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Progress in *Chlamydia psittaci* vaccine development in poultry

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***Chlamydia psittaci*, depending on its serovar, can infect humans, birds, and other animals and livestock. Its economic impact on poultry production, especially on turkeys and chickens, and potential zoonotic risk have driven the search for an effective vaccination protocol to prevent and control the infection and shedding of the organism. Currently, no vaccine is approved for use against avian chlamydiosis despite efforts in the past decades. The present genomic era presents an opportunity to establish an effective vaccination scheme, taking advantage of the major outer membrane protein (MOMP) as the major protective antigen of *C. psittaci*. The plasmid DNA expressing MOMP can be coupled with optimisation of controllable factors during vaccination such as codon optimisation (through formation of polyplexes and lipoplexes), route of administration, vaccination schedule, addition of adjuvants/co-stimulatory factors such as cytokines and CpG motifs, and recombination with other poultry pathogens such as viruses. The development of an effective vaccine against *C. psittaci* will protect susceptible poultry from infection and production performance losses and reduce the zoonotic risk and minimise the emergence of antibiotic-resistant *C. psittaci* strains.**

Keywords: avian chlamydiosis; Chlamydia psittaci; vaccine

Introduction

Chlamydia psittaci, currently has 15 known genotypes, and is able to infect avian species, mammals, reptiles, and humans (Sachse *et al.*, 2015). The highest infection rates are found in psittacines and pigeons. Turkeys, ducks, and all other domestic poultry are also susceptible; however, manifestation of clinical disease differs between poultry species and the serovar and genotype present (Andersen and Vanrompay, 2000). *C. psittaci* has a great impact on poultry health and economics as well as human health. The asymptomatic status of infected poultry species that are in close contact with humans gives even higher risk for psittacosis especially to those working closely or having

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contact with birds such as farmers, veterinarians, breeders, pet shop personnel, and abattoir workers. It has been reported that the recent human cases were associated with domestic poultry, to a higher extent with the infected ones, rather than with psittacines. The infection in humans (psittacosis) may result in asymptomatic to severe disease condition and even death (Gaede *et al.*, 2008; Beeckman and Vanrompay, 2009).

The impact of the disease, especially being zoonotic, needs effective control and preventive measures such as all-in-all-out confinement, implementation of biosecurity measures, and use of antimicrobials. However, indiscriminate antimicrobial use in some countries may lead to the development of drug-resistant strains. To minimise the use of antibiotics and possible development of resistant strains, vaccination was one of the practical recommended measures (Vanrompay *et al.*, 2007), combined with preventive measures (Longbottom, 2003). Several vaccination protocols against *C. psittaci* in poultry have been studied, but the results showed variable quality of protection.

This paper aims to assess the progress of *C. psittaci* vaccine development for domestic poultry and discuss the prospects of improving efficiency of existing but not-yet-approved vaccine and vaccination protocols for avian species. The review is based on investigations published in peer-reviewed journals from 1999 to present related to *C. psittaci* vaccination regimen for domestic poultry such as chickens, ducks, and turkeys.

Pathogenesis of and immunity against *Chlamydia psittaci*

C. *psittaci* is a Gram-negative, obligate intracellular pathogen with a biphasic developmental cycle consisting of an infectious elementary body (EB) and a non-infectious reticulate body (RB) (Wyrick, 2000). *Figure 1* shows the pathogenesis of *C. psittaci* infection based on the work of Andersen and Vanrompay (2000) and the corresponding innate immune response base on gene expression of cytokines (IL-1β, IL-6, MIF-macrophage inhibitory factor, LITAF (lipopolysaccharide-induced TNF factor), IL-12p35 and IL-10), caspase-1, GSM-CSF (granulocyte macrophage colony-stimulating factor), iNOS (inducible nitric oxide synthase), chemokines (CXCLi1, CXCLi2, CCLi3, IL-16) and TLRs (toll-like receptors; TLR2, TLR3, TLR4, TLR5, TLR7, TL21) (Lagae and Vanrompay, 2015).

During the entry of *C. psittaci* and formation of EBs, cytokines (MIF, LITAF, IL-12p35 and IL-10), caspase-1, GM-CSF, iNOS, chemokine (IL-16), and TLRs (TLR2, TL3, TLR4, TLR5, TLR7 and TLR21) are down-regulated. In the mid-cycle period, there is an increased gene expression of NF-κB-regulated pro-inflammatory cytokines (IL-6, IL-1β, MIF, LITAF, IL12p35 and IL10), and chemokines (IL-16, CXCLi1, CXCLi2 and CCLi30 and iNOS are up-regulated. However, among the TLRs, TLR2 gene expression is absent even up to mid-cycle when *C. psittaci* forms the RBs. The up-regulation and down-regulation of genes associated with the innate immune system contribute to the survival of the organism by enhancing spread to predilection site of infection while evading phagocytosis and the immune system (Lagae and Vanrompay, 2015).

