine. Vaccination with this strain appears to offer some protection in turkeys and has been reported to elicit a secretory immune response (IgA) and to evoke a protective response against experimental *O. rhinotracheale* challenge. Three weeks after vaccination in the drinking water or by oculo-nasal drops, antibodies were detected. Moreover, the Ts-mutant vaccine colonized the upper respiratory tract but not the lower respiratory tract (Lopes et al., 2002a). Sprenger et al. (2000b) vaccinated 6-week-old turkeys intranasally with a live vaccine and challenged them intratracheally with live *O. rhinotracheale* at 14 or 21 weeks of age. Lesion such as airsacculitis and pneumonia occurred less frequently in vaccinated birds than in unvaccinated birds after challenge. Furthermore, serum samples from turkeys vaccinated with live *O. rhinotracheale* vaccines contained antibodies to the organism within one week of vaccination. These antibodies were detected for 14 weeks after vaccination (Sprenger et al., 2000b).

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4. PUBLICATIONS

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4.1 Publication 1

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RESEARCH ARTICLE

Influence of Different Storage Media, Temperatures and Time Duration on Susceptibility of *Ornithobacterium rhinotracheale*

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ABSTRACT

Ornithobacterium rhinotracheale (ORT) is an important respiratory pathogen of chickens and turkeys. Isolation of the bacterium from diseased birds is necessary for serotyping, to determine the antimicrobial susceptibility for an effective therapy and to produce autogenous vaccines. A series of experiments was carried out to determine optimal conditions for storage of swabs soaked in ORT suspension. Swabs were immersed in viable ORT suspensions with different bacterial counts and then stored under different conditions. At several time points the viable ORT count in the swabs was determined. Dry cotton swabs as well as three transport media, namely Amies gel medium (AG), Amies gel medium with charcoal (AC), and Stuart gel medium (SG) were tested. ORT could be reisolated from dry swabs stored at room temperature for up to five days and from swabs stored in the media at room temperature for more than seven days. Differences among the transport media were minor. The minimal number of cfu in the ORT-suspension, in which the swabs were soaked, was 10^5 cfu/ml for successful reisolation of ORT one day post immersion from swabs stored at room temperature in AC medium, and 10^6 cfu/ml was successful for reisolation from dry swabs. Higher inoculation doses and storage at 4°C prolonged the period in which ORT could be reisolated. Storage of dry swabs at -20°C allowed reisolation of ORT at a constant level for at least 5 d.p.i. Inoculation of swabs with ORT and *E. coli* reduced the period for which ORT could be reisolated.

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INTRODUCTION

Ornithobacterium rhinotracheale (ORT) is an important respiratory pathogen of chickens and turkeys with worldwide distribution (Chansiripornchai *et al*., 2007; Murthy *et al*., 2008; Tabatabai *et al*., 2008; Ghanbarpour and Salehi, 2009). ORT is a fastidious, Gram negative, oxidase positive rod. It grows slowly but can be isolated on blood agar at 37°C under microaerophilic conditions (Van Empel and Hafez, 1999, Hafez and Vandamme, 2011). By agar gel precipitation test 18 serotypes can be distinguished (Van Empel and Hafez, 1999; Chin *et al*., 2008). Since clinical signs and post-mortem lesions of ORT infections are not sufficiently specific to allow diagnosis, laboratory methods are needed for definite diagnosis. While detection of nucleic acids by PCR is reliable and fast (Hassanzadeh *et al*., 2010), isolation of the bacterium is necessary for serotyping, to determine the antimicrobial susceptibility for an effective therapy, and to produce autogenous vaccines.

However, many factors can interfere with isolation of ORT such as the time of sampling, presence of secondary infections and shipment from farm to the diagnostic laboratory. While ORT can readily be isolated from infected birds in an early stage of the infection, the recovery of ORT in later stages may fail (Kilic *et al*., 2009). After an ORT infection, other bacteria, especially *E. coli* (Sakai *et al*., 2000; Sprenger *et al*., 2000), can induce secondary infections. Because these bacteria have a higher tenacity and grow faster, they may overgrow the fastidious and slowly growing ORT when isolation is tried.

Mostly tracheal swabs or swabs taken from lungs or air sacs at post mortem are sent instead of organs. Swabs for microbiological analysis are usually placed in various media for transport to the laboratory. Swabs could be placed into a nonnutritive transport medium, which keeps

the bacteria viable, but does not permit overgrowth of one pathogen by other bacteria present in the sample (Rosa-Fraile *et al*., 2005, Morosini *et al*., 2006). A large number of studies evaluated different swabs and transportation systems with a variety of anaerobes and fastidious aerobes (Thompson and French, 1999; Morosini *et al*., 2006). However, there is no information in the literature on the comparative performance of various transport systems in regard to ORT.

Thus the objective of this study was to determine optimal conditions for collection, and storage conditions for the samples to be transported to laboratory for successful ORT detection.

