A Strategic Approach for Immunity-Based Selection of Cross-Protective *Ornithobacterium rhinotracheale* Antigens

Daniëlle Schuijffel

PhD thesis, Faculty of Veterinary Medicine, Utrecht University, The Netherlands
ISBN: 90-8559-032-9

Cover design: Saskia de Hoog, Langenboom, The Netherlands
Printed by: OPTIMA Grafische Communicatie, Rotterdam, The Netherlands

Printing of this thesis was supported by Intervet International BV, Boxmeer, The Netherlands, and the Graduate School of Animal Health (GSAH), Utrecht, The Netherlands.

A Strategic Approach for Immunity-Based Selection of Cross-Protective *Ornithobacterium rhinotracheale* Antigens

Een strategische aanpak voor immuniteit gebaseerde selectie van kruisbeschermende *Ornithobacterium rhinotracheale* antigenen

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

Ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. W. H. Gispen,
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen op
donderdag 17 maart 2005 des middags te 12:45 uur

door

Daniëlle Francisca Schuijffel

Geboren op 26 februari 1976 te Boxmeer, Nederland

Promotor: Prof. dr. J.P.M. van Putten[1]

Co-promotor: Dr. P.J.M. Nuijten[2]

[1] Utrecht University, Faculty of Veterinary Medicine, Department of Infectious Diseases and Immunology, Division of Bacteriology, Utrecht, The Netherlands
[2] Nobilon International BV, Boxmeer, The Netherlands

The research described in this thesis was fully funded by Intervet International BV, Boxmeer, The Netherlands and performed at Intervet International BV, Bacteriology R&D, Boxmeer, The Netherlands.

Aan mijn ouders

CONTENTS

CHAPTER ONE

General Introduction

D.F. Schuijffel

Intervet International BV, Bacteriology R&D, Boxmeer, The Netherlands

INTRODUCTION

Both the human and the animal kingdom are continuously threatened by pathogens, known microbes as well as newly identified and emerging microbial species. Therefore, there is a constant need for quick and effective therapies to control or eradicate infectious diseases caused by these pathogens. For the treatment of bacterial infections, the use of antibiotics is often successful. However, treatment with antibiotics does not prevent the occurrence of subsequent infections with the same pathogen, and since inadequate use of antibiotics can also result in microbial resistance there is an increasing pressure to reduce the use of antibiotics as much as possible, in particular in the veterinary field.

A good alternative for the use of antibiotics is the active stimulation of the host's immune system to induce a protective immune response against a given pathogen. Immunization by vaccination is a cost-effective and successful approach to prevent infectious diseases. Morbidity and mortality due to several human and veterinary infectious diseases have dramatically decreased by the widespread use of vaccines. The vaccines that are applied today are mostly developed according to an empirical approach, based on trial-and-error. Although these vaccines have proven to be effective, the use of current knowledge and technologies makes it possible to develop new and improved vaccines. Present-day knowledge concerning pathogen and host in combination with the availability of numerous new techniques in the field of immunology, biochemistry and molecular biology, offers the opportunity to follow a rational and strategic approach in vaccine development.

This chapter describes 1) the development of vaccines in the past, present and future, 2) the need for veterinary vaccines to prevent bacterial respiratory tract infections of poultry, and 3) the aim of this study: to test the applicability of a strategic approach for the development of an improved vaccine against the emerging poultry pathogen *Ornithobacterium rhinotracheale*. At the end of this chapter the outline of this thesis is formulated.

1 The development of a vaccine

Vaccines can be divided in 3 types: live attenuated micro-organisms, whole inactivated micro-organisms, and subunit preparations (2, 49). In the past, these vaccines have been produced through relatively conventional technologies that lead to empirical attenuation of pathogens (40), the inactivation of micro-organisms via chemical and/or physical treatment (16), or the inactivation of toxins (39, 205) that were known to play a major role in the disease. Although these types of vaccines have proven to be efficacious they all have their pros and cons (2, 49).

Live attenuated bacterial vaccines replicate to a limited extent within the host and can stimulate both humoral and cellular protective responses. These vaccines have a complete antigenic repertoire and are suitable for mass-application. Unfortunately, they also have the potential to contra-indicate in immune-compromised patients, or to revert to a virulent wild-type form. Inactivated or killed vaccines are not able to replicate and are therefore non-infectious and considered safer than the live attenuated formulations. However, these non-replicating vaccines are not as immunogenic as the live vaccines and therefore large-dose,

booster injections and the addition of adjuvants are required to stimulate immunity (177). Furthermore, non-replicating vaccines are known to stimulate the humoral immune response but are very poor inducers of the cellular immune response. Subunit preparations containing purified bacterial polysaccharides from a pathogenic organism, or detoxified proteins, are inducers of the humoral immune response but, like inactivated and killed preparations, contain a limited antigenic composition, and are less suitable for mass-application. Subunit vaccines also need adjuvants or conjugates to render them more immunogenic.

Currently, there are two main reasons for innovative vaccine development: 1) the need for vaccines against new or emerging infectious agents, and 2) the need for improved vaccines to replace some existing vaccines. Despite the success of the currently used vaccines there is considerable room for improvement in vaccinology, for example:

- the possibility to induce broad cross-protection within species and among immunotypes
- the possibility to induce the most efficient type of immune response
- the possibility to control the onset of immunity and duration of immunity
- the possibility to distinguish between a genetically stable, attenuated, live vaccine strain and an infectious virulent wild-type strain (marker-vaccines)
- a clear(er) definition of the protective component(s) contained within a vaccine
- the reduction of non-protective components that may interfere with the degree of protection
- the reduction of undesirable vaccine components such as bacterial endotoxin that can cause negative (systemic) side effects such as fever and malaise
- the reduction of unwanted side effects, such as adverse reactions at the site of injection, caused by the presence of different adjuvants
- a more convenient delivery of the vaccine
- the transfer of immunity to progeny by maternal antibodies when desired
- a safe(r) and cheap(er) production of the vaccine or its components

Insight in host immunology can pave the way to a better understanding of acquired immunity to a certain pathogen and therefore aid in the design of effective vaccines. Characterization of microbial pathogenesis, the identification of highly conserved protective antigens, and a means to deliver the antigen(s) to the host are also important factors that each contribute to the development of a successful vaccine. Today, the rise of new technologies has created the ability to enlarge and improve our understanding of the host immune system and bacterial pathogenesis which allows a more rational approach to vaccine design.

1.1 Characterization of the host immune system

A vaccine should be able to induce immunity that protects the host against a pathogen at the right time and at the right place. As well as clearance of the infectious agent, the immune system should also prevent the host against re-infection with the pathogen. When, despite physiological barriers as skin and mucosa, pathogens enter the host, the first line of defence is provided by innate immune mechanisms (91, 104) such as phagocytic cells (152), natural

killer cells (64) and the complement system (112). Pathogens that pass physical barriers and are not controlled by innate defence mechanisms, initiate a specific immune response: the adaptive immunity (91, 104). Whereas non-adaptive or innate immunity is mostly non-specific, the adaptive immunity is highly specific to the infectious agent. Characteristic is that cells of the adaptive immune system retain "memory" on their encounter with the pathogen. These memory cells respond to re-exposure with the same pathogen by initiating a rapid and highly effective and specific immune response (4). Therefore, stimulation of the adaptive immune system to induce long-lived immunological memory is the most important function of a vaccine.

T-cells, B-cells, and antigen presenting cells are the principal cells of the adaptive immune system which can be divided into the cell-mediated immunity and antibody-mediated (or humoral) immunity (60, 91, 104, 168, 170, 171). In the cell-mediated immunity, 2 important functional subsets of T-cells can be distinguished: helper T-cells, or CD4+ T-cells, and cytotoxic T-cells, or CD8+ T-cells. Antigens derived from pathogens that multiply within the host cells are recognized by cytotoxic T-cells that upon activation kill the infected cells. Antigens derived from extracellular pathogens or pathogens multiplying in intracellular vesicles are recognized by T-helper cells. In humans, and in most mammals that have been studied, these T-helper cells can differentiate into CD4+ T_H1 cells that can stimulate infected macrophages to destroy internalized pathogens, or CD4+ T_H2 cells that can stimulate B-cells, the effector cells of the humoral immune response, to produce specific antibodies that react with these micro-organisms and hasten their destruction (115). In avian species this T_H1 versus T_H2 dichotomy has not yet been fully established (60). Nevertheless, the effector mechanisms of activated T-helper cells leading to the destruction of the antigen or target cell appear to be very similar in birds and mammals (170). Cytokines, proteins secreted by immune cells, are important regulators of the immune responses by signalling between cells, thereby often stimulating the effector function of a target cell (111, 128).

Although most micro-organisms stimulate both cell-mediated and humoral immunity, the type of immunity that is most important in protection often varies with the micro-organism. Identification of the protective immune response during infection can be used as a basis for vaccine development. Many approaches have been successfully applied to study the role of the different branches of the host immune system during microbial infection. Removal of immunological relevant organs by surgery (134, 154), specific antibody treatment (122, 206), or immunosuppressive therapy (41, 201), have demonstrated to be functional techniques that can be used to study the effect of immune-depletion on the onset and course of infection. The effect of passive transfer of immune components such as antibodies (38, 208), B-cells (172, 190) or subsets of T-cells (14) from immune donors to non-immune recipients has also been analyzed.

1.2 Microbial pathogenesis and identification of vaccine targets

Microbial pathogens posses a repertoire of virulence determinants that each make unique contributions to infection (138, 175). These virulence determinants are required and produced during different stages of the infectious process and can be involved in adherence to host tissues, production of host-specific toxins, invasion into host cells, resistance to host

defense mechanisms, and many other interactions (87, 129). Virulence factors, particularly surface-located molecules, frequently make good vaccine antigens as neutralization of these essential factors by the immune system of the (vaccine-stimulated) host may offer protection against infection with that specific pathogen (88).

In the search for potential vaccine targets one could use a conventional approach, novel strategies, or a combination of both. The conventional approach is focused on the different bacterial components and their function. By using classical microbiological and biochemical techniques, different surface-exposed or secreted virulence factors such as fimbriae, adhesins, and toxins, and their role in host-pathogen interactions are studied (62). Based on functional analysis, such as cytotoxicity assays (35) and cell invasion studies (117), putative vaccine targets are selected which can be tested for antigenicity and immunogenicity in the pathogen's natural host or in an animal model. However, with the use of modern-day technologies there are many new opportunities that can be exploited to analyze the pathogenicity of a bacterium and to identify virulent factors and immunogenic antigens. In contrast to the conventional approach, the new approach focuses on the genome, the genes and their encoded products, and putative vaccine targets can be selected without any prior knowledge concerning protein function.

The accessibility of a pathogen's genome sequence has created the possibility to randomly test all genes and the proteins they encode for vaccine potential. However, the total number of genes is often too large to test them all as single vaccines and therefore the (combined) technologies of genomics (7, 123, 209), proteomics (29, 147) and bioinformatics (216) can be used to search for interesting genes and their encoded products (10, 31, 37, 135, 136). For example, using these tools one can select for genes predicted to encode secreted or surface-exposed membrane proteins since these proteins are potential targets for effector cells of the host immune system. Using the genome sequence of a pathogen to identify vaccine candidates is also called reverse vaccinology (133, 140). Various methods have also been developed to directly identify and characterize the specific epitopes involved in specific interaction with the antigen binding site of an antibody or a T-cell receptor (169), e.g. phage display technology (207) or *in silico* search using computational vaccinology (65) or immunoinformatics (66).

Although availability of the complete genome sequence of a pathogen can accelerate the development of pharmaceuticals (26), without prior knowledge of the basic genetics or genomics of the organism of interest there are still ample possibilities to identify vaccine targets. Recombinant DNA technology can be used to create genomic expression libraries and large numbers of clones can be screened with defined immune sera (27, 210) or T-cells (174) for the presence of reactive antigens which can be selected for further characterization and immunological analysis. Expression library immunization (ELI) has also been applied to directly study the antigenicity and immunogenicity of different clones *in vivo* (17).

Vaccine target selection can also be based on *in vivo* expression of antigens since genes which are specifically induced during infection are believed to be important to the pathogenic process (138, 175). Different technologies such as *in vivo* expression technology (IVET) (129, 139), signature-tagged mutagenesis (STM) (90), DNA microarray technology (123, 209), differential fluorescence induction (DFI) (167, 191), and *in vivo* induced antigen technology (IVIAT) (45, 80, 82, 109) were designed. These methods can provide the basis for

understanding the possible metabolic shifts of bacteria during an infection and survival in the host (81, 85, 87, 88, 129).

1.3 Antigen delivery and vaccine design

New vaccine opportunities have emerged from an improved understanding of immunological events associated with immunization, the identification of promising new antigens, and an increased capacity to produce these antigens through chemical and biotechnological methods. However, proper delivery of the antigen is crucial for achieving the desired immunological outcome (113, 144, 150, 185). For vaccine design one should consider both the route of antigen administration and the type of antigen delivery system and/or antigen formulation. In general, the final vaccination strategy depends on the onset, type, and duration of immunity one should like to induce (2, 49, 63, 121, 144, 150).

The two most important routes of antigen administration are the systemic route (e.g. intramuscular, subcutaneous, intraperitoneal, and intravenous vaccination) and the mucosal route (e.g. oral, intranasal and rectal vaccination). Although most vaccines have traditionally been administered by intramuscular or subcutaneous injection, mucosal administration of vaccines may offer important advantages such as easier administration (via aerosol, food, or drinking water), the suitability for mass application, and the potential for frequent boosting (150). Since the majority of pathogens enter the host via mucosal surfaces, immunity at the site of entry is critical for achieving optimal protection. Although parenteral delivery is very effective in inducing systemic immunity, it often poorly induces mucosal immunity what can result in prevention of disease but not always in prevention of infection. Today, a wide range of approaches are being investigated for mucosal delivery of vaccines (119, 144).

For antigen delivery systems one can mainly distinguish between live vaccines and inactive vaccines. The live vaccines can be subdivided into i) homologous strains such as live deletion mutant vaccines: virulent strains attenuated by e.g. site-directed mutagenesis that have not lost their antigenicity (40), and ii) heterologous strains such as live vector vaccines: where the protective antigen is expressed by a different bacterial host which is used as a delivery vehicle (61, 71, 107, 137). These live vaccines replicate within their host and do not need additional adjuvant activity. For inactive vaccines one could think of i) monovalent or multivalent recombinant subunit vaccines: different subunit proteins are produced (e.g. by a heterologous host) and purified immunogen(s) of one or multiple pathogens are combined in a single vaccine (121), ii) peptide vaccines: chemically synthesized peptides that can be used as multiple-epitope vaccines (169, 182), and iii) DNA vaccines: plasmid DNA expression vectors which encode the antigen(s) of interest which is (are) expressed in the host under the control of strong eukaryotic promoters (84).

Since delivery of non-replicating vaccines alone is mostly not sufficient to provide protection, an immune-stimulatory or immune-modulatory formulation is required (19, 47, 59, 150, 161). Adjuvants can be used to improve the immune response to vaccine antigens in several different ways, e.g. by i) increasing the immunogenicity of weak antigens, ii) enhancing the speed and duration of the immune response, iii) stimulating and modulating antibody mediated immunity and cell mediated immunity, iv) promoting the induction of mucosal immunity, v) decreasing antigen dose in the vaccine, or vi) help to overcome

antigen competition in combination vaccines (144, 177). To further enhance and focus the immune response specific cytokines (111) and CpG sequences (12) can be incorporated in the vaccine.

2 The need for veterinary vaccines: bacterial respiratory tract infections of poultry

2.1 Bacterial respiratory diseases of poultry

Since the use of antibiotics is more and more banned from the food-chain an increased demand for veterinary vaccines has arisen, also because infectious diseases of food production animals can cause considerable economic losses. The poultry industry, which comprises mainly chickens, turkeys and ducks, is a very intensive worldwide live stock industry and therefore very reliant on the availability of effective vaccines (42, 186). Respiratory tract infections are a significant part of the overall disease incidence in poultry. Several pathogens (such as fungi, viruses and bacteria) are indicated as causes of respiratory diseases, either alone, in combination with other micro-organisms, or influenced by non-infectious factors such as climate conditions and management-related issues (67, 73, 106, 159). Diseases of the respiratory tract are often accompanied by increased mortality, increased medication costs, increased condemnation rates, growth reduction, drops in egg production, reduction of egg shell quality and decreased hatchability. The bacterial micro-organisms known to be involved in respiratory diseases of chickens and turkeys are: *Escherichia coli* (colibacillosis) (15, 54, 73, 77), *Haemophilus paragallinarum* (infectious coryza) (22, 73), *Pasteurella multocida* (fowl cholera) (73, 74), *Riemerella anatipestifer* (165), *Bordetella avium* (bordetellosis or turkey coryza) (73, 101), and *Ornithobacterium rhinotracheale* (36, 73, 78, 202). Respiratory tract infection can also be caused by Mycoplasmata. The four most common Mycoplasma species that are involved in avian mycoplasmosis are *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, *Mycoplasma meleagridis*, and *Mycoplasma iowae* (120, 155, 213).

Not all of these pathogenic bacteria are a problem for both chickens and turkeys. *H. paragallinarum* is found only in chickens and not in turkeys (22), whereas *R. anatipestifer* does infect ducks and turkeys but not chickens (165). Both pathogens cause upper respiratory tract infections like inflammation of the mucous membranes of the nasal passages and sinuses, resulting in sneezing and coughing. The appearance of infectious coryza in chickens (caused by *H. paragallinarum*) (22) is similar to turkey coryza (caused by *B. avium*) (101). Lower respiratory tract infections as airsacculitis and pneumonia can be found in *P. multocida* infections (74) as well as in *O. rhinotracheale* infections (36), *E. coli* infections (15) and *R. anatipestifer* (165) infections but are rarely found in birds infected with *H. paragallinarum* (22). Colibacillosis, caused by avian pathogenic *E. coli* (APEC), is also named air sac disease, chronic respiratory disease (CRD), or multicausal respiratory disease (15). APEC can also cause infections in the upper respiratory tract, often referred to as the "swollen head syndrome" (15). Infection with *E. coli,* most of the time in combination with Mycoplasma species, causes lesions in air sacs, lungs, and trachea, in both chickens and turkeys (54, 73, 77).

Clinical signs caused by these pathogens are often rather variable and can show quite a resemblance. For example, the respiratory disease caused by *O. rhinotracheale* may manifest clinically similar as infection with *P. multocida*, *E. coli*, or *R. anatipestifer* (36, 196). Furthermore, most of these pathogens, e.g. *P. multocida*, *O. rhinotracheale, E. coli, R. anatipestifer* and Mycoplasmas, are known to cause not only respiratory tract infections, but can also manifest as systemic diseases such as pericarditis, hepatitis, joint lesions, and cerebrovascular pathology (15, 74, 165, 196, 213). Since the clinical signs and post-mortem lesions of these respiratory infections are often not sufficiently specific to be diagnostic, the causative agent can be easily confused. Therefore, it is very important that the pathogen(s) responsible for infection is/are correctly diagnosed by cultural isolation and analysis of morphology, biochemistry, and fluorescence of the organism. Different techniques can be used for diagnostic purposes, such as specific polymerase chain reaction (PCR) tests, or serological assays such as rapid serum agglutination (RSA) tests and enzyme-linked immunosorbent assays (ELISA) (106). Morbidity and mortality of these poultry respiratory diseases strongly depends on predisposing factors like primary infection with infectious bronchitis virus (IBV), Newcastle disease virus (NDV), or other viruses, and the presence of high (elevated) concentrations of ammonia (73). Severity of disease is often directly related to the number of predisposing agents involved.

Different vaccines already exist for prevention of infections of the poultry respiratory tract. However, the development of novel, improved vaccines always remains a challenge. Currently used vaccines are mostly based on killed (inactivated) or live (attenuated) cultures of the respiratory pathogens (15, 21, 101, 120). Bacterin formulations generally provide antibody-based protective immunity against infection with a serotype or serovar contained within the vaccine (15, 24, 102, 165). The addition of an adjuvant is necessary with killed or inactivated vaccines in order to improve the immune response. Passive protection of progeny (by maternal immunity) is obtained by immunization of breeder birds (15, 197). Live vaccination using low virulence mutant strains has demonstrated to induce long-lasting protection based on cellular and humoral immunity (101). In some cases, cross-protection against heterologous immunotypes has been described after live vaccination (22).

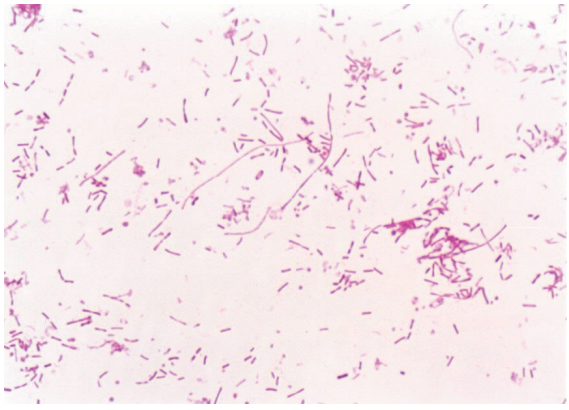

Figure 1. Microscopic view of *O. rhinotracheale* showing the pleomorphic nature of this bacterium.

2.2 An emerging pathogen: Ornithobacterium rhinotracheale

In 1991 a new respiratory disease was observed in broiler chickens in South Africa by DuPreez (192). The etiologic agent of this poultry infection was characterized in 1993 by Charlton *et al*. (34), and named *Ornithobacterium rhinotracheale* in 1994 by Vandamme *et al*. (193). Analysis of old culture collections revealed that *O. rhinotracheale* already had been isolated from respiratory tracts of diseased birds since 1981 (93). Since its assignment of being a new species, *O. rhinotracheale* has been isolated from chicken, turkey, partridge, pheasant, pigeon, rook, quail, duck, chukar, ostrich, goose, and guinea fowl from all over the world (28, 34, 36, 53, 79, 192, 196, 199). Up to now *O. rhinotracheale* has never been isolated from humans and therefore not been considered to be a threat to public health, in contrast to the zoönotic species *E. coli*, Salmonella, and Campylobacter.

O. rhinotracheale belongs to the rRNA superfamily V within the Cytophaga-Flavobacterium-Bacteroides phylum (193) and is closely related to two other poultry bacteria, *Riemerella anatipestifer* (193) and *Coenonia anatina* (194). It is a Gram-negative, non-motile, and non-sporulating bacterium with a pleomorphic nature (Fig. 1), of which no special structures or properties such as pili, fimbria, or specific toxic activities have been reported (118).

DNA and protein analysis of a large number of isolates indicate the existence of only limited diversity among different *O. rhinotracheale* strains. Although no information regarding the *O. rhinotracheale* genome is available, comparison of *O. rhinotracheale* strains by 16S rRNA sequencing and rep-PCR typing indicated considerable similarities at the genetic level and suggested that the species consists of a small group of closely related clones (9, 126). Random amplified pleomorphic DNA (RAPD) (118) and amplified fragment length polymorphism (AFLP) (195, 196) were found to be able to discriminate between different strains. SDS-PAGE and Western blot analysis of the total protein and outer membrane protein (OMP) contents of different *O. rhinotracheale* strains revealed conserved profiles with a similarity over 84% (9).

Eighteen different serotypes have been identified for *O. rhinotracheale* (A through R) by using agar gel precipitation (AGP) and enzyme linked immunosorbent assay (ELISA) (199, 200). In both tests, boiled extract antigens (BEAs) (86) were used as antigen. Serotype A is the most prevalent among both chicken ($\sim$94%) and turkey ($\sim$57%) isolates. Approximately 97% of the total number of isolates belongs to the 4 main serotypes A, B, D, and E (196, 199). Western blot analysis using isolated outer membrane proteins as antigens and hyper-immune sera of different *O. rhinotracheale* serotypes was also used to differentiate distinct serotypes (126).

O. rhinotracheale can spread horizontally by direct and indirect contact through aerosols or drinking water (51, 158). Vertical transmission through the egg has also been reported (13, 193). Clinical signs associated with *O. rhinotracheale* infection can include respiratory problems, depression, decreased food intake, reduced weight gain, drop in egg production and quality, immotility, paralysis and death. The severity of these signs, duration of the disease and mortality rate of *O. rhinotracheale* outbreaks are extremely variable and can be influenced by many environmental factors such as poor management, inadequate ventilation, stress, high stocking density, poor litter conditions, poor hygiene or concurrent infections (36, 56, 183, 188, 196, 203). The common pathological lesions found in *O. rhinotracheale*-infected chickens and turkeys include (fibrinopurulent) pneumonia, airsacculitis, sinusitis,

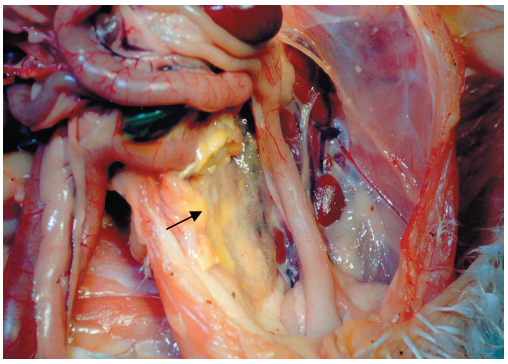

Figure 2. Airsacculitis of the abdominal air sacs of an *O. rhinotracheale*-infected chicken. The arrow indicates the characteristic foamy exudates containing large clots of fibrin.

tracheitis, pleuritis, pericarditis, hepatomegaly, and splenomegaly (13, 34, 57, 92, 158, 178, 195, 199). Characteristic of *O. rhinotracheale* infection of the air sacs is the presence of foamy exudates containing large clots of fibrin (Fig. 2) (160, 195). In addition, subcutaneous edema over the cranium with adjacent osteitis, osteomyelitis, and encephalitis has been reported in chickens (75, 76). Infections of the joints have been described for turkeys (51, 145).

Broiler chickens are most susceptible to *O. rhinotracheale* infection at 3 to 4 weeks of age and infection can result in mortality rates of 2 – 10% and condemnation rates up to 50% or more (203). Broiler-breeder and commercial layer-type chickens are most prone to infection between 24 to 52 weeks of age in the laying period at the top of their production. The mortality rates are usually low, but losses due to decreased egg production can be considerable. In turkeys over 14 weeks of age infection usually results in mortality rates of 1 – 15% although mortality rates up to 50% have been reported (50, 184).

Treatment of *O. rhinotracheale* infections with antibiotics is very difficult because of the variable susceptibility of different strains and acquired resistance to the regular antibiotics (52, 53, 130, 176, 204). In the past, vaccines based on inactivated bacteria (bacterin vaccine) have been developed and other strains were tested for vaccine potential. Vaccination of broiler-breeders with a commercially available bacterin of *O. rhinotracheale* induced long-lasting immunity and protected their progeny by maternal antibodies (30, 197). Administration of a live *O. rhinotracheale* strain to young broilers also appeared to induce protection against *O. rhinotracheale* challenge but only when maternal antibodies were low (197). In turkeys, administration of both live and killed *O. rhinotracheale* has been demonstrated to protect against infection (179). A temperature-sensitive (Ts) mutant strain was tested by Lopes *et al*. (124). The efficacy of this live test-vaccine is based on the ability to colonize the upper respiratory tract to elicit a protective secretory IgA response, and preliminary studies in turkeys appear promising (126). Although current vaccination strategies have demonstrated to be effective, no broad cross-protective immunity against the various *O. rhinotracheale* serotypes has been reported. Especially for a turkey vaccine the induction of cross-protection is important since this bird species is susceptible for a variety of *O. rhinotracheale* serotypes. The development of a new or improved vaccine that provides long lasting cross-protective immunity against all *O. rhinotracheale* serotypes would be favourable, especially for turkeys. However, additional knowledge should be obtained about the bacterium and its host regarding, both, pathogenesis and immunity.

3 The aim of this study: a strategic approach for the development of a cross-protective *O. rhinotracheale* vaccine

An *O. rhinotracheale* vaccine for poultry should induce cross-protective immunity that lasts as long as required, i.e.: at least for weeks in broilers and for months in layers, breeders, and turkeys. The vaccine should be safe, e.g. should not produce disease in animals and should not leave unwanted residues in meat and eggs. Furthermore, the vaccine should be cheap in production and easy to administer. Ideally, the vaccine should be applicable at 1 day of age since this is often the only moment birds are handled. The vaccine can be administered by injection or eye drop, or be suitable for mass application, as course particle spray, aerosol, or mixed into the food or drinking water. The present knowledge concerning host, pathogen, and the applicability of different antigen delivery systems will determine the strategic approach one should apply in order to develop a new vaccine for *O. rhinotracheale*.

3.1 Poultry immunology and O. rhinotracheale infection

Very little is known regarding the chicken immunological response to *O. rhinotracheale* infection. Birds that recovered from a natural infection with *O. rhinotracheale* or birds vaccinated with a bacterin vaccine or live strain showed elevated antibody levels directed against *O. rhinotracheale* antigens (36, 179, 196, 197). However, the importance of the humoral immune response in comparison to the importance of cell-mediated immunity in protection against *O. rhinotracheale* infection, and the role of antibodies in cross-protective immunity have not been studied yet.

3.2 Virulence factors of O. rhinotracheale

Up to now, only a few studies have been reported about the molecular basis of *O. rhinotracheale* infections and although protein profiles of different strains have been analyzed (104, 126), no virulence factors or (cross-) protective antigens have been characterized for this pathogen. Recently, Chansiripornchai *et al.* (33) studied the molecular interaction of *O. rhinotracheale* with eukaryotic cells. It was demonstrated that *O. rhinotracheale* carries multiple cell-specific adhesion factors and that both serum and LPS inhibit *O. rhinotracheale* infection of host cells *in vitro* via related but distinct mechanisms. Unfortunately, the *O. rhinotracheale* adhesions and their corresponding host cell receptors have not been identified yet.

3.3 The delivery of O. rhinotracheale antigen(s): choosing the best vaccination strategy

There are different strategies that can be applied to deliver *O. rhinotracheale* antigens. Live vaccination using genetically manipulated *O. rhinotracheale* mutant strains can be an option, as well as vector vaccine strains to express and deliver *O. rhinotracheale* antigens. Recently, a transformation system for *O. rhinotracheale* was introduced that enables genetic modification of *O. rhinotracheale* (33). However, there are still reservations regarding the use of genetically manipulated organisms (GMOs) in the food industry, mostly due to lack

of information to and knowledge of consumers. Therefore, defined recombinant subunit vaccines can be a good alternative.

3.4 The aim of this thesis

The aim of the work described in this thesis was to identify broadly cross-protective *O. rhinotracheale* antigens with vaccine potential using a state-of-the-art vaccinology approach. For this purpose, the following three-way strategy was designed (Fig. 3). First (step I), the characterization of the chicken immune response during *O. rhinotracheale* infection; second (step II), the identification of cross-protective vaccine candidates, and, finally (step III), optimisation of antigen delivery. For our studies we used experimental *O. rhinotracheale* infections in specified pathogen free (SPF) broiler chickens as a test system. This model closely resembles the natural infection in both chickens and turkeys and offers ample facilities to study the immune response of birds upon encountering *O. rhinotracheale* and to perform different vaccination experiments.

To address step I, we choose to determine the importance of antibody-mediated immunity in protection against infection by challenging healthy and immune-suppressed birds with live *O. rhinotracheale.* Reconstitution of the humoral immunity by passive transfer of immune components was envisioned to be used for verification. This complementary approach should provide sufficient prove for the role of antibodies in cross-protection against *O. rhinotracheale* infection.

Step II, the identification of those components of *O. rhinotracheale* that are involved in the induction of a cross-protective immune response was more difficult to assess. Several new techniques proven to be successful in the identification of virulence factors or potential vaccine antigens are not suitable for the detection of cross-protective vaccine antigens of *O. rhinotracheale*. The main reason for this is the lack of data concerning the genome and proteome of this pathogen and the pathogenesis of infection. Thus, we planned to screen for *O. rhinotracheale* antigens with help of a genomic expression library and the antisera obtained from birds investigated in step I. The selection for the antigen with highest cross-protective potential was continued using different vaccination experiments.

In the final step (step III), gained knowledge about the chicken immune response (step I) and the identified cross-protective vaccine antigen(s) (step II) was combined to select an appropriate means of antigen delivery.

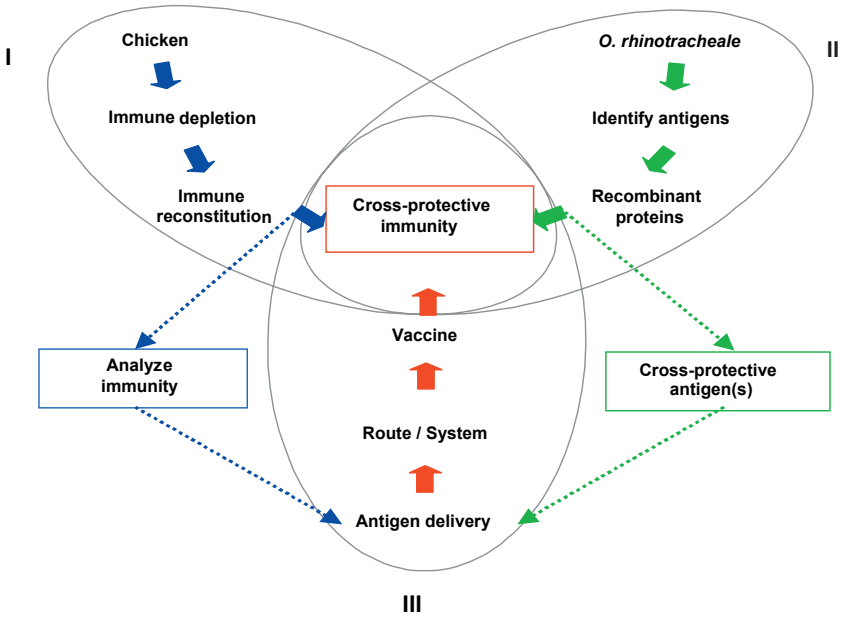

Figure 3. Summary of the strategic approach used for development of a cross-protective *O. rhinotracheale* vaccine. In step I (blue arrows), the chicken immune response during *O. rhinotracheale* infection is characterized by using an experimental method that combines selective immune depletion and passive transfer of immunity. Antigens of *O. rhinotracheale* that have cross-protective potential are identified in step II (green arrows) by using the mediators of infection as analysed in step I. Step III (red arrows) comprises the selection of an appropriate means of antigen delivery and the design of the final cross-protective vaccine using gained knowledge about the chicken immune response (step I, dotted blue line) and the identified cross-protective vaccine antigen(s) (step II, dotted green line).

4 Outline of this thesis

The results obtained with the above described strategy to identify cross-protective vaccine antigens of *O. rhinotracheale* are described in this thesis. In **Chapter 2** it is reported that the chicken antibody mediated immune response is a key component in (cross-) protection against *O. rhinotracheale* infection, and that the type of immunoglobulins produced upon encountering the pathogen depends on the infection route. In **Chapter 3** it is demonstrated that live vaccination can induce cross-protective immunity. Sera from cross-protected birds were used to select a pool of cross-reactive *O. rhinotracheale* antigens able to induce cross-protective immunity. **Chapter 4** documents the characterization of the selected *O. rhinotracheale* antigens. Vaccination studies showed a lipoprotein to be the antigen with highest vaccine potential. The efficacy of this new recombinant subunit vaccine to induce cross-protective immunity was analysed and described in **Chapter 5**. The efficacy of this new recombinant subunit vaccine was compared to live vaccination and bacterin vaccination. In **Chapter 6** the search for specifically *in vivo* expressed *O. rhinotracheale* antigens was described. Finally, the data presented in this thesis are summarized and discussed in **Chapter 7**.

CHAPTER TWO

Passive Immunization of Immune-Suppressed Animals: Chicken Antibodies Protect against *Ornithobacterium rhinotracheale* Infection

D.F. Schuijffel[1], P.C.M. Van Empel[1], A.M.M.A. Pennings[1], J.P.M. Van Putten[2], and P.J.M. Nuijten[1]

[1] *Intervet International BV, Bacteriology R&D, Boxmeer, The Netherlands,* [2] *Utrecht University, Faculty of Veterinary Medicine, Department of Infectious Diseases and Immunology, Utrecht, The Netherlands.*

Accepted for publication in Vaccine

ABSTRACT

Unravelling of the protective immunity acquired during a natural infection may contribute to vaccine development. To assess the role of antibody-mediated immunity in protection against *Ornithobacterium rhinotracheale* infection in chickens, a novel experimental method was applied that combined immune depletion and passive transfer of immunity within the same host. Administration of cyclophosphamide (CY) to broiler chickens successfully suppressed B lymphocyte development and therefore humoral immunity, as confirmed by histological and serological analysis. Challenge of CY-treated birds with *O. rhinotracheale* revealed a significantly higher pathology score in comparison to immune-competent birds that received the same bacterial challenge. Measurement of serum immunoglobulin levels of immune-competent birds revealed a positive correlation between IgA and/or IgG production and protection against infection. Passive transfer of *O. rhinotracheale*-specific antiserum to the immune-suppressed birds prior to pathogen challenge significantly decreased morbidity. This protective effect was not observed after administration of control sera containing similar concentrations of immunoglobulins. Together, these results provide firm evidence that chicken humoral immunity to *O. rhinotracheale* is a key component in protection against infection. Our data confirm that the applied immune depletion and reconstitution approach is an attractive tool to analyse the nature of the protective immune response.

INTRODUCTION

Protection against microbial infections through vaccination is a powerful tool to prevent emerging infectious diseases. In the development of novel vaccines, much can be learned from the natural protective immune response against infection. For that reason it is important to determine the relative contribution of the antibody-mediated and/or cell-mediated immune response in their capacity to resist or overcome the infection. In a laboratory setting, the host immune response can be investigated via inactivation or suppression of specific parts of the immune system. This can be achieved by, for instance, removal of organs (72, 134, 154), specific antibody treatment (122, 206), or administration of immune-suppressive chemicals (41, 154, 201). Alternatively, passive transfer of antibodies (38, 208), B-cells (172, 190), or subsets of T-cells (14, 97) from immune donors to non-immune recipients have been successfully applied in the field of immunology and for prevention and treatment of infectious diseases. A combined approach of selective immune depletion and reconstitution with distinct immune components within the same host is likely to be a very attractive tool to analyse the natural protective immune response against infection and to explore the vaccine potential of defined immune components.

Ornithobacterium rhinotracheale is increasingly recognized as a major cause of respiratory tract infections in birds. Since its discovery in 1991 and its assignment as a separate bacterial species in 1994 (192), *O. rhinotracheale* has emerged to a pathogen of great economic importance to the poultry industry with a worldwide prevalence. The bacterium primarily infects the trachea, lungs and air sacs, but can also manifest as a systemic disease with hepatitis, joint lesions, and cerebrovascular pathology (36, 73, 196). Concomitant infections with other respiratory pathogens appear to aggravate the symptoms but are not required for causing disease (202). At this time, up to 18 different serotypes of *O. rhinotracheale* have been identified (36, 198). Treatment of *O. rhinotracheale* infection is increasingly complicated since most isolates have acquired resistance against the regular antibiotics (53, 130, 176). The best strategy to protect against the pathogen is the development of a vaccine. Thus far, vaccines based on inactivated bacterin formulations have been developed (179, 197). These vaccines are efficacious against an infection with distinct serotypes but their ability to induce broad cross-protection is variable and often negligible. Development of more efficacious vaccines however, is complicated by the total lack of knowledge of the pathogenesis of the infection and the limited knowledge of the host immune response to *O. rhinotracheale* infection.

Because of the urgency to develop a broadly cross-protective vaccine against *O. rhinotracheale* infections in birds, we used a novel approach of complementary use of immune depletion and reconstitution of immunity by passive transfer of serum components within the same host to analyse the type of immunity that is involved in the protection against *O. rhinotracheale* infection. For immune depletion, the immune-suppressive drug cyclophosphamide (CY) was used in specified pathogen free (SPF) broiler chickens. CY inhibits the development of B-cells and their precursors in the Bursa of Fabricius in birds, but, well-dosed, CY does not affect T-cells, macrophages and other mediators of the cellular immune system (69, 95, 156). Passive transfer of sera from immune animals to immune-depleted recipients was used to assess the contribution of antibodies in protection against experimental challenge with the pathogen. Our results indicate that the followed

experimental approach is an attractive tool to analyse the natural host immune response against infection and to test the specificity of potentially protective antibodies.

MATERIALS AND METHODS

Chickens

Specified-pathogen-free (SPF) broiler chickens (Intervet, Boxmeer, The Netherlands) were used. All animals were placed in negative pressure isolators of 1.5 m^3, with a maximum of 20 (or 16) birds per isolator in a 3 (or 5)-week study at day of hatch, and received sterilized food (Hendrix, Boxmeer, The Netherlands) and water *ad libitum*. In each experiment, from the time point of challenge onwards, the birds of different test groups were housed in a mixed population where possible, to reduce possible isolator effects. All animal studies were approved by the committee for animal experiments of The Netherlands (DEC), according to international regulations.

Depletion of Bursa-derived lymphocytes

Cyclophosphamide (CY, Sigma, St. Louis, Missouri, USA) was administered at 3 mg/dose in a volume of 0.25 ml of phosphate buffered salt solution (PBS-0). CY was injected intramuscularly into the leg muscle at day 1 to 4 of age. During the experiment, at 2, 4 and 5 weeks of age, CY-treated and untreated control birds were sacrificed to investigate the effect of CY administration on B and T lymphocyte development in the Bursa of Fabricius and the thymus, respectively. The organs were examined both macroscopically and microscopically. Samples for histological investigation were fixed in 10% of neutral buffered formalin, routinely processed to paraffin sections, and stained with haematoxylin and eosin according to Kierman *et al*. (108). Lymphocyte depletion in bursa and thymus was scored as follows: 0-20% - no to minor lymphocyte depletion; 20-40% - minor/moderate lymphocyte depletion; 40-60% - moderate lymphocyte depletion; 60-80% - moderate/major lymphocyte depletion; 80-100% - major to complete lymphocyte depletion.

