THERAPEUTIC VACCINES AGAINST CHLAMYDIAL DISEASES

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THERAPEUTIC VACCINES AGAINST CHLAMYDIAL DISEASES

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THERAPEUTIC VACCINES AGAINST CHLAMYDIAL DISEASES

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VITA

Yihang Li, son of Bacui Li and Luobao Du, was born on February 1, 1982, in Tongcheng County, Hubei Province, People's Republic of China. In 1998, he graduated from the First High School of Tongcheng as the top student and attended Nankai University in Tianjin. In 2002, he graduated with a Bachelor's degree in Biological Sciences and was admitted for graudate studies at the Cellular and Molecular Biosciences Program at Auburn University, Alabama, USA. In 2004, he started his graduate studies in the Program of Biomedical Sciences in the College of Veterinary Medicine at Auburn University.

DISSERTATION ABSTRACT

THERAPEUTIC VACCINES AGAINST CHLAMYDIAL DISEASES

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Subclinical infections with intracellular bacteria of the family *Chlamydiaceae* cause substantial losses in animal agriculture. To validate the feasibility of therapeutic vaccination against chlamydial infection and disease, a prospective cohort study was conducted in a herd of 147 dairy cows to investigate the influence of chlamydial infection on subclinical inflammation of the bovine mammary gland. Vaccination against *Chlamydophila* spp. with an inactivated whole-organism vaccine of *C. abortus*/*C. pecorum* elementary bodies produced a highly significant decrease in milk somatic cell numbers, thus reducing bovine mastitis. It also increased antibody levels against *Chlamydophila* spp., but did not eliminate shedding of *C. abortus* in milk as detected by PCR. These results show an etiological involvement of the widespread *Chlamydophila* spp. infections in bovine mastitis, a herd disease of critical importance for the dairy industry. More importantly, this investigation shows the potential for improvement of chlamydial disease by therapeutic vaccination.

To validate the concept of unbiased selection of vaccine candidate genes from the genome of a chlamydial species, we then tested 18 *C. pneumoniae* candidate genes for immune protection. Twelve of these genes had been previously identified by expression library immunization (ELI) and *C. pneumoniae* challenge in mice. Six additional genes had been previously reported in the literature as presumptive vaccine candidates after selection by surface location of the encoded protein T cell epitopes that were identified by computer algorithms. These 18 genes were used individually to immunize mice, which were then challenged with a high-dose *C. pneumoniae* inoculum. The protective effect was evaluated by the criteria of survival, lung disease and pathogen clearance. *C. pneumoniae* genes *cutE* and Cpn0420 identified by ELI protected mice from *C. pneumoniae*-induced death, increased elimination of *C. pneumoniae*, and alleviated lung disease. Gene *oppA_2* was protective by dual criteria (disease reduction and *C. pneumoniae* elimination), whereas *ssb* (disease reduction), *ide* and Cpn0095 (*C. pneumoniae* elimination), and Cpn0020 (survival) were protective by a single criterion. Among the previously reported genes identified by other criteria, *npt1*, Momp and *gatA* protected partially by increasing survival, but none mediated further protection. Therefore, unbiased genome-wide screening by ELI proved to be the better method for identification of vaccine candidates. Given the high protection mediated by genes *cutE*, Cpn0420, and *oppA_2*, their combined use in a recombinant vaccine may mediate protection equal to that induced by previous natural infection. This may merit further investigations for use in human populations.

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Computer software used

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STATISTICA 7.1 for statistical analysis and figures

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TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 2

CHAPTER 3

LIST OF FIGURES

CHAPTER 1: LITERATURE REVIEW

BIOLOGY OF *CHLAMYDIACEAE*

Developmental biology. Organisms of the family *Chlamydiaceae* are obligate intracellular bacteria that parasitize an extremely wide range of eukaryotic cells. Since Bedson and Bland (1932) first described the biphasic developmental cycle of *Chlamydiaceae*, which is characterized by two morphologically distinct structures, it has also been confirmed by a number of microscopic studies (Eb et al., 1976, Matsumoto, 1988, Soloff et al., 1982). It is universally accepted that all *Chlamydiaceae* species have an infectious but metabolically inert elementary body (EB) phase and a metabolically active but non-infectious reticulate body (RB) phase. EBs, approximately 0.3 μm in diameter with osmotically stable and poorly permeable cell envelopes, are adapted for survival in the extracellular environment, due to their physical and chemical resistance. EBs become metabolically inert by completely shutting down their metabolism and condensing their DNA into hyperpyknotic nucleoids (Hatch, 1999). EBs attach to susceptible host cells and enter via a type III secretion system that allows for the recruitment of actin to the internalized vesicle (Miyashita et al., 1993, Rockey and Matsumoto, 2000). The internalized EBs, within this membrane-bound compartment, then modify their membrane-bound compartment to prevent phagosome-lysosome fusion, and differentiate into RBs. As the vacuole containg the chlamydiae (termed inclusion)

expands, the RBs replicate by binary fusion and then convert into infectious EBs, which are released from the host cell to initiate a new round of infection (Everett et al., 1999, Stephens, 1988).

Due to the intracellular niche of C*hlamydiaceae*, no cell free culture or gene transfer system has yet been developed. The metabolically active RBs, which have approximately the same density as host mitochondria (McClarty, 1999), are difficult to extract without host contamination. This poses an extreme difficulty for study of metabolic activities in *Chlamydiaceae*. Nonetheless, a number of researchers have made significant discoveries by demonstrating metabolic pathways of *Chlamydiaceae* (Moulder, 1979, Moulder, 1988, Stephens, 1993, McClarty, 1994). With the sequencing of chlamydial genomes, a large body of additional information has been extracted that suggest the existence of virtually all metabolic pathways of bacteria, even if those pathways are poorly utilized or inactive (McClarty, 1999).

Traditionally, *Chlamydiaceae* were cultured in yolk sacs of chicken embryos (Storz et al., 1971), but cultivation in cell culture is easier and faster. Li et al. (2005) have achieved maximum EB production by growing *C. pneumoniae*, *C. trachomatis*, *C. pecorum*, or *C. abortus* in a saturated equilibrium monolayer cell culture system. *Chlamydiaceae* EBs were used to infect Buffalo Green Monkey Kidney (BGMK) cells cultivated in Iscove's Modified Dulbecco's Medium (IMDM) with a multiplicity of infection (MOI) of 10 or above. Approximately 4×10^8 *Chlamydiaceae* genome copies per ml culture were produced after 10 days of cultivation (Li et al., 2005).

Taxonomy of *Chlamydiaceae*. All chlamydial species belong to the family *Chlamydiaceae*, which is the only family in the order *Chlamydiales*. Prior to 1980, the single genus *Chlamydia* of the family *Chlamydiaceae* had been separated into two species, *C. trachomatis* and *C. psittaci*, based on the accumulation of glycogen in inclusions and the sensitivity to sulfadiazine of *C. trachomatis*, but not *C. psittaci*. The development of DNA-based classification prompted identification of *C. pneumoniae* (Graystone et al., 1989) and *C. pecorum* (Fukushi and Hirai, 1992). However, this differentiation was still insufficient because it failed to recognize profound genetic sequestration of strains within *C. psittaci* and *C. trachomatis* that clearly amounted to different species (Tanner et al., 1999, Palys et al., 1997).

In 1999, Everett et al. proposed dividing the family *Chlamydiaceae* into two genera, *Chlamydia* and *Chlamydophila* based on genome size, 16S and 23S ribosomal RNA gene sequence similarity, DNA-DNA reassociation, coding sequence similarity, and number of ribosomal operons (Everett et al., 1999). This approach placed three species in the genus *Chlamydia*: *C. trachomatis, C. suis* and *C. muridanum*. New species diverged from existing species mainly based on differences in host tropism, and six species were placed in the genus *Chlamydophila*: *C. pneumoniae*, *C. psittaci*, *C. abortus*, *C. pecorum*, *C. caviae* and *C. felis* (Fig. 1.1). While the separation into additional species has found wide acceptance, the division of the family *Chlamydiaceae* into two genera has been controversial and some researchers argue that the available biological evidence contradicts the genus separation (Schachter et al., 2001).

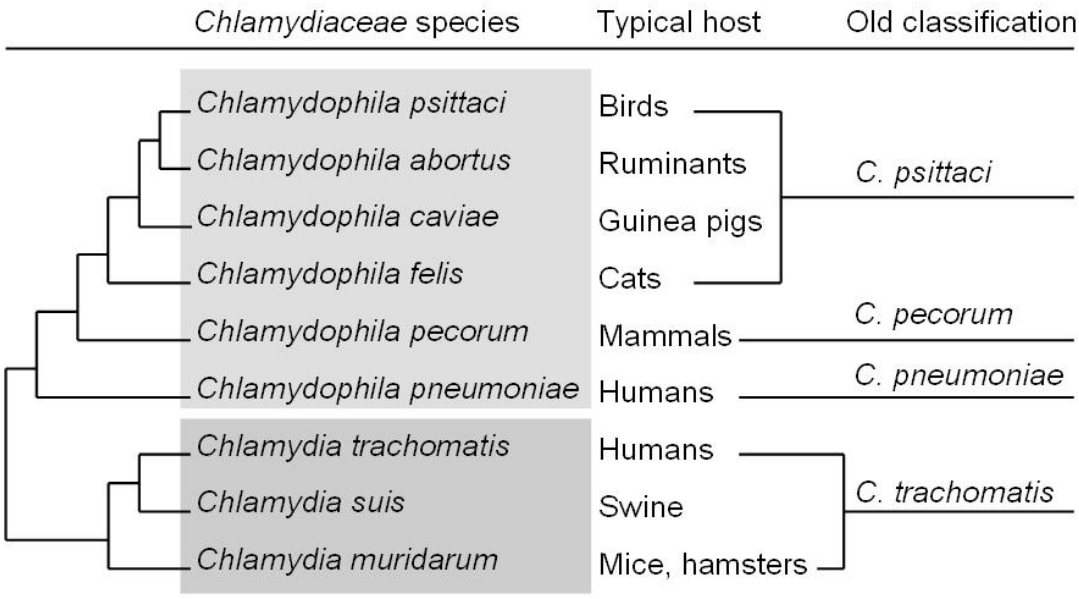

Figure 1.1. Phylogenetic relationships among chlamydial species in Everett's classification of *Chlamydiaceae* (Everett et al., 1999) and comparison with chlamydial species recognized before 1999. Everett introduced 5 new species to the *Chlamydiaceae* family and separated these 9 species into two genera, *Chlamydia* and *Chlamydophila*. The branches represent cladistic separation of chlamydiae based on the ribosomal RNA operons and *ompA* genes, but do not indicate phylogenetic distances.

Genomics and bioinformatics. The last decade has witnessed a wide application of molecular techniques, such as quantitative PCR and genomic sequencing. In 1997, Stephens et al. published the genome sequence of *C. trachomatis* serovar D, and identified key genes related to interconversion of metabolites between *C. trachomatis* and its mammalian host cells as well as potential virulence-associated genes. A large number of genes were also characterized with phylogenetic origins from eukaryotes, implying a complex evolution for adaptation to obligate intracellular parasitism (Stephens et al., 1998). Since the publication of the first chlamydial genome sequence, 13 genomes of chlamydiae from 6 different species (*C. trachomatis*, *C. pneumoniae*, *C. caviae*, *C. muridanum*, *C. abortus*, and *C. felis*) have been sequenced and are currently available to the public. More species and strains are being sequenced.

As one of two bacterial lineages whose members are exclusively intracellular parasites (the other is *Rickettsia*), the order of *Chlamydiales* (*Chlamydiaceae*, *Parachlamydiaceae*, *Waddliaceae*, *Simkaniaceae*) represents a unique group of prokaryotes (Everett et al., 1999). Based on ribosomal RNA sequences, it has been estimated that *Chlamydiales* diverged from the other lineages approximately 2 billion years ago, whereas the pathogenic species of *Chlamydiaceae* diverged from environmental chlamydiae 700 million years ago (Schachter, 1999). Genome sequencing also indicated that one *Chlamydiales* species, *Acanthamoeba* sp. UWE25, shares the unique ribosomal superoperon structure with pathogenic chlamydiae, cyanobacteria, and chloroplasts, suggesting a possible evolutionary relationship between *Chlamydiales* and cyanobacteria (Horn et al., 2004).

CHLAMYDIAL DISEASES

Epidemiology. Bacteria of the family *Chlamydiaceae* are parasites in a broad spectrum of hosts including reptiles, amphibians, birds, and mammals, as well as invertebrates, and cause a variety of acute and chronic diseases. In humans and livestock most *Chlamydia* / *Chlamydophila* infections are asymptomatic, but still pose a serious challenge to public health and lead to huge economic losses.

Worldwide, 72.6% of all countries and regions report chlamydial infections in humans, predominantly with *C. trachomatis* (Resnikoff et al., 2004). The ocular infection, trachoma, is highly endemic in North Africa, the Middle East and the Southern Indian subcontinent. Worldwide, it is estimated that 400-600 million individuals have trachoma, among which about 6 million people were blinded (Thylefors et al., 1995). *C. trachomatis* is also the most common sexually transmitted bacterial agent. The World Health Organization (WHO) estimates that it infects 90 million people every year (1996), and 4 million of them occur in the United States (Center for Disease Control and Prevention, 1998). Additionally, *C. trachomatis* pulmonary infection is estimated to cause 20-30% of all hospitalized pneumonia in infants less than 6 months old (Schachter and Grossman, 1981).

An epidemiological study of *C. pneumoniae* showed that more than 60% of adults have had some exposure during their lifetimes (Schachter and Grayston, 1998). Thus, it is reasonable to infer that most people have been infected by *C. pneumoniae* at some point during their lives (Schachter, 1999). However, *C. pneumoniae*, as an opportunistic pathogen, do not always cause symptomatic disease. Generally, chlamydiae-related

pneumonia and scarring of the lung parenchyma are important causes of morbidity only in older adults (von Hertzen, 1998, Pacheco et al., 1991). Grayston (1992) estimated that every year 8 of every 10,000 people have *C. pneumoniae*-associated pneumonia.

In animals, it is recognized that chlamydial infections are ubiquitous, although they only cause sporadic diseases. Predominantly *C. abortus* has been isolated in cases of abortion in sheep, goats, and cattle, and occasionally in horses, swine, rabbits, and guinea pigs (Appleyard et al., 1983, Jain et al., 1975, McCauley et al., 1968). Usually, animals are infected by *C. abortus* by contact with infected and aborting ewes in the placenta, uterine discharge, or feces excreted by asymptomatic carrier animals.

C. pecorum infections have also been recognized in most parts of the world. Infection of cattle and pigs by *C. pecorum* is thought to be analogous to *C. trachomatis* infection in humans (Hitchcock, 1999) and can be asymptomatic while leading to chronic sequelae such as pelvic inflammatory disease and infertility.

Pathogenesis and immunity. *Chlamydia* spp. infect virtually all eukaryotic cells, from amoeba and hydra through insects, marsupials, and higher organisms (Everett et al., 1999), and cause some form of disease in virtually all infected hosts. In most cases, chlamydial disease is not the result of active injury caused by the pathogen, but by the host immune response to a persistent chlamydial infection. Whereas scarring is considered to be the hallmark of chronic chlamydial infections (Hitchcock, 1999), it is often collateral damage from exacerbated inflammation. The severity of chlamydial disease is dependent on many factors, including genetic background and immune status

of the host, virulence of the chlamydiae, as well as infection dosage and site (Ward, 1999).