MATERIALS AND METHODS

Strains used to contaminate the swabs: Gentamycin resistant ORT strain of serotype A (B3263/91) was used as standard strain for all experiments and was kindly provided by Intervet International Boxmeer, The Netherlands. Additionally the effect of the bacterial counts in the ORT suspension, in which the swabs were soaked, and storage temperatures were tested with field isolates F56/10 (serotype A), F488/10 (serotype B), and F94/09 (serotype E), which were isolated and serotyped in our laboratory as described by Hafez and Sting (1999). ORT was grown on 5% sheep blood agar plates with 10 µg/ml gentamycin. The plates were incubated microaerobically in 5% CO_2 atmosphere at 37°C for 48 hr.

Escherichia coli strain GB 1927/10/3 was used to determine the effect of storage of ORT together with *E. coli* on ORT reisolation. It was isolated from turkeys and classified as susceptible against gentamycin by agar diffusion test. It was grown on Columbia agar (Oxoid, Wesel, Germany) at 37°C for 24 h.

For preparation of the inocula plates were flooded with PBS. An initial bacterial suspension containing 10^7 – 10^8 cfu/ml in PBS was prepared for each experiment by adjusting turbidity to McFarland standard 0.5.

Viable bacterial counts: Viable bacterial counts were determined by preparing a tenfold dilution series in PBS. Then 100 µl of each dilution were streaked on plates with Drigalski spatula and incubated as described above. ORT colonies were visually counted after 48 h.

Comparison between different transport media and dry swabs: Dry cotton swabs and three different transport media, namely i) Amies gel medium (AG), ii) Amies gel medium with charcoal (AC) and iii) Stuart gel (SG) medium, (all COPAN, Brescia, Italy) were used. Swabs were immersed for 2 min in an ORT suspension with a McFarland turbidity of 0.5 and then placed into their respective transport media. Dry swabs were stored in sterile glass tubes. The swabs were held at room temperature. After various time intervals two swabs of each medium were suspended in 1 ml sterile PBS each and the bacterial counts were determined.

Effect of ORT concentrations on the viability after storage of dry cotton swab at room temperature: In a first experiment sterile dry cotton swabs were used as bacterial carriers and immersed for 2 min in an ORT suspension with a McFarland turbidity of 0.5. Then they were removed and kept in sterile glass tubes at room temperature. After various time intervals two swabs of each storing temperature were suspended in 1 ml sterile PBS each and the bacterial counts were determined.

In a second experiment with similar design swabs were stored at 4°C or -20°C for various time intervals.

Dry swabs absorbed in different ORT concentrations: Starting with an ORT suspension with a McFarland turbidity of 0.5 a tenfold dilution series was prepared in sterile PBS to a dilution of 1:10^6. Sterile, dry cotton swabs were immersed for 2 min in each dilution. Then they were removed and kept in sterile glass tubes at room temperature. After various time intervals two swabs of each concentration were suspended in 1 ml sterile PBS each and the bacterial counts were determined.

Effect of ORT concentrations on the reisolation after storage of swabs in AC media at different temperatures: Starting with an ORT suspension with a McFarland turbidity of 0.5 a tenfold dilution series was prepared in sterile PBS to a dilution of 1:10^4. Swabs of the transportation system using AC medium were immersed for 2 min in the undiluted suspension as well as in the 1:10^2, 1:10^4 and 1:10^6 dilutions. The swabs were placed into their plastic devices containing the medium and held at room temperature. After various time intervals two swabs of each combination of inoculation concentration and storage temperature were suspended in 1 ml sterile PBS each and the bacterial counts were determined. The experiment was repeated with field strains F56/10, F488/10, and F94/09, of which only the undiluted inocula and the 1:10^2 dilutions were tested.

Effect of storage of ORT in mixed culture with *E. coli* on the reisolation: Eight suspensions containing different concentrations of ORT and/or E. coli were prepared by mixing equal parts of ORT and *E. coli* suspensions. Suspensions were either adjusted to McFarland standard 0.5 (about. 10^7 cfu/ml) or diluted 1:10^2 (about 10^5 cfu/ml). Swabs of the AC transportation system were immersed for 2 min in the bacterial suspensions. The swabs were placed into their plastic devices containing the medium and held at room temperature. At various time intervals two swabs of each inoculum were streaked directly on 5% sheep blood agar plates with gentamycin and on Gassner agar (Oxoid, Wesel, Germany).

RESULTS

Comparison between different transport media: The total ORT count in the inoculum was $10^{7.3}$ cfu/ml. The viable counts of reisolated ORT from swabs kept in Amies gel medium and AC medium as well as in Stuart gel medium were similar. Until the end of the experiment on day 7 reisolation counts showed a slow but steady decline from about $10^{5.2}$ cfu to 10^3 cfu, but ORT was reisolated from all swabs stored in media throughout the experiment. In contrast the viable bacterial counts from dry swabs decreased faster, and 6 and 7 days post inoculation (d p. i.) no ORT was reisolated from dry swabs (Table 1).