Challenge

Birds were challenged with either *O. rhinotracheale* strain B3263/91 (serotype A), *O. rhinotracheale* strain TOP98056 SP3555 (serotype A) or *O. rhinotracheale* strain O-95027 nr.16279 (serotype G). The challenge was done either by aerosol spraying of 100 ml of a fresh bacterial culture containing between 10^8 and 10^9 colony forming units (CFU) per ml in Todd Hewitt (TH) medium (Difco, Detroit, MI, USA) per isolator of ± 1.5 m^3, or by administration of 0.5 ml of a similar bacterial culture intravenously (i.v.). For the preparation of this challenge culture, bacteria were first grown on 5% sheep blood agar at 37°C, in a 5% CO_2

atmosphere for 48 hours. Then, for liquid culture, single colonies were inoculated in Todd Hewitt broth (THB) and grown for 24 hours at 37°C on a 100 rpm rotating shaker. During aerosol challenge the bacterial culture was administered as a fine spray to the birds in an isolator, using a commercial paint sprayer; the developed mist was maintained for at least 10 min without air circulation.

Antisera and passive serum transfer

Two *O. rhinotracheale*-specific antisera were used in the antibody transfer studies. The first serum was obtained from 5-week old SPF-broilers that had been intravenously challenged at 4 weeks of age with live *O. rhinotracheale* serotype A strain B3263/91. The second serum was obtained from 10-week old SPF-broilers that had been vaccinated at 5 weeks of age with a bacterin formulation of *O. rhinotracheale* serotype A strain B3263/91. Two non-*O. rhinotracheale* antisera served as controls. One negative serum was collected from 5-week old SPF-broilers that had not received any treatment. The second control serum was a *Pasteurella multocida*-specific serum obtained from 10-week old SPF-broilers vaccinated at 5 weeks of age with a bacterin formulation of *P. multocida*. All *O. rhinotracheale* and *P. multocida* antisera used in the transfer experiments contained an immunoglobulin G concentration ranging from 2.3 (B3263/91 live) to 2.5 (B3263/91 bacterin) mg/ml. The negative serum contained 0.6 mg/ml IgG. Before immunization, sera were 0.22 μm filter-sterilized.

In the passive antiserum-transfer studies, 2-week old CY-treated SPF-broilers were injected intravenously with 1.0 ml of undiluted serum. Five hours after transfer, the chicks were challenged intravenously with *O. rhinotracheale* serotype A strain B3263/91, *O. rhinotracheale* serotype A strain TOP98056 SP3555, or *O. rhinotracheale* serotype G strain O-95027 nr.16279. At 3 weeks of age, 1 week post antiserum transfer and challenge, birds were sacrificed for post-mortem investigation. Unimmunized and unchallenged control birds were included in the tests.

Serum Ig determination

Blood samples were taken during all animal experiments. Total serum immunoglobulins of isotypes A, G, and M were measured with a chicken serum IgA, IgG, or IgM enzyme-linked immunosorbent assay (ELISA) kit (Bethyl Laboratories, Montgomery, TX, USA) following the manufacturer's protocol. Sera were tested in an ELISA against antigens of *O. rhinotracheale* serotype A and *O. rhinotracheale* serotype G in order to determine *O. rhinotracheale*-specific antibody ^{2}log titres as described previously (198). Semi-dry Western blotting, was performed according to Towbin *et al*. (187). In short, *O. rhinotracheale* serotype A polypeptides were electrophoresed under denaturating conditions using NOVEX NuPAGE (Invitrogen, Carlsbad, CA, USA) and electroblotted onto Immobilon PVDF 0.45μm membrane (Millipore, Billerica, MA, USA). After blocking, filters were cut into strips and each strip was incubated with pooled antiserum (1:100 dilution) obtained from CY-treated and untreated birds before and after aerosol, intravenous, or no pathogen challenge. As a secondary antibody, rabbit anti-

chicken IgG peroxidase conjugated was used (Nordic, Tilburg, The Netherlands) in a 1:1000 dilution. As substrate solution Vector SG (Vector, Burlingame, CA, USA) was used.

Post-mortem examination and parameters of infection

At the end of all experiments, post-mortem examination was performed on all animals. The birds were bled and organ lesions were macroscopically scored for typical *O. rhinotracheale* pathology using the following scoring system: for thoracic air sacs, 0 - no abnormalities, 1- one air sac seriously affected by fibrinous airsacculitis or both air sacs containing limited pin-head sized foci of fibrinous exudates, 2 - both air sacs seriously affected by fibrinous airsacculitis; for abdominal air sacs, 0 - no abnormalities, 1 - pin-head sized foci of fibrinous exudates or slight diffuse fibrinous airsacculitis, 2 - severe fibrinous airsacculitis. The airsacculitis score is given as the sum of both scores. For lungs, 0 - no abnormalities, 1 - unilateral pneumonia, 2 - bilateral pneumonia; for liver, 0 - no abnormalities, 1 - pin-head sized foci, 2 - severe hepatitis; for the joints, 0 - no abnormalities, 1 point for each joint that shows purulent exudates. The average group-scores are given as a percentage of the maximal possible score. Statistical analysis was performed using the Kruskal-Wallis non-parametric one-way ANOVA test.

During post-mortem investigation, the thoracal and abdominal air sacs, lungs, joints, liver, and any other affected organs were collected for peroxidase anti-peroxidase (PAP) staining as described by Van Empel *et al*. (195), and sampled with cotton swabs for cultivation of *O. rhinotracheale*. The swabs were inoculated on sheep blood agar plates without and with 5 μg/ ml of polymyxin and 5 μg/ ml of gentamycin. The plates were incubated at 37°C in a 5% CO_2 atmosphere for 72 hours and checked each day for suspected colonies (small, grey and not haemolytic). These colonies were subcultured and confirmed to be *O. rhinotracheale*, with use of an agar gel precipitation (AGP) test and API-20NE identification strips as previously described (198).

RESULTS

Effect of CY-treatment on bursa and thymus development

To assess the effect of CY on the development of bursa and thymus, birds were administered 3 mg CY intramuscularly during the first four days of age. At 2, 4, and 5 weeks of age, control birds of the CY-treated and untreated groups were sacrificed and the bursa and thymus were localized, removed and processed for histology. Macroscopically, the bursas of CY-treated birds remained very small or undetectable during the whole experimental period, while the bursas from control animals increased in size (data not shown). Microscopic examination of detectable organs revealed that the bursas of CY-treated animals showed major lymphocyte depletion (80-100%) (Fig. 1A). The bursas of birds that did not receive any CY treatment showed no lymphocyte depletion (Fig. 1B). No macroscopic and microscopic detectable

differences in thymus growth and tissue could be detected between CY-treated and untreated chickens (results not shown).

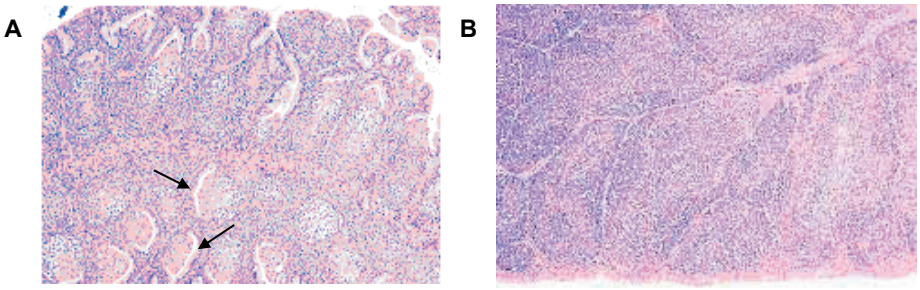

Figure 1: Haematoxylin-eosin staining of the bursa of Fabricius in CY-treated (A) and control animals (B) at 2 weeks of age. Note the atrophic regions (arrows) and subsequent B lymphocyte depletion after CY exposure. Magnification 10x.

Susceptibility of CY-treated and untreated birds to *O. rhinotracheale* infection

The role of bursa-derived immune cells in the onset of *O. rhinotracheale* infection was investigated by CY-treatment followed by *O. rhinotracheale* challenge. To study respiratory tract infections as well as systemic lesions, birds were infected with *O. rhinotracheale* via two routes: i) a natural route, i.e. aerosol spray application causing mainly respiratory lesions (air sacs and lungs) and ii) an artificial route, i.e. intravenous injection, leading to systemic lesions (joints and liver). Birds were challenged with *O. rhinotracheale* serotype A strain B3263/91 at 4 weeks of age and necropsy was done at 5 weeks of age. Organs were scored with respect to macroscopic pathology using the scoring system as described in materials and methods. As indicated in Table 1, CY-treated birds infected via i.v. injection showed approximately 10-fold higher respiratory (8.0%) and systemic (26.2%) lesion scores than birds that did not received CY injections (1.1% respiratory and 2.3% systemic). Aerosol challenged birds showed only respiratory symptoms: 57.5% in the CY-treated group compared to 14.6% in the untreated group. The unchallenged control groups showed no pathology at all. Presence of the *O. rhinotracheale* challenge strain in infected air sacs, lungs, joints, and liver was confirmed by PAP-staining and successful re-isolation of the bacterium (data not shown).

Table 1. Respiratory and systemic pathology of *O. rhinotracheale* infection in CY-treated and untreated birds.

Group	Animals	CY-treatment	Challenge	Respiratory lesion score[a]:	Systemic lesion score[a]:
A	11	+	+ (i.v.)	8.0 %	26.2 % [b]
B	12	-	+ (i.v.)	1.1 %	2.3 %
C	10	+	+ (aerosol)	57.5 % [c]	0.0 %
D	12	-	+ (aerosol)	14.6 %	0.0 %
E	12	+	-	0.0 %	0.0 %
F	11	-	-	0.0 %	0.0 %

[a] Lesions in air sacs and lungs, joints and liver, were macroscopically scored and presented as percentage of the maximum possible respiratory and systemic lesion score.
[b] Value of systemic lesion score significantly different from the value of the immune-competent challenge control group B (p=0.002)
[c] Value of respiratory lesion score significantly different from the value of the immune-competent challenge control group D (p=0.001)

Development of antibody response in control and CY-treated animals

In order to test the capacity of B-cells of CY-treated animals to produce antibodies, total serum immunoglobulin (Ig) concentrations of isotypes A, G, and M were determined at day of challenge with *O. rhinotracheale* and 1 week later at day of necropsy. This resulted in 4 important observations. First, CY-treated birds showed lower antibody levels compared to untreated birds. At the day of challenge, at 4 weeks of age, the total serum Ig level in birds that received CY-treatment was on average of 109 μg/ml, which was 6 times lower than the concentration in untreated birds (average of 658 μg/ml) (Table 2).

Second, challenge with *O. rhinotracheale* induced the production of serum antibodies. Irrespective of the CY-treatment, at 5 weeks of age the total serum Ig values of unchallenged control birds (Table 2, groups E and F) showed comparable increase factors (i.e. post-mortem values divided by pre-challenge values): 2.1 for CY-treated and 2.3 for control animals. CY-treated and challenged animals showed an average mean increase factor of 2.8 (Table 2, groups A and C) that was little higher compared to the increase factor of the unchallenged animals (2.1). In contrast, challenge of the immune-competent birds caused a strong increase in total Ig levels with an average increase factor of 5.2 (Table 2, groups B and D), which suggests an infection-related immune response.

Third, intravenous challenge induced higher serum antibody levels in comparison to aerosol challenge. Total Ig levels of immune-competent birds increased with factor 6.7 after i.v. challenge (Table 2, group B) in comparison to an increase of factor 3.6 after aerosol challenge (Table 2, group D). CY-treated birds also showed highest increase of total Ig titre after i.v. challenge (factor 4.1 versus factor 1.5) (Table 2, group A and C). However, the immune response of the CY-treated animals after i.v. challenge was still far below that observed for the immune-competent birds.

Finally, intravenous challenge of immune-competent birds primarily induced antibodies of the IgG class, whether aerosol challenge induced antibodies of both IgA and IgG class.

A high increase of serum IgG level (factor 9.7) could be observed after i.v. challenge of immune-competent birds (Table 2, group B) in comparison to i.v. challenged CY-treated birds (factor 2.6) (Table 2, group A). Aerosol challenge increased both serum IgA (3.4 in untreated birds compared to 1.0 in CY-treated birds) and IgG levels (4.1 in untreated birds compared to 1.3 in CY-treated birds). In all challenged birds, CY-treated and untreated, the serum IgM concentrations increased with a comparable factor (Table 2).

Table 2. Total immunoglobulin (Ig) concentration and serum IgA, IgG and IgM levels in sera of CY-treated and untreated birds one day before (t=4 wk) and one week after (t=5 wk) *O. rhinotracheale* challenge as determined by ELISA.

Group	Animals	CY	Total Ig[a] μg/ml (t=4 wk)	Challenge (t=4wk)	Total Ig[a] μg/ml (t=5 wk)	Increase factor[b] Total Ig	Increase factor[b] IgA	Increase factor[b] IgG	Increase factor[b] IgM
A	11	+	134.0	+ (i.v.)	551.1	4.1	4.9	2.6	5.4
B	12	-	766.6	+ (i.v.)	5145.0	6.7	4.3	9.7	4.8
C	10	+	47.0	+ (aerosol)	70.2	1.5	1.0	1.3	2.4
D	12	-	516.6	+ (aerosol)	1861.6	3.6	3.4	4.1	2.9
E	12	+	146.2	-	311.9	2.1	1.4	2.4	2.5
F	11	-	690.6	-	1561.3	2.3	2.7	2.4	1.7

[a]The sum of serum IgA, IgG and IgM concentrations is represented as the total immunoglobulin concentration.
[b]The increase factor was defined as the serum Ig concentration one week post challenge (t= 5 wk) divided by Ig concentration before challenge (t=4 wk).

Development of an *O. rhinotracheale* specific antibody response

The presence of specific *O. rhinotracheale* serum antibodies in the sera of the animals was determined in an ELISA using a boiled extract of *O. rhinotracheale* as antigen. At the day of challenge with this pathogen, all groups of birds (Table 3, groups A-F) showed minimal antibody reactivity. One week later, a considerable increase in *O. rhinotracheale* specific serum antibody titres were measured for the immune-competent birds that had been challenged with the pathogen (Table 3, groups B and D). The average *O. rhinotracheale*-specific antibody titre of i.v. challenged birds (11.7) was higher than in aerosol challenged birds (8.0). No increase in *O. rhinotracheale* antibodies was observed for the immune-compromised birds, irrespective of challenge. The development of an *O. rhinotracheale* specific antibody response in immune-competent compared to immune-compromised birds is consistent with the noted increased pathology after challenge of the immune-compromised animals with the pathogen (Table 1).

Table 3. Titres of *O. rhinotracheale*-specific antibodies one day prior (t=4 wk) and one week after challenge (t= 5 wk) of CY-treated and untreated birds with *O. rhinotracheale*, as determined by ELISA against *O. rhinotracheale* antigens.

Group	Animals	CY-treatment	Avg. ELISA titre[a] in ^{2}log (t=4 wk)	Challenge	Avg. ELISA titre[a] in ^{2}log (t=5 wk)
A	11	+	5.6 (+/- 0.5)	+ (i.v.)	5.4 (+/- 0.5)
B	12	-	5.2 (+/- 0.5)	+ (i.v.)	11.7 (+/- 1.9)
C	10	+	5.6 (+/- 0.5)	+ (aerosol)	5.2 (+/- 0.4)
D	12	-	5.0 (+/- 0.0)	+ (aerosol)	8.0 (+/- 1.7)
E	12	+	5.3 (+/- 0.5)	-	5.0 (+/- 0.1)
F	11	-	5.0 (+/- 0.0)	-	5.2 (+/- 0.4)

[a] Titres are presented as the average level for all birds in that group.

The specificity of the *O. rhinotracheale* antibody reactivity was further investigated in immunoblots with whole cell lysates of *O. rhinotracheale* separated by SDS-PAGE as antigens. As shown in Figure 2, sera from CY-treated birds (Fig. 2, lanes A, C, and E) did not exhibit immune-reactivity, irrespective of challenge. Challenge of immune-competent animals did result in specific binding of serum IgG antibodies to multiple *O. rhinotracheale* proteins (Fig. 2, lanes B and D) with the sera derived from the i.v. challenged birds showing slightly stronger and different reactivity than the sera of the aerosol challenged animals. Weak reactivity could be observed using serum from the unchallenged control birds (Fig. 2, lane F).

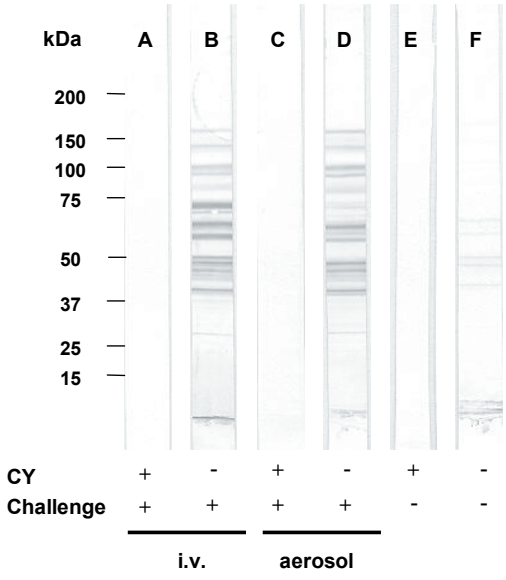

Figure 2. Western blot demonstrating the reactivity of sera from i.v. (lanes A and B) or aerosol (lanes C and D) challenged CY-treated birds (lanes A and C) and birds with a normal immune system (lanes B and D), 7 days post *O. rhinotracheale* infection, and unchallenged CY-treated and control birds (lanes E and F) to *O. rhinotracheale* antigens. Note that reactivity is primarily obtained for the challenged control animals and that different patterns are obtained for i.v. or aerosol-treated animals. Weak reactivity could be detected using serum from the unchallenged control birds (lane F).

Passive transfer of protective antisera to CY-treated animals

In order to obtain evidence that the more severe respiratory and systemic organ lesions observed in the CY-treated animals after *O. rhinotracheale* challenge were caused by the observed immune deficiency rather than effects on other organ systems, reconstitution experiments were performed. In these experiments, 2-week old CY-treated animals received *O. rhinotracheale*-specific antiserum obtained from birds that had been i.v. challenged with live *O. rhinotracheale* serotype A strain B3263/91 (Table 1, group B), antiserum from birds that had been vaccinated s.c. with inactivated *O. rhinotracheale* serotype A strain B3263/91 or with inactivated *P. multocida*, or with serum from healthy, non-challenged chicken. The total Ig concentrations of sera from challenged or vaccinated birds were comparable. Five hours after intravenous serum administration birds were i.v. challenged with *O. rhinotracheale* serotype A strain B3263/91. One week after challenge, at 3 weeks of age, birds were sacrificed and typical *O. rhinotracheale* pathology of joints and liver was scored.

As indicated in Figure 3, administration of *O. rhinotracheale* specific antisera caused a significant ($p < 0.05$) reduction of systemic lesions with scores of 9.4% and 7.3% after passive transfer of sera from animals challenged with live or inactivated *O. rhinotracheale*, compared to 25.0% after passive transfer of sera from birds immunized with *P. multocida* bacterin, 29.2% for the group that received serum from healthy chicken, and 31.8% for the birds that received no serum at all. Animals that had not been challenged with the pathogen showed no organ lesions and had a score of 0% (data not shown).

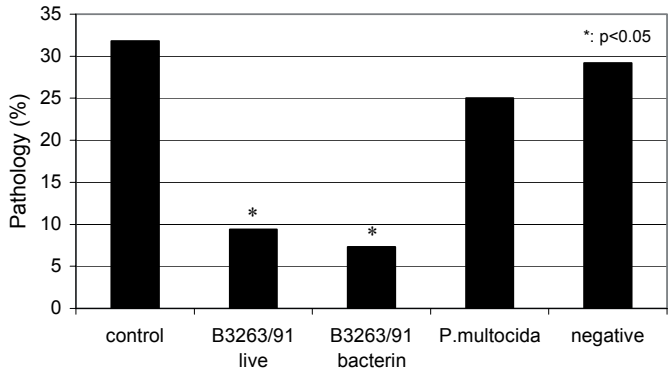

Figure 3. Protective effect of passive transfer of sera to *0. rhinotracheale* infection. Comparison of the macroscopically detectable lesions in joints and liver after challenge of CY-treated and control birds with *0. rhinotracheale* serotype A. Values are presented as the percentage of the maximum possible systemic lesion score. Note that values of groups immunized with B3263/91 live or bacterin serum were significantly different from the value of the challenge control group ($p<0.05$).

Passive transfer and cross-protection

The successful reconstitution of protection against infection in CY-treated animals by passive transfer of protective antisera paved the way to utilize this approach to assess the efficacy of other potentially protective antisera. Since 18 different *O. rhinotracheale* serotypes have been identified, in a second passive immunization experiment, we investigated whether antiserum directed against live *O. rhinotracheale* serotype A strain B3263/91 was able to cross-protect CY-treated, non-immune birds against heterologous strain or serotype challenge. Again 5 hours after administration of the serum under investigation, at 2 weeks of age, birds were i.v. challenged with serotype A (strain TOP98056 SP3555) or G. One week later birds were sacrificed and *O. rhinotracheale* pathology was scored.

As summarized in Figure 4, birds that received antiserum raised against serotype A strain B3263/91 prior to challenge with serotype A strain TOP98056 SP3555 showed a reduction in systemic organ lesion score (11.6%) in comparison to the challenge control group (32.3%) consistent with the concept that the serotype antigen may elicit protective antibodies. Significant protection ($p < 0.05$) was observed in birds that received *O. rhinotracheale* serotype A antiserum but were challenged with *O. rhinotracheale* serotype G. Organ lesion scores decreased from 33.7% in the group that did not receive the antiserum to 8.7% after passive transfer of the serum. The unchallenged groups showed no organ lesions (score of 0%).

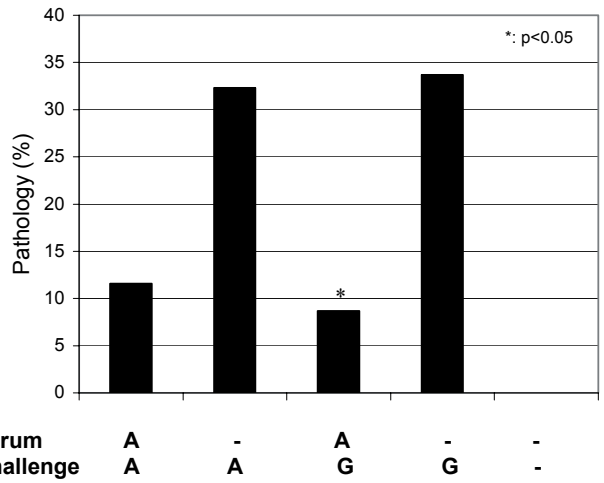

Figure 4. Cross-protective effect of passive transfer of sera to *O. rhinotracheale* infection. Lesions in joints and liver were macroscopically scored one week after challenge of passively immunized CY-treated and control birds. Values represent the percentage of the maximum possible systemic lesion score. Note that the value for the serotype A immunized and serotype G challenged groups is significantly different from the value of the serotype G challenge control group ($p<0.05$). Value of serotype A immunized and serotype A challenged groups differs from the value of the serotype A challenge control group ($p=0.11$).

DISCUSSION

Unravelling of the basis of the protection that may follow upon natural infection with a pathogen may be of great value in directing vaccine development. Here we report on a novel strategy to assess the role of antibodies in providing protection against a bacterial challenge by depletion and subsequent selective targeted reconstitution of immunity within the same natural host. This approach enables rapid determination of the contribution of humoral immunity to protection against infection, and, subsequently, easily determines the efficacy of immune sera or antibodies raised against potentially protective antigens.

In the present study, the feasibility and strength of this variant method of the classical adoptive T-cell transfer to immune-deficient animals was demonstrated using a bacterial pathogen and its natural host, *O. rhinotracheale* infection in chickens. This is an attractive test system as this bacterium seems to induce protective immunity during the natural infection via an undefined mechanism and a suitable natural host infection model is available. In addition, the immune response against *O. rhinotracheale* needs investigation as the pathogen causes an emerging disease in poultry worldwide and a novel broadly cross-protective vaccine against the pathogen is highly needed. The present findings indicate that natural protection against *O. rhinotracheale* infection is largely based on the development of a humoral immune response, that passive transfer of elicited antibodies provides protection in immune-compromised animals, and that transfer of immune sera raised against a serotype A strain provides cross-protection against a different serotype. These results illustrate the power of the developed method of immune depletion and selective reconstitution.

The first step in our work involved selective inhibition of the development of humoral immunity via the administration of the immune-suppressive agent cyclophosphamide (CY). This compound has previously been demonstrated to prevent development of the Bursa of Fabricius and has successfully been applied to study development and maturation of the immune system (69, 95) and its effect on bacterial and viral infections in chicken (11, 41, 154, 157, 201). Our histological data proved CY to be effective in inhibiting bursa development in the SPF chicken used in this study. The effect of the drug on the humoral immune system was further substantiated by the much lower total serum immunoglobulin concentrations in the CY-treated animals.

Challenge of the immune-compromised animals with *O. rhinotracheale* caused significantly more and severe lesions than in the immune-competent birds. One week post challenge, much higher serum titres against *O. rhinotracheale* were detected for the immune-competent birds compared to the CY-treated birds. These antibodies possibly limited the infection in the immune-competent birds, as subsequent passive transfer of the sera of these birds protected the immune-compromised animals against infection. The absence of an increase in *O. rhinotracheale*-specific antibodies in the CY-treated birds and the more serious pathology further indicated that the immune system of these birds was deficient. The mechanism(s) via which the elicited antibodies limit the infection remain(s) uncertain. It can be expected that the differences in pathology between immune-competent and CY-treated animals occur after the initial colonization when specific antibodies come into play. Thus the antibodies may prevent spread of the local infection and/or dissemination of the pathogen to other sites in the body.

The importance of *O. rhinotracheale*-specific antibodies in control of the infection was further demonstrated in control experiments in which sera derived from *P. multocida* bacterin vaccinated animals or healthy animals were administered before challenge. These animals were not protected against subsequent challenge with *O. rhinotracheale*. When serum from *O. rhinotracheale* bacterin vaccinated animals was transferred, protection was obtained. These sera were derived from otherwise healthy animals suggesting that *O. rhinotracheale* specific antibodies and not non-specific immune components provided the protection. The existence of cross-protection after passive transfer indicates that conserved antigens do exist and can be immunogenic. Detailed analysis of the sera may resolve the nature of the cross-protective antigen that could be a defined constituent of future vaccines.

The mechanism, via which the *O. rhinotracheale*-specific antibodies protect against infection, remains to be defined. Antibodies can mediate protection, for example, through enhanced opsonization, increased (complement dependent) bactericidal activity, blockage of attachment, neutralization of toxicity, or some combinations of these mechanisms (115, 217). In the serum of the challenged immune-competent chicken an increase in total IgA, IgG, and IgM antibodies was found. In general, antibody responses elicited during bacterial infections are heterologous and not restricted to a certain class (115, 153, 170). In the mucous layer of the respiratory organs, the first type of immunoglobulins that a pathogen like *O. rhinotracheale* encounters are from the IgA class, although IgG antibodies are capable of entering the mucosal secretions by diffusion. In previous studies, Van Empel *et al* (197) demonstrated that maternal immunity protected young broilers after vaccination of broiler-breeders. Maternally transferred antibodies are mostly IgG, as transfer of IgA and IgM occurs at substantially lower levels (115). Thus, both IgA and IgG antibodies may contribute to the protection against infection.

It should be noted that in our study, CY-treated animals that received an i.v. challenge with *O. rhinotracheale* developed respiratory pathology. This phenomenon has not been observed before in immune-competent birds. This suggests that at least part of the protection by the *O. rhinotracheale*-specific antibodies was established by preventing the migration of the pathogen from the blood into the respiratory tissue. Antibodies of the IgG class have demonstrated to be primarily induced after i.v. challenge of immune-competent birds. Since CY-treated and i.v. challenged birds lacked this increase of IgG antibodies, this Ig isotype is most probably involved in preventing this migration.

Analysis of the specificity of the *O. rhinotracheale*-specific antibodies was demonstrated on Western blot and showed reactivity with multiple bacterial proteins. Furthermore, pooled sera from i.v. challenged birds showed different reactivity profiles than pooled sera from aerosol challenged birds. One can envisage that a different infection route can result in different antigen expression profiles of the pathogen, and, subsequently, antibodies with different antigen specificity and isotype will be produced. As demonstrated in this study, i.v. challenge with *O. rhinotracheale* induced primarily immunoglobulins of the IgG class whereas aerosol challenge with the same bacterium induced the production of both IgA and IgG. Western blot demonstrated the reactivity of IgG antibodies, and not IgA antibodies, against *O. rhinotracheale* proteins. This can be an explanation for the observed differences in reactivity profiles using sera from i.v. and aerosol challenged birds. In our hands, passive transfer of serum obtained from birds that received i.v. challenge conferred cross-protective

immunity against intravenous challenge. Future studies will learn whether similar protection can be obtained with transfer of serum from aerosol-infected birds followed by an aerosol challenge. This information is relevant as it may have direct implications for the way in which novel vaccines should be administered.

Our data indicate that humoral immunity plays a crucial role in the control of *O. rhinotracheale* infection. In general both antibody-mediated and cell-mediated immunity are induced during microbial infections, although the magnitude and quality of these responses can vary greatly. The contribution of cellular immunity in protection against *O. rhinotracheale* infection is not known. Passive transfer of protective serum did not result in 100% protection, leaving space for a (minor) role for cellular immunity. To study the role of T lymphocytes in protection against *O. rhinotracheale* infection, a comparable *in vivo* depletion and reconstitution strategy can be applied with specific depletion of T-cells with drugs or monoclonal antibodies and passive transfer of specific T-cell subsets.

In conclusion, the results of this study demonstrate that chicken humoral immunity to *O. rhinotracheale* is a key component in protection against infection. This knowledge can now be further used to identify protective antigens and even cross-protective antigens, which would allow the development of a broad cross-protective vaccine. Moreover, this combined approach of depletion and transfer of immunity within the same host can be applied to other infections for identification of the protective immune response and as a basis for successful vaccine development.

ACKNOWLEDGEMENTS

We thank Virgil Schijns for helpful comments and discussions. We thank Peter Loeffen for technical biochemical assistance. We thank John Jansen, the Animal Service Department (Intervet) and the Pathology Department (Intervet) for technical assistance during animal studies, necropsy and histological procedures.

CHAPTER THREE

Successful Selection of Cross-Protective Vaccine Candidates of *Ornithobacterium rhinotracheale*

D.F. Schuijffel[1], P.C.M. Van Empel[1], A.M.M.A. Pennings[1], J.P.M. Van Putten[2], and P.J.M. Nuijten[1]

[1] *Intervet International BV, Bacteriology R&D, Boxmeer, The Netherlands,* [2] *Utrecht University, Faculty of Veterinary Medicine, Department of Infectious Diseases and Immunology, Utrecht, The Netherlands.*

Submitted for publication

ABSTRACT

Ornithobacterium rhinotracheale **is a bacterial pathogen known for causing respiratory disease in poultry. In this study we demonstrate for the first time that cross-protective immunity against different *O. rhinotracheale* serotypes can be induced by live vaccination. Sera from these live vaccinated and cross-protected birds were used to identify new vaccine targets by screening an *O. rhinotracheale* expression library. Out of 20,000 screened plaques a total of 30 cross-reactive clones were selected for further analysis. Western blot analysis and DNA sequencing identified 8 different open reading frames. The genes encoding the 8 cross-reactive antigens were amplified, cloned in an expression vector and expressed in *Escherichia coli*. Purified recombinant proteins with a molecular weight ranging from 35.9 kDa to 62.9 kDa were mixed and tested as a subunit vaccine for (cross-) protection against challenge with a homologous and heterologous *O. rhinotracheale* serotype in chickens. Subunit vaccination resulted in the production of antibodies reactive to the recombinant proteins on Western blot and conferred both homologous and heterologous protection in chickens.**

INTRODUCTION

A major challenge in vaccine development against bacterial infections is the existence of different serotypes within a pathogen species, since the use of subunits or inactivated bacterin vaccines mostly provides low or only partial cross-protection and not always for all serotypes. Therefore, the use of a directed approach in order to identify cross-protective antigens or epitopes will greatly contribute to improvement of current vaccines. Here we describe an efficient approach that allows the identification of potential cross-protective antigens of a poultry pathogen, *Ornithobacterium rhinotracheale*.

O. rhinotracheale is a gram-negative bacterial pathogen most known for causing respiratory tract infections, such as airsacculitis and pneumoniae, in birds all over the world. The pathogen may also cause systemic diseases e.g. hepatitis, joint lesions and cerebrovascular pathology (36, 73, 195, 196). The outcome of *O. rhinotracheale* infection varies from mild disease to death and can be influenced by both host factors and environmental conditions. Furthermore, other infectious agents of the respiratory tract such as *Escherichia coli* (160), *Bordetella avium* (50), and Newcastle diseases virus (188) have a triggering effect on the manifestation of *O. rhinotracheale* infection. Treatment of *O. rhinotracheale* infection is becoming more and more difficult as most isolates have acquired resistance against the regular antibiotics (53, 130, 176). Therefore, administration of a suitable vaccine inducing protective immunity against *O. rhinotracheale* infection is a good solution.

Current *O. rhinotracheale* vaccines, based on inactivated bacterin formulations, have proven to be efficacious against *O. rhinotracheale* infection with homologous serotypes (197). Because today 18 different *O. rhinotracheale* serotypes have been identified (36, 198), an immunization strategy is needed that protects birds from infections against multiple serotypes. With respect to poultry pathogens and cross-protection, vaccination with live vaccines is generally of higher quality than vaccination with killed whole cell vaccines (22, 74, 165). Most likely, cross-protective immunity is elicited by specific antigens absent in killed *in vitro* grown (bacterin) preparations but produced specifically *in vivo* after live vaccination or infection. In addition to that, a living bacterium will reach certain niches in the body of the host that are important for the induction of the proper protective immune responses (87, 138, 175). Therefore, live a-virulent *O. rhinotracheale* vaccine strains can be an option for a new immunization strategy, but for now it is still difficult to genetically engineer this bacterium and knowledge about the molecular pathogenesis of *O. rhinotracheale* infections is still scarce. An alternative strategy is the identification of cross-protective antigens in combination with a suitable vaccine application.

By means of immune depletion and reconstitution experiments, we previously demonstrated that the antibody-mediated immune response can provide cross-protective immunity against *O. rhinotracheale* infection in chickens (chapter 2). In this study we made use of those types of cross-protective antisera for the detection of relevant antigens by screening an *O. rhinotracheale* genomic expression library. Specific antisera used in immunoscreening were obtained from (partially) cross-protected birds that had experienced cross-infection by live vaccination with one serotype and subsequent challenge with a different serotype. Our hypothesis was that, as a cause of a heterologous booster, birds would produce higher titres of cross-reactive and also cross-protective antibodies. Using this approach, different cross-

reactive antigens were identified and the immunogenicity of these antigens was assessed by subunit vaccination of broiler chickens followed by homologous and heterologous *O. rhinotracheale* challenge.

MATERIALS AND METHODS

Bacterial strains and growth conditions

O. rhinotracheale serotype A strain B3263/91, *O. rhinotracheale* serotype B strain GGD 1261, *O. rhinotracheale* serotype G strain O-95029 nr.16279, and *O. rhinotracheale* serotype M strain TOP 98036 4500 were grown on 5% sheep blood agar at 37°C, in a 5% CO_2 atmosphere for 48 hours. For liquid culture, single colonies were inoculated in Todd Hewitt (TH) medium (Difco, Detroit, MI, USA) and grown for 24 hours at 37°C on a 100 rpm shaker. *E. coli* strain XL1 Blue was obtained from Clontech Laboratories (Palo Alto, CA, USA) and grown in Luria Bertani (LB) broth supplemented with 10 mM $MgSO_4$ and 0.2% maltose. *E. coli* strains TOP10 (Invitrogen, Carlsbad, CA, USA) and BL21(DE3) pLysS codon RIL (Novagen, Madison, WI, USA) were used for cloning and protein expression, respectively. Both strains were grown in Terrific Broth (TB) for protein expression supplemented with 10 mM $MgSO_4$. All *E. coli* liquid cultures were grown for 16-20 hours at 37°C on a 200 rpm shaker.

Chickens

Commercial Ross broiler chickens and specific-pathogen-free (SPF) broiler chickens (Intervet, Boxmeer, The Netherlands) were used. All animals were placed at day of hatch in negative pressure isolators of approximately 1.5 m^3 with a maximum of 12 birds per isolator in a 7-week study. The animals received sterilized food (Hendrix, Boxmeer, The Netherlands) and water *ad libitum*. In each experiment, the birds of different test groups were housed in a mixed population where possible, to diminish isolator effects. All animal studies were approved by the committee for animal experiments in The Netherlands (DEC) according to international regulations.

Live vaccination study

Ross broiler chickens were live vaccinated with *O. rhinotracheale* serotype B strain GGD 1261, *O. rhinotracheale* serotype G strain O-95029 nr.16279, or *O. rhinotracheale* serotype M strain TOP 98036 4500 at two weeks of age. Birds were vaccinated by aerosol spraying of 100 ml of a fresh bacterial culture containing approximately 10^8 colony forming units (CFU) per ml Todd Hewitt (TH) medium (Difco, Detroit, MI, USA). During aerosol spraying the bacterial culture was administered as a fine spray to the birds using a commercial paint sprayer. The developed mist in the isolators was maintained for at least 10 min with the air circulation

closed. Five days before aerosol challenge birds were triggered with Newcastle Disease (ND) strain LaSota by aerosol spray of approximately 10^6 egg infectious dose (E.I.D.) per bird. At 5 weeks of age, chickens were challenged with *O. rhinotracheale* serotype A strain B3263/91. Challenge was done either by aerosol spraying of 100 ml of a fresh bacterial culture containing approximately 10^9 CFU per ml as described above, or by administration of 0.5 ml of the same bacterial culture intravenously. Each treatment group contained 11 animals.

Post-mortem examination and parameters of infection

At the end of all animal experiments, post-mortem examination was performed on all animals. The birds were bled and organ lesions were macroscopically scored for typical respiratory and systemic pathology caused by *O. rhinotracheale* using the following scoring system: for thoracic air sacs, 0 - no abnormalities, 1 - one air sac seriously affected by fibrinous airsacculitis or both air sacs containing limited pin-head sized foci of fibrinous exudates, 2 - both air sacs seriously affected by fibrinous airsacculitis; for abdominal air sacs, 0 - no abnormalities, 1 - pin-head sized foci of fibrinous exudates or slight diffuse fibrinous airsacculitis, 2 - severe fibrinous airsacculitis (the air sacculitis score is given as the sum of both scores); for lungs, 0 - no abnormalities, 1 - unilateral pneumonia, 2 - bilateral pneumonia; for liver, 0 – no abnormalities, 1 – pin-head sized foci, 2 – severe hepatitis; for the joints, 0 – no abnormalities, 1 point for each joint that shows purulent exudates. The average group-scores are given as a percentage of the maximal possible respiratory or systemic score. Statistical analysis was performed using the Kruskal-Wallis non-parametric one-way ANOVA test.

Serological investigation and antisera preparation

Serum samples were collected at the beginning of each experiment by bleeding 10 control birds. During the animal experiments serum samples were collected after vaccination, before challenge and before post-mortem investigation. Serum samples were tested in an enzyme-linked immunosorbent assay (ELISA) against boiled extract antigens of the *O. rhinotracheale* serotypes as described by Van Empel *et al.* (198). Serum antibody levels were represented as ^{2}log-titres. For use in immunoscreening or Western blot analysis, sera from all birds within a treatment group (approximately 10) were pooled. Pre-adsorption with *E. coli* XL1 Blue cell lysate was done as described by Sambrook *et al.* (162), before use in screening the expression library in order to reduce the a-specific background signal.

Construction of a genomic expression library and immunoscreening

O. rhinotracheale serotype G genomic DNA was isolated from liquid cultured cells according to the method described by Sambrook *et al*. (162), partially digested with *Tsp*509I restriction enzyme (New England Biolabs, Beverly, MA, USA) to obtain 1 – 4 kb fragments, and cloned

into the *Eco*RI digested and dephosphorylated λTriplEx vector arms (Clontech, Palo Alto, CA, USA). Packaging was performed using the Stratagene (La Jolla, CA, USA) *in vitro* packaging extracts and phage particles containing *O. rhinotracheale* DNA were transfected into *E. coli* XL1 Blue, resulting in a genomic expression library containing 97% recombinants and a complexity of 6.9. The immunoscreening procedure was performed as described by the manufacturer (Clontech Manual, Clontech, Palo Alto, CA, USA) under native conditions. In short, phage-infected *E. coli* XL1 Blue cells were plated in LB top agar onto LB agar plates, incubated at 42°C for 4 hours during which small clear plaques became visible. These plaques were covered with nitrocellulose filters (Schleicher and Schuell, Dassel, Germany) which were saturated with 10 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated for a further 4 hours at 37°C. Filters were removed from the plates and treated with 1:250 dilution (in 0.04 M PBS-0; 0.05% polysorbate-20; 1% skim milk) of primary antiserum. Rabbit anti-chicken IgG peroxidase conjugated (Nordic, Tilburg, The Netherlands) in a 1:1000 dilution (in 0.04 M PBS-0; 0.05% polysorbate-20; 1% skim milk) was used as secondary antibody. Finally, filters were washed and the substrate solution Vector SG (Vector, Burlingame, CA, USA) was added. Positive (reactive) plaques located on the agar plates were picked and rescreened twice to obtain single purified clones.