Protective immunity against *C. psittaci* is established by the coordination of neutrophils, macrophages, CD4+ cells, CD8+ cells, and dendritic cells (DCs, which are specific antigen-presenting cells or APCs) and the involvement of major histocompatibility complex (I and II). Infected cells release IL-8 to mobilise and activate neutrophils to destroy the cell. The EBs also stimulate the cleavage of C3-complement system into C3a and C3b. C3a stimulates $CD4^+$ (Th1 and Th2) cells, CD8+ cells, and dendritic cells (DCs). DCs, which are APCs in the context of major histocompatibility I (MHC I) presents antigen to CD8+ cells; while in MHC II, antigens are presented to CD4+ cells. C3a also down-regulates regulatory T cells (Tregs) to

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minimise its inhibitory effect on T cells and DCs. Stimulation of CD4+ cells with IL-4 (Th1 stimulation) and C3b results in the production of antibodies, while Th1 stimulation triggers the release of interferon-γ (IFN-γ) to the direction towards the infected cell. CD8 + cell stimulation results in IFN-γ release for the lysis of infected cells (Kaiser, 2010; Knittler and Sachse, 2015; Radomski *et al.*, 2016).

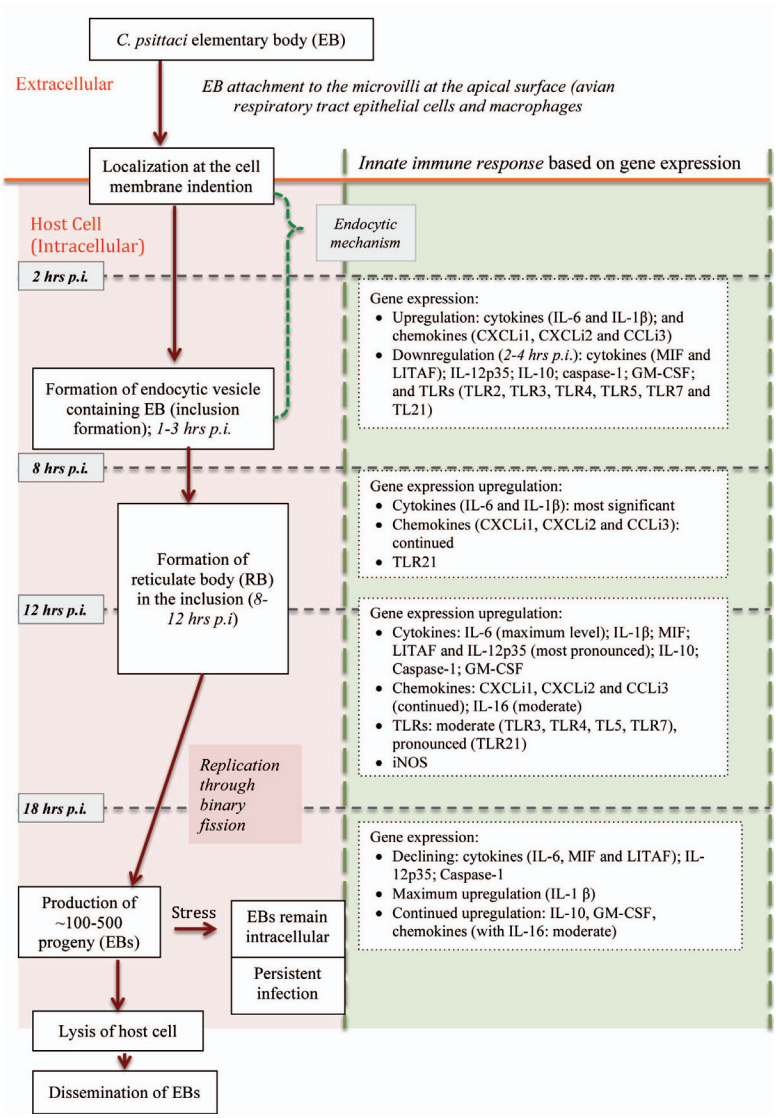


Figure 1 *C. psittaci* pathogenesis and innate immune response.
Pathogenesis of *C. psittaci* infection (left side) and the corresponding innate immune response base on gene expression of cytokines (IL-1β, IL-6, MIF-macrophage inhibitory factor, LITAF-lipopolysaccharide-induced TNF factor), IL-12p35 and IL-10), caspase-1, GSM-CSF (granulocyte macrophage colony stimulating factor), iNOS (inducible nitric oxide synthase), chemokines (CXCLi1, CXCLi2, CCLi3, IL-16) and TLRs-toll like receptors (TLR2, TLR3, TLR4, TLR5, TLR7, TL21) (Andersen and Vanrompay, 2000; Lagae and Vanrompay, 2015).