Table 1: Mean (n=2) $\log_{10}$ of cfu *Ornithobacterium rhinotracheale* (ORT) reisolated from dry swabs and swabs of transport systems with Amies gel (AG) medium, Amies gel medium with charcoal (AC) or Stuart gel (SG) medium stored at room temperature for various time intervals.

Medium	ORT count in inoculum (cfu/ml)	Storage time								
		3 h	6 h	1 d	2 d	3 d	4 d	5 d	6 d	7 d
Dry	$10^{7.3}$	6.27	5.45	5.86	4.73	2.40	2.95	3.48	-*	-
AG	$10^{7.3}$	5.36	5.66	5.34	5.11	4.39	4.10	3.70	3.11	2.98
AC	$10^{7.3}$	5.13	5.45	5.28	4.85	4.45	4.54	3.40	3.55	3.37
SG	$10^{7.3}$	5.16	5.37	5.15	4.48	4.43	4.38	3.30	3.46	3.26

*no ORT reisolated

Dry swabs stored at different temperatures: Viable ORT counts in the inocula were $10^{7.7}$ cfu/ml in the first experiment and $10^{7.6}$ cfu/ml in the second experiment. ORT counts obtained from dry swabs stored at room temperature declined quickly within the first two days p. i. to $10^{1.6}$ cfu (Table 2). In contrast viable ORT counts from dry swabs stored at 4°C stayed almost constant at about 10^{6} cfu after 2 days in the first experiment and $10^{4.5}$ cfu in the second experiment for the first three days, before decreasing sharply to about 10^{2} cfu at day 5. Storage of the dry swabs at -20°C allowed recovery of ORT at a constant level of about $10^{4.7}$ cfu (Table 3), till 2nd day.

Table 2: Mean (n=2) $\log_{10}$ of cfu *Ornithobacterium rhinotracheale* (ORT) reisolated from dry swabs stored at room temperature (RT) and at 4°C for various time intervals.

Storage temperature	ORT count in inoculum (cfu/ml)	Storage time			
		3 h	6 h	1 d	2 d
RT	$10^{7.7}$	5.99	6.26	4.15	1.63
4 °C	$10^{7.7}$	6.13	6.20	5.99	5.75

Effect of ORT storage on dry cotton swab at room temperature: The ORT count in the undiluted inoculum was $10^{7.7}$ cfu/ml. The two highest inoculation doses of $10^{7.7}$ cfu/ml or $10^{6.7}$ cfu/ml allowed viability until 1 d p. i. From swabs inoculated with $10^{5.7}$ cfu/ml or $10^{4.7}$ cfu/ml ORT could be recovered 3 hours post inoculation (h.p.i.) and 6 h p. i. From swabs inoculated with $10^{3.7}$ cfu/ml only 3 h p. i. ORT was reisolated. From swabs inoculated with $10^{2.7}$ cfu/ml or $10^{1.7}$ cfu/ml no ORT was found viable (Table 4).

Table 3: Mean (n=2) $\log_{10}$ of cfu ($10^{7.6}$) *Ornithobacterium rhinotracheale* (ORT) reisolated from dry swabs stored at 4°C and at -20°C for various time intervals.

Storage time	Storage temperature (°C)	
	4	-20
3 h	4.80	4.79
6 h	4.75	4.78
1 d	4.58	4.76
2 d	4.42	4.70
3 d	4.42	4.55
4 d	2.39	4.50
5 d	2.00	4.59

Susceptibility of ORT concentrations after storage of swabs in AC media stored at different temperatures: The ORT count in the undiluted inoculum was $10^{7.8}$ cfu/ml. From swabs inoculated with undiluted suspension and stored at room temperature reisolation counts were between 10^{4} cfu and 10^{5} cfu until 4 d p. i.. Afterwards they declined. On day 7 p. i. reisolation was still possible from reference strain A (B3263/91) as well as from field isolates F56/10 and F488/10, but not from field isolate F94/09. 14 d p. i. no reisolation was possible from all tested swabs. In contrast from swabs inoculated with undiluted suspension and stored at 4°C up to $10^{3.7}$ cfu were reisolated 14 d p. i., only field isolate F94/10 could not be reisolated from these swabs.

Inoculation with the $1{:}10^{2}$ dilution of the inoculum allowed reisolation until 2 d p. i. from swabs stored at room temperature. ORT counts reisolated from swabs inoculated with the $1{:}10^{2}$ dilution of the inoculum and stored at 4°C decreased only slowly from about $10^{3.3}$ cfu 1 d p. i. to about 10^{2} cfu 7 d p. i.. 14 d p. i. reisolation was not possible. From swabs inoculated with $10^{3.8}$ cfu/ml or $10^{1.8}$ cfu/ml of the reference strain A (B3263/91) no ORT could be reisolated 1 d p. i. (Table 5).