Like for other intracellular pathogens, the main protective immune response against infection with *Chlamydiaeceae* spp. is a Th1 driven immune response (Ferrick et al., 1995). Efficient clearance of *Chlamydia* spp. requires a strong Th1 response, typically characterized by high levels of Interleukin 2 (IL-2) and gamma interferon (IFN-γ). This response, however, often creates a cytokine storm and can cause the early death of the hosts. A potent Th2 response, on the other hand, is essential for limiting pathology caused by excessive inflammation.

Disease manifestations in humans. Three chlamydial species commonly infect humans: *Chlamydia trachomatis* causes trachoma, an ocular disease, and after sexual transmission salpingitis and pelvic inflammatory disease that may lead to infertility, as well as urinary tract infections or lymphogranuloma venereum (LGV strains). *Chlamydophila pneumoniae* causes a broad spectrum of respiratory disease manifestations and associates with exacerbation of chroninc inflammatory diseases such as atherosclerosis. *Chlamydophila psittaci* is primarily an avian pathogen that may occasionally cause severe, even fatal, interstitial pneumoniae in human subjects. (Schachter, 1999)

Discovered in 1907 by Halberstaedter and von Prowazek, *Chlamydia trachomatis* is the first chlamydial species discovered in humans. There are three biovariants of *Chlamydia trachomatis*: the trachoma biovar, which is the leading cause of infectious blindness in the world (serotypes A-C), the sexually transmitted (STD) noninvasive

biovar comprised of serotypes D-K, and the invasive LGV biovar comprised of LGV strains 1-3 (Budai, 2007, Burton, 2007, Thomson et al., 2008).

Chlamydophila pneumoniae has been shown to be associated with a number of diseases, including pneumonia erythema nodosum (Kousa et al., 1980), (Saikku et al., 1985), respiratory tract infections (Grayston et al., 1986), coronary heart disease (Saikku et al., 1988), asthma (Hahn et al., 1991), sarcoidosis (Gronhagen-Riska et al., 1992), chronic obstructive pulmonary disease (COPD; Blasi et al., 1993), reactive arthritis and myocarditis (Gran et al., 1993), meningitis (Sundelof et al., 1993), renal failure (Marchant et al., 1995), cerebrovascular diseases (Wimmer et al., 1996), abdominal aortic aneurysms (Ong et al., 1996), valvular stenosis (Juvonen et al., 1996), central nervous system infections (Koskiniemi et al., 1996), and lung cancer (Laurila et al., 1997). While *C. pneumoniae* is often found at high prevalence with these diseases, the cause and effect relationship has yet to be determined in most cases. Therefore, many researchers have found the association between *C. pneumoniae* and many of the chronic diseases listed above unconvincing. Schachter (1999) argue that the prevalence might be attributed to the ubiquitous existence of *C. pneumoniae* in the environment.

Numerous *Chlamydophila pneumoniae* strains are also found in marsupials, reptiles, and amphibian including koalas, emerald tree boa, iguanas, turtles, chameleons, and frogs (Kaltenboeck et al., 1992, Reed et al., 1975).

Chlamydophila psittaci causes endemic avian chlamydiosis in a wide range of domestic and feral bird species, epizootic outbreaks in mammals, and respiratory psittacosis in humans. All avian species can be natural hosts for *C. psittaci* (Schachter,

1999). In humans, symptoms of *C. psittaci* infection are mainly a severe atypical pneumonia and a systemic toxic/septic form without respiratory involvement. In chronic cases, recovery may be prolonged and complications including encephalitis, meningitis, myelitis and liver involvement may arise (Vanrompay *et al*., 1995).

Disease manifestations in livestock. Two major chlamydial pathogens in livestock are *Chlamydophila* (*C.*) *abortus* and *C. pecorum*. Both species are highly prevalent in dairy species. In a study with a herd of 51 virgin Holstein heifers, 53% of the vaginal cytobrush swabs taken once a week throughout a month from these heifers were found to be positive by 23S and *omp1* FRET PCRs (DeGraves et al., 2003a, DeGraves et al., 2004). The positive percentages of *C. abortus* and *C. pecorum* were 24% and 39%, respectively, although all the heifers were clinically healthy.

C. abortus (formerly termed *Chlamydia psittaci* strain B577) causes abortion and fetal loss in sheep, cattle and goats. Infection with *C. abortus* also causes abortion and mastitis in cattle, and pneumonia in pigeons, turkeys and sparrows. There are reports of human abortion, or severe respiratory disease, derived from chlamydiae from aborting sheep (Mare, 1994).

C. pecorum is another chlamydial species that infects only mammals. In cows, it represents two thirds of all chlamydiae detected and sometimes causes severe diseases such as pneumonia, polyarthritis, nephritis, encephalomyelitis, enteritis, diarrhea and urogenital infection as well as fertility disorders and kerato-conjunctivitis. It is also

subclinically associated with airway obstruction, pulmonary inflammation and reduced weight gains (Fukushi and Hirai, 1992, Everett et al., 1999).

Diagnosis. Chlamydial infections are usually asymptomatic, while still impairing the health of the host. Therefore, it is important to note that apparent clinical symptoms and pathological lesions should not be deciding factors in diagnosis. Traditionally, diagnosis of trachoma is based either on demonstration of characteristic *C. trachomatis* inclusions in the cytoplasm of cells from the conjunctival scrapings, or as identified by Giemsa stain or immunofluorescent staining (Schachter and Stamm, 1999). Genital infection of *C. trachomatis* is often diagnosed by culture, but direct fluorescent antibody tests and enzyme immunoassays are more commonly used now. For serodiagnosis in humans, the microimmunofluorescence (MIF) test is still the gold standard (Dowell et al., 2001, Wang, 2000). In animals, the complement fixation test (CFT) is used as a standard procedure, although the enzyme-linked immunosorbent assay (ELISA) is also receiving wide applications (Kaltenboeck et al., 1997a).

Methods for direct detection of *Chlamydiaceae* by nucleic acid amplification such as PCR and LCR (ligase chain reaction) are also widely used. Most importantly, the high-sensitivity and high-specificity method of PCR is becoming increasingly common as its cost decreases (Kaltenboeck and Wang, 2005). DeGraves et al. (2003a) have developed a real-time PCR platform which is capable of detecting single genome copies of chlamydiae. This platform has been used to detect *C. abortus* and *C. pecorum* in bovine vaginal cytobrush specimens in a field project (DeGraves et al., 2003b).

Animal models in chlamydial research. Many laboratory animal models of chlamydial infection are available (Vanrompay et al., 2006, Sumito and Tadayoshi, 2001). For example, mouse and hamster models are widely used for a broad range of chlamydial research, such as the study of pathogenesis, host-pathogen interaction, and vaccination strategy. The use of specific pathogen free (SPF) mice, and genetically engineered mice, further widens the application of the mouse models. Other than rodents, experimental inoculations and clinical trials with ruminants and swine are used for the study of veterinary chlamydial diseases, and non-human primates are used to model human diseases. Although the results from animal models cannot be directly applied to humans, they offer insights into different aspects of chlamydial pathogenesis (Jee et al., 2004, Finco et al. 2005, Li et al., 2006).

VACCINATION AGAINST *CHLAMYDIACEAE*

The need for an effective chlamydial vaccine is emphasized by the fact that antibiotic therapy is usually ineffective after chlamydial infection has been established. Commercial vaccines against *Chlamydia* / *Chlamydophila* spp. have been prepared from live attenuated organisms, but they have not been fully evaluated and do not provide a safe and consistent effect. In one vaccination field trial, fewer children in the *Chlamydia*-vaccinated group progressed to severe trachoma than children in the mock-vaccinated group, but paradoxically the average severity of the disease of the vaccinated group was higher. This undoubtedly underscores the immunopathological nature of chlamydial disease (Sowa et al., 1969).

In addition to the traditional prophylactic vaccines that prevent diseases, there is a growing trend of using therapeutic vaccines to alleviate disease symptoms. Efforts to develop therapeutic vaccines have progressed considerably against cancer, AIDS, hepatitis B, tuberculosis, scherosis and autoimmune diseases. Whereas preventive vaccines carry antigenic epitopes to induce antigen-specific immune responses, they usually require vaccination before exposure to be effective. Therapeutic vaccines attempt to reduce disease and facilitate elimination of the pathogen, often by breaking the host immune tolerance, shutting down the infectious cycle, or silencing specific genes of the pathogen (Sela and Hilleman, 2004).

In recent years, it has been recognized that an inexpensive therapeutic vaccine capable of reducing the disease and providing some protection from re-exposure would be the best way to control *Chlamydia* (Woldehiwet, 2006). Vaccination with chlamydial peptides or recombinant proteins has been largely unsuccessful (Taylor et al., 1988). Therefore many researchers now explore DNA as the agent directing the expression of chlamydial subunit vaccines (Longbottom and Livingstone, 2006). One of the main problems of pure recombinant or synthetic vaccines is that they are generally less immunogenic than live or killed whole bacterial vaccines (Petrovzky and Aguilar, 2004). As a result, adjuvants and other immunomodulators are often used to address this weakness.

For a successful therapeutic vaccination strategy, three critical issues must be carefully considered: identification of protective antigens, selection of optimum adjuvants and choice for delivery route of the antigens. These issues will be explored and discussed in this dissertation.

RESEARCH OBJECTIVES

The long-term goal of this project is to create a therapeutic vaccine that effectively protects humans from *C. pneumoniae* infections. This overall goal will be approached in the following specific research objectives:

1. To evaluate the protective effect of a therapeutic vaccine in a field trial, in which dairy cows vaccinated with *Chlamydophila* vaccine or a combination vaccine against bovine viral diseases will be individually monitored in health status, milk yield, antibody levels and pathogen shedding, and compared against mock vaccinated animals.

2. To test 12 *C. pneumoniae* vaccine candidate genes that were previously identified from a full genome exression library immunization (ELI) scan for protection against *C. pneumoniae* infection in a mouse model. These genes will be constructed in a pCMVi-UB vaccine vector, and used to immunize groups of 10 mice with a gene gun. After a *C. pneumoniae* high-dose challenge, day-10 survival of the mice will be assessed; and all surviving mice will be sacrificed to obtain post-challenge lung weight, as well as *C. pneumoniae* lung loads. These will be three primary criteria for evaluation of protection.

3. To test 6 vaccine candidate genes arbitrarily selected from literature that have been reported to confer protection against chlamydiae in rodent hosts. These genes will be processed similarly and their protective effect in comparison to the ELI-selected vaccine candidates will be evaluated.

CHAPTER 2: THERAPEUTIC *CHLAMYDIA* VACCINES TRANSIENTLY REDUCE BOVINE MASTITIS

INTRODUCTION

Mastitis, the inflammation of the mammary gland, is the most prevalent production disease in dairy cows and is among the livestock diseases that cause the greatest economic losses in animal agriculture (Seegers et al., 2003). In the United States, mastitis is estimated to cause an annual loss approaching 2 billion dollars (Schroeder, 1997). Losses are mainly due to reduction in milk quantity, and to a lesser extent in quality. Classically, infections with bacteria such as *Streptococcus agalactiae*, *Staphylococcus aureus*, and *Escherichia coli* have been the main cause of bovine mastitis (Schukken et al., 2003). Intensive husbandry practices have been associated with an increased incidence of mastitis caused by atypical bacterial agents such as *Streptococcus dysgalactiae* and *Mycoplasma bovis* (Pfützner and Sachse, 1996, Schukken et al., 2003). Despite decades of intensive research in bovine mastitis and extensive prophylactic and therapeutic measures, this disease remains a major problem in the dairy industry, and causal agents remain undiagnosed in a large proportion of cases ("sterile mastitis").

Exposure to infection with obligate intracellular *Chlamydophila* spp. bacteria is probably ubiquitous in cattle worldwide, with high seroprevalence rates approaching 100% in some investigations (Cavirani et al., 2001, Kaltenboeck et al., 1997a, Wang et al., 2001). Two *Chlamydophila* species are routinely detected in cattle, *C. abortus* and *C. pecorum* (Everett et al., 1999, Schachter et al., 1975). Acute infections with these bacteria have been associated with numerous distinct clinical disease entities in cattle, most prominently abortion and fertility disorders, sporadic encephalomyelitis, kerato-conjunctivitis, pneumonia, enteritis, and polyarthritis, (Bowen et al., 1978, Griffiths et al., 1995, McKercher et al., 1966, McNutt and Waller, 1940, Otter et al., 2003, Storz et al., 1968, Twomey et al., 2003, White, 1965, Wilson and Thomson, 1968, Wittenbrink et al., 1993). However, the vast majority of *Chlamydophila* spp. infections in cattle, particularly in the case of low-level infections more frequently detected since the introduction of sensitive PCR methods, are not associated with obvious clinical disease (Danesh et al., 1997, Jee et al., 2004). A well balanced host-parasite relationship appears to represent the common nature of chlamydial infection (Shewen, 1980). Thus, while it is clear that high-dose experimental inoculations and natural infections with *Chlamydophila* spp. result in defined disease manifestations, the health impact of the ubiquitous subclinical infections remains unknown.

Experimental inoculation of *C. abortus* via the teat canal produces a severe acute mastitis of the inoculated mammary glands accompanied by fever and anorexia (Corner et al., 1968, Mechlinger et al., 1980, Rank, 1999). After initial fibrinous and serous secretion and pronounced swelling of the udder in the first week, the disease appears self-limiting, and leads to a state of reduced milk production and mammary gland atrophy. *C.*

abortus has also been sporadically associated with naturally occurring bovine mastitis (Kaltenboeck et al., 1992, Kaltenboeck et al., 1997b, Wehnert et al., 1980); however, systematic investigations of the involvement of *C. abortus* in bovine mastitis have not been reported. In a recent study on the epidemiology of *Chlamydophila* spp. infection in calves, Jee et al. (Jee et al., 2004) detected *C. abortus* in the milk of 15% of the dams without any signs of disease. One-hundred microliter milk samples from a single udder quarter were tested per week for 12 weeks post partum. Thus sampling intensity was low, and higher prevalence of *Chlamydophila* spp. in milk may be detected with higher sampling intensity. Nevertheless, these results indicate that low-level natural infection of the bovine mammary gland with *Chlamydophila* spp. is most likely common.

For obvious economic reasons, bovine mastitis has been intensely studied since the advent of culture of bacteria on artificial media, and numerous parameters have been established for routine monitoring of udder health (Erskine, 2001, Green et al., 2004, Quinn, 1998). Uniformly accepted among these parameters is the number of somatic cells in milk (somatic cell count, SCC). Milk from a healthy bovine mammary gland contains less than 100,000 somatic cells per milliliter, and there is consensus that more than 10^5 SCC per ml bovine milk indicate inflammation of the mammary gland. Milk with 200,000 or more cells per ml is of reduced value because manufacturing properties are compromised, particularly for cheese production (Dohoo et al., 1991, Kaltenboek et al., 1992, Seegers et al., 2003). Clinically manifested mastitis is typically associated with SCC above 10^6 (Green et al., 2004). Milk SCCs of individual dairy cows are routinely monitored as one of several determinants of raw milk quality and cost. This well established parameter for continuous, non-invasive monitoring of inflammation of the

mammary gland offers an intriguing potential for the study of the effects of clinically inapparent chlamydial infections. Continuous simultaneous detection of chlamydial infection and inflammatory status of the mammary gland by PCR and SCC, respectively, would allow for long-term assessment of the impact of chlamydial infection on the health of an isolated organ. This is important not only for cattle, but also for the understanding of human chronic inflammatory diseases such as pelvic inflammatory disease and reactive arthritis, or coronary heart disease, for which a strong association with *C. trachomatis* and *C. pneumoniae* infection, respectively, has been found (Danesh et al., 1997, Saikku et al., 1988, Schachter, 1999).