Polymerase chain reaction and sequencing

Oligonucleotide primers used for both polymerase chain reaction (PCR) amplification and (partial) sequencing of the DNA inserts of the selected plaques were specifically designed for the λTriplEx vector arms and synthesized by Life Technologies (Invitrogen, Carlsbad, CA, USA). The 5′ primers used was 5′-GCG CCA TTG TGT TGG TAC-3′, the 3′ primer used was 5′-TTT TTC TCG GGA AGC GCG-3′. PCR was performed in an automated thermal cycler (GeneAmp 9700, Perkin Elmer, CA, USA). The final PCR reaction volume was 50 µl containing 50 µM of dNTP's (Promega, WI, USA), 10 pmol of both primers, 20 U/ml of Supertaq plus polymerase and 10X of Supertaq buffer (Both HT Biotechnology Ltd, Cambridge, UK) in water. Phage DNA was added to the reaction mix by picking a freshly plated plaque. The following conditions were used: denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 2 min, and elongation at 68°C for 2 min 30 sec followed by a final extension at 68°C for 10 min.

To determine the nucleotide sequence of the DNA inserts a sequence reaction was done (94°C 10 sec; 50°C 5 sec; 60°C 2 min for 25 cycles in an automated thermal cycler as described) using Big Dye Terminator Ready reaction mix, 50 ng PCR product and 2.4 pmol primer in a 20 µl reaction volume. Sequencing was done on an ABI 310 automated sequencer (Perkin Elmer, CA, USA). Data were collected using ABI 310 Collection Software version 1.0.4 and analysed with Sequence Analysis version 3.1 (Perkin Elmer, CA, USA). Using the partial sequences from the ends, new internal primers were designed to obtain the complete nucleotide sequence of the inserts. Contigs and alignments were made using Sequencer version 4.1.4 (Gene Codes Corporation, USA).

To determine the 5′end sequence of the open reading frames cloned in fusion with the λTriplEx vector, chromosomal DNA (2 µg) of *O. rhinotracheale* serotype G, internal 30-mers

primers and the following sequence reaction was used: 95°C 5 min; 95°C 30 sec, 65°C-0.5°C/cycle 4 min 20 sec for 10 cycles; 95°C 30 sec, 60°C-0.5°C/cycle 20 sec, 60°C 4 min 20 sec for 10 cycles; 95°C 30 sec, 55°C 30 sec, 60°C 4 min 20 sec for 80 cycles.

Nucleotide and amino acid sequences

Newly determined sequence data were deposited at EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk) and accession numbers were assigned. For Or01: AJ748732; Or02: AJ748733; Or03: AJ748734; Or04: AJ748735; Or11: AJ748736; Or77: AJ748737; Or98A: AJ748738; Or98B: AJ748739.

Bio-informatical analysis

Hydrophilicity was analysed by the method of Kyte-Doolittle (114) using Sci Ed Central 2002 software. Signal sequence prediction was performed with SignalP (http://www.cbs.dtu.dk/services/SignalP) (141). Analysis for sequence homologies, protein families and conserved domains was done using Prosite (http://au.expasy.org/prosite/) (99), Pfam (http://www.sanger.ac.uk/cgi-bin/Pfam), NCBI BLAST (http://ncbi.nlm.nih.gov/) (8, 132), and TIGR CRM (http://www.tigr.org/tdb/) (149).

PAGE and Western blot analysis

NOVEX NuPAGE was used for protein electrophoresis under denaturating conditions in 4-12% polyacrylamide gels according to manufacturers' instructions (Invitrogen, Carlsbad, CA, USA). Protein bands were made visible by using Coomassie Brilliant Blue. Polypeptides were electro- blotted onto Immobilon PVDF 0.45 μm membrane (Millipore, Bedfort, MA, USA), by semi-dry Western blotting according to Towbin *et al.* (187) and blocked, washed, and incubated with primary antisera as described above.

Cloning and protein expression

PCR amplified gene products were cloned in TOPO-TA cloning system (Invitrogen, Carlsbad, CA, USA) and digested with the appropriate restriction enzymes (obtained from New England Biolabs, Beverly, MA, USA) for directional cloning in the expression vector of interest. Ligation products were initially transformed into *E. coli* TOP10 competent cells (Invitrogen, Carlsbad, CA, USA) and were subsequently transformed to *E.coli* BL21(DE3)codonRILpLysS host cells (Novagen, Madison, WI, USA) for expression. Genes were cloned into pET plasmid vector pET22b and therefore the recombinant proteins were expressed with an *E. coli* PelB leader peptide fused at the amino terminal portion (*O. rhinotracheale* leader peptides were replaced) and 6 histidine residues at the carboxy terminal portion of the protein. *E. coli*

strain BL21(DE3)codonRILpLysS (Novagen, Madison, WI, USA) was used for high level expression as described in the pET system manual. Recombinant antigens were isolated from supernatant or purified by metal affinity chromatography, using talon resin (Clontech Inc., Palo Alto, CA, USA) in the presence of 8 M urea as described by the manufacturer, or by repeated freeze-thawing, sonification and centrifugation steps, in 5 mM Tris; 2 mM EDTA pH 7.5.

Subunit vaccination study

The 8 purified cross-reactive antigens were blended into one subunit vaccine in a water-in-oil emulsion, and tested for (cross-) protective capacity in SPF broilers. At 2 weeks of age, SPF-broilers were injected subcutaneously with 0.5 ml vaccine, containing approximately 25 μg of each present antigen per dose. At 5 weeks of age birds were primed with ND LaSota by aerosol spraying of approximately 10^6 E.I.D. per bird. At 6 weeks of age, birds were challenged with either *O. rhinotracheale* serotype G strain O-95029 nr.16279 (homologous challenge) or *O. rhinotracheale* serotype A strain B3263/91 (heterologous challenge). The challenge was done by aerosol spraying of 100 ml of a fresh bacterial culture containing approximately 10^9 colony forming units (CFU) per ml Todd Hewitt (TH) medium (Difco, Detroit, MI, USA) as described above. One week after challenge, at 7 weeks of age, birds were sacrificed and organ lesions were scored. Each treatment group contained 11 birds.

RESULTS

Generation of cross-reactive antibodies

In order to obtain *O. rhinotracheale*-specific antiserum containing potentially cross-protective antibodies directed against the complete repertoire of *in vivo* expressed antigens, the following animal experiment was performed. Two-week-old broiler chickens were live vaccinated by aerosol spraying with an *O. rhinotracheale* serotype B, G, or M strain. Although natural infection was mimicked, no abnormalities were observed and 3 weeks later, at 5 weeks of age, only minor serological responses could be detected. (Table 1, column 1). The average serotype-specific serum IgG antibody titres of vaccinated birds (which ranged from 5.7 and 5.8 to 6.1, 3 weeks after serotype M, B, and G infection respectively) remained close to the average levels of unvaccinated birds (5.2). To boost the production of cross-reactive antibodies and to determine cross-protection by live vaccination (see below), at 5 weeks of age birds were re-infected with a heterologous *O. rhinotracheale* serotype A strain via the natural route, i.e. aerosol spraying (after an additional ND priming), or via an artificial route, i.e. intravenous injection. One week later the average, serotype-specific IgG titres of serotype B vaccinated birds increased from 5.8 to 9.8 and 12.9 after aerosol and intravenous challenge, respectively (Table 1). Serotype G and serotype M vaccinated birds aerosol challenged with serotype A showed no elevation but a minor reduction of serotype-specific IgG antibody

levels: from 6.1 to 5.9 and from 5.7 to 5.4 (Table 1). However, intravenous challenge with serotype A did result in increased titres: from 6.1 to 8.0 in serotype G vaccinated birds and from 5.7 to 7.5 in serotype M vaccinated birds (Table 1). Furthermore, unvaccinated but serotype A challenge control birds also showed elevated antibody titres (Table 1) which were comparable to the serotype-specific titres of birds that received serotype B vaccination before challenge.

The serological cross-reactivity was analysed in 4 different ELISA's, specific for *O. rhinotracheale* serotypes A, B, G, and M. 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 (Table 1, columns 2 and 3), 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.

Table 1. Serological responses after live vaccination and (cross-) reactivity between different serotypes before and after challenge

	Average *O. rhinotracheale* serotype specific ELISA titres (in ^{2}log)								
	3 wks pv[a]	**Aerosol challenge[b]**				**Intravenous challenge[b]**			
Treatment	***[c]**	**A**	**B**	**G**	**M**	**A**	**B**	**G**	**M**
Vaccination with serotype B	**5.8**	11.2	**9.8**	6.5	6.2	14.6	**12.9**	8.1	8.5
Vaccination with serotype G	**6.1**	8.5	6.8	**5.9**	5.4	13.5	10.8	**8.0**	8.0
Vaccination with serotype M	**5.7**	8.4	6.2	5.2	**5.4**	11.9	9.7	7.2	**7.5**
Challenge control	5.2[d]	9.9	8.6	5.8	5.9	13.4	11.2	7.0	7.9

[a] Serum samples were obtained 3 weeks post live vaccination (pv) with *O. rhinotracheale* serotype B, G, or M
[b] Serum samples were obtained 1 week after challenge with *O. rhinotracheale* serotype A
[c] Serotype-specific ELISA titres are presented in bold

Live vaccination and cross-protection

Cross-protection due to live vaccination was determined in the experiment described above. Before aerosol challenge, birds were primed with Newcastle disease (ND) strain LaSota in order to make the birds more susceptible for infection after the second challenge, since they already received a primary challenge by means of live vaccination. One week after challenge, at necropsy, organs were scored with respect to macroscopic pathology. Respiratory pathology (scored in air sacs and lungs) observed after aerosol infection is summarized in Figure 1A.

Birds that were live vaccinated with *O. rhinotracheale* serotypes B, G, or M followed by an aerosol challenge with *O. rhinotracheale* serotype A showed significantly lower ($p < 0.05$) (45.5%, 18.2%, and 27.8% respectively) organ lesion scores than unvaccinated but aerosol challenged birds (77.8%). The unchallenged ND control group showed background respiratory pathology (11.1%).

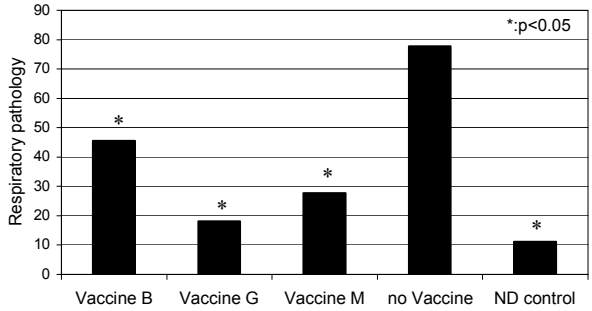

Figure 1A. Live vaccination and cross-protection. Respiratory pathology after aerosol challenge with *O. rhinotracheale* serotype A. Lesions in air sacs and lungs were macroscopically scored and represented as the percentage of the maximum possible respiratory score. Values of groups live vaccinated with *O. rhinotracheale* serotype B, G, or M were significantly different compared to the value of the unvaccinated challenge control group ($p<0.05$).

Systemic pathology (scored in joints and liver) observed after intravenous infection is summarized in Figure 1B. Birds live vaccinated with *O. rhinotracheale* serotype B before i.v. serotype A challenge showed minimal reduction in organ lesion score (19.7%) compared to the unvaccinated challenge control group (20%). Systemic symptoms of birds live vaccinated with *O. rhinotracheale* serotypes G (8.3%) and M (10%) showed a 2-fold but statistically not significant ($p>0.05$) reduction in comparison to the challenge control group.

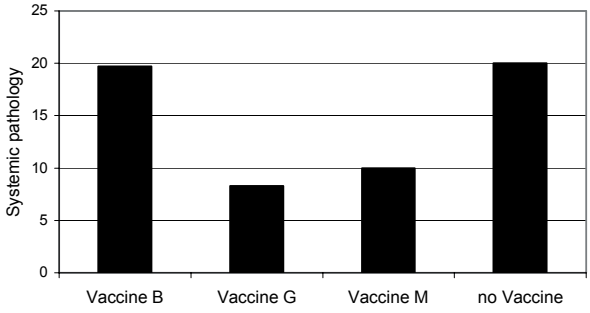

Figure 1B. Systemic pathology after i.v. challenge with *O. rhinotracheale* serotype A. Lesions in joints and liver were macroscopically scored and represented as the percentage of the maximum possible systemic score.

Screening of genomic expression library and selection of cross-reactive clones

Since live vaccination with *O. rhinotracheale* serotype G induced the highest level of cross-protection against serotype A challenge, this serotype was selected for the construction of a genomic expression library. Approximately $2*10^4$ plaques (representing the total genome) of this recombinant DNA library were screened under native conditions using the cross-protective sera obtained from *O. rhinotracheale* serotype G live vaccinated and *O. rhinotracheale* serotype A i.v. challenged chickens. Using serum from i.v. challenged birds

was preferred over using serum from aerosol challenged birds since this serum contained the highest levels of *O. rhinotracheale*-specific IgG antibodies (Table 1). The primary screening of the complete library resulted in a selection of 200 most intense reactive plaques, which were rescreened twice resulting in 175 single, pure positive plaques.

Antiserum, obtained from *O. rhinotracheale* serotype B or serotype M live vaccinated and *O. rhinotracheale* serotype A i.v. challenged birds (Table 1), was used in rescreening of the 175 plaques for cross-reactivity. Thirty plaques reacted positive in immunoscreening for all 3 serotypes G, B, and M.

Identification of open reading frames contained within inserts

The 30 cross-reactive clones were analysed for the presence of cloned insert DNA by PCR: inserts ranged in size between 1.2 and 3.6 kb (data not shown). Sequence analysis of these inserts revealed that the total selection represented 7 different sequence groups or loci with a variable number of matching clones. These groups were encoded Or01 (6 clones), Or02 (5 clones), Or03 (12 clones), Or04 (4 clones), Or11 (1 clone), Or77 (1 clone), and Or98 (1 clone). Analysis of the DNA sequences of the cloned inserts revealed multiple open reading frames (ORFs) per sequence group.

To determine which ORFs were translated into the proteins recognized by antibodies during screening, clones were induced and phage plaques were collected for Western blot analysis under denaturating conditions. With a 1:1:1 mixture of the 3 sera previously used to select the cross-reactive plaques, protein products of Or01, Or02, Or03, Or04, and Or77 could be detected (Table 2, column 1-3).

The molecular weights of the expressed proteins encoded by members of these different groups varied between 37 and 65 kDa (data not shown). Protein sizes of a certain clone varied within a 5 kDa range, indicating that proteins were expressed as a fusion protein with the λTriplEx vector. The size of the expressed fusion proteins detected on Western blot was comparable to the deduced proteins size of predicted ORFs present in the 5 different DNA sequences. As expected, these ORFs were in frame with coding sequences of the expression vector. The bands of cross-reactive proteins Or11 and Or98 could not be visualized using Western blot analysis. The DNA sequence of fragment Or11 also contained an ORF cloned in fusion with the vector. Only fragment Or98 contained two complete ORFs of comparable sizes (Or98A and Or98B) that were not cloned in frame with the expression vector. Therefore, both ORFs were considered to encode a possible cross-reactive antigen.

Since 6 out of 8 proteins were expressed as a fusion protein, the 5'-end of the ORFs was missing. Therefore, sequence reactions were performed using internal primers on genomic DNA of *O. rhinotracheale* serotype G as a template. Sequencing was done until the most upstream ATG start codon, corresponding to the ORF of interest, was reached. The sizes of the 8 complete ORFs and encoded proteins were: Or01: 1614 bps 59.8 kDa; Or02: 1572 bps 58.2 kDa; Or03: 1242 bps 46.0 kDa; Or04: 1023 bps 37.9 kDa; Or11: 1230 bps 45.6 kDa; Or77: 1140 bps 42.2 kDa; Or98A: 918 bps 34.0 kDa; Or98B: 888 bps 32.9 kDa (Table 3).

Table 2. Summary serological analysis cross-reactive antigens

	Immunoscreening	Western blot*		Western blot*	Western blot*
Plaque	Serum live[a]	Serum live[a]	**Rec. protein**	Serum live[a]	Serum subunit[b]
Or01	+	+	Or01	+	+
Or02	+	+	Or02	+	+
Or03	+	+	Or03	+	+
Or04	+	+	Or04	+	+
Or11	+	-	Or11	-	-
Or77	+	+	Or77	+	+
Or98	+	-	Or98A	-	-
			Or98B	-	+

* Western blot analysis was performed under denaturating conditions

[a] Serum live: 1:1:1 mixture of sera obtained from birds live vaccinated with *O. rhinotracheale* serotype B, G, or M and i.v. challenged with serotype A

[b] Serum subunit: Serum obtained from birds vaccinated with the subunit vaccine containing all 8 recombinant proteins

Characterization of identified open reading frames

The amino acid sequences of the identified ORFs were compared with published microbial genome sequences and analysed for protein families and conserved domains (8, 99, 114, 132, 141, 149). The results of this analysis are summarized in table 3.

Sequence analysis indicated that Or01 codes for a dihydrolipoamide acetyltransferase (E2) component of a 2-oxo acid dehydrogenase with 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.

Or02 shows similarity to a putative outer membrane protein of the bacterium *Leptospira interrogans* (31% identity, 43% similarity). The protein has a hydrophobic N-terminal end with the characteristics of a Gram-negative signal peptide. A lipoprotein attachment site could also be identified.

Or03 shows similarity to a hypothetical protein with unknown function of Gram-negative *Bacteroides thetaiotaomicron* (identity 23%, similarity 39%). The hydrophobic N-terminus of the protein has the characteristics of a Gram-negative signal peptide.

Or04 codes for a protein with unknown function with closest homology to a hypothetical protein of the bacterium *Clostridium perfringens* (identity 25%, similarity 38%). No signal sequence or transmembrane regions could be detected.

Domain search of Or11 detected an OmpA-like transmembrane domain and among the BLAST hits several outer membrane proteins were found. The most significant similarity was found with the OmpP1/FadL/TodX family from the bacterium *Desulfovibrio vulgaris* (27% identity, 41% similarity). Therefore the sequence of Or11 probably represents an outer membrane protein for long chain fatty acid transport. The hydrophobic N-terminus of the protein has the characteristics of a Gram-negative signal peptide.

Table 3. Identification and characterization open reading frames

Gene	Size ORF (bps)	Protein	Size protein (aa)	MW protein (kDa)	Signal sequence	Similar protein in database (organism [% identity])
Or01	1614	Or01	537	59.8	No	Dihydrolipoamide acetyltransferase *(Bradyrhizobium japonicum* [35%]*)*
Or02	1572	Or02	523	58.2	Yes	Putative outer membrane protein *(Leptospira interrogans* [31%]*)*
Or03	1242	Or03	413	46.0	Yes	Hypothetical protein *(Bacteroides thetaiotaomicron* [23%]*)*
Or04	1023	Or04	340	37.9	No	Hypothetical protein *(Clostridium perfringens* [25%]*)*
Or11	1230	Or11	409	45.6	Yes	OmpA-like outer membrane protein *(Desulvibrio vulgaris* [27%]*)*
Or77	1140	Or77	379	42.2	Yes	Hypothetical membrane-associated lipoprotein *(Ureaplasma urealyticum* [27%]*)*
Or98A	918	Or98A	305	34.0	No	Hypothetical protein *(Actinobacillus pleuropneumoniae* [30%]*)*
Or98B	888	Or98B	295	32.9	No	RecT protein *(Clostridium tetani* [44%]*)*

No significant homologies were found for Or77. Domain search identified a cleavage site for a lipoprotein signal peptidase II and a lipoprotein associated domain of approximately 100 amino acids length (position 164 – 266), which has a 27% identity with a conserved hypothetical membrane lipoprotein of *Ureaplasma urealyticum.* Or98A showed closest similarity to a hypothetical protein of the bacterium *Actinobacillus pleuropneumoniae* (identity 30%, similarity 44%). No significant domains could be detected.

Domain search of Or98B revealed a region characteristic for the RecT family with most significant similarity with the RecT protein of the bacterium *Clostridium tetani* (identity 44%, similarity 66%). A signature for a Gram-negative bacterial RTX-toxin activating protein C is found in position 153 to 175.

Cloning, expression, and purification of recombinant proteins

In order to amplify the ORFs for expression of the different antigens, PCR primers were designed containing specific restriction sites for directional cloning (Table 4). PCR products were cloned in pET22b and recombinant proteins were expressed in *E. coli* with an *E. coli* PelB leader secretion signal peptide fused at the amino terminal portion (putative *O. rhinotracheale* leader peptides were replaced in Or02, Or03, Or11, and Or77), see Table 4, and 6 histidine residues at the carboxy terminal portion of the protein (except for Or02).

Even though a PelB leader peptide was cloned at the N-terminus of all 8 proteins, microscopic analysis of the induced *E. coli* cultures showed inclusion bodies, except for *E. coli* cells containing the pET22b-Or77 expression construct which secreted the protein into

the supernatant. Recombinant proteins Or03, Or04, Or98A, and Or98B were purified by metal affinity chromatography, facilitated by the confirmed presence of the cloned histidine tag, solubilized in 8 M urea and dialyzed against PBS. After dialysis of the affinity-purified proteins, protein aggregates were formed which were collected and used for formulation of a vaccine and immunization of birds. Recombinant proteins Or01, Or02, and Or11 were partially purified by repeated freeze-thawing, sonification, centrifugation and ultrafiltation. Purity of the recombinant proteins was assessed by polyacrylamide gel electrophoresis (PAGE) and Coomassie Brilliant Blue staining (Fig. 2).

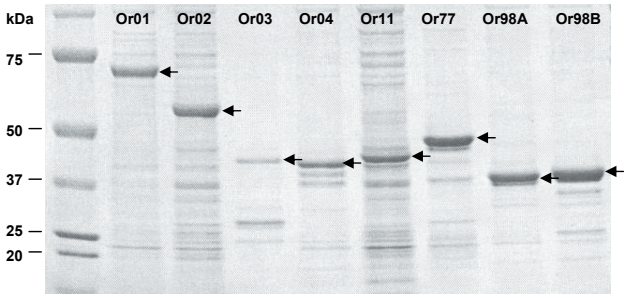

Figure 2. PAGE analysis of 8 expressed recombinant vaccine proteins.

It could be observed that recombinant proteins Or01, Or77, and Or98B migrated slower during electrophoresis since their molecular weights on PAGE were higher as expected to be, based on cloned sequence lengths (62.9 kDa, 43.7 kDa, and 35.9 kDa for recombinant Or01, Or77, and Or98B respectively).

Serological reaction of recombinant proteins was determined on Western blot using the 1:1:1 mixture of sera that was initially used to screen the library and recognized the initial 30 cross-reactive λTriplEx clones. Recombinant proteins Or01, Or02, Or03, Or04, and Or77 reacted positive. No reactivity could be detected with recombinant proteins Or11, Or98A, and Or98B (Table 2, column 4 and 5).

Antigenic analysis of the 8 proteins

To test the antibody-inducing capacity of the 8 proteins, a subunit vaccine was formulated in a water-in-oil emulsion that contained all 8 proteins in approximately equal concentrations of 25 µg per antigen per dose. Two-week-old SPF-broiler chickens were subcutaneously injected with the vaccine and subsequent production of serum antibody levels directed against the vaccine was tested 4 weeks later by Western blot analysis. The proteins in the water phase of the subunit vaccine were run on an SDS-PAGE, blotted and incubated with pooled serum from vaccinated birds or unvaccinated control birds. Serum obtained from animals that were vaccinated showed protein-specific IgG reactivity to all antigens except Or11 and Or98A (Fig. 3, Table 2 column 6). No reactivity could be detected using sera from

Table 4. Oligonucleotide primer sets for the amplification of selected *O. rhinotracheale* genes

gene	5′ oligonucleotide	3′ oligonucleotide	PCR product size (bp)	5′ start[c] – MW expression product (kDa)[a],
Or01	5′-GCTGGCC**ATG***GCTGAAATTATAAAAATGCC*-3′ MscI	5′-CCGCTCGAG*CACAAGCATAGACATTGG*-3′ XhoI	1627	M_{1} - 62.9
Or02	5′-CAGTCC**ATG***GCATGTAGCGATTTTGAT*-3′ NcoI	5′-CCGCTCGAG*GTGGTCTTTATAAAAATG*-3′ XhoI	1578	A_{19} - 58.2[b]
Or03	5′-CAGTCC**ATG***GCGATGATAATCAGTTCTTATG*-3′ NcoI	5′-CCGCTCGAG*AATAAATTCATCATTAAGC*-3′ XhoI	1092	D_{31} - 43.1
Or04	5′-CGATGGCC**ATG***AAAGATATATTTGAAT*-3′ MscI	5′-CCGCTCGAG*TTCTTCACTTGGTATTTTGA*-3′ XhoI	1034	M_{1} - 41.0
Or11	5′-CGATGGCC**ATG***GGGGCACAAGGTGTAGC*-3′ MscI	5′-GCGGCCGC*TACGATAAACCTAGACCAAA*-3′ NotI	1129	M_{35} - 44.5
Or77	5′-CATGCC**ATG***GTCTGTAGCAGTGATGATTAC*-3′ NcoI	5′-CCGCTCGAG*GTTAATTGAAACTCTTAAGC*-3′ XhoI	1107	C_{17} - 43.7
Or98A	5′-CAGTCC**ATG***GTAAAAGACTTTTCAG*-3′ NcoI	5′-CCGCTCGAG*TGCTATTAATTCTAATCG*-3′ XhoI	927	V_{3} - 37.0
Or98B	5′-CAGTCC**ATG***GAATTAGCGAAAAACGAC*-3′ NcoI	5′-CCGCTCGAG*TTTTAATTCATTTTTTCTG*-3′ XhoI	897	E_{3} - 35.9

Restriction site: underlined; ATG start codon: **bold**; Gene of interest: *italic;*

[a] Including *E.coli* PelB leader peptide and HIS-tail

[b] Cloned without C-terminal HIS-tail

[c] First amino acid of the *O. rhinotracheale* protein sequence

unvaccinated control animals (data not shown), indicating that subunit vaccination induced the humoral immune system to produce protein-specific antibodies.

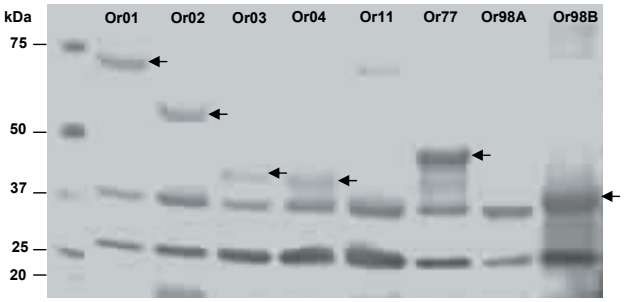

Figure 3. Western blot demonstrating the reactivity of sera from birds vaccinated with the subunit vaccine to the 8 recombinant vaccine proteins. Note that reactivity against protein Or11 and Or98A could not be detected.

Reactivity of IgG antibodies induced by vaccination against *in vitro* expressed *O. rhinotracheale* proteins was determined by Western blotting of serotype A and serotype G liquid (challenge) cultures. Specific reactivity against 4 different *O. rhinotracheale* proteins with a molecular weight of approximately 40, 45, 55, and 65 kDa could be detected at both serotypes (Fig. 4), although the 40 kDa protein of *O. rhinotracheale* serotype G showed very weak reaction. No reactivity could be observed using sera from unvaccinated control animals.

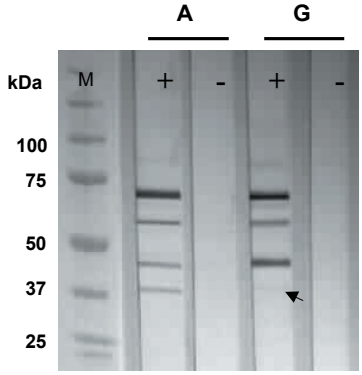

Figure 4. Western blot demonstrating the reactivity of sera from vaccinated and unvaccinated birds against 4 *O. rhinotracheale* serotype A and G *in vitro* expressed antigens with molecular weights ranging from 40 to 65 kDa. Note that the reactivity of the 40 kDa protein of *O. rhinotracheale* serotype G is very weak (indicated with an arrow).

Subunit vaccination and cross-protection

To test the protective capacity of the induced protein-specific antibodies, the birds that were vaccinated (see above) were primed with Newcastle Disease (ND) virus at 5 weeks of age, and challenged with *O. rhinotracheale* at 6 weeks of age. Challenge was performed using serotype G, to study homologous protection, or serotype A, to study heterologous (cross-) protection. Birds were infected via the natural route, i.e. aerosol spray application, causing mainly respiratory lesions in air sacs and lungs. Necropsy was performed at 7 weeks of age, one week after challenge. Organs were scored with respect to macroscopic pathology and the results are summarized in Figure 5.

Birds that received the subunit vaccine containing the 8 antigens showed complete and significant ($p = 0.002$) protection (0.0% pathology) against homologous serotype challenge compared to the unvaccinated challenge control group (25.8% pathology). The average organ lesion-score of the birds that did receive subunit vaccine before challenge with heterologous serotype A was also significantly lower ($p = 0.0004$) (11.1%) compared to the unvaccinated birds (39.8%). The ND control group showed minor respiratory background pathology (6.1%).

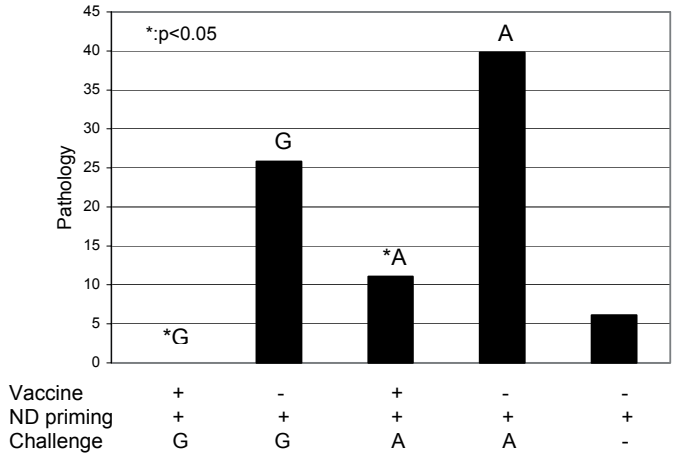

Figure 5. Protective effect of subunit vaccination to *O. rhinotracheale* serotype G (homologous) and serotype A (heterologous) challenge. Lesions in air sacs and lungs were macroscopically scored one week after challenge. Values are represented as the percentage of the maximum possible respiratory score. Note that the values of the vaccinated groups were significantly different compared to their respective challenge control groups ($p<0.05$).

DISCUSSION

In this paper we describe an effective approach for the identification of potential vaccine targets using the poultry pathogen *O. rhinotracheale.* By screening an *O. rhinotracheale* expression library using polyclonal antiserum obtained from cross-protected birds, eight different cross-reactive clones were selected. The corresponding open reading frames were identified, subsequently expressed in *E. coli* and protein antigens were purified for formulation into one multicomponent subunit vaccine. Vaccination of chickens induced the humoral immune system to produce antibodies reactive against most of the recombinant vaccine proteins. Moreover, vaccinated birds showed a high level of protection against infection with different *O. rhinotracheale* serotype strains.

In this study we demonstrate for the first time that vaccination of broilers with live *O. rhinotracheale* cells induced cross-protective immunity against heterologous challenge. Previously, cross-protection by live vaccination was observed against infection with heterologous serotypes or serovars of other poultry pathogens such as *Pasteurella multocida* (74), *Riemerella anatipestifer* (165), and *Haemophilus paragallinarum* (22). In general, inactivated vaccines do not provide high-level protection against a different immunotype not contained within the vaccine. This suggests that antigens involved in cross-protection are expressed *in vivo* and are either not expressed or expressed at low levels *in vitro,* or that live bacteria reach a certain niche essential for the induction of a proper immune response (87, 138, 175).

We screened an expression library of *O. rhinotracheale* serotype G with chicken antiserum exhibiting cross-reactive IgG antibodies induced by live vaccination followed by infection with different *O. rhinotracheale* serotypes. This allowed us to select for clones encoding proteins that are expressed during an infection and are able to induce an immune response. The observation that antibodies can induce cross-protection (chapter 2) was the most important evidence for following this strategy. In this screening we decided to use antisera from intravenously challenged animals since these showed higher IgG antibody titres compared to aerosol challenged animals, although the latter group showed a higher level of protection. We reasoned that until the day of challenge both groups were treated identical and should have identical antibody populations. Intravenous challenge appeared to be more severe than aerosol challenge and therefore caused lower protection levels, but it could give a better booster of cross-reactive antibodies. On the contrary, there is a reasonable chance that interesting proteins are missed in our immunoscreening approach. For example, some potentially cross-protective antigens might be immunorecessive during *O. rhinotracheale* infection resulting in low serum antibody titres and therefore those genes will not be identified. Furthermore, when screening the *O. rhinotracheale* expression library, antibodies of the IgA and IgM class were not detected because as a secondary antibody anti-chicken IgG was used.

Detailed analysis of the 30 selected clones revealed 8 different ORFs encoding cross-reactive proteins. As most ORFs were expressed as translational fusions with a peptide encoded by the expression vector, the 5' sequence of the gene was lacking. This 5' end of the ORFs was obtained by means of sequence analysis using genomic DNA of *O. rhinotracheale* serotype G as template. Since nothing is known about translation initiation sites of *O.*

rhinotracheale, the N-terminal prediction of the encoded protein from DNA sequence was in some cases ambiguous as true start codon had to be selected from a set of possible start sites close to each other. For example, consensus sequences like AGGA of ribosome binding sites were rarely found. For our experiments we selected the most upstream ATG start codon within the selected ORF as being the N-terminal end of the encoded protein. It should be noted that the recombinant proteins can differ from the wild type *O. rhinotracheale* proteins by the introduction of extra amino acids and epitopes.

Bio-informatical analysis was performed in order to characterize the proteins encoded by the identified ORFs. The amino acid sequences of Or02, Or03, Or11, and Or77 were predicted to contain a prokaryotic Gram-negative signal sequence (141) which is involved in the transport of the protein across the cell inner membrane. Comparison of amino acid sequences with available databases showed that the functions of 5 identified ORFs - Or02, Or03, Or04, Or77, and Or98A - are neither defined nor exhibit significant similarity to any sequence in the databases. Analysis for protein families and conserved domains did reveal that Or02 and Or77 contain the characteristics of a lipoprotein. Only the ORFs encoding Or01, Or11, and Or98B showed strong similarities with published sequences of characterized proteins: protein Or01 showed high similarity with dihydrolipoamide acetyltransferase, the E2 component of the pyruvate dehydrogenase complex which is involved in energy production and conversion (48); Or11 has the characteristics of an outer membrane fatty acid transport protein containing an OmpA-like transmembrane domain (110); and Or98B has a 66% similarity with the RecT protein family which members are involved in DNA recombination pathways (142). The suitability of each of these (partially) identified proteins to serve as vaccine antigen will be further investigated in a next study.

Although antibodies were used for detection, the selected antigens do not necessarily have to be surface-exposed or secreted proteins. They can also be intracellular or periplasmic proteins usually inaccessible for antibodies, unless, during infection, they are released by cell lysis. Whether the identified antigens were localized on the bacterial surface has to be determined. Surface components of bacteria can play an important role in virulence and therefore, they are also of interest for studying the molecular pathogenesis of *O. rhinotracheale* infection. Analysis of virulence of gene-specific *O. rhinotracheale* mutant strains can possibly indicate the role of the 8 proteins in pathogenesis, but, as mentioned before, genetic manipulation of this bacterium is still difficult.

Cloning of the 8 ORFs in an expression vector and expression of the cross-reactive proteins in *E. coli* resulted in the production of recombinant proteins. Only protein Or77 was secreted into the supernatant of the growth medium. Despite the presence of a PelB leader peptide cloned at the N-terminus of all recombinant proteins, expression of 7 out of 8 proteins in *E. coli* resulted in the formation of inclusion bodies. Active proteins are usually recovered from inclusion bodies by solubilization of the aggregated protein in urea, and subsequent refolding by dialysis (46). Unfortunately, renaturation of the protein can result in inactive, misfolded proteins and new aggregates. The proteins Or03, Or04, Or98A and Or98B were purified by metal affinity chromatography in the presence of urea followed by dialysis which resulted in formation of precipitates. However, if the protective epitope(s) is (are) in a correct conformation, aggregate-formation or inclusion bodies do not necessarily affect the protective capacity of a protein. Since Or77 was secreted in the growth medium no

additional purification steps were needed and thereby the possibility of isolating a misfolded protein was reduced.

The purified recombinant antigens were clearly visible on PAGE. However, not all recombinant proteins reacted on Western blot, even when positive serum was used from the previous expression library screening. This could be explained by presence of antibodies cross-reactive against conformational epitopes that are destroyed under denaturating conditions of PAGE and Western blotting. Initial expression library screening was done using the same antiserum but under native conditions.

The antisera used for the library screening were obtained from cross-protected birds. However, it was important to realize that the antibodies that recognized the cross-reactive clones in expression library screening were not necessarily the antibodies that conferred cross-protective immunity. The capacity of the selected proteins to induce protective immunity was studied in an animal experiment. Chickens were injected with a subunit vaccine containing all 8 recombinant proteins. Again not all vaccine proteins could be detected on Western blot using antiserum obtained from vaccinated birds. Surprisingly, one protein, Or98B, that was previously undetectable (after *E. coli* protein expression and purification) using the immunoscreening serum from cross-protected birds, reacted positive with protein-specific serum obtained after subunit vaccination. This can be explained by presence of other or different epitopes on the recombinant protein in comparison to the same antigen expressed *in vivo* during infection, which affirms conformational issues as described above.

Successful vaccination using recombinant subunit vaccines depends on the induction of antibodies that recognize the wild type proteins as expressed by the infectious pathogen. Reactivity of antibodies induced after subunit vaccination with the recombinant proteins against *in vitro* expressed *O. rhinotracheale* antigens was assessed on Western blot. Specific reactivity against 4 different *O. rhinotracheale* proteins could be observed, but whether these antigens are the cross-reactive proteins selected by our immunoscreening approach is unclear. Future studies using antigen-specific sera should give a better indication whether the expressed *O. rhinotracheale* antigens detected on Western blot correspond to the 8 identified antigens.

The (cross-) protective capacity of the selected proteins was determined in an animal experiment whereby vaccinated chickens were challenged with *O. rhinotracheale* serotype G (homologous challenge) and *O. rhinotracheale* serotype A (heterologous challenge). Vaccination followed by challenge resulted in significantly less respiratory organ lesions compared to unvaccinated challenged birds. These results indicated that the selection of 8 cross-reactive proteins contain new vaccine candidate(s) with cross-protective capacity. However, as most recombinant proteins showed to induce a specific antibody response, it is unclear which antigen(s) is (are) responsible for the observed cross-protection.

So far, several approaches have been reported that allowed *in vitro* identification of antigens out of proteomic samples or expression libraries by using specific antiserum raised upon encountering a given pathogenic microorganism. For example: the detection of Enteropathogenic *Escherichia coli* antigens using secretory immunoglobulin A antibodies isolated from human breast milk (131); the application of *in vitro* protein selection methods such as ribosome display, to identify and map immunologically relevant proteins of

Staphylococcus aureus (210), and the characterization of *Vibrio vulnificus* antigens preferentially expressed during infection by using *in vivo*-induced antigen technology (IVIAT) (109). The application of an immunoscreening approach as described in this paper - using antisera that contain antibodies with proven protective capacity - has also been described (27, 94, 214, 218). However, when prophylactic studies were performed, animal models were used instead of the natural host-pathogen interaction. The major advantage of this study is the use of *O. rhinotracheale* and its natural host, the chicken. Consequently, this approach is applicable for infectious diseases in animals and less for humans.

Based on the results presented in this paper it can be concluded that immunoscreening of an *O. rhinotracheale* expression library with cross-protective antisera and subsequent analysis of the (cross-) protective capacity of selected proteins within the natural host, was a successful method for the identification of potential vaccine antigens. Further vaccination and antigen characterization studies will have to determine which of the 8 identified antigens have the highest cross-protective capacity and have the best potential for further use in vaccine development.

ACKNOWLEDGEMENTS

We like to thank Hon Tran (Intervet Schwabenheim) for bio-informatical assistance, and the Animal Service Department and the Pathology Department (Intervet Boxmeer) for technical assistance during animal studies.

CHAPTER FOUR

Characterization of Cross-Protective Vaccine Candidates of *Ornithobacterium rhinotracheale*

D.F. Schuijffel[1], P.C.M. Van Empel[1], R.P.A.M. Segers[1], J.P.M. Van Putten[2], and P.J.M. Nuijten[1]

[1] *Intervet International BV, Bacteriology R&D, Boxmeer, The Netherlands,* [2] *Utrecht University, Faculty of Veterinary Medicine, Department of Infectious Diseases and Immunology, Utrecht, The Netherlands.*

Manuscript in preparation

ABSTRACT

Ornithobacterium rhinotracheale **is a pathogen involved in respiratory infection and systemic disease in poultry. In a previous study we identified 8 potential vaccine candidates that, when administered to chickens as a multi-component vaccine, conferred good cross-protection against challenge with different serotypes. In the current study we characterized these 8 proteins more thoroughly by sequencing, *in vitro* expression analysis, and localization experiments. In addition, we analyzed the antigenicity and immunogenicity of the purified recombinant proteins by vaccination using 1-component and 4-component subunit vaccines. All genes encoding these antigens were highly conserved among different *O. rhinotracheale* serotypes. At the protein level, variability in expression among different antigens and serotypes was detected *in vitro*. Cellular fractionation experiments indicated that the majority of the antigens are predominantly located in the outer membrane fraction. Vaccination of chickens with single-antigen vaccines demonstrated that Or77 was the only selected protein that induced cross-protective immunity by itself, making this antigen the most suitable candidate for the development of a cross-protective vaccine. Evaluation of different 4-component subunit vaccines indicated the existence of immunogenic synergism between the candidate vaccine antigens.**

INTRODUCTION

Respiratory tract infections are a major factor in the overall disease incidence in poultry. Several pathogens are indicated as possible causes of respiratory diseases, either alone or in synergy with other microorganisms. The severity of clinical signs, duration of disease, and mortality rate are often influenced by non-infectious factors such as climate conditions and management-related issues. In general, respiratory diseases of poultry can be associated with high economical losses due to increased mortality and condemnation rates, decreased egg production, and decreased growth rate (67, 73, 159).