Effect of storage of ORT in mixed culture with *E. coli* on the reisolation: From swabs inoculated with $10^{7.0}$ cfu/ml ORT without *E. coli* ORT was reisolated until 6 d p. i., from the swabs inoculated with $10^{5.0}$ cfu/ml ORT without *E. coli*, ORT was reisolated until 3 d p. i.. Absorbing the swabs additionally with *E. coli*, regardless of the bacterial counts used, shortened the period in which ORT was reisolated to 3 days and 2 days p. i., respectively. *E. coli* was reisolated from all swabs whose inoculum had contained *E. coli* throughout the whole experiment, and it even frequently grew on the blood agar containing gentamycin (Table 6).

Table 4: Mean (n=2) $\log_{10}$ of cfu *Ornithobacterium rhinotracheale* (ORT) reisolated from dry swabs inoculated with suspensions containing different ORT counts and stored at room temperature for various time intervals.

ORT count in inoculum (cfu/ml)	Storage time at room temperature			
	3 h	6 h	1 d	2 d
$10^{7.7}$	6.83	6.08	5.15	-*
$10^{6.7}$	5.31	5.09	2.45	-
$10^{5.7}$	3.82	3.77	-	-
$10^{4.7}$	3.46	3.35	-	-
$10^{3.7}$	2.15	-	-	-
$10^{2.7}$	-	-	-	-
$10^{1.7}$	-	-	-	-

*no ORT reisolated

DISCUSSION

A series of experiments was conducted to determine optimal conditions for storage of swabs absorbed with ORT. These conditions should help to determine the possible optimal conditions for shipment of the swabs from farm to the diagnostic laboratory.

Three transport media, namely Amies gel medium, Amies gel medium with charcoal (AC) and Stuart gel medium were included in the investigation and compared to dry swabs. Amies medium is a variation of Stuart medium containing further additives (Amies, 1967). Charcoal can be added to the medium to help neutralize compounds which are toxic to the bacteria (Gästrin *et al.*, 1968; Khursheed and Lang, 1996), but its addition to media is not necessarily correlated with better performance (Human and Jones, 2004). Stuart medium was originally intended as transport medium for gonococci (Stuart, 1946). All three media have been shown suitable for transport of a variety of different bacteria (Barber *et al.*, 1998).

Table 5: Mean (n=2) $\log_{10}$ of cfu *Ornithobacterium rhinotracheale* (ORT) reisolated from swabs inoculated with suspensions containing different ORT counts and stored at room temperature and at 4 °C in Amies gel medium with charcoal for various time intervals.(reshuffle the following indicated data to make it in proper descending order of ORT count in the inoculums column)

ORT strain	ORT count in inoculum (cfu/ml)	Storage temperature	Storage time							
			1 d	2 d	3 d	4 d	5 d	6 d	7 d	14 d
B3263/91	$10^{7.8}$	RT	4.6	4.7	4.6	4.0	3.1	2.1	1.5	-*
		4 °C	5.4	5.3	5.5	5.4	4.8	4.3	4.3	3.7
F56/10	$10^{7.4}$	RT	4.5	4.5	4.4	4.2	3.5	2.9	2.6	-
		4 °C	5.2	5.0	5.4	5.3	4.5	4.2	4.1	3.4
F488/10	$10^{7.2}$	RT	4.8	4.7	4.6	4.5	3.5	2.6	2.5	-
		4 °C	5.5	5.3	5.4	5.2	4.7	4.3	4.2	3.8
F94/09	$10^{7.5}$	RT	4.4	4.5	4.3	3.9	3.6	2.5	-	-
		4 °C	5.3	5.2	5.2	5.0	4.5	4.1	3.7	-
B3263/91	$10^{5.8}$	RT	2.8	2.6	-	-	-	-	-	-
		4 °C	3.3	2.7	2.8	2.5	2.4	2.5	2.6	-
F56/10	$10^{5.4}$	RT	2.8	2.4	-	-	-	-	-	-
		4 °C	3.3	2.9	2.9	2.6	2.3	2.2	2.2	-
F488/10	$10^{5.2}$	RT	2.4	2.0	-	-	-	-	-	-
		4 °C	3.3	2.5	2.5	2.4	2.2	2.0	2.0	-
F94/09	$10^{5.5}$	RT	2.8	2.6	-	-	-	-	-	-
		4 °C	3.2	2.6	2.4	2.2	2.0	1.9	1.9	-
A (B3263/91)	$10^{3.8}$	RT	-	-	-	-	-	-	-	-
		4 °C	-	-	-	-	-	-	-	-

Table 6: Reisolation of *Ornithobacterium rhinotracheale* (ORT) and *E. coli* from swabs inoculated with suspensions containing different ORT and *E. coli* concentrations, stored at room temperature in Amies gel medium with charcoal for various time intervals.