The investigation described here was conducted as a prospective study in a herd of 147 dairy cows of the interrelation between chlamydial infection and subclinical inflammation of the bovine mammary gland. To maximize the potential for significant outcomes, the study was designed with an intervention approach by perturbation of the *Chlamydophila*-specific immune response. For this purpose, an inactivated, whole organism vaccine composed of *C. abortus* and *C. pecorum* elementary bodies with the adjuvants Quil-A and aluminum hydroxide was used. Quil-A, a partially purified *Quillaja saponaria* saponin, elicits strong, Th1:Th2 balanced immune responses resulting in high antibody as well as cell-mediated immunity, while aluminum hydroxide effectively targets antigens to antigen presenting cells and mediates prolonged immune stimulation by deposition of the antigen (Cox et al., 1997, Ioannou et al., 2002, McInerney et al., 1999). We report here frequent *C. abortus* infection of the bovine mammary gland, a significant inflammatory response to the inapparent infections indicated by increased milk SCC, and a highly significant, 3 month-long reduction of

milk SCC in dairy cows with *Chlamydophila* spp. infection that were vaccinated against *Chlamydophila* spp.

MATERIALS AND METHODS

Experimental animals. A herd of 147 Holstein (91%) and Red Holstein (9%) cows in Germany was used in this study. Cows had a mean age of 4.8 (2.3-10.4) years (range) and 2.4 lactations (1-8). Cows were maintained in box stalls and fed ad libitum hay and corn silage, supplemented with dried beet shavings, molasses and minerals. Consumption of a grain-based concentrate was transponder-controlled. Replacement heifers were acquired from other producers. Milking was performed twice daily in a 15-cow herringbone milking parlor using standard hygiene and teat-dipping procedures. Forty-two percent of cows after first delivery had milk SCC higher than 1×10^5/ml, and 31% of all cows had milk SCC above 4×10^5/ml. *Staphylococcus aureus* mastitis, a common cause of bovine mastitis herd problems, was not observed in the herd. The average interval to first insemination was 124 days, the average interval between calves was 448 days, and the insemination index was 1.9. Lameness caused by arthritis, tendonitis, or digital dermatitis required frequent intervention.

Experimental design. This investigation was designed as a prospective intervention study (Dohoo et al., 2003). A total of 140 cows were enrolled in the study, with 70 cows each randomly assigned to the *Chlamydophila* vaccine or the mock control vaccine groups. Cows were immunized on days 0 and 35 of the study by subcutaneous

administration of a 2 ml vaccine dose. In addition, all animals received an intramuscular dose of an IBRV-BRSV-PI3V (infectious bovine rhinotracheitis - bovine respiratory syncytial virus - parainfluenza 3 virus) combination live attenuated vaccine (Bayer AG, Leverkusen, Germany) on days 104 and 133, and on days 104 inactivated BVDV (bovine viral diarrhea virus) vaccine followed by live attenuated BVDV vaccine (Merial, GmbH, Hallbergmoos, Germany) on day 140. The clinical status of all cows was determined in the week prior to the first vaccination (day 0), and the body condition relative to the body condition expected for the time of lactation (relative body score, RBS) was scored by a combination of measures of body fat. The RBS determination was repeated in week-long examination periods ending on days 28, 70, and 174. Conjunctival and vaginal swab specimens were collected for *Chlamydophila* PCR assays in the week prior to day 0. Serum samples for determination of anti-*Chlamydophila* antibodies were collected on days 0, 41, 68, and 194. Combined quarter milk samples for SCC determination were obtained from all cows during determination of milk yield on days 0, 12, 44 and subsequently in monthly intervals. Additional quarter milk samples for *Chlamydophila* PCR assays were collected from random subsets of *Chlamydophila*- and mock-vaccinated cows on days 0, 1, 4, 7 10, 94, and 109. All animal experimental procedures were performed by veterinarians, followed federal and state laws, and were supervised by state veterinarians.

***Chlamydophila* vaccine.** *C. abortus* BovEnd 19/88 (Bayer AG, Leverkusen, Germany) and *C. pecorum* LW613 (Spears and Storz, 1979) strains were cultivated in Madin-Darby Bovine Kidney cell monolayer cultures maintained in Eagle's Minimal Essential Medium

supplemented with 10% fetal bovine serum (Li et al., 2005) and partially purified. Chlamydial elementary bodies were inactivated with ethylene imine (Caro et al., 2003). These chlamydiae (10^6 IFU of mixed *C. abortus* and *C. pecorum* per vaccine dose) were used to prepare an aqueous vaccine with partially purified *Quillaja* saponin (Quil-A) and aluminium hydroxide as adjuvants (Caro et al., 2003, Stemke-Hale et al., 2005). A mock vaccine was prepared from identically treated cell medium of uninfected cells.

Clinical and laboratory analyses. Milk SCCs were determined by fluoro-opto-electronic cell counting by use of a Fossomatic FC (Foss A/S, Hillerød, Denmark) somatic cell counter (Schimidt-Madsen, 1975; Schukken et al., 2003). Standard bacterial cultures of milk were performed for cows that showed consistently high SCC or clinical mastitis (Dinsmore et al., 1992). Body condition relative to the expected lactation-dependent body condition (RBS) was determined by the scoring method of Edmondson et al. (Edmondson et al., 1989). Data are shown as actual minus expected body score, therefore a score of 0 indicates no difference between the actual and expected body conditions, a negative score indicates underconditioning, and a positive score indicates overconditioning. Anti-*Chlamydophila* spp. IgG1 serum antibody levels were determined by binding to inactivated *C. psittaci* antigen in an enzyme immunoassay by use of the CHEKIT-*Chlamydia* kit (Bommeli Diagnostics AG, Liebefeld-Bern, Switzerland). Antibody levels were expressed as percentage of a positive control serum.

***Chlamydophila* PCR.** *Chlamydophila* infection status was assessed in vaginal and conjunctival swab specimens and in combined quarter milk specimens by nested *Chlamydophila omp*A PCR (Kaltenboeck et al., 1992; Sachse and Hotzel, 2003). Swab tips were transferred to microcentrifuge tubes containing 500 µl of lysis buffer (0.05 % Tween 20, 0.1 M Tris-HCl, pH 8.5), vortexed, and inserted into 1 ml pipette tips for recovery of residual lysis buffer by centrifugation at 12,000×g for 1 min. The combined liquid was sedimented at 12,000×g for 15 min, the sediments were resuspended in 50 µl lysis buffer, and digested with proteinase K (10 mg/ml) at 60°C for 2 h. After inactivation of proteinase K (97°C, 15 min), samples were centrifuged at 12,000 g for 5 min to remove debris, and 5 µl of the supernatant was used for PCR. Milk specimens were processed using the QIAamp DNA Stool Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions and subjected to PCR as described above.

Variable domains III and IV of the *Chlamydophila omp*A gene were targeted using a nested PCR (Kaltenbeock et al., 1992) modified by Sachse and Hotzel (Sachse and Hotzel, 2003). In the first round, 5 µl of DNA extract were amplified using primer pair 191CHOMP/CHOMP371. Subsequently, 1 µl of the PCR product served as template in the second round, which used primers 201CHOMP/CHOMP336 (Sachse and Hotzel, 2003). For species differentiation, first-round PCR products of all positive samples were subjected to *C. psittaci/abortus/caviae/felis*- and *C. pecorum*-specific nested amplification using primer pairs 218PSITT / CHOMP336s and 204PECOR / CHOMP336s, respectively.

Statistical analysis. All statistical analyses were performed with the Statistica 7.0 software package (StatSoft, Inc., Tulsa, OK). SCC, milk yield, RBS, and anti-*Chlamydophila* antibody levels for the *Chlamydophila*- and mock-vaccinated groups were normalized to the population mean such that the means of the day 0 results were identical for both *Chlamydophila* and mock-vaccinated animals. To identify confounding factors, the data were also stratified for age of the cows, lactation number and stage, and *Chlamydophila* PCR detection. SCC data were logarithmically transformed. During the study period, 10 *Chlamydophila*- vaccinated cows and 8 mock-vaccinated cows progressed from late lactation through a 6-week dry period, then delivered a calf and entered a new lactation. Because of the fundamentally different lactation characteristics, these cows were treated as separate cases for the late lactation and the new, early lactation period. The set of data just prior to parturition was considered day 0 data for the new lactation. Normal distribution of data was confirmed by Shapiro-Wilk's W test, and homogeneity of variances by Levene's test. Data were analyzed by repeated measures analysis of variance (ANOVA). Comparisons of means under the assumption of no *a priori* hypothesis were performed by the Tukey honest significant difference (HSD) test. *Chlamydophila* PCR data were also analyzed by the Fisher exact two-tailed test. Differences at $p \leq 0.05$ in all tests were considered significant.

Table 2.1. Association of milk somatic cell counts with PCR detection of, and antibodies against *Chlamydophila* spp.

		Milk SCC on day 0[a]			Milk SCC on day 12		
		n	Mean (10^3/ml)	*p*	*n*	Mean (10^3/ml)	*p*
Conjunctival PCR, day 0[b]	Negative	82	140.0	0.288	75	119.2	***0.027***[c]
	Positive	33	199.3		31	221.2	
Vaginal PCR, day 0	Negative	78	126.0	***0.012***	73	116.3	***0.017***
	Positive	37	239.4		33	224.8	
Conjunctival + vaginal PCR, day 0	Negative	61	127.3	0.083	57	106.0	***0.011***
	Positive	54	193.4		49	202.1	
Anti-*Chlamydophila* serum IgG1	High	66	147.3	***0.036***	56	121.8	***0.014***
	Low	65	245.8		61	222.1	

[a] Data represent antilog of the mean of the log-transformed SCC.

[b] Cows with bacteriologically positive mastitis were excluded.

[c] Bold and italicized *p*-values indicate significant difference between the two means (Tukey HSD).

RESULTS

Clinically inapparent *Chlamydophila* spp. infection is associated with increased inflammation of the bovine mammary gland. At the initiation of the study, the *Chlamydophila* spp. infection status of all cows was determined by *Chlamydophila* PCR of vaginal and conjunctival swab specimens obtained on day 0, and by anti-*Chlamydophila* spp. serum IgG1 antibody enzyme immunoassay. All cows had anti-*Chlamydophila* serum antibodies, and 49% of all cows were positive in at least one of the day 0 *Chlamydophila* PCRs. PCR-typing revealed that all positive PCRs from milk specimens amplified *C. abortus* DNA fragments. Cows were stratified into *Chlamydophila* PCR-positive and -negative animals on day 0, and into animals with high (above median, more than 75% OD of positive control serum) and low (equal to or below median) anti-*Chlamydophila* antibody levels. Milk SCCs of these groups were analyzed by factorial ANOVA, and cows with bacterial culture-positive, i.e. non-chlamydial, clinical mastitis were excluded from the analysis.

Table 2.1 indicates that cows infected with *Chlamydophila* on day 0 consistently had significantly ($p \leq 0.027$), higher SCCs than non-infected cows on days 0 or 12. Later, the milk SCC differences between day 0-PCR positive and -negative cows disappeared (data not shown). Also, cows with high anti-*Chlamydophila* antibody levels had significantly higher SCCs than cows with low antibody levels ($p \leq 0.036$, Table 2.1). Animals that had low anti-*Chlamydophila* antibody levels had higher SCC throughout the observation period ($p = 0.013$ for combined repeated measures data) than animals with high antibody levels (data not shown). The effect of the interaction between day 0

Chlamydophila PCR reactivity and anti-*Chlamydophila* antibody levels on the combined day 0 and day 12 repeated measures SCC data is presented in Fig. 2.1. Cows that have low *Chlamydophila* antibody levels and are *Chlamydophila* PCR-positive before vaccination have highly significantly higher somatic cell counts than the cows that have high *Chlamydophila* antibody levels and are *Chlamydophila* PCR-negative ($p = 0.001$). Stratification of the animals for age, lactation stage and number, relative body score, and *Chlamydophila*- or mock-vaccination, did not change the trends of the results. Thus, these parameters were not confounding the influence of *Chlamydophila* infection on milk SCC. Overall, SCC data as indicator of udder health indicate that this infection has a significant negative effect on the health of the bovine mammary gland.

Vaccination against *Chlamydophila* reduces milk SCC. To further examine the influence of *Chlamydophila* spp. infection on the inflammatory status of the bovine mammary gland, the anti-*Chlamydophila* immune response of the herd was modified by vaccination with an inactivated whole-organism *C. abortus*-*C. pecorum* vaccine or a control vaccine without chlamydial antigen. Experimental cows were vaccinated on days 0 and 35 with either *Chlamydophila*- or mock-vaccine, and differences between animals vaccinated and animals with unmodified anti-*Chlamydophila* immunity in the kinetics of milk SCC, milk yield, anti-*Chlamydophila* serum antibodies, and relative body condition (RBS) were monitored. Data for these parameters were normalized for *Chlamydophila*- and mock-vaccine groups to the day 0 population mean.

Chlamydophila vaccination elicited a strong, specific immune response resulting in significantly (p = 0.018) increased anti *Chlamydophila* IgG1 antibody levels as compared to mock-vaccinated cows (Fig. 2.2A). The effect of *Chlamydophila* vaccination on milk SCC is shown in Fig. 2.2B. *Chlamydophila*-vaccinated cows had a highly significant (p = 0.007) decrease in SCC, with an average of 123,000 cells/ml milk at all time points after vaccination as compared to mock-vaccinated cows with an average of 230,000 cells/ml milk. Peak reduction was observed on day 76 from 230,000 in mock-vaccinated to 83,000 in *Chlamydophila*-vaccinated cows. Effects of *Chlamydophila* vaccination on milk yields shows a trend toward higher yields beginning 44 days after vaccination; however, the results are not statistically significant (p = 0.471; Fig. 2.3A). Similarly, the relative body condition of *Chlamydophila*-vaccinated cows late after vaccination tends to be better that that of mock-vaccinated cows (Fig. 2.3B). Again, these results fail to reach significance (p = 0.069).

Vaccination against *Chlamydophila* spp. briefly increases, but fails to eliminate, *Chlamydophila* shedding. The influence of day 0 vaccination on the PCR detection of *Chlamydophila* spp. was analyzed in milk samples of a random subset of *Chlamydophila*- and mock-vaccinated cows. Administration of both vaccines were associated with significant ($p \leq 0.01$), one week-long increases in the percentage of cows in which *C. abortus* DNA was detected in milk (Fig. 2.4); no significant difference between the vaccines was observed. Vaccination with live anti-IBRV-BRSV-PI3V vaccine combined with inactivated BVDV vaccine on day 94 was associated with a similar increase in chlamydial shedding in milk. While milk excretion of *C. abortus* organisms reverted to

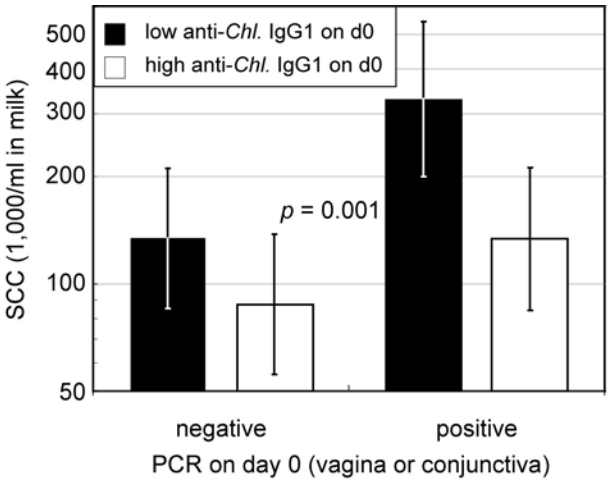

Figure 2.1. Effect of the interaction between day 0 *Chlamydophila* PCR and anti-*Chlamydophila* serum IgG1 on milk SCC on days 0 and 12. *Chlamydophila* PCR-positive cows with low *Chlamydophila* antibody levels before vaccination have significantly higher somatic cell counts on days 0 and 12 than cows that are *Chlamydophila* PCR-negative and have high anti-*Chlamydophila* antibody levels (p = 0.001; combined day 0 and 12 data in repeated measures ANOVA, Tukey HSD). Data are shown as antilog of mean log SCC ± 95% confidence interval.