Ornithobacterium rhinotracheale is a bacterial microorganism known to be involved in causing respiratory tract infections such as airsacculitis, tracheitis, and pneumoniae, and has been isolated from wild birds and commercial fowl throughout the world (36, 195, 196). Furthermore, infection with this pathogen can also manifest as systemic disease such as pericarditis, hepatitis, joint lesions and cerebrovascular pathology (36, 196). Treatment of *O. rhinotracheale* infection is increasingly complicated since most isolates have acquired resistance against antibiotics (52, 53, 130, 176, 204). Therefore, the best strategy to protect poultry against this pathogen is immunization with a suitable vaccine to induce long-lasting immunity.

In the past, vaccines based on inactivated bacterin formulations have been developed with proven efficacy against *O. rhinotracheale* infections with homologous serotypes in both chickens and turkeys (179, 197). At this time, up to 18 different serotypes have been identified (36, 199) which makes it more urgent to develop a broadly cross-protective vaccine. We previously demonstrated that the humoral immune response is important for protection (chapter 2) and that birds which were live vaccinated with one serotype were cross-protected against challenge with another, heterologous serotype (chapter 3). Based on these results, live a-virulent *O. rhinotracheale* vaccine strains could be a new immunization strategy, but for now it is still very difficult to genetically engineer this bacterium and the behavior of these strains in the environment needs detailed investigations. Therefore, an alternative strategy was applied in order to identify cross-protective vaccine targets: immunoscreening of an *O. rhinotracheale* serotype G expression library using polyclonal antisera obtained from cross-protected birds through live vaccination (chapter 3). This screening resulted in the selection of genes encoding 8 different cross-reactive antigens: 2 lipoproteins and 3 hypothetical proteins, all with unknown function; a protein with similarity to the E2p component of the pyruvate dehydrogenase complex; a putative outer membrane protein with an OmpA-like domain; and a protein with similarity to RecT protein. Vaccination of broiler chickens with a multicomponent subunit vaccine containing these 8 purified recombinant proteins resulted in a high level of protection against *O. rhinotracheale* challenge with a homologous and heterologous serotype (chapter 3).

In this study we analyzed the ability of each individual recombinant antigen to induce a cross-protective immune response to *O. rhinotracheale* serotype A challenge in broiler chickens. We characterized the genes and their corresponding protein products by sequence analysis and immunological assays using antigen-specific sera, and cellular localization experiments. Possible synergistic effects on the vaccination efficacy between the different recombinant proteins were investigated in a second animal experiment.

MATERIALS AND METHODS

Bacterial strains and growth conditions

O. rhinotracheale serotype A strain B3263/91, *O. rhinotracheale* serotype B strain GGD 1261, *O. rhinotracheale* serotype G strain O-95029 nr.16279, and *O. rhinotracheale* serotype M strain TOP 98036 4500 were grown on 5% sheep blood agar at 37°C, in a 5% CO_2 atmosphere for 48 hours. For liquid culture, single colonies were inoculated in Todd Hewitt (TH) medium (Difco, Detroit, MI, USA) and grown for 24 hours at 37°C on a 100 rpm shaker.

Chickens

Specified-pathogen-free (SPF) broiler chickens (Intervet, Boxmeer, The Netherlands) were used. All animals were placed at day of hatch in negative pressure isolators of approximately 1.5 m^3 with a maximum of 11 birds per isolator in a 7-week study. The animals received sterilized food (Hendrix, Boxmeer, The Netherlands) and water *ad libitum*. In each experiment, the birds of different test groups were housed in a mixed population where possible, to diminish isolator effects. All animal studies were approved by the committee for animal experiments in The Netherlands (DEC) according to international regulations.

Vaccine preparation

All vaccines were prepared by blending the previously purified cross-reactive recombinant antigens (chapter 3) in a water-in-oil emulsion to a final concentration of each antigen of approximately 50 µg/ml (Table 3). The antigenic mass of the single-component vaccines was 50 µg/ml. The antigenic masses of the multi-component vaccines containing 4 or 8 different recombinant proteins were 200 µg/ml and 400 µg/ml, respectively.

Vaccination studies

At 2 weeks of age, SPF-broilers were injected subcutaneously with 0.5 ml vaccine, containing approximately 25 µg of each present antigen per dose. At 5 weeks of age birds were primed with Newcastle Disease (ND) strain LaSota by aerosol spraying of approximately 10^6 egg infectious dose (E.I.D.) per bird. At 6 weeks of age, birds were challenged with *O. rhinotracheale* serotype A strain B3263/91. The challenge was done by aerosol spraying of 100 ml of a fresh bacterial culture containing approximately 10^9 colony forming units (CFU) per ml Todd Hewitt (TH) medium (Difco, Detroit, MI, USA). During aerosol challenge the bacterial culture was administered as a fine spray to the birds using a commercial paint sprayer. The developed mist was maintained in the isolators for at least 10 min with the air

circulation closed. One week after challenge, at 7 weeks of age, birds were sacrificed and organ lesions were scored. Each treatment group contained 11 birds.

Post-mortem examination and parameters of infection

At the end of all animal experiments, post-mortem examination was performed on all animals. The birds were bled and organ lesions were macroscopically scored for typical respiratory pathology caused by *O. rhinotracheale,* using the following scoring system: for thoracic air sacs, 0 - no abnormalities, 1 - one air sac seriously affected by fibrinous airsacculitis or both air sacs containing limited pin-head sized foci of fibrinous exudates, 2 - both air sacs seriously affected by fibrinous airsacculitis; for abdominal air sacs, 0 - no abnormalities, 1 - pin-head sized foci of fibrinous exudates or slight diffuse fibrinous airsacculitis, 2 - severe fibrinous airsacculitis (the air sacculitis score is given as the sum of both scores); for lungs, 0 - no abnormalities, 1 - unilateral pneumonia, 2 - bilateral pneumonia. The average group-scores are given as a percentage of the maximal possible respiratory score minus background pathology of the unchallenged ND priming control group. Statistical analysis was performed using the Kruskal-Wallis non-parametric one-way ANOVA test.

Polyacrylamide gel electrophoresis (PAGE) and Western blot analysis

NOVEX NuPAGE was used for protein electrophoresis under denaturating conditions in 4-12% polyacrylamide gels according to manufacturers' instructions (Invitrogen, Carlsbad, CA, USA). Protein bands were made visible by using Coomassie Brilliant Blue. Polypeptides were electro-blotted onto Immobilon PVDF 0.45 μm membrane (Millipore, Bedfort, MA, USA), by semi-dry Western blotting according to Towbin *et al.* (187). Membranes were blocked using 0.04 M PBS-0; 0.5% polysorbate-20; 1% skim milk, washed with 0.04 M PBS-0; 0.5% polysorbate-20, and incubated with a 1:250 dilution of primary antiserum in 0.04 M PBS-0; 0.05% polysorbate-20; 1% skim milk. For use in Western blot, sera from all birds within a treatment group (approximately 10) were pooled. Rabbit anti-chicken IgG peroxidase conjugated (Nordic, Tilburg, The Netherlands) was used as secondary antibody in a 1:1000 dilution (in 0.04 M PBS-0; 0.05% polysorbate-20; 1% skim milk). Finally, filters were washed and signal was visualized by addition of Vector SG substrate solution (Vector, Burlingame, CA, USA).

Polymerase chain reaction and sequencing

Oligonucleotide primers, used for polymerase chain reaction (PCR) amplification of the complete open reading frames, were designed using *O. rhinotracheale* serotype G sequences as a template (Table 1) and synthesized by Life Technologies (Invitrogen, Carlsbad, CA, USA). PCR was performed in an automated thermal cycler (GeneAmp 9700, Perkin Elmer, CA, USA). The final PCR reaction volume was 50 μl containing 200 ng of genomic DNA isolated from liquid culture cells according to the method as described by Sambrook *et al.*

(31), 50 μM of dNTP's (Promega, WI, USA), 10 pmol of each primer, 20 U/ml of Supertaq plus polymerase and 10X of Supertaq buffer (Both HT Biotechnology Ltd, Cambridge, UK) in water. The following conditions were used: denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 2 min, and elongation at 68°C for 2 min 30 sec followed by a final extension at 68°C for 10 min.

To determine the nucleotide sequence of the complete genes sequence reactions were performed (94°C 10 sec; 50°C 5 sec; 60°C 2 min for 25 cycles in an automated thermal cycler as described) using Big Dye Terminator Ready reaction mix (Applied Biosystems, Foster City, CA, USA), 50 ng PCR product and 2.4 pmol primer (Table 1) in a 20 μl reaction volume. Sequencing was done on an ABI 310 automated sequencer (Perkin Elmer, CA, USA). Data were collected using ABI 310 Collection Software version 1.0.4 and analysed with Sequence Analysis version 3.1 (Perkin Elmer, CA, USA). Contigs and alignments were made using Sequencer version 4.1.4 (Gene Codes Corporation, USA).

Nucleotide and amino acid sequences

Sequence data of the *O. rhinotracheale* serotype G strain O-95029 nr.16279 antigens are deposited at EMBL Nucleotide Sequence Database (http:/www.ebi.ac.uk). The assigned accession numbers are for Or01: AJ748732, Or02: AJ748733, Or03: AJ748734, Or04: AJ748735, Or11: AJ748736, Or77: AJ748737, OR98A: AJ748738, OR98B: AJ748739.

Triton X-114 detergent-phase partitioning

Triton X-114 phase separation was performed essentially as described by Bordier (24) and Pryde (151). In short, 100 ml of liquid O/N culture of *O. rhinotracheale* serotype G strain O-95029 nr.16279 was pelleted and cells were resuspended in 25 ml of 0.04 M PBS-0, pH 7.2 of 4°C. Triton X-114 (Sigma-Aldrich, St. Louis, MO, USA) solution was added to a final concentration of 0.3% (w/v). Samples were first incubated overnight at 4°C (while gently shaking) followed by a 1 hour incubation step at 37°C. Subsequently, the solution was centrifuged for 10 min at >10,000 x g at 37°C to produce an upper aqueous phase (containing the hydrophilic proteins) and a lower detergent phase (containing the integral membrane proteins). Phase fractions were separated and 0.04 M PBS-0 was added until the 1x dilution factor was regained. Analysis of the antigenic content of the different fractions was performed using Western blot.

Preparation of cellular fractions by differential centrifugation partitioning

Two 50 ml batches of fresh liquid O/N culture of *O. rhinotracheale* serotype G strain O-95029 nr.16279 were used for subcellular fractionation. Cells of one batch were pelleted and the supernatant was stored. Collected cells were osmo-shocked by resuspension in 2 ml of lysisbuffer (0.04 M PBS-0; 1 M NaCl; 1 mM EDTA, pH 8.0), and after 15 min of incubation

on ice the suspension was centrifuged for 10 min at >10,000 x g at 4°C. The supernatant was transferred to a clean tube and $MgCl_2$ was added to a final concentration of 1 mM. This supernatant contained the periplasmic fraction. Cells of the other 50 ml batch were collected by centrifugation at >10,000 x g at 4°C for 15 min, resuspended in an equal volume of 5 mM Tris pH 7.5; 2 mM EDTA, sonicated, and centrifugated at 4,000 x g at 4°C for 5 min. To separate the inner membrane from the outer membrane, sarcosine (Sigma-Aldrich, St. Louis, MO, USA) was added to the supernatant to a final concentration of 1%, and this solution was incubated overnight at room temperature on a rollerbank. A 2 hours ultracentrifugation step (200,000 x g) was used to separate the outer membrane (pellet) from the cytoplasmic/inner membrane (supernatant). To regain the 1x dilution factor of the different fractions, 0.04 M PBS-0 was added. Analysis of the antigen content of the different fractions was performed using Western blot.

RESULTS

Antigenic properties of the 8 recombinant proteins

Immunization of two-week old SPF-broilers with a subunit vaccine containing 8 different recombinant proteins has been shown to elicit antigen-specific antisera reactive against 6 out of the 8 proteins (chapter 3). To test the antibody-inducing capacity of each individual protein, in this study, SPF-broiler chickens were subcutaneously injected with 25 μg per dose of a single recombinant antigen formulated in a water-in-oil emulsion. Subsequent serum antibody levels directed against the vaccine antigens were tested 4 weeks later by Western blot analysis. The proteins in the water phase of the subunit vaccines were run on PAGE, blotted, and incubated with serum from vaccinated or unvaccinated birds. Sera obtained from vaccinated animals showed protein-specific reactivity for each of the 8 vaccine antigens (Fig. 1). Minor reactivity against antigens Or02 and Or77 was also detected for sera from unvaccinated control animals (data not shown).

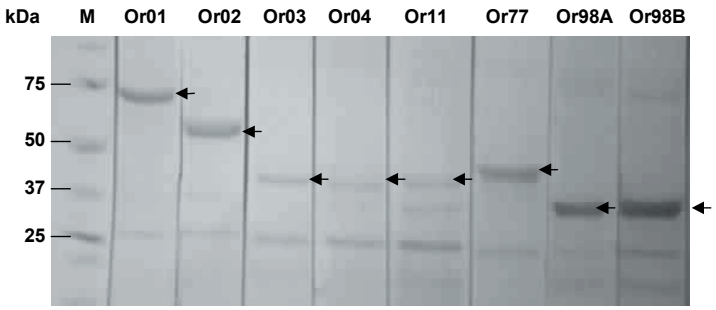

Figure 1. Western blot demonstrating the reactivity of sera from immunized birds with the individual proteins. Reactivity against the recombinant vaccine proteins is indicated with an arrow.

Cross-protection induced by subunit vaccination with single antigens

To test the cross-protective capacity of the protein-specific antibodies, the birds that were vaccinated at 2 weeks of age (see above) were primed with Newcastle Disease (ND) Virus at 5 weeks of age, and challenged with a heterologous *O. rhinotracheale* strain (serotype A) at 6 weeks of age. As a positive control the cross-protective subunit vaccine consisting of all 8 proteins was included. A subunit vaccine containing proteins obtained from the *Escherichia coli* expression strain used to produce the different recombinant proteins served as a negative control. Challenge was performed with *O. rhinotracheale* serotype A to study cross-protection. Birds were infected via the natural route, i.e. aerosol spray application, causing mainly respiratory lesions in air sacs and lungs. Necropsy was performed at 7 weeks of age, one week after challenge. Organs were scored with respect to macroscopic pathology and the post-mortem results, corrected for background pathology due to ND priming, are summarized in Figure 2.

Birds that received the positive control combi vaccine containing all 8 proteins showed a significant ($p < 0.05$) protection (pathology score 19.4%) in comparison to the unvaccinated, challenge control group (pathology score 46.1%) and the group vaccinated with the negative control vaccine containing the *E. coli* proteins (pathology score 40.6%). Of the birds vaccinated with the subunit vaccines containing the single antigens, only the group that received antigen Or77 showed a respiratory pathology score (25.4%) that was significantly ($p < 0.05$) lower compared to the a score of the unvaccinated challenge control group. Pathology levels within a 10% range of the unvaccinated group could be observed in birds vaccinated with Or01 (44.4%), Or02 (41.7%), Or03 (41.7%), Or04 (50.0%) and Or11 (49.4%). Birds vaccinated with vaccines Or98A and Or98B showed over 25 % increase in pathology (59.1% and 60.1% respectively).

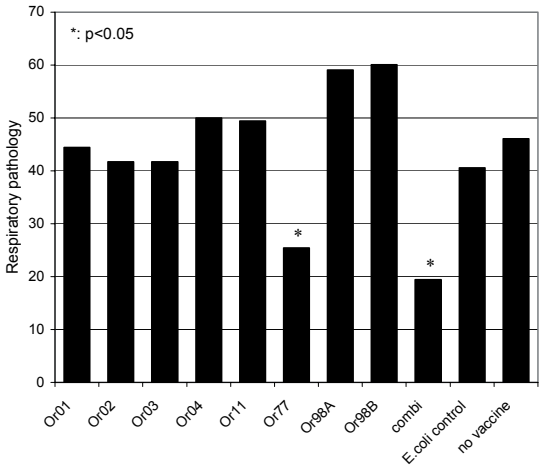

Figure 2. Subunit vaccination with individual proteins and cross-protection. Lesions in air sacs and lungs were macroscopically scored one week after *O. rhinotracheale* challenge. Values are represented as the percentage of the maximum possible respiratory score. Note that the score of the groups vaccinated with the Or77 or 8-component combi vaccine were significantly different compared to the unvaccinated challenge control group ($p<0.05$).

Reactivity of serum antibodies with *O. rhinotracheale* antigens

In order to assess the cross-reactivity of the antigen-specific antibodies raised against each of the 8 recombinant proteins (see above), whole cell lysates of *O. rhinotracheale* serotype A, B, G, and M were electrophoresed, blotted and incubated with the antisera (Fig. 3). Antisera obtained from birds vaccinated with recombinant proteins Or01, Or02, Or04, Or11, or Or77 reacted specifically to *O. rhinotracheale* serotype G antigens of similar sizes as predicted for the wild type antigens. Strong reactivity was observed against Or01 and Or02, while moderate to little reactivity was noted against Or04, Or11, and Or77. No reactivity was observed against *O. rhinotracheale* serotype G antigens Or03, Or98A, and Or98B.

When tested against *O. rhinotracheale* serotypes A, B, and M, both Or01- and Or11-specific antisera reacted with antigens of all 4 serotypes, although the reactivity of Or11 was not strong (Fig. 3). The Or02-, Or04-, and Or77-specific antisera reacted with antigens of serotypes A, B, and G, however, no reactivity could be observed against antigens of serotype M. Furthermore, as found for serotype G, no reactivity could be observed against proteins of any of the 3 other serotypes for the Or03-, Or98A, or Or98B-specific antisera.

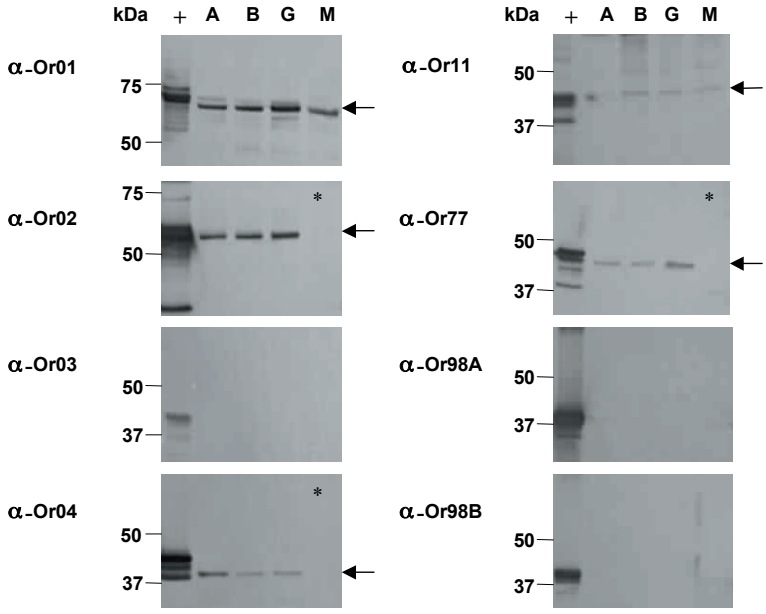

Figure 3. Western blot demonstrating the reactivity of sera from vaccinated birds against the corresponding recombinant vaccine proteins (+) and wild type *O. rhinotracheale* serotype A, B, G, and M antigens. Reactivity against *O. rhinotracheale* antigens is indicated with an arrow, serotype-specific lack of reactivity is indicated with *.

Gene distribution and genetic diversity.

The observed differences in antigen reactivity among proteins of the 4 tested serotypes may originate from the presence or absence of the respective genes, a variable expression, and/or antigenic diversity among the serotypes. To verify the presence of the different genes and to compare the genetic code of the genes encoding the 8 *O. rhinotracheale* antigens, chromosomal DNA was isolated from *O. rhinotracheale* serotype A, B, and M strains and used as a template to amplify the open reading frames using specific primers (Table 1) designed on the basis of the *O. rhinotracheale* serotype G sequences.

PCR products of the 8 different genes were obtained for all serotypes confirming the presence of the genes in these serotypes. Purified PCR products were further used for sequencing using external and internal primers and then compared to the *O. rhinotracheale* serotype G gene sequences. All genes showed to be highly conserved among these 4 serotypes (Table 2) with identities varying from 92% or 99% to 100% at the nucleotide level and 94% or 99% to 100% at the amino acid level. Observed amino acid substitutions were scattered over the different regions of the proteins of the different serotypes (data not shown).

Table 1. Oligonucleotide primer sets for the amplification of full length open reading frames

Gene	ORF (bps)	5'oligonucleotide	3'oligonucleotide	PCR-product (bps)
0r01	1614	5'-GCGACTGAAGAAGAGTTGAA-3'	5'-CTCCGATTAGTACTGCAACA-3'	1978
0r02	1572	5'-CGAGAGATTTCGTGTAGATG-3'	5'-CAAGAAGCCCGTGAAATGAT-3'	1936
0r03	1242	5'-TAGAGCAACCAACTTAGATA-3'	5'-CATCAACTCAAACTGTGATA-3'	1388
0r04	1023	5'-ATATCCATGAAAGATATATT-3'	5'-TGTGTAAAAACAAAACTATG-3'	1140
0r11	1230	5'-CCCACGGAGTTACACTCAAG-3'	5'-GCGATATAAGGCTCCGTCTG-3'	1600
0r77	1140	5'-TGTTTCCCGGTAGTAAATTG-3'	5'-GATGAGCTTATTCGCTTTGA-3'	1538
0r98A	918	5'-TAGACATTGAAGCTGAAAGT-3'	5'-ATTTCCTATGGCTGTTAAGT-3'	1122
0r98B	888	5'-AAGGTAAGATGTATTGTATC-3'	5'-AATTCACCATAAGTGATGAT-3'	1195

Table 2. Genetic diversity of *O. rhinotracheale* genes

		% sequence identity				% sequence identity	
gene	**serotype**	**nucleotide**	**amino acid**	**gene**	**serotype**	**nucleotide**	**amino acid**
Or01	A	99	100	**Or11**	A	99	99
	B	100	100		B	99	99
	M	92	94		M	99	99
Or02	A	99	99	**Or77**	A	99	100
	B	100	100		B	100	100
	M	99	100		M	99	100
Or03	A	100	100	**Or98A**	A	ND	ND
	B	100	100		B	99	99
	M	100	100		M	100	100
Or04	A	99	100	**Or98B**	A	ND	ND
	B	100	100		B	100	100
	M	100	100		M	100	100

ND: not determined yet
O. rhinotracheale serotype G was used as a reference strain

Localization of the *O. rhinotracheale* antigens

The apparent lack of protection of some of the antigen-specific antisera may have been caused by a poor accessibility of antigen. To determine the location of the candidate vaccine antigens, subcellular fractions of *O. rhinotracheale* serotype G were prepared by differential centrifugation partitioning, and integral membrane lipoproteins were isolated using Triton X-114 extraction. Western blot analysis using the antigen-specific antisera was performed to detect the presence of antigens Or01, Or02, Or04, Or11 and Or77 within the different fractions. Subcellular localization of the antigens Or03, Or98A, and Or98B was not performed since these antigens could not be detected on Western blot (see above). The results are summarized in Table 3.

All antigens could be detected in more than one subcellular fraction. However, variable intensity against the different fractions on Western blot made it possible to indicate the major location of the different antigens within the *O. rhinotracheale* cell. Protein Or01 was predominantly present in the cytoplasmic-inner membrane fraction, although a positive reaction was also observed for the outer membrane fraction. Strongest reactivity to proteins Or02, Or04, Or11 and Or77 was observed in the outer membrane and/or lipoprotein fraction. Proteins Or02 and Or77 appeared also present in the cytoplasmic-inner membrane fraction. For all 5 antigens, minor to no reactivity was observed for both the supernatant and the periplasmic fraction.

Table 3. Subcellular localization *O. rhinotracheale* antigens

	Pellet	supernatant	Periplasm[1]	Cytoplasm/inner membrane[1]	Outer membrane[1]	Lipoprotein[2]
Or01	++	+/-	+/-	++	+	-
Or02	++	+/-	+/-	+	++	++
Or03	ND	ND	ND	ND	ND	ND
Or04	+	-	-	+/-	+	+
Or11	+	-	-	+/-	+	+
Or77	++	+/-	-	+	+	++
Or98A	ND	ND	ND	ND	ND	ND
Or98B	ND	ND	ND	ND	ND	ND

[1] fraction obtained by differential centrifugation partitioning
[2] fraction obtained by Triton X-114 detergent-phase partitioning
Reactivity of antigens on Western blot is indicated by -, +/-, +, ++
- : no reactivity; +/-: minor reaction + : positive reaction; ++: strongly positive reaction; ND : not determined

Subunit vaccination with combined antigens and cross-protection

Of all 8 individual proteins, only vaccination with the subunit vaccine composed of Or77 induced a cross-protective immune response against *O. rhinotracheale* serotype A challenge. To analyze whether a combination of several proteins would be able to induce protection, 4 new subunit vaccines were designed (Fig. 4, vaccine I to IV, each containing approximately equal concentrations of 4 different antigens (25 μg per antigen per dose). The cross-protective capacity of these combined vaccines was tested in a similar animal experiment as described above. Again the subunit vaccine containing all 8 proteins (Fig. 4, vaccine V) was included as positive control.

	Recombinant proteins							
Vaccine	**Or01**	**Or02**	**Or03**	**Or04**	**Or11**	**Or77**	**Or98A**	**Or98B**
I	+	+	+	+				
II			+	+	+	+		
III					+	+	+	+
IV	+	+					+	+
V	+	+	+	+	+	+	+	+

Figure 4. Composition of combined subunit vaccines. Recombinant proteins contained within the vaccines are indicated with +. Note that the total antigenic load of vaccine I, II, III, and IV is approximately 200 μg/ml. The total antigenic load of positive control vaccine V is approximately 400 μg/ml.

As summarized in Figure 5, a reduction in respiratory pathology was observed in all vaccinated birds. Significant cross-protection ($p < 0.05$) was observed in birds that received vaccine I, II, III or V. Respiratory pathology decreased from 35.3% in the unvaccinated control group to 12.2% after administration of vaccine I ($p = 0.03$); 8.0% after administration of vaccine II ($p = 0.003$), 15.0% after administration of vaccine III ($p = 0.03$), and 3.9% after

administration of vaccine V (p = 0.0004). Pathology observed after administration of vaccine IV (23.5%) was not significantly different (p = 0.19) in comparison to the challenge control group.

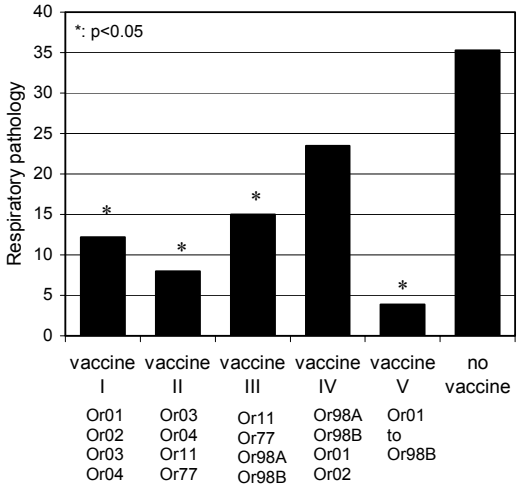

Figure 5. Subunit vaccination with different protein combinations and cross-protection. Lesions in air sacs and lungs were macroscopically scored one week after *O. rhinotracheale* challenge. Values are represented as the percentage of the maximum possible respiratory score. Note that the values of the groups that were vaccinated with vaccines I, II, III, and V were significantly different compared to the unvaccinated challenge control group (p<0.05).

DISCUSSION

A major challenge in the development of a vaccine against bacterial infections is the existence of different immunotypes within a pathogen species. For *O. rhinotracheale*, a bacterin vaccine has been developed which has proven to induce homologous protection against infection with the specific serotype contained within the vaccine (179, 197). However, 18 different serotypes have been identified for this pathogen and therefore an immunization strategy is needed that protects birds from infections with heterologous serotypes (36, 196, 199). In a previous study, live vaccination with *O. rhinotracheale* serotype B, G, or M has demonstrated to induce cross-protective immunity against a challenge with serotype A (chapter 3). Sera from these cross-protected birds were used for immunoscreening of an *O. rhinotracheale* expression library which resulted in the selection of 8 different antigens. Immunization of chickens with a multicomponent subunit vaccine containing these 8 different recombinant proteins induced the production of antibodies against 6 out of 8 vaccine proteins. Moreover, vaccinated birds showed a high level of protection against infection with a different *O. rhinotracheale* serotype strain. However, which specific antigen(s) was (were) responsible for the observed cross-protection remained unclear. In this study we analyzed the ability of the 8 different antigens to induce a cross-protective immunity, individually or in combination with others.

Initial bio-informatical analysis of these 8 different antigens (chapter 3) revealed that proteins Or01, Or11, and Or98B were the only antigens for which a putative function could be predicted based on significant similarities with published protein sequences. Protein Or01 showed similarity to dihydrolipoamide acetyltransferase (E2p), a membrane-associated component of the pyruvate dehydrogenase (PDH) complex (48). This E2p component is usually very immunogenic and therefore repeatedly identified by serological screenings (6, 89, 103). However, the use of E2p as a vaccine antigen is questionable, since this component is highly conserved across prokaryotic and eukaryotic species and it is not known whether antibodies raised against prokaryotic E2p react against conserved parts shared with their host. If so, this will pose the risk of breaking immune tolerance and causing autoimmune disease (70).

The Or11 protein showed similarity to FadL, an outer membrane protein associated with long-chain fatty acid transport, an important process in cell maintenance (20). High concentrations of long-chain fatty acids are found in the extracellular inflammatory milieu and transport, activation and degradation of these fatty acids has been predicted to suppress the local inflammatory response, providing the pathogen an important advantage during the early stages of colonization (20). Furthermore, Or11 contains an OmpA-like transmembrane structure. OmpA is the major outer membrane protein expressed by Gram-negative bacteria (110) and one of the functions attributed to OmpA include maintenance of structural cell wall integrity by stabilizing the outer membrane (181, 212). For many pathogenic species OmpA has shown strong immunogenicity and was proposed as a candidate in the design of anti-infectious vaccines (105), although the possibility of antigenic variation of OmpA and other surface exposed antigens should be considered (23, 43).

The Or98B protein showed similarity to RecT, a protein involved in homologous DNA recombination, a fundamental process in the biochemistry of DNA repair and replication (143). DNA repair mechanisms are also very important for the survival of pathogens when exposed to oxidative stress (180). It was demonstrated that RecT shares fundamental properties with the well-studied RecA (142, 143), a protein with a similar function, and to which much attention has been directed for the development of live vaccine strains (163, 164).

Although no significant similarities with published sequences could be predicted for proteins Or02 and Or77, conserved domain search revealed that these proteins contain the characteristics of a prokaryotic lipoprotein (chapter 3). When surface exposed, lipoproteins are recognized by the immune system (32) and they can have a variety of functions involved in pathogenesis, such as adherence and immune avoidance (i.e. adaptive variation of antigenic membrane) (25). Lipoproteins are known to be potent activators of the humoral and / or cellular immune system and they can function as a natural adjuvant (19). Unfortunately, for proteins Or03, Or04, and Or98A no possible functions could be attributed based on sequence analysis.

In this study the antigenicity of the individual proteins was investigated in an animal experiment whereby chickens were injected with one single recombinant protein. On Western blot it was shown that each individual protein successfully induced the production of antigen-specific antibodies, since all vaccine proteins could be detected using antiserum obtained from immunized birds. Previously, no reactivity was observed against recombinant proteins Or11 and Or98A using serum obtained from birds vaccinated with the 8-component

vaccine, even though this vaccine contained equal concentrations of specific antigen per dose (chapter 3). Possibly, these proteins are immune-recessive when administered along with other antigens.

Significant cross-protection was only observed after vaccination with the Or77 vaccine, even though all recombinant proteins were capable of inducing the humoral immune system to produce antigen-specific antibodies. The pathology score of these Or77-vaccinated birds was comparable to the pathology score of the birds that received the 8-component vaccine. The most important factors that can be responsible for the lack of protection seen for the other antigens are: i) the absence of the gene or its encoded immunogen, in particular on the surface of the pathogen, and ii) the inability of the recombinant antigens to elicit antibodies that recognize the native antigen as expressed by the pathogen *in vivo*.

The presence of the genes encoding the different antigens on the genome of *O. rhinotracheale* serotypes A, B, G, and M was confirmed by PCR amplification. Western blot analysis was performed to determine expression of the native *O. rhinotracheale* serotype A, B, G, and M antigens *in vitro*. Using antigen-specific antisera, proteins Or01 and Or11 could be detected for all 4 serotypes whereas protein Or02, Or04, and Or77 were only found in serotype A, B, and G, but not in serotype M. These observed differences in reactivity against antigens Or02, Or04, and Or77 could be the result of antigenic variation or variable expression between the 4 serotypes. Sequence analysis revealed that the *O. rhinotracheale* serotype A, B, G, and M genes encoding these antigens were highly conserved with an amino acid sequence identity of 99 to 100%. Since antigenic variation can be neglected, antigens Or02, Or04, and Or77 are most likely not expressed by this serotype M strain and therefore not present in the protein lysate of *in vitro* grown *O. rhinotracheale* that was used for analysis. Variable expression can also be an explanation for lack of reactivity against proteins Or03, Or98A, and Or98B for each of the 4 serotypes. At this time, it is not possible to mimic *in vivo* growth conditions for *O. rhinotracheale* in order to study *in vivo* expression of these antigens. However, different other techniques for example IVIAT (*in vivo* induced antigen technology) (80), STM (signature-tagged mutagenesis) (90) and DFI (differential fluorescence induction) (167) have demonstrated to be very useful for analysis of *in vivo* antigen expression (87).

All antigens were identified by the use of convalescent antisera, however, they do not necessarily have to be surface-exposed or secreted by *O. rhinotracheale* during infection. They can also be intracellular or periplasmic proteins usually inaccessible for antibodies, unless they are released into the extracellular environment by cell lysis which makes them available for the immune system. Therefore, insight in the localization of these antigens may partly explain protection levels. Two procedures were used to study the presence of 5 out of 8 antigens in the *O. rhinotracheale* outer membrane: sarcosine-insoluble outer membrane fractionation and Triton X-114 lipoprotein extraction. By using antigen-specific antiserum and Western blotting it was found that proteins Or02, Or04, Or11, and Or77 were predominantly present in the outer membrane and/or lipoprotein fractions whereas Or01, although detected in the outer membrane fraction, was most dominant in the cytoplasmic-inner membrane fraction. These results are consistent with the previous prediction of Or11 being an outer membrane protein (chapter 3), Or02 and Or77 being lipoproteins (chapter 3), and previous publications where the Or01 homolog, the E2p component of the pyruvate dehydrogenase complex, was described to be associated to the plasma membrane (6, 89).

Interestingly another E2p homolog, the *Mycoplasma gallisepticum* membrane protein P52, was also described to be membrane-associated and its immunogenicity was attributed to exposure of hydrophilic domains containing epitopes protruding outside of the membrane to the extracellular environment (103).

Although the recombinant antigens induced a specific antibody response, in order to be protective these antibodies should also recognize the wild-type antigens expressed by the pathogen during infection. Therefore the antigenic and protective epitopes of both the recombinant and native proteins should have the same conformation. In this respect, it is remarkable that protective antigen Or77 was the only recombinant antigen which needed no additional purification steps due to secretion into the supernatant of the growth medium of the *E. coli* expression strain. The other non-protective antigens had to be purified from inclusion bodies by freeze-thawing and repeated sonication cycli or by using denaturating conditions followed by dialysis (chapter 3). Furthermore, the biological activity of the native *O. rhinotracheale* proteins may depend on specific post-translational modifications e.g. glycosylation, phosphorylation, and lipidation. When the recombinant antigens are not modified these proteins might be inactive which explains the lack of protection observed after vaccination. Future studies, e.g. immune-fluorescence or whole cell ELISA, will have to demonstrate the ability of the recombinant antigen-specific antibodies to bind the native, *in vivo* expressed and surface-exposed native *O. rhinotracheale* antigens.

Previously, Hanson *et al.* (83) demonstrated vaccine synergy between different *Borrelia burgdorferi* antigens. The authors showed that vaccination of mice with a combination of 2 different antigens resulted in a 100-fold increased protection against different (heterologous) Borrelia isolates in comparison to single-antigen vaccination. In this study possible immunological synergism between different *O. rhinotracheale* antigens was analyzed by testing the efficacy of 5 different subunit vaccines each containing a different antigen composition. After a heterologous *O. rhinotracheale* serotype A challenge, only the multicomponent vaccine containing all 8 antigens induced almost complete protection (3.9% pathology) in comparison to the unvaccinated control birds (35.3% pathology). As expected, the 2 vaccines that contained antigen Or77 showed a significant reduction in respiratory pathology (to 8.0% and 15.0%). Surprisingly, the 2 vaccines that did not contain Or77 also showed reduced pathology (12.2%, and 23.5%). These results indicate that Or77 is the only selected protein that can induce cross-protective immunity by itself, whereas the other proteins can induce protection in combination. The lack of protection by a single protein could be explained by a too low antibody-titre. By combining several proteins in one single vaccine, it may be possible to raise the total antibody-titre above a certain threshold, needed for protection. Moreover, the protective capacity of the multi-component vaccines can be explained by the presence of (a) antigenic but non-immunogenic antigen(s) with immunestimulatory properties. Additional animal studies have to be performed in order to analyze the contribution of the relative antibody titres against the different antigens in protection, and to analyze which antigenic combination is the most effective.

Based on the results presented in this paper it can be concluded that Or77 is the most suitable candidate for the production and development of a cross-protective vaccine. However, immune-reactivity, conservation of this antigen over the other *O. rhinotracheale* serotypes, and the ability to induce cross-protective immunity against these serotypes have

to be confirmed. Although the other 7 antigens did not generate protective immunity by themselves, they are still interesting candidates for a multicomponent vaccine and should therefore not be neglected.

ACKNOWLEDGEMENTS

We like to thank Hon Tran (Intervet Schwabenheim) for bio-informatical assistance, and the Animal Service Department and the Pathology Department (Intervet Boxmeer) for technical assistance during animal studies.

CHAPTER FIVE

Vaccine Potential of the *Ornithobacterium rhinotracheale* Or77 Lipoprotein

D.F. Schuijffel[1], M.A.N. Rits[1], P.C.M. Van Empel[1], R.P.A.M. Segers[1], J.P.M. Van Putten[2], and P.J.M. Nuijten[1]

[1] *Intervet International BV, Bacteriology R&D, Boxmeer, The Netherlands,* [2] *Utrecht University, Faculty of Veterinary Medicine, Department of Infectious Diseases and Immunology, Utrecht, The Netherlands.*

Manuscript in preparation

ABSTRACT

Analysis of the avian immune response against infection with *Ornithobacterium rhinotracheale* has shown that antibodies are key components in protection. Previously, antisera from immune birds were used to identify a protective antigen, Or77. In this study we further analyzed the vaccine potential of this highly conserved *O. rhinotracheale* lipoprotein. Subunit vaccination of chickens with recombinant Or77 induced antibodies that cross-protected against a challenge with different *O. rhinotracheale* serotypes that expressed Or77 *in vitro*. No protection was observed in birds challenged with a serotype that did not express Or77. The Or77 vaccine also showed to be more effective in the ability to induce cross-protective immunity than vaccination with an *O. rhinotracheale* bacterin formulation or the live, wild type strain. Preliminary studies indicated that lipidation of recombinant Or77 can increase the immunogenicity of the antigen. In conclusion, the results of this study demonstrate that Or77 is an excellent candidate antigen for a cross-protective poultry vaccine against *O. rhinotracheale* infection.

INTRODUCTION

Ornithobacterium rhinotracheale is a gram-negative bacterium associated with contagious respiratory disease in poultry (36, 73, 196). This pathogen, represented by 18 identified serotypes (36, 199), has been isolated from poultry and wild birds all over the world. Infection of the respiratory tract caused by *O. rhinotracheale* is characterized by pneumonia, tracheitis, and airsacculitis (92, 178, 198), but the bacterium can also disseminate to other sites of the body resulting in local pathology such as hepatitis, meningitis and joint-infections (36, 178, 183, 196). Because of the economical losses due to decreased growth, increased mortality, increased condemnation rates, drops in egg production and decreased hatchability, *O. rhinotracheale* infections are increasingly recognized as a health problem in the poultry industry (203). The best strategy to prevent or control *O. rhinotracheale* infection is vaccination, since most *O. rhinotracheale* isolates have acquired resistance against the regularly used antibiotics (53, 130, 176, 204) and the general policy is to ban antibiotics from the food chain as much as possible (3).

In the past, vaccines based on inactivated bacterin formulations have been developed and shown to induce protective immunity in both chickens and turkeys (179, 197). The major problem with these vaccines is that they do not provide broad cross-protection against the various serotypes of *O. rhinotracheale*. Vaccination with live *O. rhinotracheale* also appeared to induce protection against challenge of broilers but only when maternal antibody levels were low (197). In turkeys, a live, temperature-sensitive mutant strain has been reported to evoke a protective response against *O. rhinotracheale* infection (124, 125). However, the development of cross-protective immunity using a live vaccine strain has not been reported.

Recently, we demonstrated that antibodies contribute for a major part to protection against *O. rhinotracheale* infection (chapter 2), and that live vaccination with *O. rhinotracheale* serotypes B, G, or M induced cross-protective immunity against *O. rhinotracheale* serotype A challenge (chapter 3). Antisera from these cross-protected birds were used to identify 8 cross-reactive *O. rhinotracheale* serotype G antigens, and vaccination of broilers with different subunit vaccines, containing mixtures of the purified recombinant proteins, protected the birds against challenge with a heterologous serotype (chapter 3). Antigen Or77, a lipoprotein of unknown function, was identified as the most immunogenic vaccine candidate of these 8 antigens (chapter 4). However, the ability of the Or77 subunit vaccine to protect against infection with heterologous *O. rhinotracheale* serotypes other than serotype A was not examined, neither was the efficacy of the Or77 subunit vaccine compared to live or bacterin vaccination.