ORT suspension	E. coli suspension	Agar for reisolation	Storage time (d)							
			1	2	3	4	5	6	7	14
10^7 cfu/ml	PBS[1]	Blood	ORT	ORT	ORT	ORT	ORT	ORT	-*	-
		Gassner	-	-	-	-	-	-	-	-
10^7 cfu/ml	10^5 cfu/ml	Blood	ORT	ORT	ORT,*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
		Gassner	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
10^7 cfu/ml	10^7 cfu/ml	Blood	ORT	ORT, *E. coli*	ORT, *E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
		Gassner	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
10^5 cfu/ml	PBS[1]	Blood	ORT	ORT	ORT	-	-	-	-	-
		Gassner	-	-	-	-	-	-	-	-
10^5 cfu/ml	10^5 cfu/ml	Blood	ORT, *E. coli*	ORT, *E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
		Gassner	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
10^5 cfu/ml	10^7 cfu/ml	Blood	ORT, *E. coli*	ORT, *E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
		Gassner	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
PBS[1]	10^7 cfu/ml	Blood	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
		Gassner	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
PBS[1]	10^5 cfu/ml	Blood	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
		Gassner	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*

[1]instead of the bacterial suspension sterile PBS was added; *no bacterial growth

Differences of the transport media in regard to viable ORT count were minor. Similar results were obtained by Barber *et al.* (1998), who tested ten different systems with several bacterial species. However, transport systems using the same medium from different manufacturers may produce different results (Morosini *et al*., 2006). Dry swabs kept ORT viable between 2 and 5 d p. i. at room temperature, but their performance was variable. Dry swabs in aerobic tubes previously had been shown suitable for transport of bacteria (Roelofsen *et al*., 1999). Possible advantages are the cost and that dry swabs do not allow multiplication of other bacteria that might overgrow ORT.

Higher inoculation doses and storage at 4°C prolonged the period in which ORT could be reisolated. The same influence of storage temperature on viability has been described for some bacterial species (Human and Jones, 2004), while other combinations of bacteria and media yielded similar results at 4°C and room temperature (Tvede and Hoiby, 1992; Human and Jones, 2004). Surprisingly, storage of dry swabs at -20°C allowed reisolation of ORT at a constant level for at least 5 d p. i. The recovery rates were similar, regardless of whether the reference strain or a field isolate was tested.

Inoculation of swabs with ORT and *E. coli* showed that additional immersion of swabs with secondary pathogens can compromise reisolation of ORT. This experiment also underlined the low viability of ORT compared to *E. coli*. which explained the lower isolation rate compared to high detection rate using PCR or immunohistochemistry (van Veen *et al*., 2000; Hafez and Vandamme, 2001). From second day after p. i. it was also possible to isolate *E. coli* on blood agar containing gentamycin. This indicated that *E. coli* multiplied in the transport medium to such numbers that it could overcome the adverse effect of the gentamicin used into the blood agar.

In conclusion for a successful isolation of ORT swabs may be stored in transport medium and brought to the laboratory as earlier as possible. Moreover, swabs may be refrigerated during transportation and at the laboratory, if they are not to be processed immediately. There is no data about the counts of ORT in organs of naturally infected birds, and probably they vary depending on the involved strain, intensity of infection and stage of the infection.

Therefore, several ORT counts in the inoculum were tested and the results showed that this parameter was the most influential. So the selection of a sample for swabbing that contains a high amount of ORT with as few other bacterial load is important for a successful reisolation in the laboratory.

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4.2 Publication 2

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5. CONCLUDING REMARKS

Respiratory disease conditions are continuing to cause heavy economic losses in the poultry industry. The gram negative rod shaped bacterium (*O. rhinotracheale*, ORT) is a causative agent of respiratory disease either alone (mono-causal) or in synergy with different other micro-organisms (multi-causal).

Since clinical signs and post-mortem lesions of *O. rhinotracheale* infections are not sufficiently specific to allow diagnosis, laboratory methods are needed for definite diagnosis, e. g. by isolation of *O. rhinotracheale*. However, many factors can interfere with isolation of *O. rhinotracheale* such as the time of sampling, presence of secondary infections by other bacteria, which may overgrow *O. rhinotracheale*, and shipment from farm to the diagnostic laboratory.

First optimal conditions for sample shipment from farm to the diagnostic laboratory were determined. Tested parameters were the transport medium, temperature during transport, inoculation dose, and additional contamination of transport swabs with *E. coli*.

Three transport media, namely Amies gel medium, Amies gel medium with charcoal (AC) and Stuart gel medium were included in the investigation and compared to dry swabs. Differences of the transport media in regard to viable *O. rhinotracheale* count were minor. Viable *O. rhinotracheale* counts 7 d p. i. from swabs stored in Stuart medium or in AC medium were similar, whereas viable *O. rhinotracheale* counts in transport swabs stored in Amies medium without charcoal were slightly less.