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Research on vaccination against *Chlamydia psittaci* in poultry

Reverse vaccinology produces vaccines based on genomic information for antigenic proteins and does not require the culture of microorganisms (Serruto and Rappuoli, 2006; Rappuoli *et al.*, 2011). DNA vaccines can elicit humoral and cell-mediated immunity, and are easy to produce especially when organisms are difficult or dangerous to culture in the laboratory. Other advantages of using DNA vaccines include genetic and temperature (heat) stability, being free of contaminants, the possibility of combining with several protective components from multiple pathogens in one vaccine, the possibility of adding adjuvant and/or immunostimulatory properties such as CpG motifs and safety and efficacy despite the presence of maternal antibodies upon administration. In the case of *C. psittaci*, it is disadvantageous to use whole chlamydial agents for vaccination because of the possibility of the presence of immunopathogenic components (Eko *et al.*, 2004), which can result in the production of lesions in target organs. However, DNA subunit vaccines produce weak cell-mediated immunity, require adjuvants that can result in unwanted reactions at the injection site and leave residues that can affect meat quality and safety (van Drunen Littel-van den Hurk *et al.*, 2000; Dunham, 2002).

The major outer membrane protein (MOMP) of *C. psittaci*, representing most of the surface exposed protein, has been identified as an important entity in infecting mucosal epithelial cells. As such, it became the target vaccine candidate because of its capability of eliciting a strong antibody response (Longbottom, 2003). The protective antigens are expected to induce strong $CD4^+$ T cell-motivated Th1 response and, thus, produce antibody response for fast and strong memory response to reinfections (Igietseme and Black, 2013).

Research on *C. psittaci* vaccine (*Table 1*) demonstrated the potential in developing a vaccination protocol that can be applied to poultry.

Vanrompay *et al.* (1999b; 1999a; 2001) utilised plasmid DNA expressing MOMP (pcDNA1/MOMP) as vaccine type with different routes of administration - IM+IN, IM, aerosol - and gene gun delivery. The combination of IM+IN takes advantage of the formers efficient transfection and the latter delivering high amounts of MOMP to activate $CD4^+$ and $CD8^+$ cells. Gene gun on one hand requires a smaller amount of DNA component and favours efficient transfection, that is, more cells are transfected especially when administered with smaller bead size. Furthermore, smaller beads tend to stay at the epidermal layer of the skin that is abundant in antigen presenting cells. Aerosol administration produces comparable results and is cost effective when applying in a huge flock; however, there was a need to increase the particle size to reduce evaporation, thus improving vaccination outcome.

Vaccination of MOMP in addition of CpG motif or branched polyethyleneimine polyplexes was conducted by He *et al.* (2007) and Verminnen *et al.* (2010). The addition of CpG motif increases the immune reaction of vaccinated chickens while branched pcDNA1/MOMP was not easily destroyed when given through aerosol. Lastly, Qiu *et al.* (2010) and Liu *et al.* (2015) worked on making MOMP stable against inactivation by combining with other viral pathogens vaccinated to poultry and achieved promising results. This method will reduce stress in birds, due to handling and cost incurred on vaccine production, as well as minimise the conduct of vaccination for separate pathogens.

The process of stimulating the immune system with DNA vaccines is similar to that produced by natural infection in which the proteins, in their original form, are presented to the immune system (Oshop *et al.*, 2002). Specific antigens are identified, isolated, and used to produce subunit vaccines (Brunham *et al.*, 2000; van Drunen Littel-van den Hurk

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et al., 2000). Antigens present on bacterial surface are identified by mass spectrometry, and the functions are made known through technologies such as *in vivo* expression technology (IVET), recombination-based *in vivo* expression (RIVET), *in vivo* induced antigen technology (IVIAT), serological proteome analysis, and DNA microarray protocols (Grandi, 2001; Scarselli *et al.*, 2005). Reverse vaccinology involves high throughput analysis of all proteins; thus, the best antigenic protein is being identified for vaccine production. Furthermore, this technique is of advantage because the sequence of all antigens regardless of immunogenic properties is available, the most conserved immunogenic proteins can be identified, and the presence of operons coding for the formation of polysaccharides can be identified (Sette and Rappuoli, 2010).