Bacterial lipoproteins are a diverse group of structurally and functionally distinct proteins (25). Some lipoproteins are known to be potent activators of the immune system and they can function as a natural adjuvant (59). The lipid modification of these lipoproteins has shown to be essential to this activity (58, 211). In previous studies (chapters 3 and 4) we used a non-lipidated version of Or77, expressed by an *Escherichia coli* expression strain with an *E. coli* secretion signal. Secreted, non-lipidated Or77 was purified from the supernatant of the culture medium and used for immunization, which resulted in significant protection levels. However, lipidation of recombinant Or77 might

evoke a stronger response of the immune system resulting in increased level of protection as compared to the secreted, non-lipidated form.

The objectives of this study were: i) to analyze whether the Or77 subunit vaccine can induce (cross-) protective immunity against multiple *O. rhinotracheale* serotypes, ii) to study expression and genetic diversity of the Or77 antigen for different *O. rhinotracheale* serotypes, iii) to compare the cross-protective efficacy of Or77 subunit vaccination with bacterin vaccination and live vaccination, and iv) to compare the efficacy of recombinant lipidated Or77 with secreted non-lipidated Or77.

MATERIALS AND METHODS

Bacterial strains and growth conditions

O. rhinotracheale serotype A strain B3263/91, *O. rhinotracheale* serotype B strain GGD 1261, *O. rhinotracheale* serotype G strain O-95029 nr.16279, and *O. rhinotracheale* serotype M strain TOP 98036 4500 were grown on 5% sheep blood agar at 37°C, in a 5% CO_2 atmosphere for 48 hours. For liquid culture, single colonies were inoculated in Todd Hewitt (TH) medium (Difco, Detroit, MI, USA) and grown for 24 hours at 37°C on a 100 rpm shaker. *Escherichia coli* strains TOP 10 (Invitrogen, Carlsbad, CA, USA) and BL21(DE3) codonRILpLysS (Novagen, Madison, WI, USA) were used for cloning and protein expression, respectively. Both strains were grown in Terrific Broth (TB) for protein expression supplemented with 10 mM $MgSO_4$. All *E. coli* liquid cultures were grown for 16-20 hours at 37°C on a 200 rpm shaker.

Chickens

Specified-pathogen-free (SPF) broiler chickens (Intervet, Boxmeer, The Netherlands) were used. All animals were placed at day of hatch in negative pressure isolators of approximately 1.5 m^3 with a maximum of 11 birds per isolator in a 7-week study. The animals received sterilized food (Hendrix, Boxmeer, The Netherlands) and water *ad libitum*. In each experiment, the birds of different test groups were housed in a mixed population where possible, to diminish isolator effects. All animal studies were approved by the ethical committee for animal experiments in The Netherlands (DEC) according to international regulations.

Polymerase chain reaction and sequencing

Oligonucleotide primers used for both polymerase chain reaction (PCR) amplification and sequencing of the complete open reading frames encoding the Or77 cross-protective antigen were designed using Or77 *O. rhinotracheale* serotype G sequence (EMBL accession number AJ748737) as a template (chapter 3). Primers used for Or77 amplification and sequencing were: forward 5'-TGTTTCCCGGTAGTAAAATTG-3' and reverse 5'-

GATGAGCTTATTCGCTTTGA-3' (Invitrogen, Carlsbad, CA, USA). PCR was performed in an automated thermal cycler (GeneAmp 9700, Perkin Elmer, CA, USA). The final PCR reaction volume was 50 μl containing 200 ng of genomic DNA isolated from liquid culture cells according to the method as described by Sambrook *et al.* (162), 50 μM of dNTP's (Promega, WI, USA), 10 pmol of each primer, 20 U/ml of Supertaq plus polymerase and 10X of Supertaq buffer (Both HT Biotechnology Ltd, Cambridge, UK) in water. The following conditions were used: denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 2 min, and elongation at 68°C for 2 min 30 sec followed by a final extension at 68°C for 10 min.

To determine the nucleotide sequence of the complete ORFs, sequence reactions were performed (94°C 10 sec; 50°C 5 sec; 60°C 2 min for 25 cycles in an automated thermal cycler as described) using Big Dye Terminator Ready reaction mix, 50 ng purified PCR product and 2.4 pmol primer in a 20 μl reaction volume. Sequencing was done on an ABI 310 automated sequencer (Perkin Elmer, CA, USA). Data were collected using ABI 310 Collection Software version 1.0.4 and analysed with Sequence Analysis version 3.1 (Perkin Elmer, CA, USA). Contigs and alignments were made using Sequencer version 4.1.4 (Gene Codes Corporation, USA).

Cloning, expression, and purification of lipidated Or77

Primers used for amplification followed by directional cloning of the Or77 gene were designed based on the same *O. rhinotracheale* serotype G sequence as described above: forward primer 5'-TGGCGCATATGAGCAGTGATGATTACCATC-3' and reverse primer 5'-GCGGCCGCTTAGTT AATTGAAACTCTTAAGC-3'. PCR amplified Or77 gene product was cloned with the TOPO-TA cloning system (Invitrogen, Carlsbad, CA, USA) and digested with NdeI and NotI restriction enzymes (New England Biolabs, Beverly, MA, USA) for directional cloning in the expression vector of interest. Ligation products were initially transformed into *E. coli* TOP 10 competent cells (Invitrogen, Carlsbad, CA, USA) and were subsequently transformed to *E. coli* BL21(DE3) codonRILpLysS host cells (Novagen, Madison, WI, USA) for expression. The Or77 gene was cloned into pET plasmid vector pETlip3 (private collection) and therefore the recombinant proteins were expressed with a *Borrelia burgdorferi* OspA leader peptide fused at the amino terminal portion (the *O. rhinotracheale* leader peptide sequence was replaced). High level expression of Or77 by *E. coli* strain BL21(DE3) codonRILpLysS was done as described in the pET system user manual. Recombinant lipidated Or77 (Or77-lip) was extracted from the outer membrane of the *E. coli* expression cells by Triton X-114 essentially as described by Bordier (24) and Pryde (151). Briefly, a liquid 16 h culture of *E. coli* strain BL21(DE3) codonRILpLysS containing the pETlip3-Or77 expression construct was pelleted and cells were resuspended 0.04 M PBS-0, pH 7.2 of 4°C. Triton X-114 (Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 0.3% (w/v). Samples were first incubated overnight at 4°C (with gently shaking) followed by a 1 hour incubation step at 37°C. The solution was centrifuged (10,000 x g for 10 min) to produce an upper aqueous phase (containing the hydrophilic proteins) and a lower detergent phase (containing the integral membrane proteins). The detergent-phase containing the Or77-lip protein was collected. Triton X-114 was removed

from the solution by adsorption to Biobeads (Biorad, Hercules, CA, USA) according to the manufacturer's directions. The quality of the final protein Or77-lip solution was assessed using PAGE.

Polyacrylamide gel electrophoresis (PAGE) and Western blot analysis

NOVEX NuPAGE was used for protein electrophoresis under denaturating conditions in 4-12% polyacrylamide gels according to manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Protein bands were made visible by using Coomassie Brilliant Blue (CBB). Polypeptides were electro-blotted onto Immobilon PVDF 0.45 µm membrane (Millipore, Bedfort, MA, USA) by semi-dry Western blotting according to Towbin *et al*. (187). Membranes were blocked using 0.04 M PBS-0 0.5% polysorbate-20 containing 1% skim milk, washed with 0.04 M PBS-0 with 0.5% polysorbate-20, and incubated with a 1:250 dilution of primary antiserum in 0.04 M PBS-0 containing 0.05% polysorbate-20 and 1% skim milk. For use in Western blot, sera from all birds within a treatment group (approximately 10) were pooled. Rabbit anti-chicken IgG peroxidase conjugated (Nordic, Tilburg, The Netherlands) in a 1:1000 dilution (in 0.04 M PBS-0; 0.05% polysorbate-20; 1% skim milk) was used as secondary antibody. Finally, filters were washed and the substrate solution Vector SG (Vector, Burlingame, CA, USA) was added.

Vaccine preparation

The secreted Or77 (Or77-sec) vaccine antigen was isolated from culture supernatants of the *E. coli* strain that produced the protein (chapter 3). The lipidated Or77 (Or77-lip) vaccine antigen was expressed by the same *E. coli* strain and extracted from the cell membrane as described above. The Or77-sec and Or77-lip subunit vaccines were prepared by blending the recombinant antigens in a water-in-oil emulsion to a final concentration of approximately 10 or 50 µg antigen per ml. The amount of antigen for each vaccine was determined by PAGE and densitometric analysis using GeneSnap software version 6.01 (SynGene, Cambridge, England).

The *O. rhinotracheale* serotype G strain O-95029 nr.16279 bacterin vaccine was prepared by inactivation of a bacterial cell suspension in sterile 0.04 M PBS-0 by the addition of formalin to a final concentration of 0.5%. The killed bacterial suspension was formulated in a water-in-oil emulsion to a final concentration of approximately $2*10^7$ cells per ml. The *O. rhinotracheale* serotype G strain O-95029 nr.16279 live vaccine was composed of a fresh bacterial culture in TH medium (Difco, Detroit, MI, USA) containing approximately 10^8 colony forming units (CFU) per ml.

Vaccination studies

At 2 weeks of age, SPF-broilers were vaccinated by subcutaneous injection in the neck of 0.5 ml Or77-sec or Or77-lip subunit (approximately 5 or 25 µg / dose) or bacterin vaccine (10^7 CFU / dose), or by aerosol spray application of 100 ml live vaccine (10^8 CFU per ml) per isolator of approximately 1.5 m^3. During aerosol spraying the live vaccine was administered as a fine spray to the birds using a commercial paint sprayer. The developed mist in the isolators was maintained for at least 10 min with the air circulation closed. At 5 weeks of age birds were primed with Newcastle Disease (ND) strain LaSota by a course spray of approximately 10^6 egg infectious dose (E.I.D.) per bird. At 6 weeks of age, birds were challenged with *O. rhinotracheale* serotype A strain B3263/91, *O. rhinotracheale* serotype B strain GGD 1261, *O. rhinotracheale* serotype G strain O-95029 nr.16279, or *O. rhinotracheale* serotype M strain TOP 98036 4500. The challenge was done as described above, by aerosol spraying of 100 ml of a fresh bacterial culture containing approximately 10^9 colony forming units (CFU) per ml Todd Hewitt (TH) medium (Difco, Detroit, MI, USA). One week after challenge, at 7 weeks of age, birds were sacrificed and organ lesions were scored. Each treatment group contained 11 birds.

Post-mortem examination and parameters of infection

At the end of each animal experiment, post-mortem examination was performed on all animals. The birds were bled and organ lesions were macroscopically scored for typical respiratory pathology caused by *O. rhinotracheale* using the following scoring system: for thoracic air sacs, 0 - no abnormalities, 1 - one air sac seriously affected by fibrinous airsacculitis or both air sacs containing limited pin-head sized foci of fibrinous exudates, 2 - both air sacs seriously affected by fibrinous airsacculitis; for abdominal air sacs, 0 - no abnormalities, 1 - pin-head sized foci of fibrinous exudates or slight diffuse fibrinous airsacculitis, 2 - severe fibrinous airsacculitis (the airsacculitis score is given as the sum of both scores); for lungs, 0 - no abnormalities, 1 - unilateral pneumonia, 2 - bilateral pneumonia. The average group-scores are given as a percentage of the maximal possible respiratory score minus background pathology of the unchallenged ND priming control group. Statistical analysis was performed using the Kruskal-Wallis non-parametric one-way ANOVA test.

RESULTS

Antigenic analysis of Or77

To test the ability of recombinant Or77 to induce cross-reactive antibodies against the different *O. rhinotracheale* serotypes, 2-week old SPF-broiler chickens were subcutaneously injected with a subunit vaccine containing 25 µg per dose of recombinant Or77 formulated in a water-in-oil emulsion. Production of Or77-specific antibodies was first analyzed by

Western blot analysis with *in vitro* grown *O. rhinotracheale* serotypes A, B, G, and M, and the Or77 recombinant vaccine as antigens. This showed a specific band of approximately 42 kDa (Fig. 1) in serotypes A, B, and G, but not in the lane with serotype M proteins. Subsequently, the reactivity of Or77-specific serum with the other *O. rhinotracheale* serotypes A to Q was determined on Western blot: all *O. rhinotracheale* serotypes exhibited the Or77-specific band of 42 kDa, except serotypes F, K, and M. The results are summarized in Table 1. Serum from mock-treated birds showed no reactivity against Or77 (data not shown).

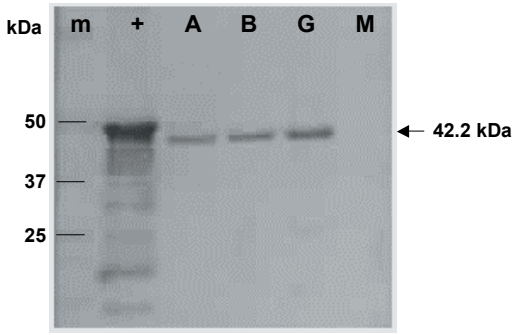

Figure 1. Western blot demonstrating the reactivity of pooled sera obtained from birds vaccinated with the Or77 subunit vaccine to the recombinant vaccine protein (+) and *in vitro* grown *O. rhinotracheale* serotypes A, B, G, and M. The 42.2 kDa wild-type Or77 is indicated by an arrow.

Genetic diversity of Or77 ORFs

In an attempt to explain the absence of reactivity of the serotypes F, K, and M with Or77-specific antiserum, the presence of an Or77-like ORF in the various serotypes was determined by PCR. For this purpose, chromosomal DNA was isolated and used as a template to amplify the open reading frame encoding Or77. All tested *O. rhinotracheale* serotypes showed an Or77-specific PCR product that was used for further sequencing. The previously determined *O. rhinotracheale* serotype G gene sequence (chapter 3) was used as a reference. The results are summarized in Table 1.

The Or77 ORF showed to be highly conserved among the different serotypes with similarities between 98 and 100 % at nucleotide level and 96 and 100 % at amino acid level. The Or77 sequences of the serotypes B, C, G, and H, and of the serotypes A, E, F, I, and M were identical. The serotypes D and L showed different point mutations that did not cause any amino acid substitutions. The serotypes J, K, N, O, and Q showed different amino acid substitutions which are indicated below Table 1.

No consistencies were found regarding reactivity against *in vitro* expressed Or77 and differences in amino acid sequences, suggesting that some serotypes indeed do not express the Or77 gene *in vitro*.

Table 1. Genetic diversity and *in vitro* expression 0r77 among different *O. rhinotracheale* serotypes

Serotype	Strain	Origin	Bird species	*In vitro* expression	% similarity nucleotide	% similarity amino acid
A	B3263/91	South Africa	Chicken	+	99	100
B	GGD 1261	Germany	Turkey	+	100	100
C	K91-201	USA	Chicken	+	100	100
D	ORV 94108 nr.2	France	Turkey	+	99	100
E	O-95029 nr.12229	France	Chicken	+	99	100
F	ORV 94084 K858	The Netherlands	Turkey	-	99	100
G	O-95029 nr.16279	France	Chicken	+	100	100
H	E-94063 4.2	The Netherlands	Turkey	+	100	100
I	BAC 96.0334 # minn 18	USA	Turkey	+	99	100
J	O-97091 HEN 81-2	The Netherlands	Chicken	+	98	98 [1)]
K	BAC 97.0321 # 101 small	USA	Chicken	-	99	99 [2)]
L	O-97071 B.U.T. 2237	UK	Turkey	+	99	100
M	TOP 98036 4500	France	Turkey	-	99	100
N	TOP 99023 LMG 13114	Belgium	Guinea Fowl	+	98	98 [3)]
O	TOP 99023 LMG 11553	Germany	Rook	+	98	96 [4)]
P	TOP 99090 may 71	UK	Turkey	+	ND	ND
Q	O-95256 sp 1507	The Netherlands	Chicken	+	99	98 [5)]
R	GGD steck	Germany	Turkey	ND	ND	ND

[1)] $Phe_{10} \rightarrow Leu$; $Thr_{67} \rightarrow Ala$; $Arg_{262} \rightarrow Lys$; $Phe_{332} \rightarrow Leu$; $Leu_{346} \rightarrow Ser$
[2)] $Phe_{10} \rightarrow Leu$; $Thr_{67} \rightarrow Ala$; $Arg_{262} \rightarrow Lys$
[3)] $Phe_{10} \rightarrow Leu$; $Ala_{25} \rightarrow Val$; $Thr_{26} \rightarrow Ala$; $Asp_{60} \rightarrow Asn$; $Arg_{262} \rightarrow Lys$; $Phe_{332} \rightarrow Leu$; $Leu_{346} \rightarrow Ser$
[4)] $Phe_{10} \rightarrow Leu$; $Thr_{26} \rightarrow Ala$; $Asp_{44} \rightarrow Glu$; $Asp_{60} \rightarrow Asn$; $Thr_{67} \rightarrow Ala$; $Ala_{114} \rightarrow Thr$
[5)] $Phe_{10} \rightarrow Leu$; $Ala_{25} \rightarrow Val$; $Thr_{26} \rightarrow Ala$; $Tyr_{84} \rightarrow Leu$
ND: Not determined yet

Or77 subunit vaccination: protection against homologous and heterologous serotypes

To test the (cross-) protective capacity of the induced Or77-specific antibodies against a challenge with the 4 different *O. rhinotracheale* serotypes, birds that were vaccinated at 2 weeks of age (see above) were primed with Newcastle Disease (ND) virus at 5 weeks of age and challenged with a homologous (G) or heterologous (A, B, M) *O. rhinotracheale* serotype at 6 weeks of age. Birds were infected via the natural route, i.e. aerosol application, causing mainly respiratory lesions in air sacs and lungs. Necropsy was performed at 7 weeks of age, one week after challenge. Organs were scored with respect to macroscopic pathology using the scoring system as described in materials and methods. Respiratory pathology is summarized in Figure 2.

Birds vaccinated with the Or77 subunit vaccine showed a significant reduction ($p = 0.03$) of respiratory pathology after challenge with the homologous serotype G strain (6.5%) in comparison to the birds of the unvaccinated challenge control group (26.7%). Of the vaccinated birds that received a challenge with the heterologous serotype strains, the groups that received *O. rhinotracheale* serotype A or B showed reduced pathology compared to the challenge control groups: 15.0% versus 45.0% for serotype A ($p = 0.003$) and 0.03% versus 8.4% for serotype B ($p = 0.32$) respectively. Vaccinated birds challenged with *O. rhinotracheale* serotype M showed no reduction in pathology (8.4% versus 6.7%), consistent with the observed lack of expression of Or77 by this serotype as determined by Western blotting.

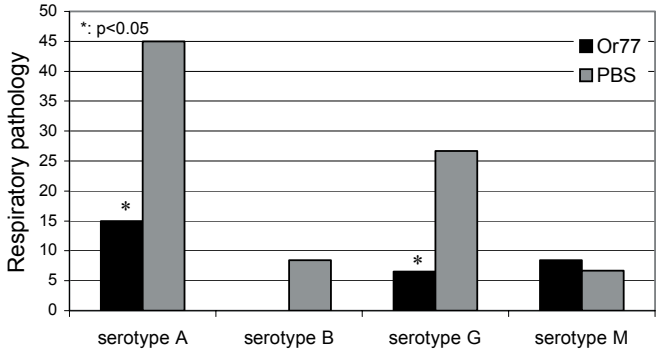

Figure 2. Or77 subunit vaccination and cross-protection. Respiratory pathology after aerosol challenge with *O. rhinotracheale* serotypes A, B, G, or M. Lesions in air sacs and lungs were macroscopically scored and presented as the percentage of the maximum possible respiratory score. Note that the values of vaccinated groups challenged with *O. rhinotracheale* serotype A or G were significantly different (*: $p<0.05$) to the values of unvaccinated serotype control groups.

Comparison of (cross-) protective immunity after Or77 subunit, bacterin and live strain vaccination

To compare the efficacy of Or77 subunit vaccination with other strategies, 3 vaccines were tested for their capacity to protect against homologous and heterologous serotype challenge: the Or77 subunit vaccine, a bacterin vaccine, and a live, wild type *O. rhinotracheale* strain. In this experiment, 2-week old SPF-broilers were vaccinated, either by subcutaneous injection of the Or77 subunit vaccine, by subcutaneous injection of an *O. rhinotracheale* serotype G bacterin vaccine, or by aerosol administration of an *O. rhinotracheale* serotype G live bacterial culture. At 5 weeks of age birds were primed with Newcastle Disease (ND) virus and birds were challenged with *O. rhinotracheale* at 6 weeks of age. Challenge was performed using serotype G, to study homologous protection, or serotype A, to study heterologous (cross-) protection. Again, birds were infected via the natural route by aerosol spray application and post-mortem analysis of air sacs and lungs was done at 7 weeks of age. The results are summarized in Figures 3 and 4.

After serotype G challenge (Fig. 3), the highest level of protection ($p = 0.002$) was observed in birds exposed to the live vaccine. This group lacked respiratory pathology (0.0%) in comparison to the challenge control group (26.7%). A reduction in respiratory pathology was observed in birds vaccinated with the Or77 subunit vaccine (6.5%, $p = 0.026$). The average lesion-score of the bacterin-G vaccinated birds (16.7%, $p = 0.195$) was not significantly different from that of the challenge control group.

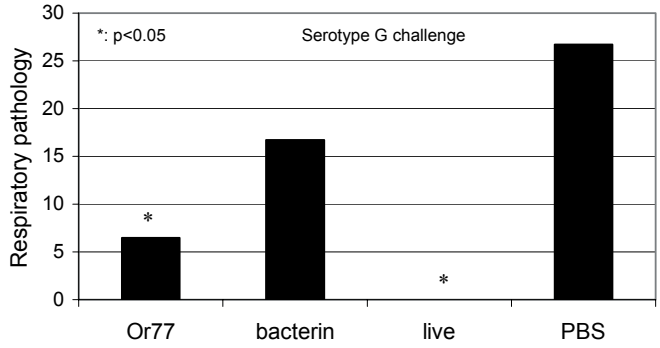

Figure 3. Vaccination and protection. Respiratory pathology after aerosol challenge with *O. rhinotracheale* serotype G. Lesions in air sacs and lungs were macroscopically scored and presented as the percentage of the maximum possible respiratory score. *: value significantly different ($p<0.05$) compared to mock-treated control group.

After serotype A challenge (Fig. 4), the only apparent reduction in respiratory pathology was observed in Or77 vaccinated birds (15.0%, p = 0.003), whereas bacterin-G vaccinated (36.7%) and live serotype G-vaccinated (41.7%) birds yielded pathology scores that were slightly reduced or comparable to the challenge control group (45.0%).

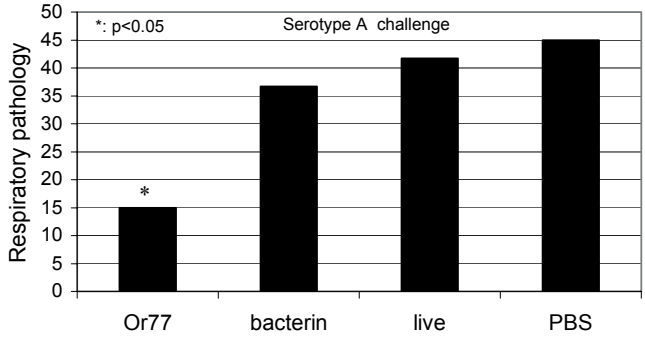

Figure 4. Vaccination and cross-protection. Respiratory pathology after aerosol challenge with *O. rhinotracheale* serotype A. Lesions in air sacs and lungs were macroscopically scored and presented as the percentage of the maximum possible respiratory score. *: value significantly different (p<0.05) compared to mock-treated control group.

Antigenic analysis of lipidated recombinant Or77

To analyze the effect of protein lipidation on the efficacy of the Or77 recombinant subunit vaccine, the Or77 protein was expressed in *E. coli* with an N-terminal lipoprotein leader peptide, and lipidated Or77 was extracted with Triton X-114 from the cell membrane of the expression strain. PAGE and CBB-staining demonstrated that the recombinant lipidated Or77 protein extract was (LIP) of rather pure quality as only the Or77 protein band was visible (Fig. 5A). The recombinant secreted Or77 protein preparation (SEC) contained additional protein material, especially in the 40-50 kDa range (Fig. 5A, indicated with {), possibly composed of Or77 break-down products or proteins of the *E. coli* expression strain. Both Or77-sec and Or77-lip proteins were used for vaccine formulation. Since the exact identity of the different bands of the Or77-sec protein solution was uncertain, the concentration of the Or77-lip protein was held equal to the concentration of the upper protein band of the Or77-sec protein solution (Fig. 5A, indicated with an asterix).

To test the antibody-inducing capacity of the Or77 vaccine proteins, 2-week old SPF-broiler chickens were subcutaneously injected with a subunit vaccine containing approximately 5 or 25 μg per dose of either secreted Or77 or lipidated Or77 formulated in a water-in-oil emulsion. Subsequent serum antibody levels directed against the vaccine antigens were tested 4 weeks later by Western blot analysis. The proteins in the water phase of the vaccines were run on PAGE, blotted, and incubated with serum from vaccinated or unvaccinated birds. Sera obtained from birds vaccinated with 25 μg per dose of lipidated or secreted Or77 showed reactivity against both vaccine antigens (Fig. 5B). Reactivity against the additional

Or77-sec proteins could also be observed. Similar reactivity was obtained with sera from birds vaccinated with 5 µg Or77 per dose (data not shown). No reactivity against Or77 could be observed using sera from mock-treated birds (Fig. 5B).

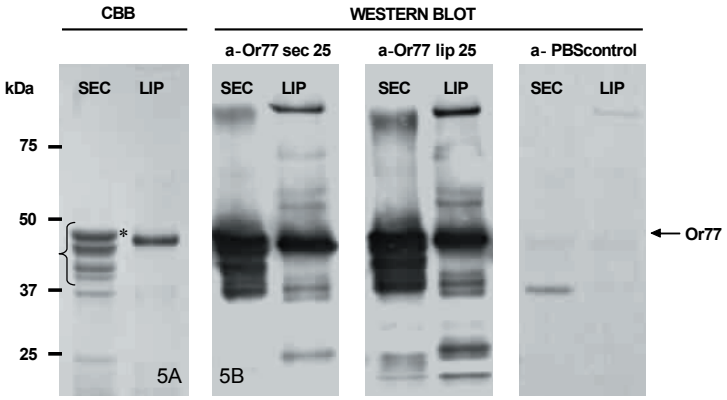

Figure 5. Analysis of purified recombinant secreted Or77 (SEC) and lipidated Or77 (LIP). 5A: CBB-staining demonstrating the purity of vaccine antigens, and 5B: Western blot demonstrating the reactivity of sera from vaccinated birds (25 µg SEC or LIP per dose) against the Or77 vaccine antigens. The molecular weight of Or77 is indicated with an arrow. *: The assumed Or77 protein in the Or77-sec protein solution of which vaccine formulation was based on. {: the 4 protein bands in the 40-50 kDa range.

Comparison of cross-protective immunity induced by secreted and lipidated Or77

To test the effect of lipidation of Or77 on the immunogenicity of this antigen, the birds that were vaccinated at 2 weeks of age (see above) were primed with Newcastle Disease (ND) Virus at 5 weeks of age and birds were challenged with an aerosol of *O. rhinotracheale* serotype A at 6 weeks of age. Post-mortem analysis of air sacs and lungs was done at 7 weeks of age. The results are summarized in Figure 6.

After challenge the highest level of protection was observed in birds vaccinated with lipidated Or77 using a 25 µg dose. This group showed a significant ($p = 0.01$) reduction of respiratory pathology (14.9%) in comparison to the challenge control group (44.9%). A reduction in pathology was also noted for birds vaccinated with the same lipidated Or77 antigen using a 5 µg dose (26.0%, $p = 0.04$) and for birds that received the vaccine containing 25 µg secreted Or77 per dose (21.2%, $p = 0.01$). No significant reduction of respiratory pathology was found for birds that received 5 µg of secreted Or77 (28.8%, $p = 0.10$). Furthermore, no significant differences in pathology score could be detected between the Or77-sec and Or77-lip vaccinated groups.

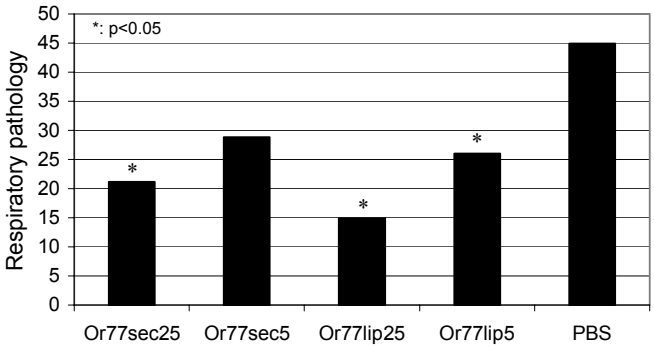

Figure 6. Subunit vaccination and cross-protection. Respiratory pathology after aerosol challenge with *0. rhinotracheale* serotype A. Lesions in air sacs and lungs were macroscopically scored and presented as the percentage of the maximum possible respiratory score. *: value significantly different (p<0.05) compared to mock-treated control group.

DISCUSSION

The development of an effective vaccine against an infectious disease will be facilitated through understanding of the immune response associated with protection after natural infection, the identification of highly conserved protective antigen(s), and antigen delivery studies. For *O. rhinotracheale*, the antibody-mediated immune response was demonstrated to be a key factor in protection against infection in chickens (chapter 2). Application of this knowledge led to the discovery of a novel lipoprotein, Or77, which was identified by screening an *O. rhinotracheale* genomic expression library using sera from protected birds (chapter 3). Vaccination of chickens with a recombinant Or77 antigen induced the production of antibodies that protected the birds against *O. rhinotracheale* challenge with a heterologous serotype (chapter 4). The efficacy of this vaccine in comparison to previously described vaccination strategies (197, chapter 3) however, remained to be determined.

In this paper we describe that i) vaccination of chickens with the Or77 subunit vaccine protects these birds against a challenge with different, but not all, *O. rhinotracheale* serotypes, ii) the Or77 antigen is not expressed by all *O. rhinotracheale* serotypes even though the Or77 gene is present and highly conserved in all tested serotypes, iii) the Or77 subunit vaccine induces significant cross-protective immunity against challenge with a heterologous *O. rhinotracheale* serotype in contrast to bacterin and live vaccination, and iv) the Or77-lip subunit vaccines induce a higher level of cross-protection in comparison to the Or77-sec vaccines.

For the development of a cross-protective vaccine, it is important that the elicited antibodies recognize the bacterial cells of different infectious serotypes. Our data indicate that the Or77 subunit vaccine induces antibodies that cross-react with different *O. rhinotracheale* serotypes and protect against challenge with *O. rhinotracheale* serotypes A, B, and G. No protection was obtained against challenge with serotype M. This serotype has previously been reported to lack Or77 expression, at least under *in vitro* growth conditions (chapter 4). Since the Or77 amino acid sequence, and therefore antigenic epitopes, were 100% identical for all four serotypes, the most logical explanation for the lack of protection against serotype

M challenge is the absence of Or77 expression not only *in vitro* but also *in vivo*. The factors that regulate of Or77 transcription, such as promoter sequences, are still unknown.

A lack of Or77 expression, despite the presence of an intact gene, was also noted for *O. rhinotracheale* serotypes F and K. Whether these serotypes also lack expression of Or77 during *in vivo* growth, is unknown. The existence of Or77 negative serotypes implies that Or77 may not protect against all serotypes, limiting its potential as a candidate vaccine antigen. Here it should be noted that serotypes F, K, and M are only occasionally isolated from chicken and turkeys and together represent less than 3% of a collection of more than 2100 serotyped strains isolated from birds all over the world (unpublished data). This suggests that serotypes F, K and M may be less virulent, making them less important to be covered by a vaccine. In general, the most frequently isolated strains are of serotype A in chickens, and of serotypes A, B, D and E in turkeys (36, 196) and these serotypes were demonstrated to express Or77.

The efficacy of the Or77 subunit vaccine to protect against challenge with a homologous (G) and heterologous (A) *O. rhinotracheale* serotype was compared to vaccination with a live vaccine and vaccination with an inactivated bacterin formulation, both prepared from *O. rhinotracheale* serotype G cultures. Since in a previous study only cross-protection was analyzed (chapter 4), this is the first time that homologous protection was demonstrated using the Or77 subunit vaccine. Birds that were vaccinated with live bacteria by means of aerosol spray showed the highest level of protection against an aerosol challenge with the same strain. Birds that received the bacterin vaccine showed reduced pathology but the difference was not statistically significant. Although not previously tested, a serotype G bacterin was expected to induce significant homologous protection in line with the results of a serotype A bacterin formulation that was shown to be a potent stimulator of the immune response (36, 196, 197).

With respect to serotype A challenge, only the Or77 subunit vaccine was able to induce cross-protective immunity. In this experiment, live vaccination did not induce cross-protection. This was remarkable since in a previous study birds vaccinated with live *O. rhinotracheale* serotype G were protected against a challenge with *O. rhinotracheale* serotype A, and sera from these cross-protected birds was used to identify the Or77 antigen (chapter 3). Furthermore, the bacterin vaccine did not protect against heterologous challenge, even though the Or77 antigen was demonstrated to be expressed *in vitro* and therefore will be present in the bacterin vaccine. Possibly, the concentration of Or77 antigen within the bacterin formulation was too low to stimulate the production of cross-protective antibodies. Another explanation may be that the level of Or77-specific antibody titres produced after bacterin vaccination was insufficient to protect the birds against *O. rhinotracheale* challenge.

The results of these different animal studies imply that *O. rhinotracheale* lipoprotein Or77 is a vaccine target with high potential. The use of purified antigen as a vaccine component generally requires a strong adjuvant to trigger the host immune system. This may cause adverse side effects such as local reactions at the site of injection (177). Some lipoproteins are able to induce a strong protective response without adjuvants or apparent toxicity (19, 59). The specific chemical structure of the Pam_3Cys lipid moiety which is attached during post-translational modification of the protein (25) probably stimulates the immunogenicity of the antigen, as was demonstrated for the *Burrelia burgdorferi* OspA lipoprotein (58, 211). It

is known that the Pam_3Cys lipid is a ligand for Toll-like receptor 2 (TLR2), which is expressed by antigen presenting cells such as macrophages and B-cells, but also by epithelial cells (5). These TLRs play an essential role in recognition of microbial components, play a central role in innate immune defense, and can fulfill an important role in regulation of adaptive immunity (5, 189).

Previously, we demonstrated that it was possible to isolate Or77 from the *O. rhinotracheale* cell membrane by Triton X-114 extraction (chapter 4). However, the antigenicity and immunogenicity of native lipoprotein Or77 was not studied. The cross-protective Or77 antigen used in our previous animal experiments (chapter 3 and 4) was a non-lipidated, secreted recombinant protein that was cloned and expressed in *E. coli*, devoid of the *O. rhinotracheale* lipoprotein signal peptide. To analyze whereas lipidation of the recombinant Or77 antigen would increase its immunogenicity and thereby the efficacy of the recombinant subunit vaccine, the Or77 gene was cloned and expressed with the *B. burgdorferi* OspA lipoprotein signal peptide fused to the N-terminus of the protein. In our experiment both expression of recombinant Or77 by *E. coli* and purification of lipidated Or77 from the cell membrane were successful, indicating that the recombinant protein was present in the membrane fraction accessible for detergent extraction. However, it should be noted that even though Or77 partitioned in the Triton X-114 detergent phase, lipidation of the protein was not confirmed by [^{3}H] palmitic acid incorporation or mass spectrometric analysis.

Vaccination of broilers with lipidated Or77 induced an antibody response and protected the birds against challenge with a heterologous serotype, even when a small dose of 5 μg was used. However, it did not result in a significant increase of protection compared to vaccination with secreted Or77. PAGE and CBB-staining (Fig. 5) suggested that the total protein content of the Or77-sec solution was higher in the Or77-sec than in the Or77-lip solution. Furthermore, on Western blot Or77-lip-specific antibodies showed strong reactivity to multiple Or77-sec protein bands. These data suggest that the concentration of total Or77 in the Or77-sec vaccine may have been higher than in the Or77-lip vaccine, and thus that the Or77 antigen dose may have been different in both vaccines. This hypothesis is probable as for vaccine formulation only the upper Or77-sec protein band was used for calculation. Densitometric analysis of both protein solutions revealed that when all four protein bands in the 40-50 kDa range of the Or77-sec solution (Fig. 5, indicated with {) are included in calculation, the Or77-sec vaccine contained a factor 2.8 more protein material in comparison to the Or77-lip vaccine (data not shown). Considering the difference in Or77 antigen dose, and that less Or77-lip than Or77-sec was needed to induce comparable protection, it can be concluded that lipidation of the antigen indeed increases its immunogenicity. However, the development of antigenic mass assays in order to determine the exact antigen concentrations should be considered for future experiments. Furthermore, the vaccine antigens used in our experiments were formulated in a water-in-oil emulsion and their immunogenicity in the absence of this potent adjuvant was not studied yet. Thus, further investigation is needed to analyze the immune-stimulatory activity of lipidated Or77.

Based on the results of these experiments it can be concluded that *O. rhinotracheale* lipoprotein Or77 is an excellent candidate for the development of a cross-protective vaccine. However, additional studies have to be performed to determine the optimal dose and host response in combination with the appropriate vaccine delivery strategies for poultry.

ACKNOWLEDGEMENTS

We like to thank the Animal Service Department and the Pathology Department (Intervet Boxmeer) for technical assistance during animal studies.

CHAPTER SIX

Screening for *In Vivo* Expressed Antigens of *Ornithobacterium rhinotracheale* by *In Vivo* Induced Antigen Technology (IVIAT)

D.F. Schuijffel[1], M.T.J. Van Ampting[1], J.P.M. Van Putten[2], R.P.A.M. Segers[1], and P.J.M. Nuijten[1]

[1] *Intervet International BV, Bacteriology R&D, Boxmeer, The Netherlands,* [2] *Utrecht University, Faculty of Veterinary Medicine, Department of Infectious Diseases and Immunology, Utrecht, The Netherlands*

ABSTRACT

***In vivo* induced antigen technology (IVIAT) was used to screen for specific *in vivo* expressed antigens of *Ornithobacterium rhinotracheale,* that may serve as new vaccine candidates and increase the understanding of *O. rhinotracheale* pathogenesis. Pooled convalescent chicken sera were adsorbed with *in vitro* grown *O. rhinotracheale* and then used to probe a genomic expression library. Out of 90,000 screened plaques a total of 34 positive clones were selected for further analysis. DNA sequencing of the 5'-end of the PCR-amplified inserts of the reactive clones identified 7 different groups. Two groups were identical to 2 previously identified *O. rhinotracheale* antigens: Or02, a putative outer membrane lipoprotein, and Or98B, a RecT homologue. One group showed high similarity with the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which has been found in the cytoplasm as well as on the surface of several bacterial pathogens. The 4 other groups did not reveal any similarities with sequences in published databases. Rescreening of the clones with sera obtained from birds infected via different routes showed different reactivity, indicating that the environment of the bacterium influences antigen expression or that the host response generates a different antibody profile. Whether these antigens are specifically or preferentially expressed during *in vivo* growth and have vaccine potential awaits further study.**

INTRODUCTION

Pathogenic bacteria possess a repertoire of virulence determinants that each make unique contributions to infection. To understand the mechanism(s) by which a pathogen causes disease and circumvents the immune system, the gene products should be identified that are specifically required during different stages of the infectious process. In many instances, virulence gene expression is modulated in response to the changing environment encountered at the site of infection and different infection routes might lead to different gene expression profiles (129, 138, 175). It is reasonable to assume that genes specifically expressed during infection are likely to be important to the pathogenic process. Different molecular approaches such as *in vivo* expression technology (139), signature-tagged mutagenesis (90), and differential fluorescence induction (1) have been developed to identify microbial genes specifically expressed *in vivo* (81, 85, 87), thereby filling gaps in the understanding of microbial pathogenesis.

In vivo induced antigen technology (IVIAT) is another useful approach to identify microbial antigens expressed specifically during infection. In contrast to other techniques, *in vivo* gene expression can be analyzed without genetic manipulation of the pathogen and therefore this technique is particularly useful in the study of new and emerging pathogens of which no genomic information is present (80). IVIAT uses convalescent sera adsorbed with the *in vitro* grown pathogen, to probe for genes specifically expressed *in vivo* by screening a genomic expression library of this specific organism. Consequently, antigens are identified that are exclusively expressed during infection. This technique has been successfully applied for the identification of *in vivo* expressed (*ive*) antigens of different pathogens such as *Mycobacterium tuberculosis* (45), *Vibrio cholerae* (82), and *Vibrio vulnificus* (109).

Ornithobacterium rhinotracheale is a gram-negative bacterium associated with respiratory infections in poultry (36, 196). In experimental infections, respiratory disease such as airsacculitis and pneumoniae can be induced by aerosol administration of *O. rhinotracheale* whereas intravenous application induces systemic disease such as hepatitis and joint infections. The route of aerosol application is believed to be the natural infection route of the bacterium (195, 196). Up to now, little is known about the pathogenesis of *O. rhinotracheale* infection, although severity of infection, duration of disease and mortality rate have been found to be extremely variable and influenced by various environmental and host factors (36, 197). Therapeutic treatment of *O. rhinotracheale* infections can be difficult due to acquired resistance against the regularly used antibiotics (130, 176). The alternative is to prevent infection by administration of an efficient vaccine (197).

The purpose of this study was to discover specific *in vivo* expressed antigens of *O. rhinotracheale* in order to increase our understanding in the pathogenesis of the infection. Since it is very difficult to genetically engineer this bacterium, IVIAT was selected to be the most efficient approach for antigen identification. An additional benefit of this technique is that IVIAT does not rely on the isolation of *in vivo* grown *O. rhinotracheale* or on the availability of laboratory animals. Pooled serum from *O. rhinotracheale* infected chickens was adsorbed with *in vitro* grown *O. rhinotracheale* (Fig. 1). The resulting adsorbed serum containing the subpopulation of antibodies reactive against *ive* antigens was used to probe a genomic expression library. Sera obtained from previous challenge studies (chapter 2 and

3) were used for the IVIAT screenings in order to study the effect of different infection routes on subsequent gene expression of *O. rhinotracheale*. The results of this study may increase the understanding of *O. rhinotracheale* infections in chickens and may facilitate the development of new, potent vaccines.