Dry swabs kept *O. rhinotracheale* viable between 2 and 5 days post infection (d p. i.) at room temperature, and their performance was volatile. This was shown by the uneven decline of *O. rhinotracheale* counts reisolated from dry swabs and by the differences between the different experiments. Possible advantages are the cost and that dry swabs do not allow multiplication of other bacteria that might overgrow *O. rhinotracheale*.

Inoculation dose and storage temperature had the most important impact on the recovery of *O. rhinotracheale*. For successful reisolation 1 d p. i. dry swabs stored at room temperature needed to be contaminated with at least 10^6 cfu/ml and swabs in AC medium with at least 10^5 cfu/ml. Higher inoculation doses and storage at 4°C prolonged the period in which *O. rhinotracheale* could be reisolated. Surprisingly, storage of dry swabs at -20°C allowed reisolation of *O. rhinotracheale* at a constant level for at least 5 d p. i..

Inoculation of swabs with *O. rhinotracheale* and *E. coli* showed that contamination of swabs with secondary pathogens can compromise reisolation or *O. rhinotracheale*, since additional contamination with *E. coli* shortened the period, during which *O. rhinotracheale* was isolated, one day.

In conclusion for a successful isolation of *O. rhinotracheale* swabs should be stored in transport medium and brought to the laboratory as fast as possible. If possible, swabs should be refrigerated during transport and at the laboratory, if they cannot be processed immediately. However, most important is the selection of a sample for swabbing that contains a high amount of *O. rhinotracheale* with as few other bacteria as possible.

Besides "only" diagnosis, isolation of *O. rhinotracheale* allows serotyping. Currently 18 different serotypes designated A to R have been identified by AGP.

Between 2009 and 2011 372 *O. rhinotracheale* isolates were typed by AGP at the Institute for Poultry Diseases. 310 isolates from turkeys and 62 isolates from unknown origin were typed using agar gel precipitation test. 56.1 % of isolates from turkeys belonged to serotype A and 20.6 % to serotype E. The prevalence of other isolates was below 10 %. Serotypes D, F, and K were not detected. Eleven isolates were not typable with reference sera against serotypes A – L and might belong to serotypes M – R or to undescribed serotypes. The three serotypes most often found in the isolates from unknown origin were A (35.5 %), B (19.4 %), and C (12.9 %). The prevalence of other isolates was below 10 %. Serotypes F and K were not detected. Seven isolates were not typable with reference sera A – L. In previous investigations turkey isolates belonged mainly to serotype A, B and D (van Empel et al., 1997). Here also serotype A was the most prevalent, followed by type E, while serotypes B and D were rarely encountered. The difference might be attributable to regional differences, which have been noted (van Empel et al., 1997), or to a change in the prevalence of serotypes during the last 15 years.

Cross reactions, especially of serotype A isolates with serotypes I, H and J, were common. Cross reactions, especially involving serotypes A, H, i, and J were more common than reported by Hafez and Sting (1999) and by van Empel et al. (1997), who found that cross reactions between serotype A and B as well as between B and E exist. One reason might be that in the older studies only reference sera against serotypes A – G were used.

The relevance of the serotypes is controversial. It seems clear, that serotypes are not associated with virulence for different hosts (van Empel et al., 1996), and there are indications that vaccines are cross protective against several serotypes (Schuijffel et al.,

2005a). However, many poultry veterinarians in Germany prefer their *O. rhinotracheale* isolates to be typed before they are used for the production of autogenous vaccines. However, the test requires a relatively large amount of different antisera and the results can be subjective, while the cross-reactions can cause some confusion. Therefore molecular biological tools have been investigated as possible means of further characterization of *O. rhinotracheale* isolates.

In the present study the partial 16S rRNA gene and the complete Or01 gene of *O. rhinotracheale* reference strains and field isolates were sequenced. Identities between the Or01 genes was between 94 % and 100 % and thus lower than between the partial 16S rRNA PCR amplicons (98-100%). Both trees showed some similarities, e. g. reference strain F and a certain field isolate belonging to serotype H were set apart from the other strains. Further reference strains D and H also were grouped closely together in both trees. However, there was no apparent correlation between reference strains and isolates belonging to one serotype, so sequencing of 16S rRNA or of the Or01 gene does not seem to be a suitable method to replace the AGP for serotyping.

However, due to the difficulties to isolate *O. rhinotracheale* as outlined above, diagnosis of *O. rhinotracheale* often is done by PCR. Between 2009 and 2011. 714 dry swabs taken from diseased turkeys, broilers, broiler breeders, layers, or from unknown origin were investigated by PCR for the presence of *O. rhinotracheale*. One hundred ninety seven out of 481 swabs from turkeys (41.0 %), 10 out of 144 swabs from broilers or broiler breeders (6.9 %), 17 out of 28 swabs from layers (60.7 %), and 26 out of 61 swabs from unknown origin (42.6%) were tested positive. The results of three swabs from turkeys were suspect.