Van Drunen Littel-van den Hurk *et al.* (2002) outlined the factors that influence the quality of immune response stimulated by DNA vaccines. These factors include route of administration in relation to animal species, type of eukaryotic promoter or enhancer, type and location of the plasmid encoded antigen in the cell, presence of unmethylated CpG motifs within the vector, additional plasmids in a multivalent DNA vaccine, cytokine-encoding plasmid in the vaccine, vaccination regimen (dose and interval of vaccination), and presence of maternal antibodies and cytokines.

The route of administration influences the induction of mucosal, cellular, and/or humoral immune system. The most common route in birds is intramuscular. Other routes of administration include incorporation into cationic liposomes, use of gene gun, intravenous, intradermal, intranasal, intraocular, mucosal (tracheal), oral, topical, intraperitoneal, intrabursal, in utero, subcutaneous, epidermal and *in ovo* (Oshop *et al.*, 2002; Dunham, 2002).

The intramuscular route is commonly used because it is simple and effective, and promotes Th1 response that is characterised by the production of IFN-γ by T-helper cells and of immumoglobulins of the IgG2a isotype. On the other hand, the gene gun method makes use of gold particles coated with DNA that are being fired into the skin. This procedure prevents degradation of DNA and enables delivery to target cells such as somatic cells and antigen-presenting cells, making vaccination more effective (van Drunen Littel-van den Hurk *et al.*, 2000; Singh and O'Hagan, 2003). In addition to unmethylated CpG motifs, according to Singh and O'Hagan (2003), other possible adjuvants include monophosphoryl lipid A from lipopolysaccharide of *Salmonella enterica* serovar Minnesota and tripenoid glycosides or saponins that are cytokine inducers, from Chilean tree (*Quillaja saponiria*) bark. Furthermore, particulate antigen delivery systems (emulsions, microparticles, immune stimulating complexes (ISCOMS), liposomes, virosomes and virus-like particles) were considered as an addition or substitute to immunostimulatory adjuvants to stimulate the Th1 pathway.

The amount of DNA to be administered will differ based on delivery method and the size of the animal to be vaccinated. Gene gun technique requires less DNA than intramuscular vaccination (Dunham, 2002; Oshop *et al.*, 2002). The vaccination schedule is another aspect to be considered. In some instances, prime-boost vaccines are being combined with a different immunogen, which is not DNA-based. Another regime is the so-called co-stimulation or the administration of cytokines as secondary signal after the injection of DNA antigen (primary signal). Other genetic adjuvants can also be used as co-stimulator such as genes encoding cytokines (granulocyte macrophage colony-stimulating factor-GM-CSF, interferon-γ and interleukins), chemokines, and co-stimulatory molecules (B7.1 and B7.2) (Dunham, 2002).

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Conclusions

A combination of routes for first and booster shots need to be further investigated to explore which combination achieves the best result coupled with modifications on the vaccine type and adjuvants added. The utilisation of other viral pathogens of poultry as DNA vectors (Kaiser, 2010) should be explored, especially those that have the same point of entry into susceptible hosts and predilection site of infection as *C. psittaci*. The unique pathogenesis of *C. psittaci,* especially its ability to penetrate target cells and survive intracellularly by evading cellular reactions, and the existence of several genotypes pose a challenge in controlling the infection especially through vaccination. On one hand, the influence of polymorphisms of immune system gene(s) of the host can be evaluated according to the vaccine type given. Genetic variations on the host immune system such as single-nucleotide polymorphisms (SNPs) may influence the efficacy of avian chlamydial vaccines. Nonetheless, the work of Qiu *et al.* (2010) and Liu *et al.* (2015) need to be refined and validated to be ready for the next steps in vaccine development and implementation.

Experiments conducted in domestic poultry showed potential in developing an applicable vaccination protocol against C. *psittaci*. In this genomic era, much can be done to optimise the best combination of vaccine form, adjuvant, codon optimisation, vector design, and route and frequency of administration. The production of a cost-effective vaccination protocol is vital to the prevention and control of chlamydia infection in birds and humans.

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Table 1 Different vaccines studied in poultry species, their route of administration, target species, vaccine dose and schedule, and outcomes.