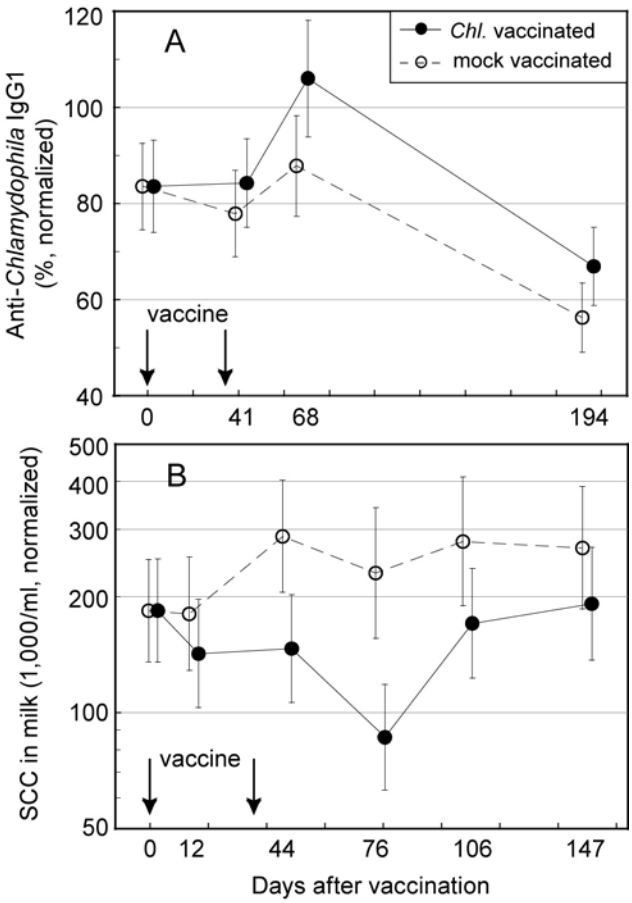

Figure 2.2. Effect of *Chlamydophila* vaccination on anti-*Chlamydophila* serum antibodies and milk somatic cell counts. Data are shown as antilog of mean log SCC ± 95% confidence interval and were normalized for identical day 0 means of *Chlamydophila*- and mock-vaccinated animals (vaccine on days 0 and 35). **A**. *Chlamydophila*-vaccinated cows have significantly higher anti-*Chlamydophila* serum IgG1 levels than mock-vaccinated cows ($p = 0.018$; combined time points after day 0 in repeated measures ANOVA, Tukey HSD). Levels of anti-*Chlamydophila* serum IgG1 antibodies are shown as percent optical density in comparison to a low-positive control serum. All cows had positive pre-vaccination antibody levels. **B**. *Chlamydophila*-

vaccinated cows have significantly lower milk SCC than mock-vaccinated cows (p = 0.007 for all combined time points after day 0, repeated measures ANOVA, Tukey HSD).

baseline shedding on day 10 after vaccination, shedding of chlamydiae never completely stopped, and no difference in shedding was evident between *Chlamydophila*- and mock-vaccinated cows. Thus, any vaccination induced *Chlamydophila* shedding in milk for approximately one week, and the *Chlamydophila* vaccine does not eliminate *Chlamydophila* spp. more effectively than the mock vaccine.

A subset of cows responds to *Chlamydophila* vaccination with increased SCC. The risks of enhancing immune-mediated chlamydial disease by anti-chlamydial vaccination have been well described (Ward, 1999). In a final analysis, we screened only *Chlamydophila*-vaccinated cows that responded with increases rather than decreases in milk SCC. Hyper-responder cows were identified by a 2-fold or higher increase in day 76 over day 0 milk SCC. Four hyper-responders (7%) were identified among the 67 cows remaining by day 76 in the study (Fig. 2.5). These cows show a trend in milk SCC over time that significantly differs from the rest of the herd ($p = 0.002$). The milk SCC of the standard-responders declined until day 76, while an increase in milk SCC was observed in the hyper-responders. Differences in antibody levels, milk production, and RBS between hyper-responding and standard-responding cows were not significant throughout the observation period.

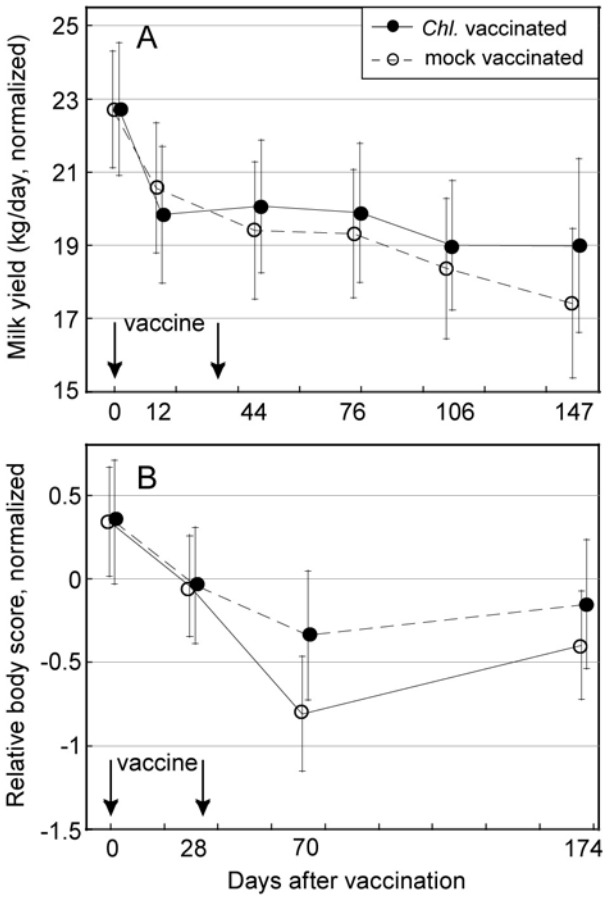

Figure 2.3. Effect of *Chlamydophila* vaccination on milk production and body condition. **A**. *Chlamydophila*-vaccinated cows do not produce significantly more milk than mock-vaccinated cows (p = 0.471; days 44-147 repeated measures ANOVA). **B**. *Chlamydophila*-vaccinated cows tend to have a better body condition on days 70 through 174 than cows that were mock-vaccinated, but the difference does not reach statistical significance (p = 0.069; days 70-174 repeated measures ANOVA; Tukey HSD).

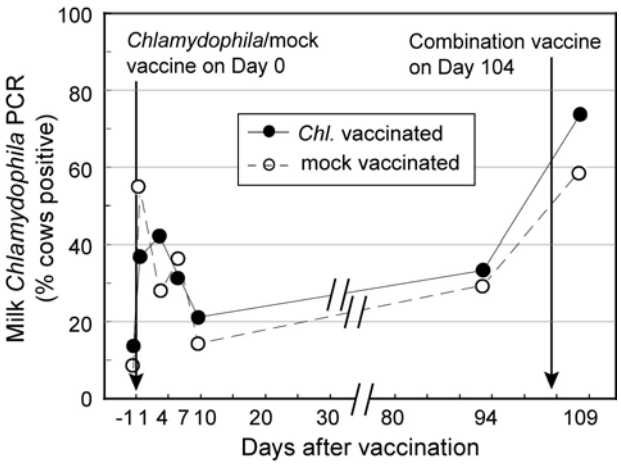

Figure 2.4. Effect of vaccinations on detection of *Chlamydophila* in milk. Cows were vaccinated on day 0 with *Chlamydophila*- or mock vaccine, and all cows on day 104 with a combination of live attenuated IBRV-BRSV-PI3V vaccine and inactivated BVDV vaccine. After both vaccinations, the percentage of cows with positive *Chlamydophila* milk PCR in the combined PCR-tested *Chlamydophila*- (n = 22) and mock-vaccine (n = 19) cows increased significantly. The difference between percent *Chlamydophila* milk PCR positive animals on day 0 versus days 1, 4, or 7 ($p < 0.01$, Fisher exact two-tailed test) or on day 94 versus day 109 ($p = 0.01$) is highly significant. No significant difference of the *Chlamydophila* milk PCR results of any test day was observed between *Chlamydophila*- and mock-vaccinated cows. Both *Chlamydophila* and irrelevant vaccinations therefore increase *Chlamydophila* detection in milk for approximately one week, but the *Chlamydophila* vaccine does not eliminate or reduce *Chlamydophila* spp. shedding significantly as compared to an irrelevant mock vaccine.

DISCUSSION

In this experimental herd, the initial epidemiological survey found 100% seroprevalence and, using conjunctival, vaginal, and milk samples obtained at a single time point, 49% PCR prevalence of *Chlamydophila* spp. infection. These data indicate that every cow is continuously exposed to *Chlamydophila* spp. Cows likely cycle through a period of relative resistance after an infection episode, characterized by increased anti-*Chlamydophila* antibody levels and PCR negativity. This is followed by relative susceptibility to *Chlamydophila* spp. associated with lower antibody levels and increased PCR positivity (DeGraves et al., 2003b, Jee et al., 2004).

The increased milk SCC on days 0 and 12 in *Chlamydophila* PCR-positive animals demonstrate that the inapparent *Chlamydophila* spp. infection, and the inability of the immune response to efficiently eliminate it, is not innocuous to the host. The high SCCs clearly indicate that the *Chlamydophila* infection stimulates a subtle, but quantifiable inflammatory response. This is particularly true for animals with the highest susceptibility to *Chlamydophila*, that are *Chlamydophila*-PCR-positive and have low anti-*Chlamydophila* antibody levels (Fig. 2.1). Cyclicity of anti-*Chlamydophila* immunity might explain the later disappearance of the day 0-12 difference in milk SCC between day 0 *Chlamydophila*-PCR-positive and -negative cows.

Perturbation of the herd anti-*Chlamydophila* immunity corroborated the inflammatory effect of clinically inapparent *Chlamydophila* infection (Fig. 2.2).

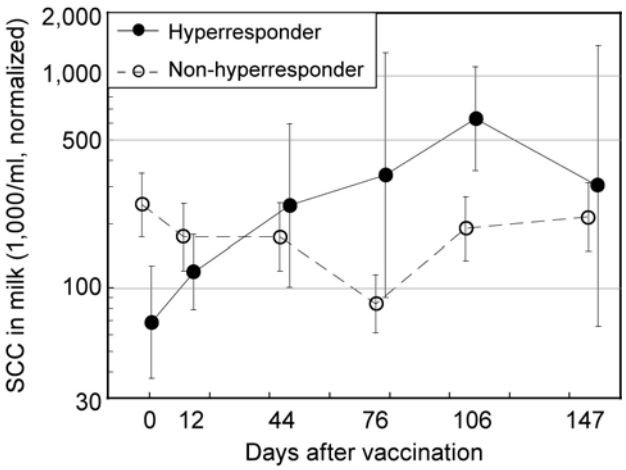

Figure 2.5. Hyper-responders identified among *Chlamydophila*-vaccinated cows. Hyper-responders among *Chlamydophila*-vaccinated cows were identified by a 2-fold or higher increase of day 76 milk SCC over pre-vaccination SCC. Data are shown as antilog of mean log SCC ± 95% CI. The difference in the trend of milk SCC over time between the hyper-responders (n = 4) and standard-responders (n = 63) is highly significant ($p = 0.002$, repeated measures ANOVA, Tukey HSD).

Vaccine-mediated immune stimulation, evident in increased serum anti-*Chlamydophila* antibodies, showed a highly significant association with decreased milk somatic cells in *Chlamydophila*-vaccinated cows (SCC 123,000/ml) as compared to mock-vaccinated animals (SCC 230,000/ml). Even subtle inflammation, in the context of the bovine mammary gland, has major negative consequences for the quality and quantity of milk, and results in economic losses for dairy industry. While the trend in vaccine-mediated increase in milk yield is not significant (Fig. 2.3A), it is consistent with a large body of evidence that links SCC reduction with higher milk production. Data on estimated milk gains in relation to milk SCC suggest approximately 200 kg milk gain per year for a cow with a SCC of 120,000/ml in milk versus a cow with 230,000/ml (Schroeder, 1997, Seegers et al., 2003). *Chlamydophila* vaccination also potentially improves overall health, as suggested by the trend for higher relative body scores in *Chlamydophila*-vaccinated cows, although it fails to reach significance. Clearly, larger studies are required to conclusively demonstrate improvement in milk yields and body condition.

The reduction of milk SCC by the *Chlamydophila* vaccine disappears between days 76 and 106, as evident in Fig. 2.2B, and even more clearly in the serial correlation between SCCs. Between day 76 and 106, anti-*Chlamydophila* antibody levels in *Chlamydophila*-vaccinated cows are still significantly higher than those of mock-vaccinated cows (Fig. 2.2A). These data support the notion that antibody effects are not the protective mechanism of the *Chlamydophila* vaccine. Rather, a body of experimental and epidemiological data suggests that antibodies are only surrogate markers for an immune mechanism that protects the vaccinated animals against *Chlamydophila*-induced disease. Presumably, this mechanism is the Th1 cellular immunity that is required to

clear chlamydial infection (Huang et al., 1999, Rank, 1999). It is likely that the limited time frame of the protective effect is the corollary of the limited life span of immune effector cells.

The modified anti-*Chlamydophila* immune response elicited by therapeutic vaccination of infected animals does not eliminate *C. abortus*, as indicated by consistently positive results of milk *Chlamydophila* PCRs of *Chlamydophila*- and mock-vaccinated cows (Fig. 2.4). Nevertheless, it may well be that the *Chlamydophila* vaccine-induced immune response reduces chlamydial loads, but does not completely eliminate the organisms. The nested PCR method used in this study does not allow discrimination between different chlamydial burdens. It will be interesting to quantify chlamydial milk loads with quantitative PCR methodology in future studies (DeGraves et al., 2003a, Jee et al., 2004).

An intriguing observation is the antigen-independent, week-long increased *C. abortus* shedding in milk after *Chlamydophila*-, mock-, or multivalent vaccination against unrelated bovine viruses (Fig. 2.4). While the mechanism triggering this burst of chlamydial discharge is unknown, a likely candidate for the trigger is the adjuvant content of the vaccines. It is well established that adjuvants mimic pathogen-associated molecular patterns (PAMPs), bind receptors such as toll-like receptors, and initiate a signaling cascade that results in activation of innate immune effector mechanisms that ultimately direct and augment antigen-specific immunity (Schijns, 2003). Changes in host cell metabolism associated with adjuvant action may initially enhance chlamydial replication or release from infected cells. However, this chlamydial release does not provide a specific antigenic stimulus that modulates adaptive immunity such that *C.*

abortus-mediated inflammation of the mammary gland is eventually mitigated. Only the *Chlamydophila* vaccine acted as "therapeutic vaccine" and modulated the existing *Chlamydophila*-specific host response such that inflammation of the mammary gland was reduced for approximately 100 days (Fig. 2.2B).

It is tempting to speculate about the mechanisms involved in the anti-inflammatory, therapeutic effect of *Chlamydophila* immunization of animals with significant immunity to, and concurrent infection by *C. abortus* (Hilleman, 2004, Sela and Hilleman, 2004). The Quil-A saponin adjuvant component of the *Chlamydophila* vaccine strongly stimulates both Th1 and Th2 immune responses, unlike many other adjuvants that mainly stimulate antibody production and Th2 immunity. The aluminum hydroxide component is thought to enhance the antigenic stimulus by antigen deposition and preferentially stimulates Th2 immunity (Cox and Coulter, 1997). As stated above, Th1 immunity is thought to be an absolute requirement for clearance of chlamydial infections, while Th2 immunity mitigates Th1-associated inflammation, but prevents chlamydial clearance. Thus, the precise mechanisms of disease protection are unclear, be it either i) Th1-mediated elimination of *C. abortus*, or ii) Th2-mediated mitigation of *C. abortus*-induced inflammation, iii) a balanced combination of both mechanisms, or iv) an enhanced cell-mediated immune response associated with one of the previous mechanisms.