These results show that in turkeys *O. rhinotracheale* can be regarded as one of the main reasons of respiratory disease of turkeys, as the bacterium was detected in 41 % of investigated swabs from flocks with respiratory signs. In contrast the prevalence of *O. rhinotracheale* in broilers seems to be overestimated, since only 7 % of swabs were tested positive. Interestingly the detection of *O. rhinotracheale* in swabs taken from layers was very high, although only comparatively few swabs from layers were investigated. This might indicate that infections of layers with *O. rhinotracheale* are more prevalent than assumed by veterinarians in the field.

6. REFERENCES

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7. SUMMARY

Investigations on the viability as well as on the molecular and serological characterization of *Ornithobacterium rhinotracheale* (ORT) isolates

Ornithobacterium rhinotracheale (*O. rhinotracheale*, ORT) is a gram-negative staining rod. In chickens and turkeys *O. rhinotracheale* causes a respiratory disease. Isolation of the bacterium from infected flocks is necessary for serotyping, to produce autogenous vaccines, and to determine the antimicrobial susceptibility for an effective therapy.

Therefore, in the first part of the thesis, a series of experiments was carried out to determine optimal conditions for storage of swabs soaked in *O. rhinotracheale* suspension to simulate the transport of swabs for isolation of *O. rhinotracheale* to the laboratory. Swabs were immersed in *O. rhinotracheale* suspensions with different bacterial counts and then stored under different conditions. At several time points the viable *O. rhinotracheale* count in the swabs was determined. Dry cotton swabs as well as three transport media, namely Amies gel medium, Amies gel medium with charcoal (AC), and Stuart gel medium were compared. *O. rhinotracheale* was reisolated from dry swabs stored at room temperature for up to five days and from swabs stored in the media at room temperature for more than seven days. Differences among the transport media were minor. The minimal number of cfu in the *O. rhinotracheale* suspension in which the swabs were soaked was 10^5 cfu/ml for successful reisolation of *O. rhinotracheale* one day post immersion from swabs stored at room temperature in Amies gel medium with charcoal, and 10^6 cfu/ml was successful for reisolation from dry swabs. Higher inoculation doses and storage at 4°C prolonged the period in which *O. rhinotracheale* could be reisolated. Storage of dry swabs at -20°C allowed reisolation of *O. rhinotracheale* at a constant level for at least five days. Inoculation of swabs with *O. rhinotracheale* and *E. coli* reduced the period during which *O. rhinotracheale* was reisolated.

In the second part of the thesis information about diagnosis and serotyping of *O. rhinotracheale* isolates at the Institute of Poultry Diseases of the Free University Berlin was compiled and analyzed. Between 2009 and 2011 714 dry swabs taken from diseased turkeys, broilers, broiler breeders, layers, or from unknown origin were investigated by PCR for the presence of *O. rhinotracheale*. One hundred ninety seven out of 481 swabs from turkeys (41.0 %), 10 out of 144 swabs from broilers or broiler breeders (6.9 %), 17 out of 28 swabs from layers (60.7 %), and 26 out of 61 swabs from unknown origin (42.6%) were tested positive. The results of three swabs from turkeys were suspect.

Furthermore 310 isolates from turkeys and 62 isolates from unknown origin were typed using agar gel precipitation test with antisera prepared for this study. 56.1 % of isolates from turkeys belonged to serotype A and 20.6 % to serotype E. The prevalence of other serotypes was below 10 %. Serotypes D, F, and K were not detected. Eleven isolates were not typable with reference sera against serotypes A – L. The three serotypes most often found in the isolates from unknown origin were A (35.5 %), B (19.4 %), and C (12.9 %). The prevalence of other isolates was below 10 %. Serotypes F and K were not detected. Seven isolates were not typable with reference sera A – L. Cross reactions, especially of serotype A isolates with serotypes I, H and J, were common.

Further the partial 16S ribosomal RNA (rRNA) and the complete Or01 genes of reference strains A – H and of nine field isolates were cloned and sequenced. Identity scores of 16S rRNA fragments were between 98 % and 100 %. Identities of the Or01 sequences were between 94 % and 100 %. Phylogenetic trees of both genes showed similarities. However, there was no apparent correlation between reference strains and isolates belonging to one serotype, so sequencing of 16S rRNA or of the Or01 gene does not seem to be a suitable method to replace the AGP for serotyping.

8. ZUSAMMENFASSUNG

Untersuchungen über die Viabilität sowie über die molekulare und serologische Charakterisierung von *Ornithobacterium rhinotracheale* (ORT) Isolaten

Ornithobacterium rhinotracheale (*O. rhinotracheale*, ORT) ist ein a gram-negatives, stäbchenförmiges Bakterium, das bei Hühnern und Puten Erkrankungen der Atemwege hervorrufen kann. Die Isolierung des Bakteriums ist von großer Bedeutung zur Serotypisierung, zur Herstellung stallspezifischer Impfstoffe sowie zur Testung der Resistenzenlage für eine erfolgreiche Therapie.