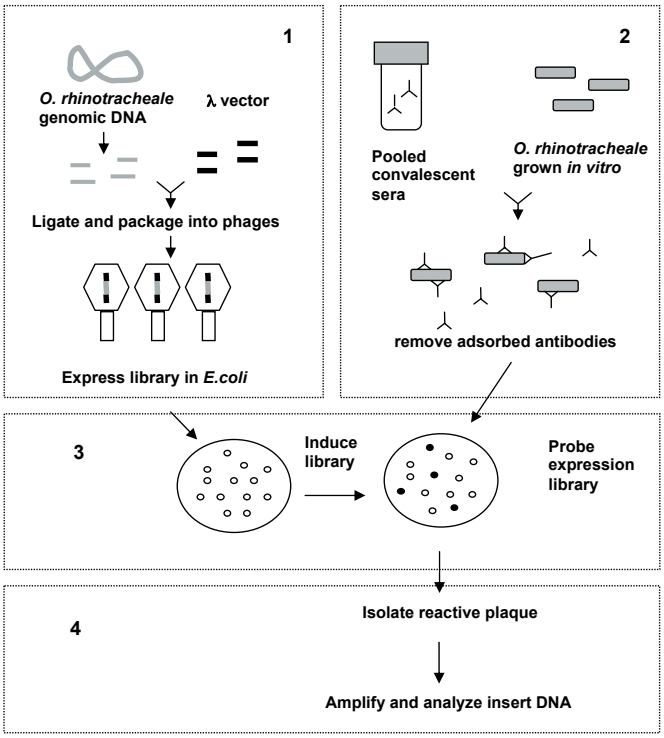

Figure 1. *In vivo* induced antigen technology. 1: An expression library of *O. rhinotracheale* is created in a λTriplEx expression system. 2: Sera from chickens that have experienced an infection with *O. rhinotracheale* are pooled and extensively adsorbed with *in vitro* grown *O. rhinotracheale,* resulting in a pool of antibodies reactive against specifically *in vivo* expressed antigens. 3: The library is expressed in *E. coli* and induced plaques are probed with adsorbed sera. 4: Reactive plaques are selected and purified, and cloned insert DNA is sequenced and analyzed.

MATERIALS AND METHODS

Bacterial strains and growth conditions

O. rhinotracheale serotype G strain O-95029 nr.16279 was grown on 5% sheep blood agar (BA) plates at 37°C, in a 5% CO_2 atmosphere for 48 hours. For liquid culture, single colonies were inoculated in Todd Hewitt (TH) medium (Difco, Detroit, MI, USA) and grown for 24 hours at 37°C at 100 rpm. *Escherichia coli* strain XL1 Blue was obtained from Clontech Laboratories (Palo Alto, CA, USA) and grown on Luria Bertani (LB) agar plates supplemented with 10 mM

$MgSO_4$ and 15 µg/ml tetracycline, or for liquid culture, grown in LB broth supplemented with 10 mM $MgSO_4$ and 0.2% maltose. XL1 Blue was incubated for 16-20 hours at 37°C, for liquid culture on an orbital shaker at 200 rpm.

Antisera

The antisera used in this study were obtained from specified-pathogen-free (SPF) broiler chickens that were vaccinated and/or challenged with *O. rhinotracheale* (Table 1). Each type of antiserum consisted of a pool of sera from approximately 10 different birds that received equal treatment. Serum samples were tested in an enzyme-linked immunosorbent assay (ELISA) against boiled extract antigens of the *O. rhinotracheale* serotypes A and G as described (195). *O. rhinotracheale*-specific serum IgG antibody levels were represented as ^{2}log-titres. Total serum immunoglobulin concentrations of isotypes A, G, and M were measured with a chicken serum IgA, IgG, or IgM ELISA kit (Bethyl Laboratories, Montgomery, TX, USA) following the manufacturer's protocol.

The first antiserum (NR1) was obtained from 5-week old birds that were vaccinated with an aerosol of live *O. rhinotracheale* serotype G at 2 weeks of age. At 4 weeks of age the chickens were challenged with *O. rhinotracheale* serotype A (strain B3263/91) either intravenously (NR1iv) or by aerosol (NR1ae) following the standard challenge procedures (chapter 2 to 5). The ELISA IgG antibody titres against *O. rhinotracheale* serotype A were 10.3 for antiserum from i.v. challenged birds and 7.4 for aerosol challenged birds. The antibody titres against *O. rhinotracheale* serotype G were 7.2 and 6.6, respectively. The immunoglobulin concentration per isotype was not determined for these sera. Live vaccination with *O. rhinotracheale* serotype G previously demonstrated to induce cross-protective immunity against *O. rhinotracheale* serotype A challenge (chapter 3).

The second antiserum (NR2) was obtained from 5-week old unvaccinated birds that were i.v. challenged with *O. rhinotracheale* serotype A (strain B3263/91) at 4 weeks of age. The ELISA IgG antibody titres against *O. rhinotracheale* serotype A and G were 11.7 and 6.0, respectively. The total immunoglobulin concentration of serum NR2 was 5144 µg/ml. This serum has demonstrated to significantly ($p < 0.05$) reduce pathology when administered i.v. to immune-suppressed animals 4 hours prior to i.v. challenge with homologous or heterologous *O. rhinotracheale* serotypes (chapter 2).

The third serum (NR3) was obtained from 5-week old control birds that were not vaccinated and not challenged. ELISA IgG antibody titres against serotype A and G were 5.1 and 5.0. The total immunoglobulin concentration of serum NR3 was 1561 µg/ml. Previously, these immunoglobulins yielded no reduced pathology when administered i.v. to immune-suppressed animals 4 hours prior to i.v. challenge with homologous or heterologous *O. rhinotracheale* serotypes (chapter 2).

Preadsorption of antisera

O. rhinotracheale serotype G used for preadsorption of sera was grown both in TH medium and on BA plates as described above. A batch of preadsorption material was prepared by collecting 100 ml of liquid culture (OD_{600} approximately 1.0) cells by centrifugation and subsequent resuspension in 100 ml of 0.04 M PBS-0; 0.05% polysorbate-20, and by collecting confluent grown colonies of 5 BA plates in 0.04 M PBS-0; 0.05% polysorbate-20 followed by a washing and resuspension step in 100 ml of the same buffer. Both cell suspensions were pooled and divided into two equal parts. One part was used to make lysate. This was done by several freeze-thawing and sonification cycli followed by a final centrifugation step (15 min; 10,000 x g; 4°C). The other part of the cell suspension was kept intact. The preadsorption material was stored at -20°C until use. *E. coli* XL1 Blue preadsorption material was made as described above for *O. rhinotracheale*.

Preadsorptions were started 2 days before use of the antisera in Western blot or immunoscreening. An incubation volume of 15 ml consisted of 2.5 ml of *O. rhinotracheale* cell lysate, 3 ml of *O. rhinotracheale* intact cells, 2.5 ml of *E. coli* cell lysate, 3 ml of *E. coli* intact cells, 4 ml of 0.04M PBS-0; 0.05% polysorbate-20, and 150 µl of undiluted antiserum (1:100 final concentration total antibody). Sera were adsorbed at 4°C for approximately 2 days while lightly shaking. At least 1 hour before use, preadsorption was continued at 37°C. The complete preadsorption mixture was used for Western blot analysis and expression library screening.

Polyacrylamide gel electrophoresis (PAGE) and semi-dry Western blotting

NOVEX NuPAGE was used for protein electrophoresis under denaturating conditions in 4-12% polyacrylamide gels according to manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Polypeptides were electro-blotted onto Immobilon PVDF 0.45 µm membrane (Millipore, Bedfort, MA, USA), by semi-dry Western blotting according to Towbin *et al*. (187). Membranes were blocked by incubating for 1 hour at 37°C in 0.04 M PBS-0; 0.5% polysorbate-20; 1% skim milk while shaking. After washing with 0.04 M PBS-0; 0.5% polysorbate-20, filters were treated with adsorbed antiserum (1:100 dilution), washed again, and incubated with rabbit anti-chicken IgG peroxidase conjugate (Nordic, Tilburg, The Netherlands) in a 1:1000 dilution (in 0.04 M PBS-0; 0.05% polysorbate-20; 1% skim milk). Both incubations were done at 37°C for 1 hour while shaking. Finally, filters were washed with 0.04 M PBS-0; 0.5% polysorbate-20 and the substrate solution Vector SG (Vector Laboratories, Burlingame, CA, USA) was added to perform chromogenic peroxidase staining.

Construction of a genomic expression library

O. rhinotracheale serotype G genomic DNA was isolated from liquid cultured cells according to the method described by Sambrook *et al*. (22), partially digested with *Tsp*509I restriction enzyme (New England Biolabs, Beverly, MA, USA) to obtain 1 – 4 kb fragments, and cloned

into the *Eco*RI digested and dephosphorylated λTriplEx vector arms (Clontech, Palo Alto, CA, USA). Packaging was performed using the Stratagene (La Jolla, CA, USA) *in vitro* packaging extracts and phage particles containing *O. rhinotracheale* DNA were transfected into *E. coli* XL1 Blue, resulting in a genomic expression library containing 97% recombinants and a complexity of 6.9.

Immunoscreening

The expression library immunoscreening procedure was performed under native conditions as described by the manufacturer (Clontech Manual, Clontech, Palo Alto, CA, USA). In short, phage-infected *E. coli* XL1 Blue cells were mixed with LB 10 mM $MgSO_4$ top agar and plated onto LB 10 mM $MgSO_4$ agar, incubated at 42°C for 4 hours during which small clear plaques became visible. These plaques were covered with nitrocellulose filters (Schleicher and Schuell, Dassel, Germany) which were saturated with 10 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated for a further 4 hours at 37°C. Filters were removed from the plates and treated exactly as described above for Western blot analysis. Positive (reactive) plaques located on the agar plates were picked and rescreened with the same serum to obtain single purified clones.

Polymerase chain reaction and sequencing

Oligonucleotide primers used for both polymerase chain reaction (PCR) amplification and (partial) sequencing of the DNA inserts of the selected plaques were specifically designed for the λTriplEx vector arms and synthesized by Life Technologies (Invitrogen, Carlsbad, CA, USA). The 5' primer used was 5'-GCG CCA TTG TGT TGG TAC-3', the 3' primer used was 5'-TTT TTC TCG GGA AGC GCG-3'. PCR was performed in an automated thermal cycler (GeneAmp 9700, Perkin Elmer, CA, USA). The final PCR reaction volume was 50 µl containing 50 µM of dNTP's (Promega, WI, USA), 10 pmol of both primers, 20 U/ml of Supertaq plus polymerase and 10X of Supertaq buffer (Both HT Biotechnology Ltd, Cambridge, UK) in water. Phage DNA was added to the reaction mix by picking a freshly plated plaque. The following conditions were used: denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 2 min, and elongation at 68°C for 2 min 30 sec followed by a final extension at 68°C for 10 min.

To determine the nucleotide sequence of the DNA inserts a sequence reaction was done (94°C 10 sec; 50°C 5 sec; 60°C 2 min for 25 cycles in an automated thermal cycler as described) using Big Dye Terminator Ready reaction mix, 50 ng PCR product and 2.4 pmol primer in a 20 µl reaction volume. Sequencing was done on an ABI 310 automated sequencer (Perkin Elmer, CA, USA). Data were collected using ABI 310 Collection Software version 1.0.4 and analysed with Sequence Analysis version 3.1 (Perkin Elmer, CA, USA). Contigs and alignments were made using Sequencer version 4.1.4 (Gene Codes Corporation, USA). Analysis for sequence homologies was done using NCBI BLAST (http://ncbi.nlm.nih.gov/) (8).

RESULTS

Description of different antisera used for IVIAT screening

Antisera used for the IVIAT immunoscreening are listed in Table 1. These sera were obtained from broiler chickens that were vaccinated and/or challenged with *O. rhinotracheale* via 2 different infection routes: aerosol or intravenous. Each type of antiserum consisted of a pool of serum from approximately 10 different birds that received equal treatment.

The first antiserum (NR1) was a 1:1 mixture of 2 different pools of sera (NR1iv and NR1ae) obtained from 5-week old birds, vaccinated with an aerosol of live *O. rhinotracheale* serotype G at week 2 and challenged either intravenously (NR1iv) or by aerosol (NR1ae) with *O. rhinotracheale* serotype A at week 4. This protocol has previously demonstrated to generate a humoral immune response (chapter 2) and to induce cross-protective immunity against *O. rhinotracheale* infection (chapter 3).

The second antiserum (NR2) was obtained from 5-week old unvaccinated birds that were intravenously challenged with *O. rhinotracheale* serotype A at 4 weeks of age. This serum contained increased IgA titres and highly elevated serum IgG and IgM levels and conferred protection against i.v. *O. rhinotracheale* challenge when administered to immune-suppressed birds (chapter 2).

The third antiserum (NR3) was obtained from 5-week old birds that were not vaccinated and not challenged with *O. rhinotracheale*. This control serum showed background *O. rhinotracheale*-specific ELISA titres, and yielded no protection against i.v. *O. rhinotracheale* challenge when administered to immune-suppressed birds (chapter 2).

Table 1. Overview of sera used for IVIAT

Serum	Treatment		Serum ELISA titre[3]		Serum Ig concentration[3]		
	Vaccination[1]	Challenge[2]	α-OrA titre	α-OrG titre	IgA	IgG	IgM
	serotype / route		(^{2}log)		(μg/ml)		
NR1ae	G / aerosol	A / aerosol	7.4	6.6	ND	ND	ND
NR1iv	G / aerosol	A / i.v.	10.3	7.2	ND	ND	ND
NR2	-	A / i.v.	11.7	6.0	786	3087	1271
NR3	-	-	5.1	5.0	329	902	330

ND: not determined
[1] Birds were vaccinated at 2 weeks of age
[2] Birds were challenged at 4 weeks of age
[3] Sera were collected at 5 weeks of age

Preadsorption of antisera with *in vitro* grown bacteria

Extensive preadsorption of the above described sera with a mixture of cell lysate and fresh culture of *in vitro* grown *O. rhinotracheale* serotype G was expected to remove antibodies reactive against *O. rhinotracheale* antigens expressed *in vitro*. Western blot analysis using adsorbed and unadsorbed NR1 and NR2 sera and *in vitro* grown *O. rhinotracheale* serotype G showed a strongly reduced reaction after preadsorption (Fig. 2, lanes 2 and 4). It was not further confirmed whether the antibodies in the adsorbed sera were able to recognize *O. rhinotracheale* bacteria *in vivo* since a test system suitable for this purpose was not available. No reactivity could be observed after incubation with the unadsorbed control serum NR3 (Fig. 2, lane 5).

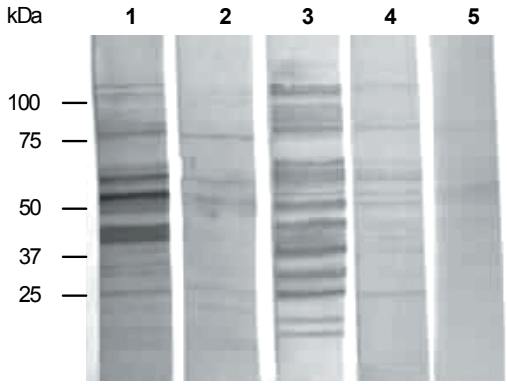

Figure 2. Western blot demonstrating the reactivity of adsorbed and unadsorbed sera against *in vitro* grown *O. rhinotracheale* serotype G. Lane 1: Serum NR1 unadsorbed, lane 2: serum NR1 adsorbed, lane 3: serum NR2 unadsorbed, lane 4: serum NR2 adsorbed, lane 5: serum NR3 unadsorbed.

Selection of reactive clones by screening of a genomic expression library with adsorbed sera

In order to search for *in vivo* induced antigens a genomic expression library of *O. rhinotracheale* serotype G was constructed and approximately $9*10^4$ plaques (representing several fold the total genome) were screened under native conditions using the preadsorbed NR1 and NR2 sera. Prior to use, the sera were also adsorbed with the *E. coli* lysate to reduce non-specific signals. Screening of the expression library using adsorbed serum NR1 resulted in the selection of 23 reactive clones (1.1 to 1.23) and with adsorbed serum NR2, a number of 11 reactive clones were obtained (2.1 to 2.11). All positive clones were isolated and rescreened with the same serum resulting in 34 single reactive clones which was about 12% of the number of reactive clones obtained with the unadsorbed NR1 and NR2 sera (control experiments, data not shown). This indicated a 10-fold enrichment of serum IgG antibodies that are reactive against exclusively *in vivo* expressed antigens. The negative control serum NR3 yielded no reactive clones (data not shown).

Analysis of the reactive clones

PCR analysis of the 34 clones using primers specific for the λTriplEx vector arms indicated that the cloned insert DNA ranged in size between 1.5 and 3.5 kb (data not shown). Sequence analysis of the 5'-end of 29 inserts revealed that the total selection represented 7 different sequence groups or loci with a variable number of matching clones (see Table 2). These groups were encoded OR*ive*1 (3 clones), OR*ive*2 (12 clones), OR*ive*3 (1 clone), OR*ive*4 (10 clones), OR*ive*5 (1 clone), OR*ive*6 (1 clone), and OR*ive*7 (1 clone). Sequence group OR*ive*4 consisted of clones selected by screening with both sera NR1 and NR2.

BLAST homology searches with the different sequences showed that the nucleotide sequence of OR*ive*1 was identical to a recently identified cross-reactive antigen of *O. rhinotracheale*, designated Or02 (chapter 3 and 4). Antigen Or02 shows similarity to a putative outer membrane protein of the bacterium *Leptospira interrogans* (31% identity, 43% similarity). Furthermore this protein has a hydrophobic N-terminal end with the characteristics of a gram-negative signal peptide having a lipoprotein lipidation site. OR*ive*3 was identical to another previously identified cross-reactive antigen of *O. rhinotracheale*, designated Or98B (chapter 3 and 4). A conserved domain search of this protein sequence revealed a region characteristic for the RecT family with most significant similarity with the RecT protein of *Clostridium tetani* (identity 44%, similarity 66%). OR*ive*4 showed high similarity at amino acid level with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from several bacterial species (identity > 50%, similarity > 65%). No similarities were found for OR*ive*2, OR*ive*5, OR*ive*6, and OR*ive*7.

Infection route and *in vivo* protein expression

For the selection of *ive* clones 1.1 to 1.23 antiserum NR1: a mixture of sera from birds infected via 2 different infection routes was used. These 2 sera were expected to contain different antibody populations since it was assumed that infection route influences the antigen expression profile of the pathogen (chapter 2, Fig. 2). Therefore all positive identified clones were rescreened with preadsorbed sera obtained from birds that were live vaccinated with O. rhinotracheale serotype G but challenged either intravenously (NR1iv) or by aerosol with *O. rhinotracheale* serotype A. (NR1ae). In this immunoscreening, 2 negative controls were used: a clone that did not react positive during previous immunoscreenings (negative control 1) and a clone without *O. rhinotracheale* insert DNA (negative control 2). Reactivity of the expressed proteins with the 2 different sera were compared with the negative controls and indicated moderate positive (+), strong positive (++), or negative (-). The results of this screening are summarized in Table 2.

Table 2. Overview of clones selected by IVIAT.

Sera used in IVIAT	Sequence group	Number of clones	Similar protein in database Organism, % identity / % similarity	Sera from aerosol challenged birds [4]	Sera from i.v. challenged birds [5]
NR1 [1]	OR*ive*1	3	Putative outer membrane protein *Leptospira interrogans*, identity 31% / similarity 43% Or02 – *O. rhinotracheale*, identity/similarity 100% [3]	++	+
	OR*ive*2	12	No similarities found	-	++
	OR*ive*3	1	RecT protein *Clostridium tetani*, identity 44% / similarity 66% Or98B – *O. rhinotracheale*, identity/similarity 100% [3]	-	-
	OR*ive*4	5	GAPDH Different bacterial species, identity>50% / similarity>65%	++	++
	OR*ive*5	1	No similarities found	++	+
	ND	1 (1.8)	ND	-	-
NR2 [2]	OR*ive*4	5	GAPDH Different bacterial species, identity>50% / similarity>65%	++	++
	OR*ive*6	1	No similarities found	-	ND
	OR*ive*7	1	No similarities found	-	ND
	ND	4 (2.4)	ND	++	++
		(2.7)		-	+
		(2.8)		-	-
		(2.11)		++	++

ND: not determined
1) Serum obtained from birds vaccinated with *O. rhinotracheale* serotype G and challenged intravenously or by aerosol with *O. rhinotracheale* serotype A.
2) Serum obtained from birds challenged intravenously with *O. rhinotracheale* serotype A.
3) See chapter 3 and 4
4) Serum NR1ae: obtained from birds vaccinated with *O. rhinotracheale* serotype G and challenged by aerosol with *O. rhinotracheale* serotype A.
5) Serum NR1iv: obtained from birds vaccinated with *O. rhinotracheale* serotype G and challenged intravenously with *O. rhinotracheale* serotype A.

From clones 1.1 to 1.23, initially selected by using a mixture of sera NR1iv and NR1ae, the members of sequence groups OR*ive*1, OR*ive*4, and OR*ive*5 all reacted positive with both sera, although reaction with NR1ae was strongest for OR*ive*1 and Or*ive*5. Reactivity of clones representing group OR*ive*2 was only observed for the antiserum from birds that had been challenged intravenously. Surprisingly, during this screening no reactivity against group OR*ive*3 and clone 1.8 was observed.

Clones 2.1 to 2.11 were selected using sera from unvaccinated but intravenously challenged birds. Clones from group OR*ive*4 and clones 2.4 and 2.11 showed strong reactivity with both sera NR1iv and NR1ae. No reactivity was observed against groups OR*ive*6, OR*ive*7, and clone 2.7 using serum from aerosol challenged birds (NR1ae), while during this screening reactivity against clone 2.8 was negative either sera NR1iv and NR1ae.

DISCUSSION

Many bacterial virulence genes are expressed *in vivo* during the different phases of the infectious process in response to changes in the micro-environment and host factors (138, 175). Moreover, some bacterial structures are only expressed in the host and not when grown *in vitro*. Identification of this class of *in vivo* expressed (*ive*) genes and their products can contribute to better understanding of pathogenesis and can provide new vaccine targets. In recent years, several techniques have emerged such as *in vivo* expression technology (139), signature-tagged mutagenesis (90), and differential fluorescence induction (1) in order to identify genes expressed *in vivo* (81, 85, 87). For the identification of *O. rhinotracheale ive* genes these technologies are less suitable due to the difficulties to genetically engineer this pathogen and to efficiently recover bacteria from infected animals. Therefore, in this study *in vivo* induced antigen technology (IVIAT) (85, Fig. 1) was applied in the search for specific *in vivo* expressed genes of *O. rhinotracheale*. The use of convalescent sera collected from chickens that encountered *O. rhinotracheale* via different infection routes allowed a direct identification of antigens produced in the birds during different types of infection. Since antibodies play an important role in protection against *O. rhinotracheale* infections (chapter 2) and it was demonstrated that used antisera contain protective antibodies (chapter 2 and 3), IVIAT identified *ive* antigens are potentially suitable vaccine targets.

In this study, pooled sera from at least 10 identically treated chickens was adsorbed with total antigens from *in vitro* grown *O. rhinotracheale* cells with the expectation to remove all antibodies directed against *in vitro* expressed antigens. This procedure resulted in a strong decrease in the number of immunoreactive bands on Western blots (Fig. 2), indicating that preadsorption was effective. This was confirmed by the results of the immunoscreening of the expression library that yielded approximately 10-fold less reactive clones with the adsorbed sera than with the unabsorbed sera. Screening of the genomic expression library resulted in a total number of 34 positive clones. The antisera used for immunoscreening were derived from chickens that underwent live vaccination followed by infection with a different *O. rhinotracheale* serotype, or from non-vaccinated, *O. rhinotracheale* infected birds. This allowed us to select for genes encoding antigens expressed during an infection. In a previous study (chapter 2) we observed that both i.v. and aerosol challenge resulted in an

increase of serum IgA, IgG, and IgM with the highest titres in i.v. challenged birds. In our screenings we solely focused on reactivity of IgG class antibodies as this is the predominant immunoglobulin isotype present in serum (approximately 60%, Table 1) and therefore was expected to be least diluted during preadsorptions. It cannot be excluded that potentially interesting antigens involved in early stages or the mucosal site of infection (where IgM and IgA rather than IgG are the predominant antibody class) may have been missed. The choice to screen only with IgG antibodies however, seems justified as IgG antibodies were previously demonstrated to confer protection (chapter 4 and 5).

The *O. rhinotracheale* DNA inserts of the 34 clones selected with serum NR1 and NR2 were amplified by PCR and sequence analysis of the first 600 bps of 29 clones revealed 7 different groups (OR*ive*1 to OR*ive*7). Groups OR*ive*1 to OR*ive*5 were selected by screening with serum NR1, while groups OR*ive*4, OR*ive*6, and OR*ive*7 were obtained by screening with serum NR2. Clones of sequence group OR*ive*4 reacted with both sera. As was demonstrated in a previous study (chapter 3), cloned genes selected by immunoscreening of the *O. rhinotracheale* genomic expression library were generally expressed as translational fusions. Although this was not confirmed for the different clones identified by this study, for preliminary characterization the 5'-sequences of the *O. rhinotracheale* inserts were used to perform homology searches. Analysis with the BLAST algorithm revealed similarities for OR*ive*1, OR*ive*3 and OR*ive*4, but not for OR*ive*2, OR*ive*5, OR*ive*6, and OR*ive*7.

The nucleotide sequence of OR*ive*1 was identical to a recently identified cross-reactive antigen of *O. rhinotracheale*, designated Or02 (chapter 3 and 4). This antigen was selected by immunoscreening of the same *O. rhinotracheale* expression library using unadsorbed sera from *O. rhinotracheale* serotype B, G, or M live vaccinated and *O. rhinotracheale* serotype A challenged birds. This protein showed similarity to a putative outer membrane protein of the bacterium *Leptospira interrogans* and the N-terminus of the protein has the characteristics of a gram-negative signal peptide and lipoprotein lipidation site. Vaccination of chickens with single recombinant Or02 antigen did induce a specific antibody response but birds were not protected against an aerosol *O. rhinotracheale* challenge (chapter 4). However, it was also demonstrated in chapter 4 that Or02 is expressed during *in vitro* growth and therefore not specifically expressed during infection. Despite extensive preadsorption this antigen was selected during our IVIAT screening. Probably the amount of Or02-specific antibodies in serum NR1 was too high to achieve full depletion by the adsorption procedure.

The nucleotide sequence of the 5' end of OR*ive*3 was identical to another previously identified cross-reactive antigen of *O. rhinotracheale*, designated Or98B (chapter 3). This protein, which has strong similarity to RecT of *Clostridium tetani,* can be involved in DNA recombination and repair (142). Like recombinant Or02, recombinant Or98B did induce a specific antibody response but these birds were not protected against an aerosol challenge with *O. rhinotracheale* (chapter 4). Previous Western blot analysis using antigen-specific serum revealed no reactivity against the *O. rhinotracheale* Or98B, already indicating that this antigen is most likely not expressed *in vitro,* but specifically during *in vivo* growth (chapter 4). The results of the IVIAT screening confirm this hypothesis.

The amino acid sequence of OR*ive*4 showed strong similarity with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Mycoplasma pneumoniae*. This enzyme plays a key role in glucose metabolism and can be present as cytosolic protein as well as cell surface

protein (18, 98, 173). Surface-located GAPDH is suggested to contribute to the organism's invasiveness by its ability to capture plasmin and the binding of proteins such as fibronectin (18, 127) which has been suggested to play an important role in the adherence of several pathogens to host epithelial cells. The streptococcal surface GAPDH has also been reported to function as an ADP-ribosylating enzyme which may enable communication between host and pathogen during infection (146). Although the function of this protein in virulence of *O. rhinotracheale* awaits further investigation, this protein is expected to be expressed both *in vitro* and *in vivo* since GAPDH is one of the many housekeeping proteins essential for the viability of the bacterial cell. An explanation for its detection by the IVIAT procedure may again be an insufficient reduction of OR*ive*4-specific antibodies during the adsorption step.

Rescreening of the 34 clones selected with a mixture of different pooled sera, with serum from either aerosol challenged birds or serum from i.v. challenged birds revealed that group OR*ive*2 and clone 2.7 represent a protein that is specifically expressed after i.v. administration of the pathogen. This may suggest that this protein is not involved in the pathogenesis of respiratory tract infections. The proteins encoded by OR*ive*1 (Or02), OR*ive*4, OR*ive*5, 2.4 and 2.11 showed reactivity with both sera, but OR*ive*1 and OR*ive*5 clones showed a stronger signal with sera from aerosol challenged birds in comparison to i.v. challenged birds. This suggests that these proteins are preferentially expressed during respiratory infection and/ or play a role in the adaptation to the host environment. It remains unexplained why 3 antigens (ORive3 (Or98B), 1.8, and 2.8) showed no reactivity at all with either serum during the rescreening experiment.

Together the results presented in this study demonstrate that IVIAT, the *in vivo* induced antigen technology, can successfully be applied to select different genes encoding *in vivo* expressed antigens of *O. rhinotracheale*, providing that full depletion of antibodies by the adsorption procedure is achieved. Future analysis will determine which of the identified antigens are preferentially or exclusively expressed *in vivo,* what their role is in the pathogenic process, and whether they are suitable for future vaccine development.

CHAPTER SEVEN

Summarizing Discussion

D.F. Schuijffel

Intervet International BV, Bacteriology R&D, Boxmeer, The Netherlands

Context and aim of this study

As long as both human and animals are threatened by infectious microbial species there will be a constant need for safe and efficacious vaccines that afford protective immunity. Fortunately, with the present-day knowledge of pathogen and host biology, and state-of-the-art technologies, opportunities arise to follow a rational and strategic approach for vaccine development.

The work described in this thesis was initiated in order to evaluate a new 3-step approach for strategic vaccine development based on the induction of immunity (Fig. 1). The strategy was applied to a recently described poultry pathogen, *Ornithobacterium rhinotracheale*, for several reasons: First, in recent years, *O. rhinotracheale* has emerged as an important pathogen mainly known for causing respiratory diseases in chickens and turkeys (36, 196). Second, vaccines based on bacterin formulations protect against *O. rhinotracheale* infections with a homologous serotype (179, 197) but there is a need for a broadly cross-protective vaccine. Third, at the start of this research, all protocols for experimental infections, pathological analysis, and immunological assays were available to study the disease (198, 200). Fourth, no information regarding any, including protective, antigens of *O. rhinotracheale* was available; therefore all new information concerning its antigenic composition would be useful for vaccine development and prevention of disease. Fifth, since the experimental infection is not an animal model but rather the real disease, this choice will allow us to study the actual host-pathogen interaction.

The main objectives of the present study were to better understand the host protective immune response to *O. rhinotracheale* infection by identification of the mediators of protection (step I), to use these immune components to select and identify cross-protective *O. rhinotracheale* vaccine antigens by using a whole genome screening method (step II), and to evaluate cross-protective antigens identified in steps I and II, for their vaccine potential in order to develop suitable antigen delivery strategies (step III).

1. The chicken immune response to *O. rhinotracheale* infection

The first step in our vaccinology approach was the characterization of the chicken immune response during *O. rhinotracheale* infection. It was known that birds recovering from a natural infection with *O. rhinotracheale* showed elevated antibody levels directed against *O. rhinotracheale* antigens (36, 196). However, the exact role of antibody-mediated immunity in the (cross-) protection against *O. rhinotracheale* infection had never been analysed. Here, some aspects of our immunological studies described in the different chapters, will be discussed.

1.1 Experimental infections with O. rhinotracheale

In all animal experiments described in this thesis, the natural host-pathogen interaction was studied by using specified pathogen free (SPF) broiler chickens and 2 different *O. rhinotracheale* challenge methods. Although this test system was most suitable to achieve our goals, variations were noted in the pathology scores of the challenge control groups

between different experiments. This variation did not influence results as control groups were included in each experiment. Yet, it may illustrate the multiplicity of factors that determine the establishment of an *O. rhinotracheale* infection. One possible explanation for the observed variation is that the experimental infections were induced by either aerosol spray application, what is believed to mimic the natural infection route most closely, or by intravenous injection of the pathogen. The severity of the aerosol challenge can depend on multiple factors, such as the priming with Newcastle Disease Virus, the settings of the sprayer used for aerosol spray application, the size of the droplets, the viability of the bacterial cultures, the density of the developed mist, the actual time birds are kept in this mist, and the duration of the mist to be cleared from the isolator by ventilation. Although we used a fixed spray volume of the challenge culture per isolator, the actual dose per birds cannot be controlled. For these reasons, birds of different test groups were housed in a mixed population where possible, to diminish isolator effects. By using intravenous challenge, the individual dose can be controlled but this method is supposed to be more stressful for the birds and does not resemble the natural route of infection.

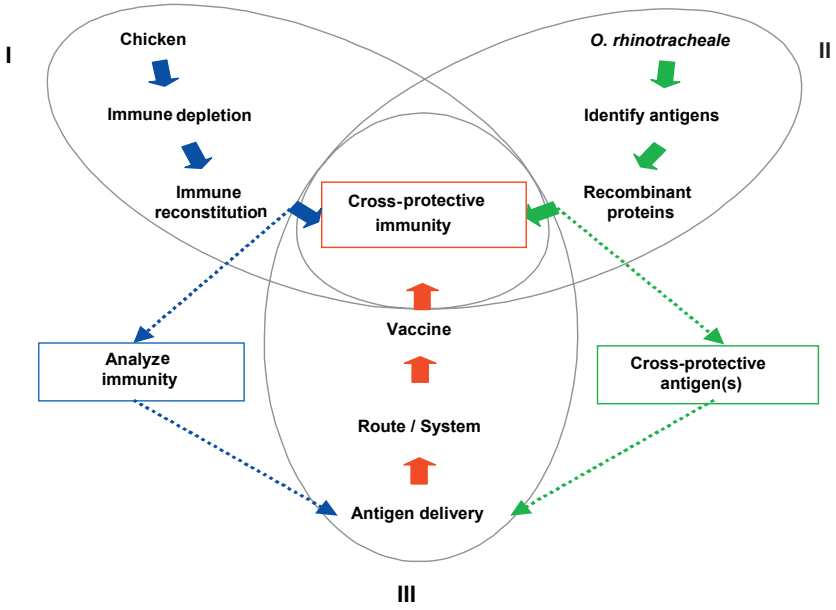

Figure 1. Summary of the strategic approach used for development of a cross-protective *O. rhinotracheale* vaccine. In step I (blue arrows), the chicken immune response during *O. rhinotracheale* infection is characterized by using an experimental method that combines selective immune depletion and passive transfer of immunity. Antigens of *O. rhinotracheale* that have cross-protective potential are identified in step II (green arrows) by using the mediators of infection as analysed in step I. Step III (red arrows) comprises the selection of an appropriate means of antigen delivery and the design of the final cross-protective vaccine using gained knowledge about the chicken immune response (step I, dotted blue line) and the identified cross-protective vaccine antigen(s) (step II, dotted green line).

The choice to the use of SPF-broilers instead of commercial broiler chickens was based on the fact that the microbial status of the SPF-parent birds regarding various poultry pathogens, was known. However, it should be noted that these SPF-birds are expected, but not confirmed, to be free of *O. rhinotracheale*. Vertical transmission of *O. rhinotracheale* or transfer of maternal immunity from infected mother birds to their progeny can seriously affect the susceptibility of the test animal to infection and influence the experimental results. Therefore, extra birds that did not encounter *O. rhinotracheale* were included in our animal experiments, to monitor for presence of *O. rhinotracheale*-specific antibodies and the development of infection during the course of the experiment. In some experiments, the presence of maternal antibodies at day of hatch was confirmed by ELISA and Western blot (data not shown), but these antibody titres rapidly declined to undetectable levels within 2 weeks, before start of the actual experiment. Despite the presence of natural antibodies in the control birds, no evidence (bacteriological of histological) for the presence of *O. rhinotracheale* in these birds was found. Yet, the presence of maternal antibodies in the young chicken indicates that the SPF-status regarding *O. rhinotracheale* needs further investigation.

Since multiple variables concerning host, pathogen and environment can influence the experimental outcome, for future studies it remains important to consider each variable and closely monitor the birds during each animal experiment.

1.2 The role of antibodies in protection

In chapter 2, we studied the role of antibodies in providing protection against *O. rhinotracheale* infection by using a strategy of selective B-cell depletion and immune reconstitution with distinct antisera within the same host. It was concluded that antibodies play a key role in (cross-) protection against *O. rhinotracheale* infection in chickens as challenge of immune-compromised birds with *O. rhinotracheale* caused significantly more and severe organ lesions than challenge of immune-competent birds. Furthermore, passive transfer of *O. rhinotracheale*-specific antiserum to immune-deficient birds specifically protected these animals against pathogen challenge. An important observation was that transfer of sera derived from *O. rhinotracheale* serotype A challenged birds protected against an *O. rhinotracheale* serotype G challenge.

Generally, in immune-competent birds lesions in air sacs and lungs are restricted to aerosol challenged birds, whereas lesions in the joints and liver usually only develop after i.v. challenge. In our experiments (chapter 2) it was noted that immune-deficient birds developed not only systemic but also respiratory pathology, when the i.v. route of experimental infection was applied. These observations imply the importance of immunoglobulins in the prevention of migration from *O. rhinotracheale* from the blood to the respiratory tissue. In contrast, development of systemic pathology after aerosol challenge of these immune-deficient birds was not observed, indicating that factors other than antibodies are involved in preventing the migration of *O. rhinotracheale* from the respiratory tissue to the circulation. This can be explained by the presence of mucus in secretions of the mucosal membranes that line the respiratory tract. This mucus layer may can, irrespective of the immune status of the birds, act as a physicochemical barrier and as a vicious matrix in which the bacterium can be trapped, making it unable to adhere and penetrate the epithelial cells (115).

1.3 Routes of infection and relevance for the selective immune depletion and reconstitution approach

As described in chapter 2, different *O. rhinotracheale* infection routes induced the production of antibodies of different immunoglobulin classes: IgG was the principal serum immunoglobulin class induced by intravenous challenge, whereas aerosol challenge mainly stimulated the production of both IgG and IgA. This observation suggests that the infection route influences the nature of the immune response what may be of importance for our challenge experiments. In our passive transfer studies, we used serum from i.v. challenged birds, containing mainly IgG antibodies, and demonstrated significant protection against i.v. challenge. It was not studied whether passive transfer of these IgG antibodies also protected birds against aerosol challenge. It can be imagined that different results may have been obtained as IgA rather than IgG is the main immunoglobulin class of the mucosal lining of the respiratory tract. We reasoned that since serum antibodies of the IgG class are induced after aerosol challenge, and since these antibodies can enter the mucosa by diffusion (115), it was likely to assume that passive transfer of systemic antibodies can also protect birds against an aerosol challenge. However, this needs further investigation.

1.4 The involvement of other immune components

Even though we concluded that antibodies are a key component in cross-protection, our findings cover only the tip of the immunological iceberg. The mechanism via which these antibodies protect against infection remains to be defined, as well as the contribution of innate immune mechanisms and cell-mediated immunity. Such additional immunological knowledge can be very helpful for final vaccine formulation and delivery. For example, the innate immune response plays an important role in the initiation of adaptive immunity and it would be very interesting to analyze whether the adaptive immune response can be triggered or manipulated by the innate immunity, e.g. by stimulation of Toll-like receptors (TLRs). At this moment, 2 types of avian TLR have been identified, both with homology to human TLR2 (68). It is known that human and mammalian TLRs, which are expressed on many cell types including B lymphocytes and antigen presenting cells, can detect specific pathogen-associated molecular patterns (PAMPs) such as bacterial lipopolysaccharides, lipoproteins, unmethylated CpG motifs, (189). Cross-linking of TLRs by PAMPs can result in T-cell independent stimulation of B-cells (185). Furthermore, triggering of TLRs can lead to maturation of the APC, resulting in the upregulation of co-stimulatory molecules, MHC class II molecules, cytokine and chemokine production, and activation of humoral and cellular immunity (5, 116).

A tool to thoroughly analyze chicken immunology is the use of DNA micro-arrays, since mRNA levels are immense informative about cell state and activity of genes, and changes in gene expression are usually related to changes in protein level (123). DNA arrays of chicken immunological genes can help understand what genes are expressed and what immunological molecules are up- or down regulated during *O. rhinotracheale* infection and immunization. This work is now possible with the recent release of the chicken genome sequence.

2. The identification of cross-protective *O. rhinotracheale* vaccine candidates

Since antibodies are important mediators of cross-protective immunity, these components were ideal for the identification of protective antigens by means of screening an expression library. In the case that antibodies were not involved in protection, we also could have used sera to screen for antigens because T-cell and B-cell epitopes can be present on the same protein and, depending on the antigen delivery strategy, one can stimulate antibody-mediated or cellular immunity (116, 185). In this section, some aspects of the immunity-based selection and characterization of cross-protective vaccine candidate antigens will be discussed.

2.1 Live vaccination and antisera used for immunoscreening

Antisera for use in immunoscreening were produced through vaccination (or a mild challenge) of chickens with *O. rhinotracheale* serotype B, G, or M strains, followed by a heterologous challenge with *O. rhinotracheale* serotype A (chapter 3). The initial challenge involved aerosol administration as this resembles a natural infection. The heterologous challenge, to measure cross-protection, was administered either intravenously or by aerosol. By using this strategy we observed for the first time cross-protective immunity against *O. rhinotracheale* infection, which implies the presence of boosted cross-protective antibodies in the different sera.