In a subset of the study population, early vaccination attempts against the human ocular disease, trachoma, caused by *Chlamydia trachomatis*, unexpectedly resulted in an increase in disease severity that resulted from a delayed-type hypersensitivity response (Ward, 1999). This has, to this day, prevented further human vaccine trials and confined

vaccine studies to animal models. We examined *Chlamydophila*-vaccinated cows for evidence of a similar exacerbation of the inflammatory response and found 4 cows that reacted with significantly increased SCC without any signs of bacterial mastitis (Fig. 2.5). SCC in these hyper-responding cows continuously increased until day 106, and subsequently decreased again. Other parameters such as anti-*Chlamydophila* antibodies, milk yield, and relative body condition were not significantly different from the standard-responders. While a hypersensitivity mechanism potentially is involved, the results may well indicate a disease mechanism that is independent of the *Chlamydophila* vaccination. Clearly, further and larger studies are required to address this question.

Clinical utility of a vaccine for medical use is contingent on the absence of serious side effects such as disease exacerbation. This has prompted a decades-long and still unsuccessful search for an effective, but also safe vaccine against human *C. trachomatis* infection (Christiansen and Birkelund, 2002). In contrast, utility of a livestock vaccine is contingent upon improvement of herd health rather than the absence of side effects. The disease-protective effect of the *Chlamydophila* vaccine makes therapeutic vaccination ("antigen-specific immune modulation") for reduction of bovine somatic milk cells an attractive choice for the livestock industries as compared to the use of antibiotics or other drugs for this purpose. The temporal restriction of the vaccine effect will require frequent re-vaccination and targeted use of this vaccine during periods of high risk, but it will also limit negative side effects. In addition, routine continuous monitoring of SCC in dairy herds will rapidly identify potentially hyper-responding cows and thus prevent their repeated vaccination. Use of a *Chlamydophila* vaccine in cattle may also aid to evaluate,

and likely mitigate, the impact of subclinical chlamydial infection on other bovine herd health problems (Stemke-Hale et al., 2005, Wehnert et al., 1980).

In addition to the intrinsic value for control of economic losses in animal agriculture, the *Chlamydophila* vaccine and its use in the natural host population against subclinical mastitis in dairy cows may offer intriguing advantages. Long-term noninvasive sampling and enhanced expression phenotyping afforded by the emerging bovine (*Bos taurus*) genome sequence (http://www.ncbi.nih.gov/Genbank) will allow sophisticated calibration of therapeutic vaccine parameters such as adjuvants, antigen composition of subunit vaccines, application dosages and intervals, and co-administration of antimicrobial, anti-inflammatory, or immunomodulatory drugs. Strategies defined for this natural disease that control chronic inflammation caused by bovine *Chlamydophila* spp. infection may suggest rational approaches to the management of human chlamydial infections, and the consequences of their association with chronic inflammatory diseases such as pelvic inflammatory disease, reactive arthritis, or atherosclerosis (Campbell et al., 1998, Danesh et al., 1997, Quinn, 1998, Schachter, 1999).

CHAPTER 3: IDENTIFICATION OF VACCINE CANDIDATES AGAINST *CHLAMYDOPHILA PNEUMONIAE*

INTRODUCTION

Chlamydophila (*C.*) *pneumoniae* is a major agent of community-acquired respiratory infection and pneumonia. It is also strongly associated with atherosclerotic coronary heart disease in developed countries, and is thought to be involved in the pathogenesis of asthma (Saikku, 2000). These public health concerns indicate a requirement for control of such infections.

Antibiotics can be successful for the treatment of acute pulmonary infection caused by *C. pneumoniae* (de Kruif et al., 2005), but once infection and pathology are established, antibiotic treatment has little effect on the outcome of chlamydial diseases (Longbottom, 2003). For instance, in large scale field trials, antibiotic treatment did not influence atherosclerosis that had been associated with increased antibody levels against *C. pneumoniae* and the presence of the agent in lesions (Hammerschlag, 2003).

Initial attempts to develop effective vaccines against *C. trachomatis* infections and diseases were made in the 1950s and 1960s (Taylor et al., 1992). Such inactivated

vaccines were only partially protective, and a subpopulation of vaccinees even had increased disease (Stagg, 1998). These results discouraged further vaccination attempts in humans, and prompted searches for vaccines in animal models (Brunham and Rey-Ladino, 2005, Murdin et al., 2000). While heat-inactivated whole *Chlamydia* organisms induced expression of co-stimulatory molecules, they failed to induce full differentiation of monocytes into antigen presenting cells and thus failed to induce full immunity (Yamaguchi et al., 2002). Attenuated live vaccines have been developed for animals, but were also inefficient in inducing protection (Brunham et al., 2000). Vaccination by transfer of dendritic cells pulsed with inactivated *C. trachomatis* induced sustained IL-12 production, and directed a protective Th1 cell-dominated response (Lu and Zhong, 1999). However, this approach is presently not practical for clinical use, and dendritic cells pulsed *ex vivo* with chlamydial major outer membrane protein (MOMP, a major vaccine candidate antigen) induced strong, but non-protective Th2 immunity (Shaw et al., 2002). Thus, attempts to date have not yielded a safe, efficacious, and clinically useful chlamydial vaccine, despite the need to develop such a vaccine for immunization against *C. pneumoniae*.

As an alternative to these whole pathogen vaccines, recent trends in vaccine development have turned to component or subunit vaccine compositions. These are far safer and more consistently manufactured, but have often shown reduced efficacy relative to live or inactivated pathogen vaccines. This has been attributed to reduced antigenic complexity and inefficient adjuvants; however another consideration is that the best antigens are rarely, if ever, established for a vaccine.

A number of arbitrarily selected *C. pneumoniae* genes have been tested for protection in rodent models. In one study, heat-aggregated CopN (chlamydial outer protein N) protein, when intranasally administered with *E. coli* heat-labile toxin (LT), induced a strong immune response and protected BALB/c mice against intranasal *C. pneumoniae* challenge. In a different study, in a Balb/c mouse system, immunization with plasmids encoding the major outer membrane protein (MOMP) or an ADP/ATP translocase (Npt1) of *C. pneumoniae* resulted in a reduced bacterial load in the lung after challenge (Murdin et al., 2000). Finco et al. (2005) showed that subcutaneous immunization with recombinant *C. pneumoniae* enolase, among other proteins, significantly decreased the amount of *C. pneumoniae* after an intraperitoneal challenge in hamsters. Svanholm et al. (1999) showed that intranasal immunization with plasmid DNA encoding chlamydial heat shock protein 60 (HSP-60) reduced the *C. pneumoniae* lung loads by 5-20 fold in C57Bl/6 mice, while also decreasing disease severity. Rodriguez et al. (2005) also showed that intranasal, but not intraperitoneal, DNA immunization with *C. pneumoniae* MOMP or HSP-60 conferred protection against *C. pneumoniae* infection, probably due to induction of cell mediated immune responses. In addition, Thorpe et al. (2007) used recombinant LcrE, a potential component of the chlamydial type III secretion system to intraperitoneally immunize BALB/c mice. The LcrE immunization induced $CD4^+$ and $CD8^+$ T cell activation, and decreased *C. pneumoniae* load in the lung tissues. Overall, none of these antigens mediated protection that is close to the protection conferred by natural immunity after asymptomatic low-level *C. pneumoniae* infection. Thus, true highly protective *C. pneumoniae* vaccine antigens

still need to be identified as components of a vaccine with reasonable probability for successful human application.

A solution to the antigen discovery problem is expression library immunization (ELI). ELI is a recombinant DNA pooling strategy that enables the assay of the full repertoire of genome-encoded components of a pathogen for protective antigens using genetic immunization. Since the original demonstration of ELI by intramuscular injection of genetic vaccine constructs for protection against *Mycoplasma pulmonis* pneumonia in mice (Barry et al., 2004, Barry et al., 2005), a number of methods have been used to deliver genes into a host to raise immune responses against the encoded product. Those most commonly used have been injection into intramuscular (IM) or intradermal (ID) sites and DNA-coated particle delivery into skin epidermis with a gene gun (Barry et al., 2004). In an ELI screen, the whole genome of a pathogen is reconstructed as gene fragments (Barry et al., 2005). The library of fragments is manipulated into mammalian expression constructs, partitioned into sublibrary pools, and then used as inocula for test animals. Following pathogen exposure, vaccine utility is evaluated by the single criterion of disease protection (Stemke-Hale et al., 2005). Another technology has been developed to speed construction and improve the quality of expression libraries. Linear expression elements (LEEs) are recombinant-DNA constructs that are built wholly *in vitro*. Requiring no amplification or propagation step that uses a live system such as bacterial cloning, LEEs are built by generating an open reading frame (ORF) by PCR, gene assembly, or some other in vitro DNA construction method, and then covalently or non-covalently attaching gene control elements such as a promoter and terminator (Sykes and Johnston, 1999a, Sykes and Johnston, 1999b). The

desired recombinant expression vector is constructed completely *in vitro* and ready to deliver directly *in vivo*.

The complete 1,230 kb genome sequence of the CDC/CWL-029 strain of *C. pneumoniae* has been published by Kalman et al. (1999). Using bioinformatic approaches, this knowledge allows identification of all putative ORFs for production of LEE vaccine constructs. Thus, all ORFS can be screened for protective candidate antigens for use in a vaccine against *C. pneumoniae*. We have used this approach for testing the complete *C. pneumoniae* genome, and have found highly protective vaccine candidate genes.

MATERIALS AND METHODS

Chlamydophila pneumoniae. *C. pneumoniae* strain CDC/CWL-029 (ATCC VR-1310) was grown with Buffalo Green Monkey Kidney cells, purified by differential centrifugation, and quantified as previously published (Li et al., 2005).

Animals. Inbred A/Jfemale mice were obtained from the Jackson Labratory (Bar Harbor, ME) at 5 weeks of age. Udel "shoebox" type cages with spun fiber filter tops were maintained in static air or ventilated cage racks. Five animals were housed per cage in a temperature-controlled room on a 12-hour light/dark cycle, with ad libitum access to water and one of two diets. Mice were fed a 19% protein/1.33% L-arginine standard rodent maintenance diet. Beginning two weeks pior to the challenge infection and during

challenge infection, a custom diet with 24% protein/1.8% L-arginine (Harlan Teklad, Madison, WI) was used because it was associated in preliminary experiments with enhanced immune responses and lower variance than the standard diet composed of non-chemically defined nutrient components. All animal protocols were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC).

Confirmatory *C. pneumoniae* vaccine candidate screen. After the round 2 screen of 46 candidates (Li et al., 2006), the highest ranked 12 candidates were cloned as full (10 candidates) or partial genes and tested individually in a high-dose *C. pneumoniae* challenge using a day-10 LD_{50} inoculum. This experiment was designed as a rigorous challenge of the protective efficacy of the final candidate genes. The readouts are evaluation of protection from disease by survival of mice and determination of lung weight increase, as well as elimination of *C. pneumoniae* organisms by determination of total chlamydial lung loads.

Genetic immunization was performed by ballistic delivery of recombinant mammalian expression vectors carrying individual bacterial genes under control of a eukaryotic promoter. This genetic immunization vector, pCMVi-UB is described in Fig. 3.1. Bacterial sequences were PCR amplified from *C. pneumoniae* genomic DNA with sets of gene-specific primers using the following two phase protocol. For Phase 1, 2.0 µl 5xiProof buffer (Bio-Rad Labratories Inc., Hercules, CA), 0.2 ul 10mM dNTP (Promega, Madison, WI), 1.0 µl 1uM forward gene-specific primer, 1.0 µl 1 µM reverse gene-specific primer, 1.0 µl genomic DNA (0.4 ng/ul), 0.1µl iProof DNA pol (5 unit/µl), and 4.7 µl water were mixed and thermally cycled as follows: 98°C, 30 sec, followed by 5 times 98°C, 10 sec, 50°C, 30 sec, and 72°C, 15 sec, 20 times 98°C, 10 sec, 62°C, 30 sec,

72°C, 15 sec/kb, followed by 72°C, 7 min. Phase 2 used the entire 10 µl volume of the phase 1 reaction, combined with 10 µl 10x Taq DNA pol buffer (Promega), 2 µl 10mM dNTP (Promega), 2.5 µl 10 µM universal forward dU primer, 2.5 µl 10 µM universal reverse dU primer, 1 µl Taq DNA pol (1 unit/µl), and 72 µl water. The thermal cycling conditions were 95°C, 2 min, followed by 5 times 94°C, 30 sec, 50°C, 30 sec, 72°C, 1.5 min, 15 times 94°C, 30 sec, 64°C, 30 sec, 72°C, 1,5 min/kb, followed by 72°C, 10 min.

The PCR generated fragments were dU cloned into the specially prepared pCMVi-UB vector. The vector was cleaved at *Bgl*II and *Hind*III sites and synthetic single stranded adapters were ligated to the imbedded 3' ends of the cleavage sites. This resulted in generation of protruded 3' ends. Adapter sequences were designed to compliment the ends of the PCR products added during the second phase of the protocol. To generate 3' protruded ends on the PCR products they were treated with UGPase. This removed the primer incorporated dU bases from the 5' ends of the PCR products and exposed complementary to the adaptors 3' ends. The prepared vector and UDGase treated PCR product were mixed together and without any additional steps used for bacterial transformation. Correct integration and sequence of the assembled expression cassettes was confirmed by sequencing.

Plasmid-coated gold particles for gene gun immunization were prepared in a standard protocol (Bio-Rad) using endotoxin free plasmid DNA preparations. Each vaccine dose contained a total of 1 µg of a plasmid DNA mix. The mix contained 0.9 µg of an antigen encoding plasmid and 0.1 µg of a genetic adjuvant. This adjuvant was a 1:4 mixture of two plasmids encoding the B and A subunits of *E. coli* heat-labile toxin (LT

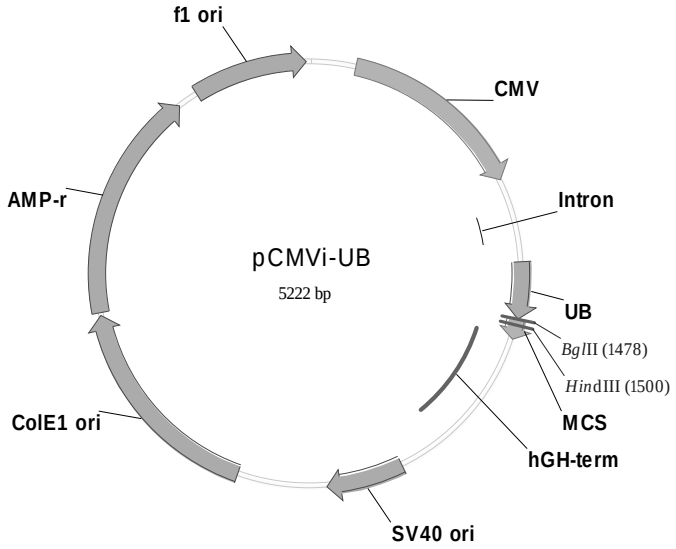

Figure 3.1. Recombinant mammalian expression vector used in round 3 immunization experiments. Vector pCMVi-UB contained individual bacterial genes under control of the eukaryotic modified cytomegalovirus immediate-early promoter enhanced by a chimeric intron (CMVi). A eukaryotic expression cassette was cloned into a generic bacterial plasmid containing pBR322, f1 and SV40 origins of replication and an ampicillin resistance gene. The eukaryotic expression cassette contains a mouse ubiquitin encoding sequence under control of the CMVi promoter and flanked by a multicloning site and a human growth hormone terminator. The bacterial protein encoding sequences were cloned into unique *Bgl*II and *Hind*III restriction site in a manner that ensured continuity of the ubiquitin into a bacterial reading frame. The recombinant cassette expressed a fusion protein comprised of mouse ubiquitin and bacterial protein separated with a linker.