Deshalb wurden im ersten Teil der vorliegenden Arbeit eine Reihe von Versuche durchgeführt, um die bestmöglichen Bedingungen für Transport und Aufbewahrung von *O. rhinotracheale* -Tupferproben zu bestimmen. Dafür wurden die Tupfer in *O. rhinotracheale* -Suspensionen mit einem unterschiedlichen Keimgehalt getränkt und unter verschiedenen Bedingungen aufbewahrt. Nach verschiedenen Lagerdauern wurde die Keimzahl von *O. rhinotracheale* in den Tupfern bestimmt. Zunächst wurden Trockentupfer aus Baumwolle sowie Tupfer in drei Transportmedien, und zwar Amies Gel Medium, Amies Gel Medium mit Aktivkohle sowie Stuart Gel Medium, getestet. Aus Trockentupfern, die bei Zimmertemperatur aufbewahrt wurden, konnte *O. rhinotracheale* über bis zu fünf Tage reisoliert werden, aus den in Medium aufgenommen Tupfern über mindestens sieben Tage. Die Unterschiede zwischen den Medien waren gering.

Für eine erfolgreiche Reisolierung von *O. rhinotracheale* nach einem Tag aus Tupfern, die bei Zimmertemperatur in Amies Gel Medium mit Aktivkohle aufgenommen wurden, musste der Keimgehalt in der *O. rhinotracheale* -Suspension zur Kontamination der Tupfer mindestens 10^5 cfu/ml betragen. Für Trockentupfer lag der Wert bei 10^6cfu/ml. Höhere Inokulationsdosen und eine Lagerung bei 4°C verlängerten die Zeit, während der *O. rhinotracheale* reisoliert werden konnte. Aus bei -20°C gelagerten Trockentupfern wurden annähernd gleich bleibende Keimzahlen von *O. rhinotracheale* über mindestens fünf Tage nachgewiesen.

Im zweiten Teil der Arbeit wurden Informationen über die Diagnose und Serotypisierung von *O. rhinotracheale* am Institut für Geflügelkrankheiten der Freien Universität Berlin zusammengestellt und analysiert. Zwischen 2009 und 2011 wurden 714 Trockentupfer aus Puten-, Broiler- oder Legehennenherden mit Atemwegserkrankungen sowie aus Herkünften, zu denen von den Einsendern keine Angaben gemacht wurden, mittels PCR auf *O. rhinotracheale* -DNA untersucht. 197 von 481 Tupfern von Puten (41,0 %), 10 von 144

Tupfern von Broilern (6,9 %), 17 von 28 Tupfern von Legehennen (60,7 %) sowie 26 der 61 Tupfer ohne nähere Angaben (42,6 %) wurden positiv getestet. Das Ergebnis der Untersuchung von drei Tupfern von Puten war fraglich.

Ferner wurden 310 Isolate von Puten und 62 Isolate ohne nähere Angaben mit für diese Arbeit hergestellten Seren im AGP serotypisiert. 56,1 % der Isolate von Puten gehörten zu Serotyp A und 20.6 % zu Serotyp E. Die Prävalenz der anderen Serotypen lag unter 10 %. Kein Isolat gehörte den Serotypen D, F und K an. Elf Isolate waren mit den Referenzseren gegen die Serotypen A – L nicht typisierbar. Die drei Serotypen, die bei den Isolaten ohne nähere Angaben am häufigsten gefunden wurden, waren A (35.5 %), B (19.4 %) und C (12.9 %). Die Prävalenz der anderen Serotypen lag unter 10 %. Kein Isolat gehörte den Serotypen F und K an. Sieben Isolate waren mit den Referenzseren gegen die Serotypen A – L nicht typisierbar. Kreuzreaktionen, insbesondere zwischen Serotyp A mit den Serotypen I, H und J traten häufig auf.

Zuletzt wurde ein Teil des Gens der 16S ribosomalen RNA (rRNA) und das gesamte Or01 Gen der Referenzstämme A bis H und von neun Feldisolaten kloniert und sequenziert. Die Homologien der 16S rRNA Sequenzen lagen zwischen 98 % und 100 %, die der Or01 Sequenzen zwischen 94 % and 100 %. Die phylogenetischen Bäume beider Gene zeigten Ähnlichkeiten, allerdings gab es keinen erkennbaren Zusammenhang zwischen den Refernzstämmen und den Feldisolaten desselben Serotyps. Insofern scheint die Sequenzierung des 16S rRNA Gens oder des Or01 Gens keine geeignete Methode zu sein, um die Serotypisierung mittels AGP zu ersetzen.

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10. SELBSTÄNDIGKEITSERKLÄRUNG

Heremit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich auschließlich die angegebenen Quellen und Hilfe in Anspruch genommen habe.

Berlin 10.01.2013 Sureerat Numee