Since a bacterin was believed not to provide high-level protection against heterologous serotypes (36), the results of the live vaccination study described above suggested that *O. rhinotracheale* reaches a certain niche in the bird essential for the induction of a cross-protective immune response, or that the cross-protective antigens are only (sufficiently) produced *in vivo* during infection. These observations opened the venue of the use of live a-virulent *O. rhinotracheale* vaccine strains as a new immunization strategy, or, alternatively, the use of defined recombinant vaccines containing (a) cross-protective antigen(s). As genetically engineering *O. rhinotracheale* is still very difficult and information on the genome and proteome are still fragmentary, the work described in thesis was focussed on the identification of the specific antigens that induce cross-protection.

2.2 Immunoscreening

The expression library used for identification of potential cross-protective vaccine antigens was constructed by using genomic DNA of *O. rhinotracheale* serotype G. The decision to use this serotype was based on the observation that live vaccination with serotype G induced the highest level of protection against a serotype A challenge (chapter 3).

Screening of the expression library was started with serum obtained from cross-protected birds, live vaccinated with an aerosol of *O. rhinotracheale* serotype G and challenged intravenously with serotype A (chapter 3). This allowed us to enrich the selection for antigens that induce a cross-protective immune response during infection. We used sera from i.v. challenged birds as these showed the highest antibody levels, even though aerosol

challenged birds showed a higher level of protection. We reasoned that until the day of challenge both groups were treated identical and should have identical antibody populations at the moment of infection when immunity should be active. Furthermore, sera from birds that encountered *O. rhinotracheale* via 2 different infection routes was also expected to contain boosted antibody levels against antigens which expression is not restricted to a certain infection route and niche within the host.

Rescreening of reactive clones was done using sera from birds live vaccinated with an aerosol of *O. rhinotracheale* serotype B, or M, and challenged intravenously with serotype A. Only the clones that showed reactivity with each of these sera were selected for further identification, clones that reacted with only B or M sera were neglected. Because birds live vaccinated with serotype B were protected against an aerosol but not against an i.v. challenge, we assumed that the cross-protective antibodies induced by live vaccination with serotype B were not boosted after i.v. challenge. This leaves the possibility that interesting clones with cross-protective potential were undetected by the serotype B serum and therefore excluded from our initial selection.

During immunoscreening we specifically selected for reactivity of IgG antibodies since this is the principal serum immunoglobulin class. Even though both IgA and IgM can fulfil an important role in mucosal defence of the respiratory tract (115) and therefore are expected to have specificity for virulence factors involved in the first phase of infection, we neglected the reactivity of these types of immunoglobulins. As described above we expected antibody class to be less important in protection against *O. rhinotracheale* infection than antibody specificity and therefore considered it no problem to screen solely for IgG reactivity.

2.3 Analysis of discovered antigens: bioinformatics and genetic diversity

Eight different antigens with cross-protective potential were selected: 2 lipoproteins: Or02 and Or77, and 3 hypothetical proteins: Or03, Or04, and Or98A, all 5 with unknown function, and 3 proteins for which a putative function could be predicted based on significant similarities with published protein sequences. Or01 showed similarity to the E2p component of the pyruvate dehydrogenase complex, Or11 with a putative outer membrane protein with OmpA-like domain, and Or98B with a protein with similarity to RecT protein (chapter 3 and 4).

We confirmed the presence of the 8 corresponding genes on the genome of *O. rhinotracheale* serotypes A, B, G, and M, and sequence analysis demonstrated that all cross-reactive antigens were highly conserved among these different serotypes (chapter 4). On Western blot, only 5 out of 8 antigens reacted with sera specific for their recombinant equivalent: no reactivity could be detected against *O. rhinotracheale* serotype G proteins Or03, Or98A and Or98B (chapter 4) probably caused by absence of *in vitro* expression. For *O. rhinotracheale* serotypes A, B, and M, we observed a variable reactivity among genes and serotypes: antigens Or01 and Or11 were detected for all 4 serotypes, whereas no reactivity was found against the serotype M antigens Or02, Or04, and Or77. Since genetic analysis revealed high protein conservation, we concluded a lack of *in vitro* expression to be the most logical explanation for this serotype-dependent reactivity. Future studies will have to determine what regulatory mechanism(s) is (are) involved in differential expression during

in vitro and *in vivo* growth. At this time, no promoter and other regulatory sequences have been identified for *O. rhinotracheale*.

2.4 Protective capacity of the antigens

Several approaches have been reported that allowed *in vitro* identification of antigens out of proteomic samples and expression libraries by using pathogen-specific antiserum (109, 131, 210). The uniqueness of our study consisted of the use of the natural host for *O. rhinotracheale* infection (the chicken) to screen for antigens and, at the same time, perform prophylactic studies.

Immunization of broiler chickens with a subunit vaccine containing the 8 different recombinant proteins induced protective immunity against both homologous and heterologous challenge, indicating the presence of cross-protective antigens within this pool (chapter 3). Immunization of broilers with single-component subunits demonstrated that all recombinant proteins were able to induce an antigen-specific antibody response. However, only recombinant Or77 induced cross-protection (chapter 4).

By combining different antigens in a 4-component vaccine, cross-protective immunity could be observed (chapter 4). Antigenic synergism between different antigens as was previously observed for different *Borrelia burgdorferi* antigens (83) can explain the enhanced immune response of the 4-component vaccines. Possibly, the selection of putative vaccine candidates includes (a) non-immunogenic antigen(s) with immunestimulatory properties. Furthermore, vaccination with multi-component vaccines might raise the total antibody-level above a certain threshold, needed for protection. However, additional studies have to be performed in order to analyse the relative contribution of each antigen in protection and to analyse which combination of antigens is the most effective. The generation of constructs that simultaneously express multiple antigens or antigen fusions can be very attractive for future vaccine design.

The vaccination studies also confirmed our hypothesis that antibody class is less important than antibody specificity in the protection against *O. rhinotracheale* infection. This conclusion was based on the observation that systemic antibodies induced by parenteral vaccination protected the birds against respiratory infection caused by aerosol challenge.

2.5 Localization of the antigens

We analyzed whether ineffective protection as was observed after immunization with the single protein vaccines (chapter 4) could be explained by the absence of the protein on the surface of the pathogen. Therefore, cellular fractionation studies were performed for 5 out of 8 proteins and this indicated that antigens Or02, Or04, Or11 and Or77 were predominantly present in the outer membrane and/or lipoprotein fractions, whereas Or01, although detected in the outer membrane fraction, was predominantly present in the cytoplasm/inner membrane of the cell. It should be noted that we did not quantify the concentrations of the antigens in the different fractions, and were unable to include markers for the different cellular fractions to determine their purity.

Even though antigens appeared to be located in the outer membrane of the cell, immune-fluorescence studies using antigen-specific or convalescent sera are necessary to verify surface-exposure of the antigen. A preliminary (unpublished) experiment already confirmed the exposure of the *O. rhinotracheale* serotype G Or77 antigen on the surface. This is consistent with the observation that recombinant Or77 can induce a protective immune response (chapter 4 and 5). However, additional studies are needed to analyze the cellular localization of the other 7 antigens, especially during *in vivo* growth.

2.6 Conformation of vaccine components

Another explanation for the absence of protection with the single subunits (chapter 4) could be the inability of the recombinant antigens to elicit antibodies that recognize the native antigen as expressed by the pathogen *in vivo*. The recombinant proteins used in our experiments were produced by an *E. coli* expression strain (chapter 3). Despite the presence of a PelB leader peptide cloned at the N-terminus of all recombinant proteins only Or77 was secreted into the supernatant of the *E. coli* growth medium and no additional purification steps were needed to isolate this antigen. Secreted Or77 was the only antigen that induced cross-protective immunity. This correlation between secretion and protection could be a co-incidence or be explained by other reasons.

Expression of the other recombinant antigens did result in the formation of inclusion bodies and recovery of proteins by dialysis resulted in the formation of new aggregates. However, as long as the protective epitopes are accessible for the immune system this aggregate formation does not necessarily have to affect the protective capacity of the recombinant protein. Since these recombinant antigens were not able to induce cross-protective immunity individually, incorrect folding of the proteins should be taken into account. Different expression and purification protocols that do not affect the final conformation of the recombinant protein should therefore be considered for use in the future.

It is also possible that the biological activity of the native *O. rhinotracheale* proteins depend on specific post-translational modifications such as glycosylation, phosphorylation and lipidation. Improper modification of the recombinant proteins might also have affected the immunogenicity of these antigens.

2.7 Vaccine potential of Or77

Since purified Or77 induced a strong cross-protective immune response (chapter 4), this antigen was considered to be the vaccine candidate with the highest potential. The *Or77* gene encoding a lipoprotein with unknown function is present and highly conserved among the different *O. rhinotracheale* serotypes (chapter 5). However, no homologues could be detected in other bacterial species. Western blot analysis revealed that this antigen was not expressed *in vitro* by *O. rhinotracheale* serotypes F, K, and M. This lack of reactivity was consistent with the observation that antibodies induced by Or77 immunization did protect against *O. rhinotracheale* serotype A, B, and G challenge but did not confer protection against *O. rhinotracheale* serotype M challenge, indicating that the Or77 antigen is not expressed by this serotype, neither *in vitro* nor *in vivo*.

In our experiments we did not analyze whether Or77 vaccination cross-protected against the other *O. rhinotracheale* serotypes. Based on the assumption that the observed Or77 expression profiles correlate with protection, antibodies induced by vaccination with Or77 would protect against infection with all but 3 *O. rhinotracheale* serotypes. Therefore, additional components are necessary for a vaccine to be effective against serotypes F, K, and M.

Analysis of the efficacy of Or77 subunit vaccination in comparison to bacterin vaccination and live vaccination demonstrated that the Or77 subunit vaccine protected birds against both homologous and heterologous challenge, whereas bacterin-G only conferred reduced pathology after homologous challenge (chapter 5). Even though we demonstrated that the Or77 antigen can be expressed by the *O. rhinotracheale* serotype G strain *in vitro* (chapter 4 and 5) and therefore expected it to be present in the bacterin vaccine, no cross-protection was induced. This could be explained by the presence of a relatively low concentration of Or77 in the bacterin vaccine in comparison to the antigen concentration of the Or77 subunit vaccine.

Analysis of the efficacy of the Or77 recombinant subunit vaccine in comparison to live vaccination revealed that the live strain induced the highest level of protection against challenge with a homologous serotype, and that only the Or77 subunit vaccine induced significant cross-protection (chapter 5). Remarkably, in this experiment no cross-protection could be observed in live vaccinated birds. These results are in contrast to observations from a previous study were live vaccination induced a high-level of cross-protective immunity (chapter 3) and sera from cross-protected birds were used to select cross-protective antigen Or77. Furthermore, live vaccination is expected to result in the expression of additional antigens that can increase the immunological response involved in cross-protection which are not present in the Or77 subunit vaccine. The reason for absence of cross-protection after live vaccination is not clear. However, serological analyses of the antisera obtained from this and different other vaccination and challenge studies could indicate the antigenicity and immunogenicity of Or77 and the other cross-reactive antigens during different types and stages of infection.

2.8 IVIAT

In chapter 6, *in vivo* induced antigen technology (IVIAT) was applied in the belief that identification of *in vivo* expressed (*ive*) antigens contributes to a better understanding of the pathogenesis of infection. Sera preadsorbed with *in vitro* grown *O. rhinotracheale,* and thus enriched with antibodies specific for *in vivo* expressed antigens, were used to probe for *ive* antigens. Preliminary characterization of the selected clones identified 7 different sequence groups, OR*ive*1 to OR*ive*7, of which 2 corresponded to previously identified antigens Or02 and Or98B, 1 showed similarity with the glycolytic enzyme GAPDH, and 4 out of 7 remained unidentified. Additional studies are needed to verify whether these sequences encode for the reactive antigens as selected during library screening.

Previous experiments (chapter 4) already revealed that the Or02 antigen is expressed by *O. rhinotracheale in vitro*, indicating that our IVIAT screening did not solely select for antigens specifically expressed *in vivo*. An explanation can be that the Or02-specific antibody titre in the serum used for screening was too high to be completely reduced during adsorption, which indicates the antigenic potential of this antigen. Antigen Or98B was already suggested

to be an *ive* antigen (chapter 4). However, for all selected antigens the specificity of *in vivo* expression has to be confirmed, for example, by immunohistology on histological sections of infected tissue using the antigen-specific antibodies.

In chapter 2 it was already demonstrated that different infection routes induced the production of antibodies reactive against different antigens. Immunoscreening also allowed us to study the effect of different infection routes on antigen expression (chapter 6). The selected IVIAT clones showed different reactivity when screened with sera obtained from birds infected via the aerosol route or by the intravenous route, indicating that the environment of the pathogen influences antigen expression. However, the data presented in this chapter are premature and future studies will have to be performed in order to draw conclusions concerning the role of these selected antigens during different phases of infection, e.g. after aerosol or i.v. challenge.

2.9 Role of genes or antigens in virulence

The role of the different genes or antigens in virulence also awaits further and more detailed investigation. Lipoproteins like Or02 (OR*ive*1) and Or77 can have a variety of functions involved in pathogenesis, such as adherence to host cells, transport of nutrients, and immune avoidance (i.e. adaptive variation of the antigenic membrane). The other antigens are, based on sequence similarities, predicted to be involved in cell's metabolism (Or01, OR*ive*4), membrane maintenance (Or11), and DNA repair and replication (Or98B (OR*ive*3)). Analysis of gene-specific mutant strains can indicate the role of the identified proteins in pathogenesis although genetic manipulation of *O. rhinotracheale* is still very difficult.

Whether the identified genes and their encoded products are specific for *O. rhinotracheale* also needs additional investigation. For several genes (chapter 3, 4, 6) no homologues were found using current databases. Genes and antigens identified specifically for *O. rhinotracheale* can be excellent targets for diagnostic purposes.

3. Delivery

An *O. rhinotracheale* vaccine for poultry should i) induce (cross-) protective humoral immunity that lasts as long as required, i.e. at least for weeks in broilers and for months in layers, breeders and turkeys, and ii) be easy to administer, preferentially by mass-application, and be safe and cheap. In vaccine design one should also consider the route of antigen administration, the type of antigen delivery system, and the type of antigen formulation.

3.1 The route of antigen administration and type of antigen delivery system

We repeatedly demonstrated that subcutaneous vaccination with Or77 induced cross-protective immunity (chapter 4 and 5). A benefit from using a recombinant subunit vaccine is that it is safe, well-defined, cheap in production, and relatively easy to administer. However, subunit vaccines are only useful in broilers when applicable for immunization at 1 day of age, since this is often the only moment that birds are handled.

Delivery of a non-replicating vaccine alone is mostly not sufficient to provide protection and therefore an immune-stimulatory or immune-modulatory formulation is required. During our studies we used an oil-in-water adjuvant for Or77 vaccine formulation. Even though we did not observe adverse reactions at the site of injection, the risk of inducing local reactions should always be reduced where possible. The data described in chapter 5 suggest that lipid modification of Or77 increases the immunogenicity of the antigen and the efficacy of the vaccine (chapter 5). As described above, the lipid moeiety of a lipoprotein can act as a ligand for Toll-like receptors, which play a central role in innate defence and might fulfil an important role in regulation and modulation of adaptive immunity (5, 189). However, additional studies are needed to analyze whether lipidated Or77 is immune-stimulatory, even without the presence of a potent adjuvant, and if increase of immunogenicity is a result of activation of TLRs. Additionally, increasing immunogenicity by protein lipidation should also be considered for the other selected antigens, especially for lipoprotein Or02, since this protein was cloned and expressed devoid of its lipoprotein signal sequence. Lack of lipid modification of the recombinant Or02 can affect the biological activity of the protein and eventually affect the protective capacity.

In search for a vaccination strategy suitable for mass application other than at day 1, one could also think of a replicating, live vaccine strain. Live, replicating vaccines do not require any adjuvant and can be administered by aerosol, course particle spray, food, or drinking water. Although we demonstrated that aerosol vaccination protected birds at the deep-mucosal level against respiratory infection (chapter 3), knowledge concerning mucosal immunity and cross-protection is still limited. Whether oral administration of a replicating vaccine induces mucosal immunity that protects birds at the respiratory level also awaits additional studies.

Even though we showed in chapter 3 that live vaccination with an aerosol of *O. rhinotracheale* can confer cross-protective immunity, the use of a live *O. rhinotracheale* vaccine strain is still challenging. Here the main problems are that knowledge concerning *O. rhinotracheale* infection is still limited and, as described in chapter 5, the efficacy of live vaccination is variable. Furthermore, it is difficult to genetically engineer this bacterium although a recently introduced transformation system that enables genetic modification of *O. rhinotracheale* can be of use in the future (33).

An alternative strategy for the delivery of Or77 is vector vaccination, whereby the protective antigen is expressed by *O. rhinotracheale* itself, or by a different bacterial or viral host, e.g. Salmonella (107), *E. coli* (137), Newcastle disease virus (96), or canarypox virus (148) which is used as a delivery system. An additional benefit of using such expression systems is the ability i) to induce immunity against both the host used for delivery and the pathogen from which the expressed antigen is derived, ii) to express and deliver fusions of antigens derived from single or multiple pathogenic species, and iii) to co-express and deliver immune-stimulatory molecules such as cytokines.

General conclusion

The results presented in this thesis demonstrate the feasibility of an immunity-based 3-step approach for vaccine development. Evidence is presented that i) antibodies are the key components of the chicken protective response to *O. rhinotracheale* infection, ii) putative vaccine antigens can be successfully identified by using the mediators of protection, and iii) different vaccination strategies can be evaluated with respect to applicability and suitability. In addition, the work described in this thesis provides a solid basis for the further development and production of a cross-protective *O. rhinotracheale* vaccine.

The benefit of using *O. rhinotracheale* infections in chicken as a model for strategic vaccine development was the ability to study the natural host-pathogen interaction. Unfortunately, this privilege does not account for all host-pathogen interactions since the performed immunological studies of immune depletion and reconstitution are technically more difficult or ethically impossible for large animals or humans. This also accounts for the different protection studies. However, the use of sera from immune or protected individuals is certainly a powerful tool in the screening for potential protective antigens of various pathogens.

CHAPTER
EIGHT

Dutch Summary
Nederlandse Samenvatting

D.F. Schuijffel

Intervet International BV, Bacteriology R&D, Boxmeer, The Netherlands

Mens en dier staan continue bloot aan een scala van micro-organismen waaronder pathogene bacteriën, virussen en parasieten. Daarom is het van groot belang dat er geschikte therapieën beschikbaar zijn ter bestrijding of voorkoming van infectieziekten veroorzaakt door deze micro-organismen. Voor de behandeling van bacteriële infecties is het gebruik van antibiotica vaak succesvol, maar antibiotica geven geen bescherming tegen een nieuwe besmetting met hetzelfde pathogen. Verder kan veelvuldig gebruik van antibiotica resulteren in resistentie. Een goed alternatief voor het gebruik van antibiotica is vaccinatie, waarbij het immuunsysteem van de gastheer wordt aangezet tot productie van cellen en moleculen welke bescherming bieden tegen microbiële indringers.

Ornithobacterium rhinotracheale is een bacterie die luchtweginfecties kan veroorzaken bij pluimvee, maar ook systemische infecties zoals gewrichts-, lever- en hersenvliesontsteking tot gevolg kan hebben. *O. rhinotracheale* infecties kunnen aanzienlijke schade aan de pluimvee industrie toebrengen. Bovendien wordt de bacterie in toenemende mate resistent tegen antimicrobiële middelen. Pluimveehouders zijn dan ook steeds meer afhankelijk van de beschikbaarheid van veilige en effectieve vaccins. De huidige bacterinvaccins, welke zijn gebaseerd op de afgedode bacterie, hebben bewezen effectief te zijn tegen infectie met een homoloog serotype. Op dit moment zijn er echter meer dan vijftien verschillende serotypen beschreven en daarom is het van belang een *O. rhinotracheale* vaccin te ontwikkelen dat een brede kruisbescherming kan bieden tegen infectie met meerdere serotypen.

Een overzicht van de verschillende aspecten van vaccinontwikkeling en een samenvatting van bacteriële respiratoire infecties in pluimvee, met in het bijzonder *O. rhinotracheale* infectie, worden gegeven in **Hoofdstuk 1**. Dit hoofdstuk wordt afgesloten met de doelstelling van het onderzoek zoals beschreven in dit proefschrift: de identificatie van antigenen welke geschikt zijn als kandidaat voor een nieuw, kruisbeschermend *O. rhinotracheale* vaccin. In het onderzoek is gebruik gemaakt van een strategische aanpak, ervan uitgaande dat drie factoren van groot belang zijn bij de ontwikkeling van een succesvol vaccin: i) inzicht in de immunologie van de infectie, ii) de identificatie van kruisbeschermende antigenen, en iii) de manier waarop deze antigenen toegediend worden aan de gastheer.

Het onderzoek is begonnen met de analyse van de bijdrage van de immunologische afweer van de kip tijdens een infectie met *O. rhinotracheale*. In **Hoofdstuk 2** staat beschreven dat een methode van selectieve depletie en reconstitutie van het antilichaam-gemedieerde immuunsysteem is toegepast om aan te tonen dat i) dieren zonder humoraal immuunsysteem na challenge met *O rhinotracheale* aanzienlijk hogere pathologiescores hebben in vergelijking tot immuun-competente dieren, ii) toediening van serum afkomstig van *O. rhinotracheale*-geïnfecteerde dieren aan immuun-deficiënte dieren bescherming biedt tegen een *O. rhinotracheale* challenge, iii) antilichaam-gemedieerde bescherming tegen *O. rhinotracheale* specifiek is; toediening van sera afkomstig van *Pasteurella multocida*-geïnfecteerde dieren of ongeïnfecteerde dieren biedt geen bescherming tegen *O. rhinotracheale* challenge, en iv) toediening van *O. rhinotracheale* serotype A antilichamen bescherming geeft tegen een *O. rhinotracheale* serotype G challenge en dus dat antilichamen betrokken zijn bij kruisbeschermende immuniteit.

De volgende stap in de strategische aanpak was de identificatie van kruisbeschermende *O. rhinotracheale* antigenen (**Hoofdstuk 3**). Omdat aangetoond was dat antilichamen een

belangrijke rol zouden kunnen spelen bij kruisbescherming, zijn antisera gebruikt voor de immunoscreening van een genomische expressiebank van *O. rhinotracheale* serotype G. De selectie van potentieel kruisbeschermende antigenen vond plaats met behulp van verschillende sera afkomstig van dieren die na levend vaccinatie met serotype B, G, of M beschermd waren tegen een serotype A challenge. Het voordeel van deze sera is dat ze antilichamen bevatten tegen antigenen die tot expressie komen tijdens een infectie en in staat zijn een kruisbeschermende immuunrespons te induceren. De screening resulteerde in acht verschillende kruisreagerende antigenen: twee lipoproteinen: Or02 en Or77, en drie hypothetische eiwitten: Or03, Or04, en Or98A, alle vijf met een onbekende functie, en drie eiwitten waarvoor op basis van homologieën met andere genen een mogelijke functie toegeschreven kan worden: Or01: een enzym betrokken bij cel metabolisme, Or11: een buiten membraan eiwit betrokken bij onderhoud van de bacteriële cel, en Or98B: een eiwit betrokken bij DNA recombinatie.

Hoewel de acht antigenen geselecteerd werden op basis van kruisreactiviteit met verschillende sera van beschermde dieren was de kruisbeschermende capaciteit van deze antigenen nog niet bevestigd. De genen coderend voor deze eiwitten werden daarom gekloneerd in een expressievector, en tot expressie gebracht in *Escherichia coli*. Kippen gevaccineerd met een mix van deze acht gezuiverde recombinante eiwitten waren beschermd tegen een serotype A challenge. Deze beschermende immuniteit was het bewijs van de aanwezigheid van een of meerdere kruisbeschermende antigenen in het vaccin.

In **Hoofdstuk 4** wordt beschreven dat vaccinatie van dieren met de afzonderlijke recombinante antigenen een specifieke antilichaamrespons induceerde. Echter, alleen de dieren gevaccineerd met Or77 bleken kruisbeschermd te zijn tegen een serotype A challenge. Opvallend was de observatie dat vaccinatie met een mix van vier recombinante eiwitten anders dan Or77 een goede kruisbeschermende immuunrespons induceerde. Synergie tussen de verschillende antigenen en/of immuunstimulatoire eigenschappen van antigenen worden gegeven als een mogelijke verklaring voor deze bescherming.

Tevens wordt in dit hoofdstuk een beschrijving gegeven van verschillende factoren die een rol zouden kunnen spelen bij het ontstaan van immuniteit of juist de afwezigheid ervan. Zo is het voor een goede humorale bescherming van belang dat de antilichamen geïnduceerd door het vaccin het natieve eiwit herkennen zoals het door de bacterie in de gastheer tot expressie wordt gebracht. Genen coderend voor de verschillende eiwitten zijn soms in hoge mate geconserveerd aanwezig op genniveau, terwijl op eiwitniveau de expressie wisselend kan zijn. Zo bleek tijdens *in vitro* studies dat antigenen Or03, Or98A en Or98B niet tot expressie kwamen. Analyse van verschillende fracties van de *O. rhinotracheale* cel heeft aangetoond dat de geteste antigenen Or01, Or02, Or04, Or11, en Or77 in meer of mindere mate in de buitenmembraan fractie van de bacterie zijn gelokaliseerd. De aanwezigheid van het beschermende antigene epitoop op het celoppervlak, en daarmee de toegankelijkheid voor beschermende antilichamen, is echter nog niet bewezen, maar lijkt een belangrijke voorwaarde voor werkzaamheid van een vaccin gebaseerd op dergelijke antigenen. Afwezigheid van post-translationele modificatie van de recombinante eiwitten en conformationele verschillen tussen het recombinante en het natieve eiwit kunnen ook van invloed zijn geweest op afwezigheid van bescherming. Opvallend was dat het beschermende eiwit Or77 het enige recombinante eiwit was wat gesecreteerd werd door *E. coli.* Daardoor

was dit antigen gemakkelijk te isoleren, zonder additionele zuiveringsstappen welke de "natieve" conformatie van het eiwit kunnen beïnvloeden.

In **Hoofdstuk 5** van dit proefschrift wordt het accent gelegd op een nadere analyse van lipoprotein Or77 met betrekking tot kruisbescherming. Allereerst is de *in vitro* expressie van *O. rhinotracheale* lipoprotein Or77 onderzocht bij de verschillende serotypes. Ook de genetische diversiteit van het open reading frame (ORF) coderend voor Or77 werd geanalyseerd. Uit deze experimenten is gebleken dat slechts drie *O. rhinotracheale* serotypes, namelijk F, K, en M, het eiwit niet tot expressie brengen, ondanks het feit dat het Or77 gen aanwezig is en in hoge mate geconserveerd is onder alle serotypes. Afwezigheid van Or77 expressie door serotype M was consistent met de observatie dat vaccinatie met het recombinante eiwit geen bescherming bood tegen een infectie met dit serotype. Tevens werd er aangetoond dat vaccinatie met recombinant Or77 resulteerde in een reductie in pathologie na challenge met *O. rhinotracheale* serotype A, B en G, drie van de veertien serotypes welke het Or77 antigen tot expressie brengen.

In ditzelfde hoofdstuk werd aangetoond dat in vergelijking met vaccinatie met een bacterin of een levende, wildtype stam het Or77 subunit vaccin de hoogste mate van kruisbescherming gaf. Een voordeel van een recombinant subunit vaccin is dat de vaccincomponenten zijn gedefinieerd in tegenstelling tot een bacterin of levend vaccin. Een nadeel is dat de effectiviteit van een niet-replicerend vaccin afhankelijk is van een bepaalde mate van immuunstimulatie of immuunmodificatie. De resultaten van een eerste studie hebben aangetoond dat de immunogeniteit van het Or77 vaccineiwit mogelijk wordt vergroot door middel van lipidering. De natuurlijke adjuverende werking van een gelipideerd vaccineiwit kan de toevoeging van een adjuvant overbodig maken.

In de zoektocht naar potentiële vaccin kandidaten en ter vergroting van de inzicht in de pathogenese van *O. rhinotracheale* infectie is tevens gebruik gemaakt van *in vivo*-induced antigen technology (IVIAT) (**Hoofdstuk 6**). Door middel van screening van een expressiebank met behulp van antisera die gepreadsorbeerd waren met *in vitro* gegroeide *O. rhinotracheale*, is getracht om antigenen te selecteren die specifiek *in vivo* - dus tijdens infectie - tot expressie worden gebracht. Een eerste analyse van de geselecteerde kloons resulteerde in de identificatie van zeven verschillende groepen. Van deze groepen correspondeerden er twee met eerder geselecteerde antigenen Or02 en Or98B, één groep toonde op basis van DNA sequentie overeenkomsten met een enzym betrokken bij cel metabolisme, en vier van de zeven bleven ongeïdentificeerd. Aangezien Or02 in eerdere hoofdstukken al had bewezen *in vitro* tot expressie gebracht te worden dient de specificiteit van *in vivo* expressie van de geselecteerde kloons nog bevestigd te worden. Tevens werd er in **Hoofdstuk 6** aangetoond dat sera afkomstig van dieren geïnfecteerd met *O. rhinotracheale* via de aerosol of intraveneuze route met verschillende IVIAT-kloons reageert wat indirect bevestigt dat het milieu in de gastheer van invloed is op de antigen expressie van *O. rhinotracheale* en dus op het pathogeen-specifieke antilichaam profiel.

In **Hoofdstuk 7** worden de resultaten zoals beschreven in dit proefschrift samengevat en bediscussieerd. Dit onderzoek kan worden beschouwd als een belangrijke stap in de ontwikkeling van een succesvol kruisbeschermend *O. rhinotracheale* vaccin. Toekomstig onderzoek zal vooral gericht zijn op optimalisatie van antigeenpresentatie aan het immuunsysteem van de gastheer. De effectiviteit van de strategische aanpak voor antigen

identificatie met betrekking tot vaccinontwikkeling is bewezen. De bruikbaarheid van deze zo succesvolle aanpak voor vaccinontwikkeling bij andere pathogenen zal echter sterk afhangen van het type gastheer of de aanwezigheid van een geschikt diermodel.

References

1. **Abu Kwaik, Y., and L.L. Pederson.** 1996. The use of differential display-PCR to isolate and characterize a *Legionella pneumophila* locus induced during the intracellular infection of macrophages. Mol. Microbiol. **21**: 543-556.
2. **Ada, G.** 1997. Overview of vaccines. Mol. Biotechnol. **8**:123-134.
3. **Ahl, A.S., and B. Buntain.** 1997. Risk and the food safety chain: animal health, public health and the environment. Rev. Sci. Tech. **16**:322-330.
4. **Ahmed, R., and D. Gray.** 1996. Immunological memory and protective immunity: understanding their relation. Science **272**:54-60.
5. **Akira, S., K. Takeda, and T. Kaisho.** 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol. **2**:675-680.
6. **Ala'Aldeen, D.A.A., A.H. Westphal, A. de Kok, V. Weston, M.S. Atta, T.J. Baldwin, J. Bartley, and S.P. Borriello.** 1996. Cloning, sequencing, characterization and implications for vaccine design of the novel dihydrolipoyl acetyltransferase of *Neisseria meningitides*. J. Med. Microbiol. **45**:419-432.
7. **Allan, E., and B.W. Wren.** 2003. Genes to genetic immunization: identification of bacterial vaccine candidates. Methods **31**:193-198.
8. **Altschul, S.F., T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D.J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**:3389-3402.
9. **Amonsin, A., J.F. Wellehan, L.L. Li, P. Vandamme, C. Lindeman, M. Edman, R.A. Robinson, and V. Kapur.** 1997. Molecular epidemiology of *Ornithobacterium rhinotracheale*. J. Clin. Microbiol. **35**:2894-2988.
10. **Ariel, N., A. Zvi, K.S. Makarova, T. Chitlaru, E. Elhanany, B. Velan, S. Cohen, A.M. Friedlander, and A. Shafferman.** 2003. Genome-based bioinformatic selection of chromosomal *Bacillus anthracis* putative vaccine candidates coupled with proteomic identification of surface-associated antigens. Infect. Immun. **71**:4563-4579.
11. **Arnold, J.W., and P.S. Holt.** 1995. Response to *Salmonella enteritidis* infection by the immunocompromised avian host. Poultry Sci. **74:**656-665.
12. **Babiuk, L.A., S. Gomis, and R. Hecker.** 2003. Molecular approaches to disease control. Poultry Sci. **82**:870-875.
13. **Back, A., D. Halvorson, G. Rajashekara, and K.V. Nagaraja.** 1998. Development of a serum plate agglutination test to detect antibodies to *Ornithobacterium rhinotracheale*. J. Vet. Diagn. Invest. **10**:84-86.
14. **Badovinac, V.P., K.A. Nordyke Messingham, S.E. Hamilton, and J.T. Harty.** 2003. Regulation of CD8+ T-cells undergoing primary and secondary responses to infection in the same host. J. Immunol. 4933-4942.
15. **Barnes, H.J., J.P. Vaillancourt, and W.B. Gross.** 2003. Colibacillosis. p. 631-652. *In* Y.M. Saif, H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald, and D.E. Swayne (ed.), Poultry Diseases, 11th ed. Iowa State Press USA.
16. **Barrow, P.A.** 1997. Novel approaches to control of bacterial infections in animals. Acta Vet. Hung. **45**:317-329.
17. **Barry, M.A., W.C. Lai, and S.A. Johnston.** 1995. Protection against Mycoplasma infection using expression library immunization. Nature **377**:632-635.

18. **Bergmann, S., M. Rohde, and S. Hammerschmidt.** 2004. Glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus pneumoniae* is a surface-displayed plasminogen-binding protein. Infect.Immun. **72**:2416-2419.
19. **Bessler, W.G., L. Heinevetter, K-H. Wiesmüller, G. Jung, W. Baier, M. Huber, A.R. Lorenz, U.V.D. Esche, K. Mittenbühler, and P. Hoffmann.** 1997. Bacterial cell wall components as immunomodulators -I. Lipopeptides as adjuvants for parenteral and oral immunization. Int. J. Immunopharmac. **19**:547-550.
20. **Black, P.N., and C.C. DiRusso.** 2003. Transmembrane movement of exogenous long-chain fatty acids: proteins, enzymes, and vectorial esterification. Microbiol. Mol. Biol. R. **67**:454-472.
21. **Blackall, P.J.** 1995. Vaccines against infectious coryza. World's Poultry Sci. J. **51**:17-26.
22. **Blackall, P.J., and M. Matsumoto.** 2003. Infectious Coryza, p.691-703. *In* Y.M. Saif, H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald, and D.E. Swayne (ed.), Poultry Diseases, 11th ed. Iowa State Press USA.
23. **Bolduc, G.R., V. Bouchet, R-Z. Jiang, J. Geisselsoder, Q.C. Truong-Bolduc, P.A. Rice, S.I. Pelton, and R. Goldstein.** 2000. Variability of outer membrane protein P1 and its evaluation as a vaccine candidate against experimental otitis media due to nontypable *Haemophilus influenzae*: an unambiguous, multifaceted approach. Infect. Immun. **68**:4505-4517.
24. **Bordier, C.** 1981. Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem. **256**:1604-1607.
25. **Braun, V., and H.C. Wu.** 1994. Lipoprotein, structure, function, biosynthesis and model for protein export. In: New Comprehensive Biochemistry, Bacterial Cell Wall (Eds Ghuysen, J.-M., and R. Hakenbeck) Elsevier Science, Amsterdam, vol **27**, pp. 319-341.
26. **Broder, S., and J.C. Venter.** 2000. Whole genomes: the foundation of new biology and medicine. Curr. Opin. Biotech. **11**:581-585.
27. **Brodeur, B.R., M. Boyer, I. Charlebois, J. Hamel, F. Couture, C.R. Rioux, and D. Martin.** 2000. Identification of group B Streptococcal sip protein which elicits cross-protective immunity. Infect. Immun. **68:** 5610-5618.
28. **Buys, S.** 1996. *Ornithobacterium rhinotracheale* and emerging disease in South Africa. Aerosols, Newsletter of the World Vet. Poul. Assoc. p.8-10.
29. **Cash, P.** 2003. Proteomics of bacterial pathogens. Adv. Biochem. Eng. Biotechnol. **83**:93-115.
30. **Cauwerts, K., P. de Herdt, F. Haesebrouck, J. Vervloesem, and R. Ducatelle.** 2002. The effect of *Ornithobacterium rhinotracheale* vaccination of broiler breeder chickens on the performance of their progeny. Avian Pathol. **31**:619-624.
31. **Chakravarti, D.N., M.J. Fiske, L.D. Fletcher, and R.J. Zagutsky.** 2001. Application of genomics and proteomics for identification of bacterial gene products as potential vaccine candidates. Vaccine **19**:601-612.
32. **Chambaud, I., H. Wroblewski, and A. Blanchard.** 1999. Interactions between mycoplasma lipoproteins and the host immune system. Trends Microbiol. **7**:493-499.
33. **Chansiripornchai, N.** 2004. Molecular interaction of *Ornithobacterium rhinotracheale* with eukaryotic cells. Ph.D. thesis. Utrecht University, The Netherlands.
34. **Charlton, B.R., S.E. Channing-Santiago, A.A. Bickford, C.J. Cardona, R.P. Chin, G.L. Cooper, R. Droual, J.S. Jeffrey, C.U. Meteyer, H.L. Shivaprasad, and R.L. Walker.** 1993. Preliminary characterization of a pleomorphic gram-negative rod associated with avian respiratory disease. J. Vet. Diagn. Invest. **5**:47-51.

35. **Chen, L.M., K. Kaniga, and J.E. Galan.** 1996. Salmonella spp. are cytotoxic for cultured macrophages. Mol. Microbiol. **21**:1101-1115.
36. **Chin, R.P., P.C.M. van Empel, and H.M. Hafez.** 2003. *Ornithobacterium rhinotracheale* infection, p.683-690. *In* Y.M. Saif, H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald, and D.E. Swayne (ed.), Poultry Diseases, 11th ed. Iowa State Press USA.
37. **Cockle, P.J., S.V. Gordon, A. Lalvani, B.M. Buddle, R.G. Hewinson, and H.M. Vordermeier.** 2002. Identification of novel *Mycobacterium tuberculosis* antigens with potential as diagnostic reagents or subunit vaccine candidates by comparative genomics. Infect. Immun. **70**:6996-7003.
38. **Cole, L.E., K.L. Toffer, R.A. Fulcher, L.R. San Mateo, P.E. Orndorff, and T.H. Kawula.** 2003. A humoral immune response confers protection against *Haemophilus ducreyi* infection. Infect. Immun. **71**:6971-6977.
39. **Confer, A.W.** 1993. Immunogens of Pasteurella. Vet. Microbiol. **37**:353-368.
40. **Connell, N., C.K. Stover, and W.R. Jacobs Jr.** 1992. Old microbes with new faces: molecular biology and the design of new vaccines. Curr. Opin. Immunol. **4**:442-448.
41. **Corrier, D.E., M.H. Elissalde, R.L. Ziprin, and J.R. DeLoach.** 1991. Effect of immunosuppression with cyclophosphamide, cyclosporing, or dezamethasone on Salmonella colonization of broiler chicks. Avian Dis. **35:**40-45.
42. **Cserep, T.** 2001. Vaccines and vaccination. p.55-70. *In* F. Jordan, M. Pattison, D. Alexander, and T. Faragher (ed.), Poultry Diseases, 5th ed. W.B. Saunders, USA.
43. **Davies, R.L., R. MacCorquodale, and B. Caffrey.** 2003. Diversity of avian *Pasteurella multocida* strains based on capsular PCR typing and variation of the OmpA and OmpH outer membrane proteins. Vet. Microbiol. **91**:169-182.
44. **De, B.K., J.S. Sampson, E.W. Ades, R.C. Huebner, D.L. Jue, S.E. Johnson, M. Espina, A.R. Stinson, D.E. Briles, and G.M. Carlone.** 2000. Purification and characterization of *Streptococcus pneumoniae* palmitoylated pneumococcal surface adhesin A expressed in *Escherichia coli*. Vaccine **18**: 1811-1821.
45. **Deb, D.K., P. Dahiya, K.K. Srivastava, R. Srivastava, and B.S. Srivastava.** 2002. Selective identification of new therapeutic targets of *Mycobacterium tuberculosis* by IVIAT approach. Tuberculosis **82**: 175-182.
46. **De Bernardez Clark, E.** 2001. Protein refolding for industrial processes. Curr. Opin. Biotech. **12:** 202-207.
47. **Degen, W.G., T. Jansen, and V.E. Schijns.** 2003. Vaccine adjuvant technology: from mechanistic concepts to practical applications. Expert Rev. Vaccines **2**:327-335.
48. **De Kok, A., A.F. Hengeveld, A. Martin, and A.H. Westphal.** 1998. The pyruvate dehydrogenase multi-enzyme complex from Gram-negative bacteria. Biochem. Bioph. Acta **1385**:353-366.
49. **Del Giudice, G.** 2003. Vaccination strategies: an overview. Vaccine **21**:83-88.
50. **De Rosa, M., R. Droual, R.P. Chin, and H.L. Shivaprasad.** 1997. Interaction of *Ornithobacterium rhinotracheale* and *Bordetella avium* in turkey poults. Proceedings 46th Western Poultry Disease Conference, 52-53.
51. **De Rosa, M., R. Droual, R.P. Chin, H.L. Shivaprasad, and R.L. Walker.** 1996. *Ornithobacterium rhinotracheale* infection in turkey breeders. Avian Dis. **40**:865-874.
52. **Devriese, L.A., P. de Herdt, and F. Haesebrouck.** 2001. Antibiotic sensitivity and resistance in *Ornithobacterium rhinotracheale* strains from Belgian broiler chickens. Avian Path. **30**:197-200.