A+B), which has been shown to induce a mixed Th1 and Th2 immune response (Arrington et al., 2002). The coding sequence for subunit A was modified to change the R at position 192 to G to detoxify the gene (Dickinson and Clements, 1995). DNA was delivered by gene gun (Bio-Rad) into each ear lobe of each mouse (10 mice/group). An accelerated vaccination schedule was used to immunize mice on days 0, 3, 6, 20, and 34. Mice were challenged with 5×10^8 *C. pneumoniae* elementary bodies 4 weeks after the last immunization.

Also cloned and used in this round were the genes encoding six *C. pneumoniae* proteins, CopN, Npt1, Enolase, Momp, GatA and HSP-60, that had been reported as protective antigens in the literature. These vaccine candidates were tested by immunizing groups of 10 female A/J mice.

Negative and positive controls. In all experiments, unvaccinated (naïve) but challenged animals served as negative protection controls, and mice immunized with $5x10^6$ genomes of viable *C. pneumoniae* one month prior to the vaccine challenge served as positive protection controls (live vaccination). Groups were scored for protection by calculating the percent lung weight increase over that of age-matched unchallenged female A/J mice (138.4 mg), and by calculating the mean logarithm of total *C. pneumoniae* per lung. These values were then converted to a relative protection score by normalizing them to the lung weight increase or logarithm of total lung *C. pneumoniae* load that was calibrated by control immune (protection score 1 = 100% protection) and naïve (protection score 0 = 0% protection) groups. A CMVi-UB LEE construct encoding the luciferase gene (LUC) served as a control for LEE-based immunizations, and a plasmid

construct pCMVi-UB carrying the same LUC insert was used as the control for plasmid-based immunizations.

***C. pneumoniae* lung challenge infection**. Mouse intranasal inoculation was performed as described (Huang et al., 1999), and optimal doses for live immunization and challenge inocula were determined in preliminary experiments. For intranasal inoculation, mice received a light isoflurane inhalation anesthesia. Vaccine protection control mice were inoculated with a low dose of 5×10^6 *C. pneumoniae* elementary bodies in 20 µl SPG buffer. In rounds 1 and 2, higher-dose challenge infection was performed 4 weeks after the last gene gun genetic vaccination or low dose inoculation of live *C. pneumoniae*, by intranasal inoculation of 1×10^8 *C. pneumoniae* elementary bodies in 20 µl SPG buffer. In round 3, mice were challenged by an LD_{50} dose of 5×10^8 *C. pneumoniae* elementary bodies in 20 µl SPG buffer. Mice were sacrificed by CO_2 inhalation 10 days after inoculation, and lungs were weighed, snap frozen in liquid nitrogen, and stored at -80°C until further processing. Percent lung weight increase was based on naïve lung weights of 138.4 mg for adult female A/J mice.

Mouse lung nucleic acid extraction. Mouse lungs were homogenized in guanidinium isothiocyanate Triton X-100-based RNA/DNA stabilization reagent in disposable tissue grinders (Fisher Scientific, Atlanta, GA) to create a 10% (wt/vol) tissue suspension. This suspension was used for total nucleic acid extraction by the High Pure® PCR template preparation kit (Roche Applied Science, Indianapolis, IN) (DeGraves et al., 2003b; Wang et al., 2004).

Analysis of *C. pneumoniae* lung loads by real-time PCR. The primers and probes used in the PCR assay were custom synthesized by Operon, Alameda, CA. The copy number of *C. pneumoniae* genomes per lung was determined by *Chlamydia* genus-specific 23S rRNA FRET (fluorescence resonance energy transfer) qPCR (DeGraves et al., 2003b).

Data analysis. All analyses were performed with the Statistica 7.1 software package (StatSoft, Tulsa, OK). Data of *C. pneumoniae* genome copies were logarithmically transformed. Normal distribution of data was confirmed by the Shapiro-Wilk's W test, and homogeneity of variances by Levene's test. Data were evaluated by mean plots ± 95% confidence intervals, and analyzed by analysis of variance (ANOVA). Post-hoc comparisons of means were performed under the assumption of no *a priori* hypothesis by the Tukey honest significant difference (HSD) test, or by Dunnett's test for determination of the significant differences between a single control group mean and the remaining treatment group means. Survival data were analyzed by one-sided Fisher Exact test.

RESULTS

The full repertoire of 1,263 ORFs had been independently screened, partitioned into 30 pools of *C. pneumoniae* ORFs, and used to immunize mice by gene gun three times at monthly intervals (Fig. 3.2 [Li et al., 2006]). All ORFs had been evaluated by calculation of a protection score, and forty-six *C. pneumoniae* ORFs were selected for further individual vaccine candidate screening in this investigation (Table 3.1). These 46 individual partial or full-length ORFs were screened as individual LEEs in round 2.

Total lung *C. pneumoniae* protection scores and the ranking of the genes based on these scores are shown in the right 2 columns of Table 1. The results of round 2 selected the following *C. pneumoniae* genes, in this ranking, as candidates for final testing and confirmation in round 3: *cutE*, Cpn0420, *ide*, *oppA_2*, *ssb*, *glgX*, Cpn0020, Cpn0509, *fabD*, *rl1*, *atoC*, and Cpn0095 (Li et al., 2006).

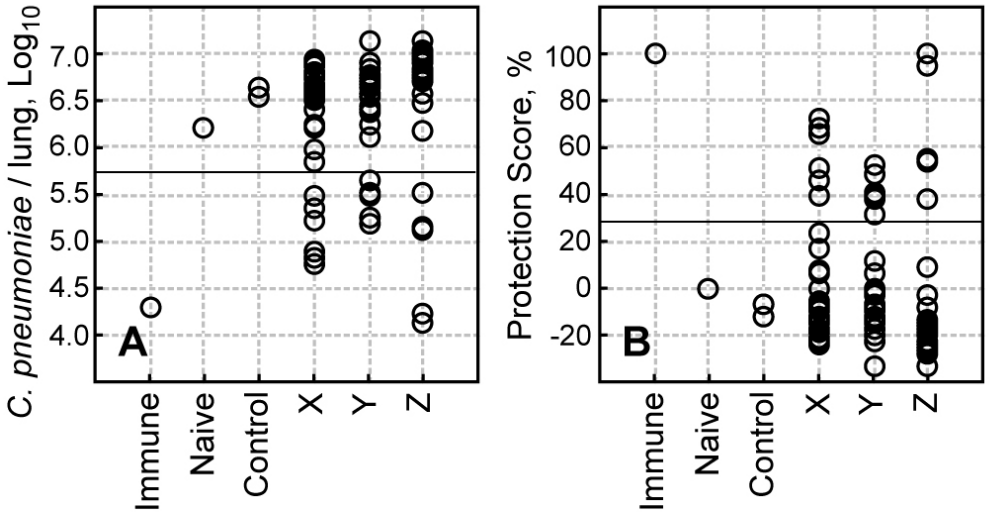

Figure 3.2. Round 1 ELI screen of the complete *C. pneumoniae* genome for protective ORFs in female A/J mice. The LEE library of *C. pneumoniae* ORFs was arranged in 90 pools (30 X-, 30 Y-, and 30 Z) of ~42 LEE constructs each that were used as inocula for 3 gene gun immunizations in 4-week intervals (n=5 mice/pool). Each test inoculum contained 200ng of a mixture of ~42 ORFs and 800ng of pUC118 carrier DNA. Four weeks after the last immunization, all mice were challenged by intranasal inoculation of $1x10^8$ *C. pneumoniae* organisms and sacrificed 10 days later. Positive control, immune mice received a low-dose inoculum of *C. pneumoniae* 4 weeks prior to high dose challenge. Immune and naïve groups (n = 20) were used to calibrate the range of possible protection. Another set of negative control animals was immunized with a construct expressing an irrelevant (LUC) gene product (n = 10). **A.** Group means of total *C. pneumoniae* lung loads (genomes) were determined by real-time PCR. The area below the horizontal line corresponds to the area above the protection threshold line in panel B. **B.** Protective capacity of all test groups. The protection scores are calibrated by a 100% protection score of the immune group and a 0% protection of the naïve group.

The area above the horizontal line contains the vaccine pools that were used to select candidate protection ORFs. ORFs were ranked using the sum of protection scores of the ORF's respective XYZ pools three-way intersection approach of pools above the protection threshold. The combined approach selected 46 *C. pneumoniae* ORFs for further testing in the individual vaccine candidate screens in rounds 2 and 3 (Table 3.1).

Table 3.1. Genetic vaccine fragments of *C. pneumoniae* genes selected in round 1 for further testing in round 2, and selected in round 2 for final testing in round 3.

Gene	Vaccine fragments	Round-1 Total Lung *C. pneumoniae* Protection Score	Round -1 Rank	Round-2 Total Lung *C. pneumoniae* Protection Score [a]	Round -2 Rank[b]
*mutL*_a	2	0.729	1	0.102	21
ldh	1	0.680	2	-0.074	36
atoC	1	0.663	3	0.349	***11***
CPn0249_b	2	0.651	4	-0.295	45
gapA	1	0.622	5	-0.273	44
*ide*_b	3	0.621	6	0.857	***3***
CPn0884	1	0.614	7	0.042	30
CPn0913	1	0.554	8	0.039	31
fabD	1	0.544	9	0.484	***9***
*cutE*_a	2	0.542	10	***1.287***	***1***
CPn0420	1	0.541	11	***1.102***	***2***
CPn0755	1	0.539	12	0.071	24
ppa	1	0.537	13	0.230	15
yigN	1	0.521	14	-0.163	40
*efp*_2	1	0.519	15	-0.098	38
*glgX*_b	2	0.514	16	0.559	***6***
CPn0330	1	0.512	17	0.125	18

Table 3.1 continued.

CPn0095_a	2	0.508	18	0.276	***13***
CPn0020_b	2	0.502	19	0.524	***7***
CPn0174	1	0.502	20	-0.017	34
*ychM*_a	2	0.496	21	-0.109	39
CPn1072	1	0.495	22	0.086	22
CPn0044	1	0.495	23	0.133	17
CPn0155	1	0.495	24	0.228	16
CPn0523	1	0.492	25	-0.214	42
*oppA*_2_a	2	0.489	26	0.762	***4***
CPn0554	1	0.488	27	0.043	28
yacE	1	0.484	28	-0.298	46
CPn0830	1	0.479	29	-0.086	37
fliI	1	0.476	30	0.118	19
rl1	1	0.472	31	0.401	***10***
CPn0509	1	0.460	33	***0.524***	***8***
CPn0981_b	2	0.458	34	0.025	33
CPn1020_b	2	0.438	37	-0.197	41
pyk	1	0.433	40	0.047	27
*ftsH*_a	2	0.416	43	0.053	26
CPn1061	1	0.414	44	0.070	25
CPn0927	1	0.405	47	0.316	12
CPn1070	1	0.405	48	0.028	32

Table 3.1 continued.

*gidA*_b	2	0.403	49	0.112	20
CPn0553	1	0.396	50	0.076	23
rs5	1	0.392	53	0.043	29
CPn0602	1	0.345	70	-0.218	43
ssb	1	0.308	94	0.582	***5***
CPn0369	1	0.299	100	-0.038	35
*pbp2*_b	3	0.290	109	0.244	14

[a] Bold and italicized numbers in this column indicate significant difference ($p < 0.05$) from naïve controls in a post-hoc Dunnett's test for determination of the significant differences between a single control group mean and the remaining treatment group means in ANOVA.

[b] Bold and italicized numbers in this column indicate genes selected for further testing in round 3.

The final 12 highest ranked candidates were cloned as full-length genes into genetic immunization plasmid CMVi-UB, except for *ide* and Cpn0095, which were cloned as fragments ide_ab and Cpn0095_a. Mice were genetically vaccinated with these constructs together with a genetic vaccine adjuvant composed of plasmids expressing mutant, non-toxic *E. coli* enterotoxin A and B subunits (Arrington et al., 2002). A 5-fold increased challenge inoculum of 5×10^8 *C. pneumoniae* elementary bodies was used that elicited severe disease and was lethal in 10 days for approximately 50% of intranasally inoculated naïve female A/J mice (LD_{50}). Cpn0095_a was not used in this challenge. The high-dose challenge was used to evaluate to total protective efficacy of the vaccine candidates for prevention of *C. pneumoniae*-induced death and lung disease, as well as the efficacy in eliminating the agent.

Survival data shown in Table 3.2 indicate that along with the calibration live vaccine, genes *cutE*, Cpn0420, and Cpn0020 prevented death of any inoculated animal while 43% of naïve mice died ($P < 0.05$, Fisher Exact test). In all groups vaccinated with the remaining constructs, one or more animals died, and the survival in these groups was not significantly different from naïve mice. Thus, genes *cutE*, Cpn0420, and Cpn0020 mediated significant protection from *C. pneumoniae*-induced death.

Table 3.2. Survival of *C. pneumoniae* high-dose-challenged mice in round 3 vaccinated with plasmid-cloned *C. pneumoniae* genes selected in round 2 for further testing.

Vaccine	day-10 survival	% day-10 survival[a]
Naïve	8/14	57
Live vaccine	15/15	***100***
Control vaccine	9/10	90
cutE	10/10	***100***
Cpn0420	10/10	***100***
*ide*_ab	9/10	90
*oppA*_2	9/10	90
ssb	9/10	90
Cpn0509	9/10	90
fabD	3/10	30
glgX	7/10	70
Cpn0020	10/10	***100***
atoC	8/10	80
rl1	8/10	80

[a] Bold and italicized numbers indicate significant difference ($p < 0.05$) from naïve controls in Fisher Exact test.

Next, the efficacy of the vaccine constructs in reducing *C. pneumoniae*-induced lung disease (interstitial bronchopneumonia) was evaluated by analyzing lung weight increases of surviving challenged mice when they were sacrificed on day 10 after inoculation. Lung weight increase over unchallenged matched animals is proportional to lung infiltration with inflammatory cells, and therefore reflects disease intensity (Huang et al., 1999). Data shown in Table 3.3 and Fig. 3.3 indicate that genes *cutE*, Cpn0420, *oppA_2*, and *ssb* significantly reduced the lung weight increase of infected mice as compared to the average 64.5% increase of naïve mice (= 0 % protection; live vaccinated mice = 32.0% increase = 100% protection), and thus mediated significant protection from lung disease ($p < 0.05$, Dunnett's test).

Table 3.3. Round-3 protection scores based on the day-10 lung weight increase (over unchallenged mice; equals protection from disease) of *C. pneumoniae* high-dose challenged mice immunized with plasmid-cloned *C. pneumoniae* genes.

Vaccine[a]	Round 3 lung weight increase protection score[b]	*P* for difference to naïve controls[c]
Naïve	0.000	
Live vaccine	1.000	***0.002***
Control vaccine	0.330	0.543
cutE	0.827	***0.029***
Cpn0420	0.948	***0.009***
*ide*_ab	0.648	0.126
*oppA*_2	1.139	***0.002***
ssb	1.033	***0.005***
Cpn0509	0.761	0.058
fabD	0.404	0.605
glgX	0.516	0.304
Cpn0020	0.530	0.230
atoC	0.558	0.231
rl1	0.350	0.526

[a] Naïve n =8, live vaccine n=15; genetic vaccine groups n=3-10.