Vaccine type	Route of vaccination	Target species and/or age	Dose/vaccination schedule	Outcome	References
Plasmid DNA expressing MOMP (pcDNA1/MOMP) of *C. psittaci* serovar A	IM + IN; gene gun delivery in gold beads	Turkeys	For IM+IN: 100 µg pcDNA1/MOMP in 100 µl saline per inoculation site For gene gun: 2 µg pcDNA1/MOMP in 1 µm gold beads	T helper memory mobilised (increased peripheral blood lymphocytes proliferative responses); B cell memory mobilised (level of IgG is higher than IgM)	Vanrompay *et al.*, 1999b
Gold beads with plasmid DNA expressing MOMP (pcDNA1/MOMP) of *C. psittaci* serovar A	ID through gene gun delivery	Turkeys	1 µg of pcDNA1/MOMP in 1 mg of o.6 µm gold beads	Enhanced T-helper and B-cell memory; reduced severity of clinical signs, lesions and excretion of organisms	Vanrompay *et al.*, 1999a
Plasmid DNA (pcDNA1::MOMP A)	IM + IN IM Aerosol	Turkeys	IM+IN: 100 µg each diluted in saline IM: 100 µg diluted in saline Aerosol: 100 µg at particle size of 0.5 µm Schedule: day 1 and day 21	Enhanced T-helper and B-cell memory; reduced severity of clinical signs, lesions and excretion of organisms	Vanrompay *et al.*, 2001
Plasmid DNA expressing MOMP (pcDNA1::MOMP)+CpG and recombinant MOMP (rMOMP)+CpG of C. psittaci serovar D	IM	Turkeys	pcDNA1::MOMP (100 µg) + CpG ON (20 µg) at day 7 rMOMP (100 µg) + CpG ON (20 µg) at day 28	Increased antibody titre but with no improved protection	Loots *et al.*, 2006
Integration of CpG motif as adjuvant for *omp-1* DNA (pcDNA3.1::MOMP)	IM	Chickens (6-wk old)	100 µg DNA 20 µg of CpG (CpG oligonucleotides with 3 GTCGTT); booster shot after 14 days	Significant leukocyte proliferation in spleen; increased NO level in macrophages; faster clearance of *Chlamydia*; decreased inflammatory cell invasion	He *et al.*, 2007

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Table 1 Continued

Vaccine type	Route of vaccination	Target species and/or age	Dose/vaccination schedule	Outcome	References
rMOMP emulsified in Freud's incomplete adjuvant			100 µg rMOMP); booster shot after 14 days	High antibody titre with moderate lymphocyte proliferation indexes; insufficient protection	
ompA codon adjusted to codon usage of birds forming pcDNA1/$MOMP_{opt}$ branched	IM	Turkeys	100 µg of brPEI-pcDNA1/$MOMP_{opt}$ with booster vaccination at day 21 with the same dose	Mobilisation of T-cell memory cells based on PBL proliferative responses; for IM vaccination-stimulation index and CD4+ T-cells are remarkably high	Verminnen *et al.*, 2010
polyethylenimine-pcDNA1/$MOMP_{opt}$ (brPEI-pcDNA1/$MOMP_{opt}$) with N/P ratio of 8	Aerosol		500 µg of brPEI-pcDNA1/$MOMP_{opt}$ with booster vaccination at day 21 with the same dose		
Chlamydial MOMP gene recombinant adenovirus (Ad-MOMP)	SC and IM	Chicks (7-day old); layers	0.2 ml ($6.8x10^9$ 50% tissue culture infective dose [$TCID_{50}$]) 0.3 ml ($1x10^{10}$ $TCID_{50}$)	Safe and effective; immunity induced is effective	Qiu *et al.*, 2010
5′-terminus of polymorphic membrane protein D (*pmpD*) gene coding N-terminal fragment combined with herpesvirus of turkey (HVT) –HVT-*pmpD*-N vaccine	SC	Chicks (day-old)	8000 PFU of rHVT-pmpD-N	Increased PmpD-specific antibody levels; vigorous PmpD-specific lymphocyte proliferation response Stable after 20 passages *in vitro* and growth comparable to HVT alone *in vivo* and *in vitro*; increases the CD4+ cell population; complete protection against highly virulent MDV RB-1B strain; decreased respiratory signs, lesions and *C. psittaci* population	(Liu *et al.*, 2015)

IM, intramuscular; IN, intranasal; SC, subcutaneous; ID, intradermal.

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