53. **Devriese, L.A., J. Hommez, P. Vandamme, K. Kersters, and F. Haesebrouck.** 1995. *In vitro* antibiotic sensitivity of *Ornithobacterium rhinotracheale* strains from poultry and wild birds. Vet. Rec. **137**:435-436.
54. **Dho-Moulin, M., and J.M. Fairbrother.** 1999. Avian pathogenic *Escherichia coli* (APEC). Veterinary Research **30**: 299-316.
55. **Donoghue, D.J.** 2003. Antibiotic residues in poultry tissues and eggs: human health concerns. Poultry Sci. **82**:618-621.
56. **El-Gohary, A.A.** 1998. *Ornithobacterium rhinotracheale* (ORT) associated with hatching problems in chicken and turkey eggs. Vet. Med. J. Giza **46**:183-191.
57. **El-Gohary, A., and M.H.H. Awaad.** 1998. Concomitant *Ornithobacterium rhinotracheale* (ORT) and *Escherichia coli* infection in chicken broilers. Vet. Med. J. Giza **45**:67-75.
58. **Erdile, L.F., M.-A. Brandt, D.J. Warakomski, G.J. Westrack, A. Sadziene, A.G. Barbour, and J.P. Mays.** 1993. Role of attached lipid in immunogenicity of *Borrelia burgdorferi* OspA. Infect. Immun. **61**: 81-90.
59. **Erdile, L.F., and Guy, B.** 1997. OspA lipoprotein of *Borrelia burgdorferi* is a mucosal immunogen and adjuvant. Vaccine **15**: 988-996.
60. **Erf, G.F.** 2004. Cell-mediated immunity in poultry. Poultry Sci. **83**:580-590.
61. **Everest, P., P. Griffiths, and G. Dougan.** 1995. Live *Salmonella* vaccines as a route towards oral immunisation. Biologicals **23**:119-124.
62. **Finlay, B.B., and S. Falkow.** 1997. Common themes in microbial pathogenicity. Microbiol. Rev. **53**:210-230.
63. **FitzGerald, D., and R.J. Mrsny.** 2000. New approaches to antigen delivery. Crit. Rev. Ther. Drug Carr. Sys. **17**:165-248.
64. **Fleischer, B.** 1980. Effector cells in avian spontaneous and antibody-dependent cell-mediated cytotoxicity. J. Immunol. **125**: 1161-1166.
65. **Flower, D.R.** 2003. Databases and data mining for computational vaccinology. Curr. Opin. Drug Discov. Devel. **6**:396-400.
66. **Flower, D.R., and I.A. Doytchinova.** 2002. Immunoinformatics and the prediction of immunogenicity. Appl. Bioinformatics **1**:167-176.
67. **Forbes, N.A.** 1998. Avian respiratory disease. Vet. Quart. **20**:S67-S69.
68. **Fukui, A., N. Inoue, M. Matsumoto, M. Nomure, K. Yamada, Y. Matsuda, K. Toyoshima, and T. Seya.** 2001. Molecular cloning and functional characterization of chicken Toll-like receptors. J. Biol. Chem. **276**:47143-47149.
69. **Fulton, R.M., W.M. Reed, H.L. Thacker, and D.B. DeNicola.** 1996. Cyclophosphamide (Cytoxan)-induced hematologic alterations in specific-pathogen-free chickens. Avian Dis. **40**:1-12.
70. **Fussey, S.P.M., S.T. Ali, J.R. Guest, O.F.W. James, M.F. Bassendine, and S.J. Yeaman.** 1990. Reactivity of primary biliary cirrhosis sera with *Escherichia coli* dihydrolipoamide acetyltransferase (E2p): characterization of the main immunogenic region. Proc. Natl. Acad. Sci. **87**:3987-3991.
71. **Gentschev, I., G. Dietrich, S. Spreng, A. Kolb-Maurer, J. Daniels, J. Hess, S.H.E. Kaufmann, and W. Goebel.** 2000. Delivery of protein antigens and DNA by virulence-attenuated strains of *Salmonella typhimurium* and *Listeria monocytogenes*. J. Biotechnol. **83**:19-26.
72. **Glick, B., and I. Olah.** 1984. Methods of bursectomy. Method. Enzymol. **108:**3-10.
73. **Glisson, J.** 1998. Bacterial respiratory diseases of poultry. Poultry Sci. **77**:1139-1142.

74. **Glisson, J.R., C.L. Hofacre, and J.P. Christensen.** 2003. Fowl Cholera, p.658-676. *In* Y.M. Saif, H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald, and D.E. Swayne (ed.), Poultry Diseases, 11th ed. Iowa State Press USA.

75. **Goovaerts, D., M. Vrijenhoek, and P. van Empel.** 1998. Immuno-histochemical and bacteriological investigation of the pathogenesis of *Ornithobacterium rhinotracheale* infection in South Africa in chickens with osteitis and encephalitis syndrome. Proc. 16th meeting of the Euro. Soc. of Vet. Path. Lillehammer p. 81.

76. **Goovaerts, D., M. Vrijenhoek, and P. van Empel.** 1999. Immuno-histochemical and bacteriological investigation of the pathogenesis of *Ornithobacterium rhinotracheale* infection in chickens with osteitis and encephalitis syndrome. Proc. 48th West. Poul. Dis. Conf. 79.

77. **Gross, W.B.** 1994. Diseases due to Escherichia coli in poultry, p.237-259. In C.L. Gyles (ed.), Escherichia coli in domestic animals and man. CAB International, Wallingford, United Kingdom.

78. **Hafez, H.M.** 1996. Current status on the role of *Ornithobacterium rhinotracheale* in respiratory disease complexes in poultry. Arch. Geflügelk. **61**:208-211.

79. **Hafez, H.M., and R. Sting.** 1999. Investigations on different *Ornithobacterium rhinotracheale* "ORT" isolates. Avian Dis. **43**:1-7.

80. **Handfield, M., L.J. Brady, A. Progulske-Fox, and J.D. Hillman.** 2000. IVIAT: a novel method to identify microbial genes expressed specifically during human infections. Trends Microbiol. **8**: 336-339.

81. **Handfield, M., and R.C. Levesque.** 1999. Strategies for isolation of *in vivo* expressed genes from bacteria. FEMS microbiology reviews **23**: 69-91.

82. **Hang, L., M. John, M. Asaduzzaman, E.A. Bridges, C. Vanderspurt, T.J. Kirn, R.K. Taylor, J.D. Hillman, A. Progulske-Fox, M. Handfield, E.T. Ryan, and S.B. Calderwood.** 2003. Use of *in vivo*-induced antigen technology (IVIAT) to identify genes uniquely expressed during human infection with *Vibrio cholerae.* Proc. Natl. Acad. Sci. **100**: 8508-8513.

83. **Hanson, M.S., N.K. Patel, D.R. Cassatt, and N.D. Ulbrandt.** 2000. Evidence for vaccine synergy between *Borrelia burgdorferi* decorin binding protein A and outer surface protein A in the mouse model of lyme borreliosis. Infect. Immun. **68**:6457-6460.

84. **Hasan, U.A., A.M. Abai, D.R. Harper, B.W. Wren, and W.J.W Morrow.** 1999. Nucleic acid immunization: concepts and techniques associated with third generation vaccines. J. Immunol. Meth. **229**:1-22.

85. **Hautefort, I., and J.C.D. Hinton.** 2000. Measurement of bacterial gene expression *in vivo*. Phil. Trans. R. Soc. Lond. **355**: 601-611.

86. **Heddleston, K.L., J.E. Gallagher, and P.A. Rebers.** 1972. Fowl cholera: gel diffusion precipitin test for serotyping Pasteurella multocida from avian species. Avian Dis. **16**:925-936.

87. **Heithoff, D.M., C.P. Conner, and M.J. Mahan.** 1997. Dissecting the biology of a pathogen during infection. Trends Microbiol. **5:** 509-513.

88. **Heithoff, D.M., R.L. Sinsheimer, D.A. Low, and M.J. Mahan.** 2000. *In vivo* gene expression and the adaptive response: from pathogenesis to vaccines and antimicrobials. Phil. Trans. R. Soc. Lond. **355**: 633-642.

89. **Hemila, H., A. Palva, L. Paulin, S. Arvidson, and I. Palva.** 1990. Secretory S complex of *Bacillus subtili*: sequence analysis and identity to pyruvate dehydrogenase. J. Bacteriol. **172**:5052-5063.

90. **Hensel, M., J.E. Shea, C. Gleeson, M.D. Jones, E. Dalton, and D.W. Holden.** 1995. Simultaneous identification of bacterial virulence genes by negative selection. Science **269**: 400-403.
91. **Higgins, D.A., and G.W. Warr** (eds.) 2000. The avian immune response to infectious diseases. Special issue. Dev. Comp. Immunol. **24**: 85-101.
92. **Hinz, K.H., C. Blome, and M. Ryll.** 1994. Acute exudative pneumonia and airsacculitis associated with *Ornithobacterium rhinotracheale* in turkeys. Vet. Rec. **135**:233-234.
93. **Hinz, K-H., and H.M. Hafez.** 1997. The early history of *Ornithobacterium rhinotracheale* (ORT). Arch. Geflügelk. **61**:95-96.
94. **Hocking, D., E. Webb, F. Radcliff, L. Rothel, S. Taylor, G. Pinczower, C. kapouleas, H. Braley, A. Lee, and C. Doidge.** 1999. Isolation of recombinant protective *Helicobacter pylori* antigens. Infect. Immun. **67:** 4713-4719.
95. **Honjo K., and Y. Hirota.** 1993. Immunohistochemical investigations of lymphocytes in the lymphoid organs of cyclophosphamide-treated chickens. J. Vet. Med. Sci. **55**:896-897.
96. **Huang, Z., S. Elankumaran, A. Panda, and S.K. Samal.** 2003. Recombinant Newcastle disease virus as a vaccine vector. Poult. Sci. **82**:899-906.
97. **Hubbard, R.D., C.M. Flory, and F.M. Collins.** 1992. T-cell immune responses in *Mycobacterium avium*-infected mice. Infect. Immun. **60**:150-153.
98. **Hughes, M.J.G., J.C. Moore, J.D. Lane, R. Wilson, P.K. Pribul, Z.N. Younes, R.J. Dobson, P. Everest, A.J. Reason, J.M. Redfern, F.M. Greer, T. Paxton, M. Panico, H.R. Morris, R.G. Feldman, and J.D. Santangelo.** 2002. Identification of major outer surface proteins of *Streptococcus agalactiae.* Infect. Immun. **70**: 1254-1259.
99. **Hulo N., C.J.A. Sigrist, V. Le Saux, P.S.Langendijk-Genevaux, L.Bordoli, A.Gattiker, E. De Castro, P. Bucher, and A. Bairoch.** 2004. Recent improvements to the PROSITE database. Nucl. Acids. Res. **32**:D134-D137.
100. **Hung, A.L., and A. Alvarado.** 2001. Phenotypic and molecular characterization of isolates of *Ornithobacterium rhinotracheale* from Peru. Avian Dis 45:999-1005.
101. **Jackwood, M.W., and Y.M. Saif.** 2003. Bordetellosis (Turkey Coryza). p705-718. *In* Y.M. Saif, H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald, and D.E. Swayne (ed.), Poultry Diseases, 11th ed. Iowa State Press USA.
102. **Jacobs, A.A., K. van den Berg, and A. Malo.** 2003. Efficacy of a new tetravalent coryza vaccine against emerging variant type B strains. Avian Pathol. **32**:265-269.
103. **Jan, G., M. Le Henaff, C. Fontenelle, and H. Wroblewski.** 2001. Biochemical and antigenic characterization of *Mycoplasma gallisepticum* membrane proteins P52 and P67 (pMGA). Arch. Microbiol. **177**:81-90.
104. **Janeway, C.A., and P. Travers.** 1996. Immunobiology: the immune system in health and disease, 2nd edition.
105. **Jeannin, P., G. Magistrelli, L. Goetsch, J-F. Haeuw, N. Thieblemont, J-Y. Bonnefoy, and Y. Delneste.** 2002. Outer membrane protein A (OmpA): a new pathogen-associated molecular pattern that interacts with antigen presenting cells – impact on vaccine strategies. Vaccine **20**:A23-A27.
106. **Jordan, F., M. Pattison, D. Alexander, and T. Faragher** (ed.). 2001. Poultry Diseases, 5th ed. W.B. Saunders, USA.

107. Kang, H.Y., J. Srinivasan, and R. Curtiss III. 2002. Immune responses to recombinant pneumococcal PspA antigen delivered by live attenuated Salmonella enterica serovar typhimurium vaccine. Infect. Immun. **70**:1739-1749.

108. Kierman, J.A. 1981. Histological and histochemical methods, theory and practice. Pergamon press London 2nd. ed. Pp. 96-97.

109. Kim, Y.R., S.E. Lee, C.M. Kim, S.Y. Kim, E.K. shin, D.H. Shin, S.S. Chung, H.E. Choy, A. Progulske-Fox, J.D. Hillman, M. Handfield, and J.H. Rhee. 2003. Characterization and pathogenic significance of *Vibrio vulnificus* antigens preferentially expressed in septicemic patients. Infect. Immun. **71**:5461-5471.

110. Koebnik, R., K.P. Locher, and P. van Gelder. 2000. Structure and function of bacterial outer membrane proteins: barrels in a nutshell. Mol. Microbiol. **37**:239-253.

111. Kogut, M.H. 2000. Cytokines and prevention of infectious diseases in poultry: a review. Avian Path. **29**:395-404.

112. Koppenheffer, T.L. 1998. Complement. In J.M. Sharma, Section Ed. Avian Immunology. In P.P. Pastoret, P. Griebel, H. Bazin, and A. Govaerts (eds.). Handbook of Vertebrate immunology. Academic Press 99-101.

113. Kyd, J., and A. Cripps. 2000. Identifying vaccine antigens and assessing delivery systems for the prevention of bacterial infections. J. Biotech. **83**:85-90.

114. Kyte, J., and Doolottle, R.F. 1982. A simple method for displaying the hydrophatic character of a protein. J. Mol. Biol. **157:** 105-132.

115. Lamm, M.E. 1997. Interaction of antigens and antibodies at mucosal surfaces. Annu. Rev. Microbiol. **51:**311-340.

116. Leclerc, C. 2003. New approaches in vaccine development. Comp. Immunol. Microb. Infec. Dis. **26**:329-341.

117. Lee, C.A. 1997. Type III secretion systems: machines to deliver bacterial proteins into eukaryotic cells. Trends Microbiol. **5**:148-155.

118. Leroy-Sétrin, S., G. Flaujac, K. Thenaisy, and E. Chaslus-Dancla. 1998. Genetic diversity of *Ornithobacterium rhinotracheale* strains isolated from poultry in France. Lett. Appl. Microbiol. **26**:189-193.

119. Levine, M.M., and G. Dougan. 1998. Optimism over vaccines administered via mucosal surfaces. Lancet **351**:1375-1376.

120. Ley, D.H. 2003. *Mycoplasma gallisepticum* infection, p722-744. *In* Y.M. Saif, H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald, and D.E. Swayne (ed.), Poultry Diseases, 11th ed. Iowa State Press USA.

121. Liljeqvist, S., and S. Stahl. 1999. Production of recombinant subunit vaccines: protein immunogens, live delivery systems and nucleic acid vaccines. J. Biotech. **73**:1-33.

122. Lloyd, J.B., H.S. Gill, D.M. Haig, and A.J. Husband. 2000. *In vivo* T-cell subset depletion suggests that CD4+ T-cells and a humoral immune response are important for the elimination of orf virus from the skin of sheep. Vet. Immunol. Immunop. **74:**249-262.

123. Lockhart, D.J., and E.A. Winzeler. 2000. Genomics, gene expression and DNA arrays. Nature **405**:827-836.

124. Lopes, V.C., A. Back, D.A. Halvorson, and K.V. Nagaraja. 2002. Minimization of pathologic changes in *Ornithobacterium rhinotracheale* infection in turkeys by temperature-sensitive mutant strain. Avian Dis. **46**:177-185.

125. Lopes, V.C., A. Back, H.J. Shin, D.A. Halvorson, and K.V. Nagaraja. 2002. Development, characterization, and preliminary evaluation of a temperature-sensitive mutant of *Ornithobacterium rhinotracheale* for potential use as a live vaccine in turkeys. Avian Dis. **46**:162-168.

126. Lopes, V., G. Rajashekara, A. Back, D.P. Shaw, D.A. Halvorson, and K.V. Nagaraja. 2000. Outer membrane proteins for serologic detection of *Ornithobacterium rhinotracheale* infection in turkeys. Avian Dis. **44**:957-962.

127. Lottenberg R., C.C. Boder, M.D.P. Boyle, S.J. Kain, B.L. Schroeder, and R. Curtiss III. 1992. Cloning, sequence analysis, and expression in *Escherichia coli* of a streptococcal plasmen receptor. J. Bacteriol. **174**: 5204-5210.

128. Lowenthal, J.W., B. Lambrecht, T.P. van den Berg, M.E. Andrew, A.D.G. Strom, and A.G.D. Bean. 2000. Avian cytokines – the natural approach to therapeutics. Dev. Comp. Immunol. **24**:355-365.

129. Mahan, M. J., J.M. Slauch, P.C. Hanna, A. Camilli, J.W. Tobias, M.K. Waldor, and J.J. Mekalanos. 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. Science. **259**: 686-688.

130. Malik, Y.S., K. Olsen, K. Kumar, and S.M. Goyal. 2003. *In vitro* antibiotic resistance profiles of *Ornithobacterium rhinotracheale* strains from Minnesota turkeys during 1996-2002. Avian Dis. **47**:588-593.

131. Manjarrez-Hernandez, H.A., S. Gavilanes-Parra, E. Chavez-Berrocal, A. Navarro-Ocana, and A. Cravioto. 2000. Antigen detection in enteropathogenic *Escherichia coli* using secretory immunoglobulin A antibodies isolated from human breast milk. Infect. Immun. **68:** 5030-5036.

132. Marchler-Bauer A, Anderson JB, DeWeese-Scott C, Fedorova ND, Geer LY, He S, Hurwitz DI, Jackson JD, Jacobs AR, Lanczycki CJ, Liebert CA, Liu C, Madej T, Marchler GH, Mazumder R, Nikolskaya AN, Panchenko AR, Rao BS, Shoemaker BA, Simonyan V, Song JS, Thiessen RA, Vasudevan S, Wang Y, Yamashita RA, Yin JJ, and Bryant SH. 2003 "CDD: a curated Entrez database of conserved domain alignments", Nucleic Acids Res. **31**:383-387.

133. Masignani, V., R. Rappuoli, and M. Pizza. 2002. Reverse vaccinology : a genome-based approach for vaccine development. Expert Opin Biol Ther. **2** :895-905.

134. Mast, J., M. Desmidt, G. Room, C. Martuin, R. Ducatella, F. Haesobrouck, T.F. Davison, B. Kaspers, and B.M. Goddeeris. 1997. Different methods of bursectomy induce different effects on leucocyte distribution and reactivity. Arch. Geflügelk. **61**:238-246.

135. McAtee, C.P., K.E. Fry, and D.E. Berg. 1998. Identification of potential diagnostic and vaccine candidates of *Helicobacter pylori* by proteome technologies. Helicobacter **3**:163-169.

136. McAtee, C.P., M.Y. Lim, K. Fung, M. Velligan, K. Fry, T.P. Chow, and D.E. Berg. 1998. Characterization of a *Helicobacter pylori* vaccine candidate by proteome techniques. J. Chromatography **714**:325-333.

137. Medina, E., and C.A. Guzman. 2001. Use of bacterial vaccine vectors for antigen delivery: potential and limitations. Vaccine **19**:1573-1580.

138. Mekalanos, J.J. 1992. Environmental signals controlling expression of virulence determinants in bacteria. J. Bacteriol. **174**: 1-7.

139. Merrell, D.S., and A. Camilli. 2000. Detection and analysis of gene expression during infection by *in vivo* expression technology. Phil. Trans. R. Soc. Lond. **355**: 587-599.

140. Mora, M., D. Veggi, L. Santini, M. Pizza, and R. Rappuoli. 2003. Reverse vaccinology. Drug Discov. Today **8**:459-464.

141. **Nielsen H., J.Engelbrecht, S.Brunak and G. von Heijne.** 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. **10**, 1-6.

142. **Noirot, P. R.C. Gupta, C.M. Radding, and R.D. Kolodner.** 2003. Hallmarks of homology recognition by RecA-like recombinases are exhibited by the unrelated *Escherichia coli* RecT protein. EMBO J. **22**:324-334.

143. **Noirot, P., and R.D. Kolodner.** 1998. DNA strand invasion promoter by *Escherichia coli* RecT protein. J. Biol. Chem. **20**:12274-12280.

144. **O'Hagan, D.T.** 2001. Recent developments in vaccine delivery systems. Curr. Drug Targets Infect. Dis. **1**:273-286.

145. **Pages Mante, A.** 1999. ORT infection. Zootecn. Int. Jan. 51-53.

146. **Pancholi, V., and V.A. Fischetti.** 1993. Glyceraldehyde-3-phosphate dehydrogenase on the surface of group A streptococci is also an ADP-ribosylating enzyme. Proc Natl Acad Sci. **90**: 8154-8158.

147. **Pandey, A., and M. Mann.** 2000. Proteomics to study genes and genomes. Nature **450**:837-846.

148. **Paoletti, E.** 1996. Applications of pox virus vectors to vaccination: an update. Proc. Natl. Acad. Sci. USA **93**:11349-11353.

149. **Peterson, J.D., L.A. Umayam, T.M. Dickinson, E.K. Hickey and O. White.** 2001. The Comprehensive Microbial Resource. Nucleic Acids Res. **29**:123-125.

150. **Potter, A.A., and L.A. Babiuk.** 2001. New approaches for antigen discovery, production and delivery: vaccines for veterinary and human use. Curr. Drug Targets Infec. Dis. **1**:249-262.

151. **Pryde, J.G.** 1986. Triton X-114: a detergent that has come in from the cold. TIBS **11**:160-163.

152. **Qureshi, M.A., C.L. Heggen, and I. Hussain.** 2000. Avian macrophage effector functions in health and disease. In D.A. Higgins and G.W.Warr (eds.) The avian immune response to infectious diseases. Special issue. Developmental and Comparative Immunology **24**: 103-119.

153. **Qureshi, M.A., I. Hussain, and C.L. Heggen.** 1998. Understanding immunology in disease development and control. Poultry Sci. **77:**1126-1129.

154. **Rautenschlein, S., H.-Y. Yeh, and J.M. Sharma.** 2002. The role of T-cells in protection by an inactivated infectious bursal disease virus vaccine. Vet. Immunol. Immunop. **89:**159-167.

155. **Razin, S., D. Yogev, and Y. Naot.** 1998. Molecular biology and pathogenicity of Mycoplasmas. Microbiol. Mol. Biol. Rev. **62**:1094-1156.

156. **Reynolds, D.L., and A.D. Maraqa.** 1999. A technique for inducing B-cell ablation in chickens by in ovo injection of cyclophosphamide. Avian Dis. **43:**367-375.

157. **Reynolds, D.L., and A.D. Maraqa.** 2000. Protective immunity against Newcastle disease: the role of cell-mediated immunity. Avian Dis. **44**:145-154.

158. **Roepke, D.C., A. Back, D.P. Shaw, K.V. Nagaraja, S.J. Sprenger, and D.A. Halvorson.** 1998. Isolation and identification of *Ornithobacterium rhinotracheale* from commercial turkey flocks in the upper Midwest. Avian Dis. **42**:219-221.

159. **Saif, Y.M., H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald, and D.E. Swayne** (ed.), 2003, Poultry Diseases, 11th ed. Iowa State Press USA.

160. **Sakai, E., Y. Tokuyama, F. Nonaka, S. Oshishi, Y. Ishikawa, M. Tanaka, and A. Taneno.** 2000. *Ornithobacterium rhinotracheale* infection in Japan: preliminary investigations. Vet. Rec. **146**:502-503.

161. **Sallovitz, J.M., M.I. Zonco Menghini, and C.E. Lanusse.** 1998. Impact of liposomes as delivery systems in veterinary medicine. Vet. Res. **5**:409-430.

162. Sambrook, J., and D.W. Russell. 2001. Molecular cloning: a laboratory manual, 3th edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.

163. Sander, P., E.C. Böttger, B. Springer, B. Streinmann, M. Rezwan, E. Stavropoulos, and M.J. Colston. 2003. A recA deletion mutant of *Mycobacterium bovis* BCG confers protection equivalent to that of wild-type BCG but shows increased genetic stability. Vaccine **21**:4124-4127.

164. Sander, P., K.G. Papavinasasundaram, T. Dick, E. Stavropoulos, K. Ellrott, B. Springer, M.J. Colston, and E.C. Böttger. 2001. *Mycobacterium bovis* BCG *recA* deletion mutants shows increased susceptibility to DNA-damaging agents but wild-type survival in a mouse infection model. Infect. Immun. **69**:3562-3568.

165. Sandhu, T.S. 2003. *Riemerella anatipestifer* infection, p.676-682. *In* Y.M. Saif, H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald, and D.E. Swayne (ed.), Poultry Diseases, 11th ed. Iowa State Press USA.

166. Schijns, V.E.C.J., K.C. Weining, P. Nuijten, E.O. Rijke, P. Staeheli. 2000. Immunoadjuvant activities of *E. coli*- and plasmid-expressed recombinant chicken IFN-α/β, INF-γ and IL-1β in 1-day- and 3-week-old chickens. Vaccine **18**:2147-2154.

167. Schneider, W.P., S.K. Ho, J. Christine, M. Yao, A. Marra, and A.E. Hromockyj. 2002. Virulence gene identification by differential fluorescence induction analysis of *Staphylococcus aureus* gene expression during infection-simulating culture. Infect. Immun. **70**:1326-1333.

168. Scott, T.R. 2004. Our current understanding of humoral immunity of poultry. Poultry Sci. **83**:574-579.

169. Sette, A., and J. Fikes. 2003. Epitope-based vaccines: an update on epitope identification, vaccine design and delivery. Curr. Opin. Immunol. **15**:461-470.

170. Sharma, J.M. 2003. The avian immune system, p.5-16. *In* Y.M. Saif, H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald, and D.E. Swayne (ed.), Poultry Diseases, 11th ed. Iowa State Press USA.

171. Sharma, J.M., and K.A. Schat. 1991. Natural immune functions. In Sharma, J.M. (ed.). Avian Cellular Immunology. CRC Press 51-70.

172. Simmons, C.P., S. Clare, M. Ghaem-Maghami, T.K. Uren, J. Ranking, A. Huett, R. Goldin, D.J. Lewis, T.T. MacDonald, R.A. Strugnell, G. Frankel, and G. Dougan. 2003. Central role for B lymphocytes and CD4+ T-cells in immunity to infection by the attaching and effacing pathogen *Citrobacter rodentium*. Infect. Immun. **71:**5077-5086.

173. Sirover, M.A. 1997. Role of the glycolytic protein, glyceraldehydes-3-phosphate dehydrogenase, in normal cell function and in cell pathology. J. Cell. Biochem. **66**: 133-140.

174. Skeiky, Y.A.W., P.J. Ovendale, S. Jen, M.R. Alderson, D.C. Dillon, S. Smith, C.B. Wilson, I.M. Orme, S.G. Reed, and A. Campos-Neto. 2000. T-cell expression cloning of a *Mycobacterium tuberculosis* gene encoding a protective antigen associated with the early control of infection. J. Immunol. 7140-7149.

175. Smith, H. 1998. What happens to a bacterial pathogen *in vivo?* Trends Microbiol. **6**:239-243.

176. Soriano, V.E., N.A. Vera, C.R. Salado, R.P. Fernandez, and P.J. Blackall. 2003. *In vitro* susceptibility of *Ornithobacterium rhinotracheale* to several antimicrobial drugs. Avian Dis. **47**:476-480.

177. Spickler, A.R., and J.A. Roth. 2003. Adjuvants in veterinary vaccines: modes of action and adverse effects. J. Vet. Intern. Med. **17**:273-281.

178. Sprenger, S.J., A. Back, D.P. Shaw, K.V. Nagaraja, D.C. Roepke, and D.A. Halvorson. 1998. *Ornithobacterium rhinotracheale* infection in turkeys: experimental reproduction of the disease. Avian Dis. **42**:154-161.

179. Sprenger, S.J., D.A. Halvorson, D.P. Shaw, and K.V. Nagaraja. 2000. *Ornithobacterium rhinotracheale* infection in turkeys: immunoprophylaxis studies. Avian Dis. **44**:549-555.

180. Storz, G., L.A. Tartaglia, S.B. Farr, and B.N. Ames. 1990. Bacterial defenses against oxidative stress. Trends Genet. **6**:363-368.

181. Subramaniam, S., B. Huang, H. Loh, J. Kwang, H-M. Tan, K-L. Chua, and J. Frey. 2000. Characterization of a predominant immunogenic outer membrane protein of *Riemerella anatipestifer*. Clin. Diagn. Lab. Immun. **7**:168-174.

182. Suhrbier, A. 1997. Multi-epitope DNA vaccines. Immunology and Cell Biology **75**:402-408.

183. Szalay, D., R. Glavits, C. Nemes, A. Kosa, and L. Fodor. 2002. Clinical signs and mortality caused by *Ornithobacterium rhinotracheale* in turkey flocks. Acta Vet. Hung. **50**:297-305.

184. Tahseen, A. 1997. *Ornithobacterium rhinotracheale* developing into a serious infection. World Poul. Misset. **13**:47-48.

185. Takahashi, H. 2003. Antigen presentation in vaccine development. Comp. Immunol. Microbiol. Infect. Dis. **26**:309-328.

186. Tinch, A.E. 2001. The poultry industry, p.3-12. *In* F. Jordan, M. Pattison, D. Alexander, and T. Faragher (ed.), Poultry Diseases, 5th ed. W.B. Saunders, USA.

187. Towbin, H., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc.Nat.Acad.Sci. **76:**43-50.

188. Travers, A.F. 1996. Concomitant *Ornithobacterium rhinotracheale* and Newcastle disease infection in broilers in South Africa. Avian Dis. **40**:488-490.

189. Underhill, D.M., and A. Ozinsky. 2002. Toll-like receptors: key mediators of microbe detection. Curr. Opin. Immunol. **14**:103-110.

190. Vainio, O., P. Toivanen, K. Granfors, and J.R.L. Pink. 1988. Antibody-forming capacity of B-cell-deficient chickens reconstituted with limiting numbers of B-cell precursors. Eur. J. Immunol. **18**:309-312.

191. Valdivia, R.H., and S. Falkow. 1997. Fluorescence-based isolation of bacterial genes expressed within host cells. Science **277**:2007-2011.

192. Van Beek, P., P. van Empel, H. van den Bosch, P.K. Storm, J.H. Bongers, and J.H. du Preez. 1994. Respiratory problems, growth retardation and arthritis in turkeys and broilers caused by a Pasteurella-like organism: *Ornithobacterium rhinotracheale* or "Taxon 28". Tijdschr. Diergeneesk. **119**:99-101.

193. Vandamme, P., P. Segers, M. Vancaneyt, K. van Hover, R. Mutters, J. Hommez, F. Dewirst, B. Paster, K. Kersters, E. Falsen, L. Devrieze, M. Bisgaard, K-H. Hinz, and W. Mannheim. 1994. *Ornithobacterium rhinotracheale* gen. nov. sp. nov. isolated from the avian respiratory tract. Inter. J. System. Bact. **44**:24-37.

194. Vandamme, P., M. Vancaneyt, P. Segers, M. Ryll, B. Köhler, and K-H. Hinz. 1999. *Coenonia anatine* gen. nov. sp. nov. a novel bacterium associated with respiratory disease in ducks and geese. Int. J. System. Bact. **49**:867-874.

195. Van Empel, P.C.M. 1996. *Ornithobacterium rhinotracheale*. PhD thesis. Utrecht University, The Netherlands.

196. Van Empel, P. and H. Hafez. 1999. *Ornithobacterium rhinotracheale*: a review. Avian Path. **28**:217-227.

197. Van Empel, P. and H. van den Bosch. 1998. Vaccination of chickens against *Ornithobacterium rhinotracheale* infection. Avian Dis. **42**:572-578.

198. Van Empel, P., H. van den Bosch, D. Goovaers, and P. Storm. 1996. Experimental infection in turkeys and chickens with *Ornithobacterium rhinotracheale*. Avian Dis. **40**:858-864.

199. Van Empel, P., H. van den Bosch, P. Loeffen, and P. Storm. 1997. Identification and serotyping of *Ornithobacterium rhinotracheale*. J. Clin. Microbiol. **35**:418-421.

200. Van Empel, P., M. Vrijenhoek, D. Goovaers, and H. van den Bosch. 1999. Immunohistochemical and serological investigation of experimental *Ornithobacterium rhinotracheale* infection in chickens. Avian Path. **28**:187-193.

201. Van Loon, A.A.W.M., W. Kosman, H.I. van Zuilenkom, S. van Riet, M. Frenken, and V.E.J.C. Schijns. 2003. The contribution of humoral immunity to the control of avian reoviral infection in chickens after vaccination with live reovirus vaccine (strain 2177) at an early age. Avian Pathol. **32**:15-23.

202. Van Veen, L., P. van Empel, and T. Fabri. 2000. *Ornithobacterium rhinotracheale*, a primary pathogen in broilers. Avian Dis. **44:**896-900.

203. Van Veen, L., E. Gruys, K. Frik, and P. van Empel. 2000. Increased condemnation of broilers associated with *Ornithobacterium rhinotracheale*. Vet. Rec. **147**:422-423.

204. Van Veen, L., E. Hartmen, and T. Fabri. 2001. *In vitro* antibiotic sensitivity of strains of *Ornithobacterium rhinotracheale* isolated in The Netherlands between 1996 and 1999. Vet. Rec. **149**:611-613.

205. Verheul, A.F., H. Snippe, and J.T. Poolman. 1993. Meningococcal lipopolysaccharides: virulence factor and potential vaccine component. Microbiol. Rev. **57**:34-49.

206. Vugmeyster Y., K. Howell, K. McKeever, D. Combs, and E. Canova-Davis. 2003. Differential *in vivo* effects of rituximab on two B-cell subsets in cynomolgus monkeys. Int. Immunopharmacol. **3:**1477-1481.

207. Wang, L.F., and M. Yu. 2004. Epitope identification and discovery using phage display libraries: applications in vaccine development and diagnostics. Curr. Drug Targets **5**:1-15.

208. Watanabe, M. and M. Nagai. 2003. Role of systemic and mucosal immune responses in reciprocal protection against *Bordetella pertussis* and *Bordetella parapertussis* in a murine model of respiratory infection. Infect. Immun. **71**:733-738.

209. Wei, Y., J.M. Lee, C. Richmond, F.R. Blattner, J.A. Rafalski, and R.A. LaRossa. 2001. High-density microarray-mediated gene expression profiling of *Escherichia coli*. J. Bacteriol. **183**:545-556.

210. Weichhart, T., M. Horky, J. Söllner, S. Gangl, T. Henics, E. Nagy, A. Meinke, A. von Gabain, C.M. Fraser, S.R. Gill, M. Hafner, and U. von Ahsen. 2003. Functional selection of vaccine candidate peptides from *Staphylococcus aureus* whole-genome expression libraries *in vitro*. Infect. Immun. **71**:4633-4641.

211. Weis, J.J., Ma, Y., and L.F. Erdile. 1994. Biological activities of native and recombinant *Borrelia burgdorferi* outer surface protein A: dependence on lipid modification. Infect. Immun. **62**: 4632-4636.

212. Weiser, J.N., and E.C. Gotschlich. 1991. Outer membrane protein A (OmpA) contributes to serum resistance and pathogenicity of *Escherichia coli* K-1. Infect. Immun. **59**:2252-2258.

213. Whitford, H.W., R.F. Rosenbusch, and L.H. Lauerman, ed. 1994. Mycoplasmosis in animals: laboratory diagnosis. First edition. Iowa State University Press, Ames, Iowa, USA.

214. Xu, Y., L. Jiang, B.E. Murray, and G.M. Weinstock. 1997. *Enterococcus faecalis* antigens in human infections. Infect. Immun. **65**:4207-4215.

215. Yang, Y.-P., R.S. Munson Jr, S. Grass, P. Chong, R.E. Harkness, L. Gisonni, O. James, Y. Kwok, and M.H. Klein. 1997. Effect of lipid modification on the physicochemical, structural, antigenic and immunoprotective properties of *Haemophilus influenzae* outer membrane P6. Vaccine **15**: 976-987.

216. Zagursky, R.J., S.B. Olmsted, D.P. Russell, and J.L. Wooters. 2003. Bioinformatics: how it is being used to identify bacterial vaccine candidates. Expert Rev Vaccines **2**:417-436.

217. Zeitlin, L., R.A. Cone, T.R. Moench, and K.J. Whaley. 2000. Preventing infectious disease with passive immunization. Microbes Infect. **2:**701-708.

218. Zhang, G., K. Kiss, R. Seshadri, L.R. Hendrix, and J.E. Samuel. 2004. Identification and cloning of immunodominant antigens of *Coxiella burnetii*. Infect. Immun. **72**:844-852.

Dankwoord

Zonder de hulp en steun van een groot aantal mensen was dit proefschrift niet tot stand gekomen. Daarom wil ik iedereen die, direct of indirect, heeft bijgedragen aan mijn promotieonderzoek daarvoor hartelijk danken. Een aantal mensen verdienen echter extra aandacht in dit dankwoord.

Allereerst natuurlijk mijn co-promotor Piet Nuijten en promotor Jos van Putten. Beste Piet, bedankt voor de kans die je me hebt gegeven om bij Intervet mijn promotieonderzoek te doen. Je hebt me zo veel mogelijk zelf mijn weg laten vinden en op de juiste momenten begeleid en bijgestuurd. Het was een erg prettige samenwerking. Beste Jos, bedankt dat je promotor wilde zijn van een AIO-op-afstand. Je interesse en vertrouwen in mijn onderzoek heb ik altijd erg gewaardeerd.

Beste Paul, Ad, Ruud en Ton. Bedankt voor alle vragen, suggesties, kritieken en discussies tijdens de verschillende projectbesprekingen en tijdens het schrijven van dit proefschrift. Simen-Jan, eerst room-mates toen buren. Bedankt voor de gezelligheid!

De mensen van Moleculaire Biologie, Bacteriologie, en Biochemie, te veel namen om allemaal te noemen. Thea en Wies, jullie waren er altijd voor advies, zowel op het lab als daarbuiten. Bedankt dat jullie mijn paranimfen willen zijn en naast me willen staan op de grote dag! Altijd terecht kon ik ook bij Katja, Henriëtte, Leonie, en vraagbaak Paul. Daniëlle, Leonie en Ria, bedankt voor de hulp tijdens de verschillende dierstudies. Peter, bedankt voor de technische hulp!

De mensen van Vaccin Technologie en Immunologie, Animal Service Department, en Pathologie. Bedankt voor de ideëen en suggesties bij de start van mijn onderzoek (Virgil), de bereiding van mijn vaccins (Marc), de verzorging van mijn kippen en betrokkenheid bij het onderzoek (Mimi, Laura, Imke, Geert, Andre en Paul) en voor hulp bij sectie (Rob, Ed, en Eva) en uitvoering van histologische analyses (John en Mieke).

I would like to thank Hon Tran from the Department of BioChemInformatics from Intervet Schwabenheim for helpful analysis of sequence data. Herzlichen Dank!

Marleen van Ampting en Maarten Rits, bedankt voor de waardevolle bijdragen die jullie hebben geleverd aan dit proefschrift. Heel veel succes met jullie verdere carrière!

Daarnaast wil ik ook familie, vrienden en kennissen bedanken voor de nodige ontspanning op zijn tijd. Voor velen was het moeilijk voor te stellen wat zo'n promotieonderzoek inhoudt. Hopelijk is dit na vandaag veranderd!
In het bijzonder wil ik mijn ouders bedanken. Jullie hebben me altijd gestimuleerd om mijn eigen plan te trekken en onvoorwaardelijk achter mijn keuzes gestaan. Daarom draag ik dit proefschrift aan jullie op.
En tot slot natuurlijk mijn allergrootste vriend Marc. Het laatste jaar was druk, erg druk. Maar jij was er altijd om even lekker uit te razen, te relativeren, te relaxen, en vooral te lachen!

Daniëlle

Curriculum Vitae

De schrijfster van dit proefschrift werd op 26 februari 1976 geboren te Boxmeer. Zij haalde haar VWO diploma in 1995 aan het Elzendaal college in Boxmeer. Hetzelfde jaar begon zij aan de studie Medische Biologie aan de Vrije Universiteit (VU) te Amsterdam. In het kader van deze opleiding liep zij twee maal stage. Haar eerste stage werd gevolgd bij de Vakgroep Medische Microbiologie en Infectiepreventie aan de faculteit Geneeskunde van de VU. Hier werkte zij onder begeleiding van dr. J.G. Kusters en dr. M.M. Gerrits aan antibioticaresistentie in de maagbacterie *Helicobacter pylori*. Haar tweede stage werd gevolgd op de afdeling Immunologie, hoofdafdeling Infectieziekten en Immunologie van de Faculteit Diergeneeskunde, Universiteit Utrecht. Hier deed zij onder begeleiding van dr. M.H.M. Wauben onderzoek naar detectiemethoden van antigen-specifieke T cellen. In september 2000 heeft zij haar studie afgerond. Van augustus 2000 tot november 2004 was zij werkzaam bij de afdeling Bacteriologie, Intervet International bv, alwaar zij haar promotieonderzoek heeft uitgevoerd zoals beschreven in dit proefschrift.

The author of this thesis was born on February 26th 1976 in Boxmeer. She graduated from the Elzendaal college in Boxmeer in 1995 and started her study Medical Biology at the Free University in Amsterdam in this same year. As part of her education she performed two practical periods. The first was at the Department of Medical Microbiology and Infection Control of the Medical Faculty of the Free University. She worked under supervision of dr. J.G. Kusters and dr. M.M. Gerrits on antibiotic resistance in the stomach pathogen *Helicobacter pylori*. The second practical period was performed at the Division of Immunology, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University. She worked on detection methods of antigen-specific T cells under supervision of dr. M.H.M. Wauben. In September 2000 she graduated as MSc in Medical Biology. From August 2000 until November 2004 she worked at the Bacteriology Department, Intervet International bv, were she performed her PhD project as described in this thesis.