[b] Dead mice were treated as missing data.

[c] Bold and italicized numbers indicate significant difference ($p < 0.05$) from naïve controls in Dunnett's post-hoc test.

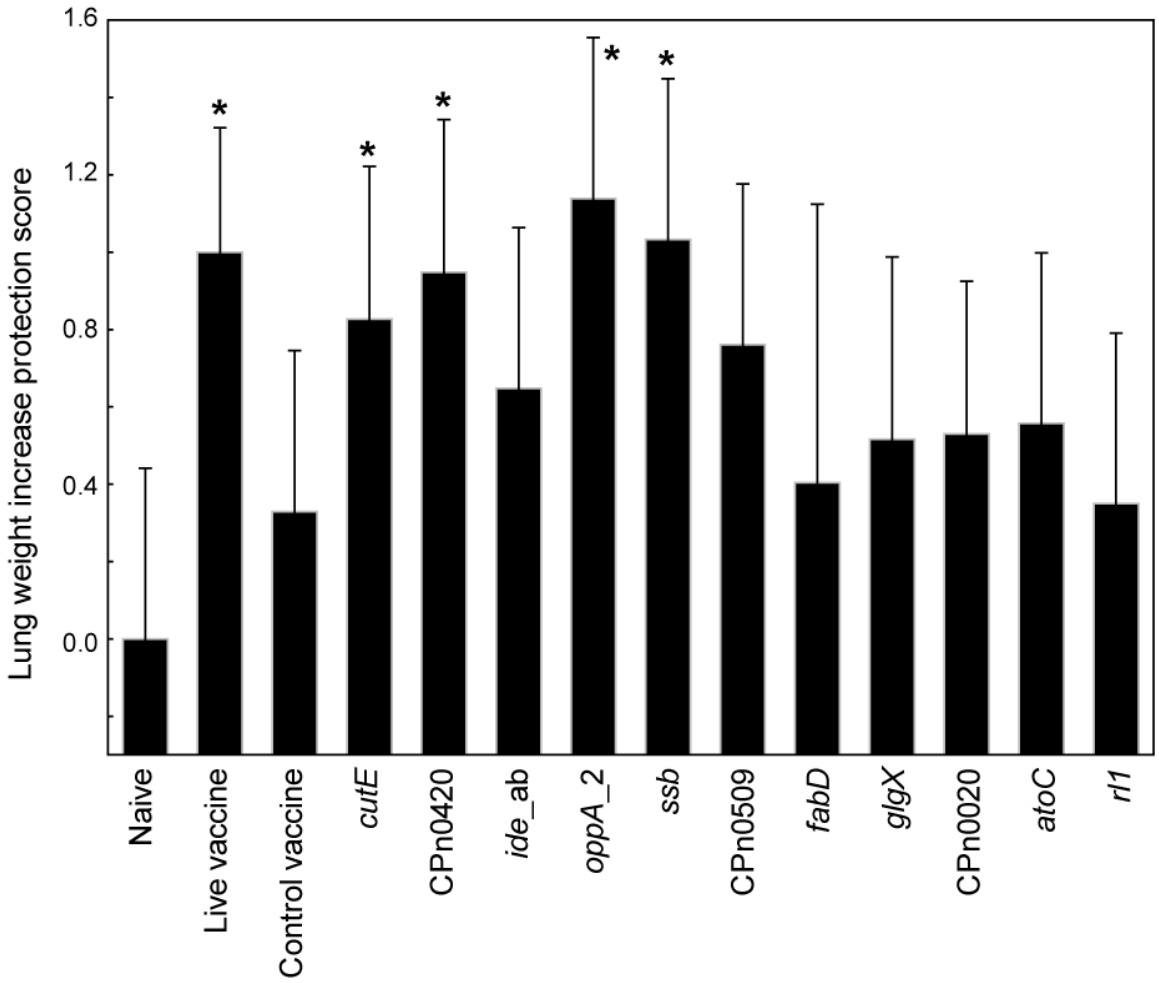

Figure 3.3. Disease protection efficacy of final vaccine candidates. After testing of 46 individual candidates in round 2, 12 of these genes were cloned as full-length genes (except *ide*_ab and Cpn0095_a) into genetic immunization plasmid CMVi-UB and used for vaccination together with genetic vaccine adjuvant LT A+B in round 3. Cpn0095_a was not included in the round-3 high-dose challenge. Vaccinated mice (*n*=10/group) were intranasally challenged with an LD_{50} of 5×10^8 *C. pneumoniae* elementary bodies. Surviving mice (see Table 3.2) were sacrificed on day 10 p.i., lungs were weighed, and the lung weight increase over the average lung weight of unchallenged age-matched female A/J mice was calculated. The lung weight increase is a reliable measure of disease intensity, and high increases reflect severe disease. Lung weight increase data

were linearly transformed into protection scores by setting the score for unprotected naïve mice at 0 and for optimally protected live-vaccinated mice at 1. Data are shown as means ± 95% confidence intervals. Asterisks indicate protected groups that are significantly different from naïve mice ($p < 0.05$; Dunnett's test). The data correspond to data shown in Table 3.3.

Finally, efficacy of the final vaccine candidates in enhancing elimination of *C. pneumoniae* as compared to naïve mice was evaluated. To maximize sample size, protection scores based on the logarithm of total *C. pneumoniae* lung loads on day 10 from rounds 2 and 3 were combined. Due to differences in mouse age, feeding, and challenge inocullum, responses of the naïve and live vaccine mice are not exactly identical in different experiments. In each individual experiment, protection scores were separately calculated so that they relate the efficacy of individual vaccines to the naïve and live-vaccine calibration groups. The adoption of protection scores eliminates differences due to separate experiments by normalizing between the three experiments, thereby making analyses of the combined dataset statistically possible. Efficacy of round-2 LEE-based vaccination with gene fragments (*cutE*_a, *ide*_b, Cpn0095_a, *oppA*_2_a, *glgX*_b, Cpn0020_b) or full-length genes (Cpn0420, *ssb*, Cpn0509, *fabD*, *atoC*, *rl1*) with the plasmid-based vaccination with gene fragments (*ide*_ab, Cpn0095_a) or full-length genes (*cutE*, Cpn0420, *oppA*_2, *ssb*, Cpn0509, *fabD*, *glgX*_b, Cpn0020, *atoC*, *rl1*) was also combined. Cpn0095_a had been used in separate round-2 experiments both as LEE and as plasmid. Data shown in Table 3.4 and Fig. 3.4 indicate that genes *cutE*, Cpn0420, *ide*, Cpn0095, and *oppA_2* mediated significantly enhanced elimination of *C. pneumoniae* ($p < 0.05$, Dunnett's test) as compared to the $10^{6.670}$ *C. pneumoniae* lung load of naïve mice (= 0 % protection; live vaccinated mice = $10^{4.044}$ *C. pneumoniae* lung load = 100% protection),.

Table 3.4. Combined protection scores based on the logarithm of the total *C. pneumoniae* lung load on day 10 after round-2 and -3 challenge infections.

Vaccines[a]	Round-3 $\log_{10}$ total Lung *C. pneumoniae* Protection Score[b]	*P* for difference to naïve controls[c]
Naïve	0.000	
Live vaccine	1.000	***< 0.001***
Control vaccine	-0.096	0.983
cutE	0.853	***< 0.001***
Cpn0420	0.702	***0.006***
*ide*_ab	0.610	***0.024***
Cpn0095	0.607	***0.011***
*oppA*_2	0.599	***0.028***
ssb	0.511	0.075
Cpn0509	0.450	0.135
fabD	0.401	0.302
glgX	0.385	0.258
Cpn0020	0.339	0.301
atoC	0.248	0.526
rl1	0.246	0.530

[a] Naïve, live vaccine groups n=60; genetic vaccine groups n=13-20.

[b] Dead mice were treated as missing data.

[c] Bold and italicized numbers indicate significant difference ($p < 0.05$) from naïve controls in Dunnett's post-hoc test.

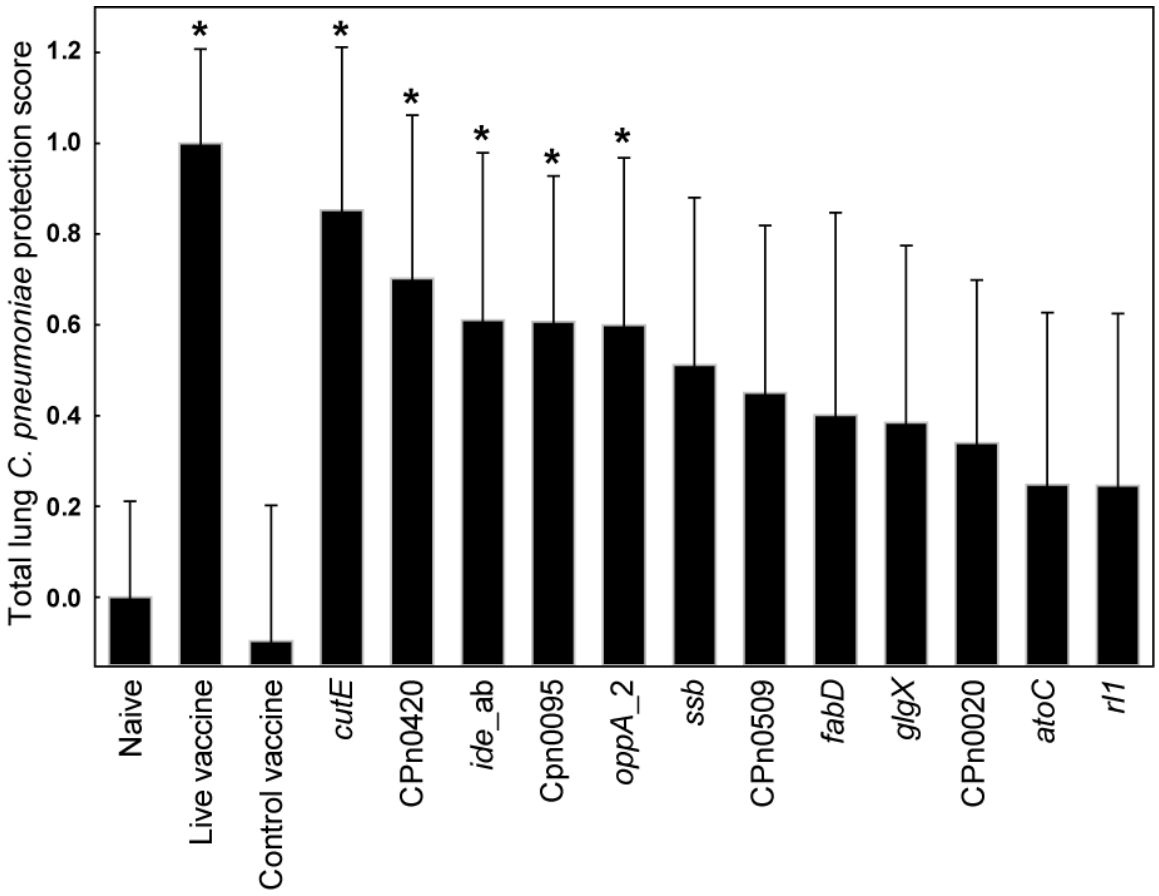

Figure 3.4. Vaccine protective efficacy of final vaccine candidates for elimination of *C. pneumoniae*. For vaccination rounds 2 and 3 of the final vaccine candidate genes, protection scores were calculated based on the logarithm of the total *C. pneumoniae* lung load on day 10. Protection score data from round 2 with the use of LEE constructs and from round 3 with plasmid-cloned genes (full-length except for partial genes ide_ab and Cpn0095_a) were pooled and analyzed by one-way ANOVA. Data are shown as means ± 95% confidence intervals (naïve, live vaccine groups $n = 60$; genetic vaccine groups $n = 13$-20). Asterisks indicate protected groups that are significantly different from naïve mice ($p < 0.05$; Dunnett's test). The data correspond to data shown in Table 3.4.

Comparative screen of 6 individual *C. pneumoniae* vaccine candidates reported in the literature. Among *C. pneumoniae* protective antigens reported in the literature, the most promising candidate genes *copN*, *npt1*, enolase, Momp, *gatA*, and *hsp60* were cloned and tested in comparison to the genes identified in the whole *C. pneumoniae* genome screen in this investigation. Survival data shown in Table 3.5 indicate that mice immunized with genes *Npt1*, Momp and *gatA* had a survival rate of 100%, significantly higher than the naïve mice ($p < 0.05$, Fisher Exact test). In all groups vaccinated with the remaining constructs, one or more animals died, and the survival in these groups was not significantly different from naïve mice. Thus, genes *npt1*, Momp and *gatA* mediated significant protection from *C. pneumoniae*-induced death.

Table 3.5. Survival of *C. pneumoniae* high-dose-challenged mice in round 3 vaccinated with plasmid-cloned *C. pneumoniae* genes selected from literature for comparative testing.

Vaccine	Day-10 survival	% day-10 survival[a]
Naïve	8/14	57
Live vaccine	15/15	***100***
Control vaccine	9/10	90
copN	9/10	90
npt1	9/9	***100***
Enolase	9/10	90
Momp	10/10	***100***
gatA	10/10	***100***
hsp60	8/10	80

[a] Bold and italicized numbers indicate significant difference ($p < 0.05$) from naïve controls in Fisher Exact test.

The efficacy of these 6 candiates in reducing *C. pneumoniae*-induced lung disease was also evaluated by analyzing day-10 lung weight increases of surviving challenged mice. Data shown in Table 3.5 and Fig. 3.5 indicate that none of the previously identified genes mediated significant protection from lung disease.

Finally, efficacy of the final vaccine candidates in enhancing elimination of *C. pneumoniae* as compared to naïve mice was evaluated. Data shown in Table 3.6 and Fig. 3.6 indicate that none of the previously identified genes mediated significantly enhanced elimination of *C. pneumoniae*.

In summary, *cutE* and Cpn0420 identified in the whole *C. pneumoniae* genome screen were genes individually protective by all criteria (survival, disease reduction, *C. pneumoniae* elimination). Gene *oppA_2* was protective by dual criteria (disease reduction, *C. pneumoniae* elimination), and single criterion-protective genes were *ssb* (disease reduction), *ide* and Cpn0095 (*C. pneumoniae* elimination), and Cpn0020, and previously reported genes *npt1*, Momp and *gatA* (survival). Given the high protection mediated by genes *cutE*, Cpn0420, and *oppA_2*, their combined use in a recombinant vaccine may mediate protection equal to previous natural infection (live vaccine) that may merit further investigations for use in human populations.

Table 3.6. Round-3 protection scores based on the day-10 lung weight increase of *C. pneumoniae* high-dose-challenged mice vaccinated with plasmid-cloned *C. pneumoniae* genes selected from literature for comparative testing.

Vaccine[a]	**Round-3 lung weight increase protection score**[b]	***P* for difference to naïve controls**[c]
Naïve	0.000	
Live vaccine	1.000	***0.001***
Control vaccine	0.330	0.415
copN	0.445	0.249
npt1	0.471	0.218
Enolase	0.065	0.807
Momp	-0.025	0.893
gatA	0.140	0.708
hsp60	0.073	0.800

[a] Naïve $n = 8$, live vaccine $n = 15$; genetic vaccine groups $n = 8$-10.

[b] Dead mice were treated as missing data.

[c] Bold and italicized number indicates significant difference ($p < 0.05$) from naïve controls in Dunnett's post-hoc test.

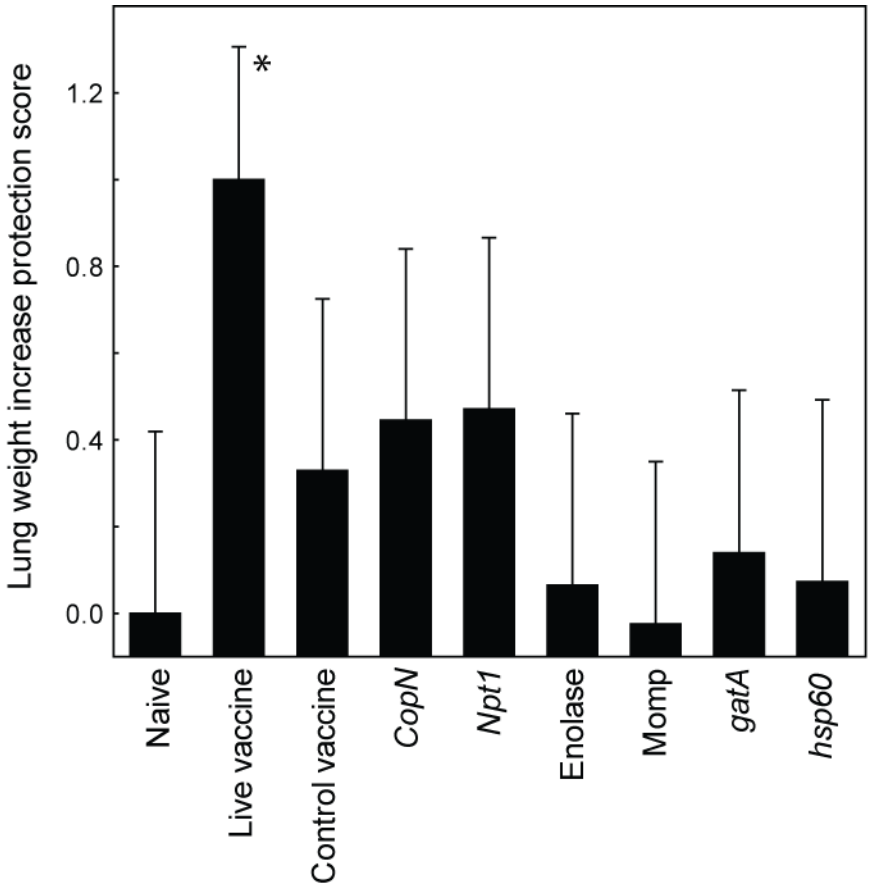

Figure 3.5. Vaccine candidates selected from literature for comparative testing do not confer protection against *C. pneumoniae* disease. Lung weight increase data were linearly transformed into protection scores by setting the score for unprotected naïve mice at 0 and for optimally protected live-vaccinated mice at 1. Data are shown as means ± 95% confidence intervals. Groups do not differ from naïve mice ($p > 0.05$; Dunnett's test). The data correspond to data shown in Table 3.6.

Table 3.7. Round-3 protection scores based on the day-10 *C. pneumoniae* lung loads of the high-dose challenged mice vaccinated with plasmid-cloned *C. pneumoniae* genes selected from literature for comparative testing.

Vaccines[a]	**Round-3 $\log_{10}$ total lung *C. pneumoniae* protection score**[b]	***P* for difference to naïve controls**[c]
Naïve	0.000	
Live vaccine	1.000	**0.001**
Control vaccine	0.065	0.415
copN	0.305	0.249
npt1	0.189	0.218
Enolase	0.148	0.807
Momp	0.095	0.893
gatA	0.124	0.708
hsp60	0.031	0.800

[a] Naïve, live vaccine groups $n = 60$; genetic vaccine groups $n = 8$-10.

[b] Dead mice were treated as missing data.

[c] Bold and italicized number indicates significant difference ($p < 0.05$) from naïve controls in Dunnett's post-hoc test.

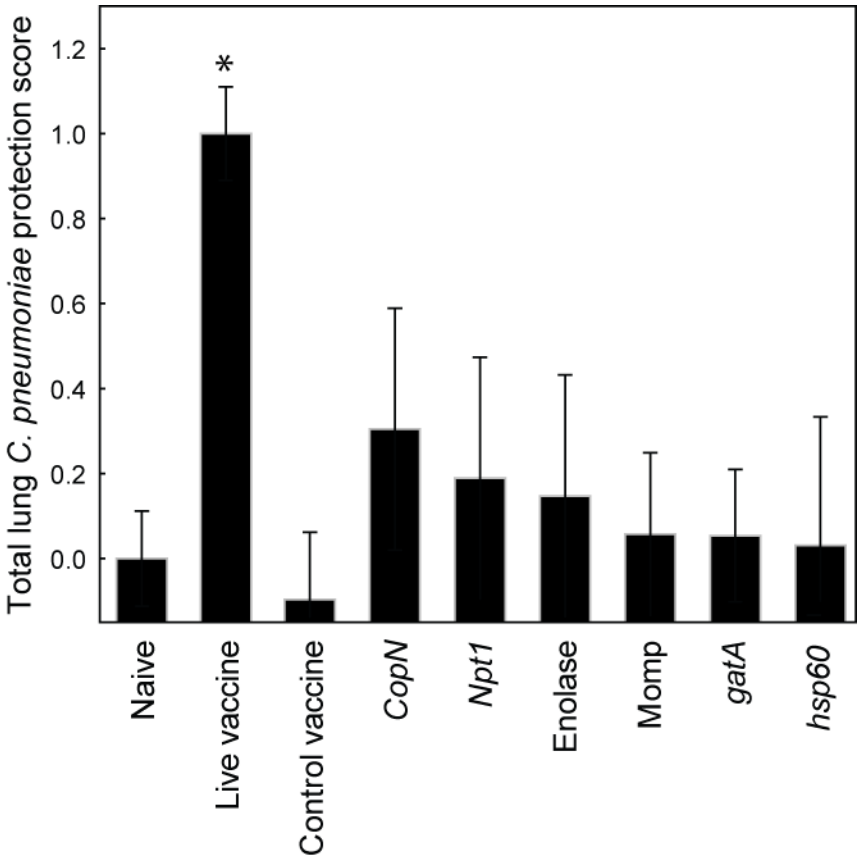

Figure 3.6. Immunization with the vaccine candidates selected from literature for comparative testing do not significantly decrease *C. pneumoniae* long load after the *C. pneumoniae* challenge. Protection scores were calculated based on the logarithm of the total *C. pneumoniae* lung load on day 10 and analyzed by one-way ANOVA. Data are shown as means means ± 95% confidence intervals. No group differs from naïve mice ($p > 0.05$; Dunnett's test). The data correspond to data shown in Table 3.7.

DISCUSSION

In this project, we have scanned all 1,263 putative ORFs of the *C. pneumoniae* genome using ELI (Li et al., 2006, McNeilly et al., 2007), and identified several candidate genes that effectively protect mice against *C. pneumoniae* lung infection. Most significantly, vaccination with *C. pneumoniae* genes *cutE* and Cpn0420 significantly protected mice against C. *pneumoniae* mediated death, reduced lung disease, and increased elimination of the *C. pneumoniae* organisms in the mouse lungs. Some genes, such as *ide*, *oppA*, *ssb*, and Cpn0095, mediated considerable protection, but not significant by all criteria. These genes may nevertheless serve as secondary choice vaccine candidate antigens that should undergo further testing.

Of the six candidate genes arbitrarily selected from the literature that have been reported to confer protection against chlamydial infections, only *copN*, Momp and *gatA* protected mice from *C. pneumoniae*-induced death, however, none ameliorated lung disease, or facilitated clearance of the pathogens.

Immune reactivity to some genes tested in this study, such as *fabD*, *glgX, atoC*, *rl1*, and *hsp60*, resulted in deaths of several mice soon after the challenge infection. This is typically the result of shock precipitated by an uncontrolled release of cytokines ("cytokine storm") during a strongly polarized Th1 immune response. However, the immune response elicited by these genes in surviving mice did not result in later protection from disease or efficient elimination of *C. pneumoniae*. Thus, use of these genes is not advisable in any vaccine.

Conversely, vaccination with genes Cpn0020, *npt1*, Momp, and *gatA* provided complete protection from death, however, they also did not reduce subsequent disease or *C. pneumoniae* lung loads. This suggests that these genes did elicit a limited Th2 response that was protective early in infection, but did not eliminate chlamydiae or the disease that resulted from the continuous chlamydial presence. Thus, incorporation of these genes in a vaccine is also not advisable.

The protective antigens, used individually or preferably in combination, must be further evaluated in vaccine formulations that are appropriate for administration to humans. It has been suggested that a multi-component vaccine would be more effective (Igietseme et al., 2003). For example, Ifere et al. (2006) have shown that a vaccine composed of MOMP and PorB (porin B) induced a higher Th1 response than single single subunit vaccines. It is likely that a combination of the candidate genes identified in this study may provide pronounced protection that is close or equal to the level of protection mediated by prior natural infection.

It is very important to identify suitable adjuvants, since they can selectively induce appropriate immune responses and improve protective efficacy by facilitate specific presentation of the antigens to macrophages or dendritic cells, or facilitate consistant release of the antigens (Longbottom and Livingstone, 2006). Aluminium salts have been shown to be effective, but are not preferable since they induce a Th2-biased humoral immune response (Moingeon et al., 2001). Liposomes and MF59 have also been tested. Arrington et al. (2002) have tested cholera toxin (CT) and the *E. coli* heat-labile enterotoxin (LT) and they elicited a strong Th1-biased immune response, which is

effective against intracellular pathogens. Our previous tests have confirmed the effect of the LT adjuvants (data not shown).

In design of an efficacious vaccine against the intracellular pathogen *Chlamydia* / *Chlamydophila* spp., an efficient delivery system is critical for mediation of a long term protective immunity (Woldehiwet, 2006). Some researchers have used bacteria or bacterial antigens as delivery vehicles and achieved considerable success. As an example, He et al. (2007) used a live attenuated recombinant influenza A/PR8/34 virus as a vaccine vector for intranasal delivery of a subunit vaccine (a chlamydial epitope) against *C. trachomatis* infection, and a strong Th1 response against chlamydial EBs was detected. Additionally, *C. trachomatis* shedding was decreased and long-term protective immunity correlated with the preservation of specific Th1 cells and elevated immunoglobulin G2a in genital secretions.

Another potential avenue for delivery of a *C. pneumoniae* vaccine is DNA vaccine formulations. DNA vaccines are a safer and more stable alternative to killed whole organism or live attenuated vaccines, although unfortunately, they have been less effective in humans and livestock than in mouse models (Longbottom and Livingstone, 2006). Nevertheless, DNA vaccination is a promising strategy, but more work still needs to be done to identify optimal strategies for delivery, adjuvantation, and antigen presentation of DNA vaccines.

In conclusion, this study has identified the best suited protein vaccine candidate antigens among all putative *C. pneumoniae* proteins. These antigens will be the basis for further formulation of an experimental vaccine in a commercially viable format. This vaccine will then be tested in animal models as well as human studies.

CHAPTER 4: OVERALL CONCLUSIONS

Infections with *C. abortus* and *C. pecorum* are highly prevalent in cattle and have been associated with bovine mastitis. To investigate the influence of *Chlamydophila* spp. infection on subclinical inflammation of the bovine mammary gland as characterized by milk SCC, a field trial was conducted in a herd of 147 Holstein dairy cows. PCR detection of *C. abortus* and low serum antibody levels against *Chlamydophila* spp. significantly associated with subclinical mastitis. To examine the effect of the infection by response modification, immune perturbation was used by two subcutaneous administrations of an Alum-Quil-A-adjuvanted preparation of inactivated *C. abortus/C. pecorum* elementary bodies. Vaccination against *Chlamydophila* significantly reduced bovine mastitis, and increased anti-*Chlamydophila* antibody levels, but was not capable of eliminating *C. abortus* shedding in milk. The disease-protective effect peaked 11 weeks after vaccination, and lasted for a total of 14 weeks.

Overall, these results demonstrate the viability of therapeutic vaccination against chlamydial infection, even if this vaccination does not prevent chlamydial infection. It has been obvious for a long time that natural immunity against chlamydiae is not sterilizing, i.e. elimination of chlamydial organisms by the host is accelerated by specific immunity, but is incomplete and requires a relatively long time (1-2 weeks). This is due to the virtually exclusive requirement for T helper cells secreting interferon-γ for immune

protection against chlamydiae. Nevertheless, the benefits of boosting T cell immunity by specific vaccination against chlamydiae are indisputable, as evident in the reduction of disease symptoms. This disease is mediated by a prolonged, but weak and relatively ineffective T cell immune response (Wang et al., 2008). Thus, by boosting protective T cell immunity, chlamydial vaccination may briefly activate the inflammatory response to chlamydiae (enhanced week-1 response in cows) and also accelerate elimination of chlamydial organisms and subsequent mucosal immune surveillance. This yields a net reduction of disease symptoms that lasts for about 3-4 months. This temporary reduction is highly significant and beneficial for animal agriculture; however, it also indicates that sustained re-vaccination may be required to maintain this benefit.

Production of a whole-organism based, inactivated chlamydial vaccine is expensive, whereas whole-organism vaccine likely contains a large load of weakly- or non-protective antigens such as LPS that nevertheless may stimulate pattern recognition receptors of the innate immune system. Therefore, the next step, after proof of principle of therapeutic vaccination, is the identification of optimally suited chlamydial vaccine candidate antigens that mediate maximum immune protection by reducing disease as well as chlamydial organism loads.

C. pneumoniae enhances widespread chronic human inflammatory diseases such as atherosclerosis, similar to the enhancement of bovine mastitis caused by *C. abortus*. Thus, therapeutic vaccination may also be a viable approach to reduce such human diseases. Towards this goal, the second part of the dissertation targeted identification of protective *C. pneumoniae* antigens. We tested twelve *C. pneumoniae* genes previously

identified by expression library immunization among all genes of the *C. pneumoniae* genome. In addition, we compared these genes to six genes that we selected from published data as the best protective genes identified by other methods. These genes had been preselected based on assumptions required for protective efficacy such as surface exposure of the encoded protein or the identification of T cell epitopes by computer algorithms. We individually tested all these genes using a DNA vaccination platform based on biolistic vaccine delivery.

Based on the three criteria of survival rate, post-challenge lung weight increase, and *C. pneumoniae* load in the lungs, seven genes conferred protection to immunized mice by one criterion (*Cpn0020*, *npt1*, Momp and *gatA* for survival; *ssb* for disease reduction; *ide* and Cpn0095 for *C. pneumoniae* elimination), one gene was protective by two of these criteria (*oppA_2* for *C. pneumoniae* elimination and disease reduction), and most significantly, genes *cutE* and Cpn0420 protected mice by all three criteria from *C. pneumoniae*-induced death, alleviated lung disease, and improved elimination of the organisms. Among the six arbitrarily selected vaccine candidates, three (*npt1*, Momp and *gatA*) protected against death mediated by *C. pneumoniae* nasal challenge, but none protected by mediating reduction of disease or chlamydial lung load. Thus, only genes indentified by unbiased screening of the whole *C. pneumoniae* genome by ELI were highly protective and are viable vaccine candidates. Therefore our data show that ELI is the method best suited for identification of chlamydial vaccine candidate genes, and that it is far superior to methodologies for selecting candidates that are based on preconceived notions of requirements for immune-protective antigens.

Our data lay the foundation for a future *C. pneumoniae* therapeutic subunit vaccine. The protective vaccine candidate genes, after being further tested individually and in combination, can be converted to a format that can be applied to humans in the future. The development of a final vaccine will require testing of different delivery platforms, and careful dose titration of vaccine antigens, preferably as a combination of the highly protective antigens *cutE*, Cpn0420, and *oppA*, and appropriate adjuvant formulation.

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