

Protection of budgerigars (*Melopsittacus undulatus*) against *Chlamydophila psittaci* challenge by DNA vaccination

Taher HARKINEZHAD, Katelijjn SCHAUTTEET*, Daisy VANROMPAY

Department of Molecular Biotechnology, Ghent University, Faculty of Bioscience Engineering,
Coupure Links 653, 9000 Ghent, Belgium

(Received 28 January 2009; accepted 29 July 2009)

Abstract – Plasmid DNA (pcDNA1::MOMP A) expressing the major outer membrane protein (MOMP) of *Chlamydophila psittaci* genotype A strain 89/1051 has been tested for its ability to induce protective immunity against *Cp. psittaci* challenge in budgerigars. Eight pairs of male and female budgerigars were housed in eight separate bird cages placed in two negative pressure isolators, four cages per group. All budgerigars were immunised twice intramuscularly with 100 µg plasmid DNA. Both groups received a primary DNA inoculation at day 0 followed by a booster inoculation 3 weeks later. Group 1 received pcDNA1::MOMP A, while group 2 received the placebo vaccine pcDNA1. Budgerigars were challenged by aerosol 2 weeks following the booster vaccination. The challenge consisted of 10⁸ TCID₅₀ of the homologous *Cp. psittaci* genotype A strain. Cloacal and pharyngeal swabs of all budgerigars, taken prior to the experimental infection were negative in both PCR and culture. However, all budgerigars showed low pre-existing serum antibody titres. This indicates that animals were previously infected. Nevertheless, DNA immunisation could significantly reduce clinical signs, macroscopic lesions, pharyngeal and cloacal excretion as well as chlamydial replication, even in the presence of pre-existing serum antibodies, as compared to the placebo-vaccinated controls.

Chlamydophila psittaci / budgerigar (*Melopsittacus undulatus*) / DNA vaccination / MOMP

1. INTRODUCTION

Chlamydophila psittaci, a Gram-negative obligate intracellular pathogen, causes chlamydiosis in *Psittaciformes* (cockatoos, parrots, parakeets, lorries) and psittacosis or parrot fever in humans. The infection is highly prevalent in *Psittaciformes* as studies in wild and captive *Psittaciformes* in North and South America, Australia, Europe and Japan have shown:

16–81% of the examined birds were positive for *Cp. psittaci* [2–4, 7, 10, 12, 25]. The outer membrane protein A (*ompA*) genotype A is found most often in these birds. The infection is also highly prevalent among captive Belgian *Psittaciformes*, as we recently demonstrated by examining 39 *Psittaciforme* breeding facilities. Fifty-nine (19.2%) of 308 *Psittaciformes* were positive for *Cp. psittaci* by nested PCR/EIA, and bacteria were isolated from 25 (42.3%) birds with PCR-positive results. Eight of 39 (20.5%) breeding facilities were positive in both nested polymerase chain reaction (PCR)/EIA and culture, and respiratory disease was

* Corresponding author: Katelijjn.Schautteet@UGent.be

present in all these facilities [23]. Interestingly, we not only discovered “the expected” genotype A, but for the first time also detected genotype E/B strains in *Psittaciformes* [6]. Moreover, we also demonstrated the transmission of genotype E/B from African grey parrots to humans. In birds, the course of infection can be rather mild but a mortality rate of 50% or even higher is not unusual¹. Faecal and nasal excretions of diseased birds are the primary source of human infections [6, 13, 23]. However, apparent clinically healthy *Psittaciformes* also present a threat to human health, since many cockatoos, parrots, parakeets and lorries never get rid of the bacterium once infected and most of them actually become *Cp. psittaci* carriers, shedding the bacteria again after being stressed [1, 11]. Thus, a considerable number of people are at risk of becoming infected with this bacterium such as people working in pet shops, garden centers, quarantine stations and zoos. But, also visitors of these facilities and people keeping *Psittaciformes* as pets can become infected. Vaccinating *Psittaciformes* could significantly reduce zoonotic risk. However, there is no commercial vaccine. However, DNA vaccination might be used for future vaccination of *Psittaciformes*, since the significance of DNA immunisation as a means of preventing severe clinical signs, lesions and bacterial excretion has already been demonstrated in a specific pathogen free turkey and chicken experimental model of *Cp. psittaci* infection [14, 21, 22, 24, 26]. Therefore, we evaluated the ability of plasmid DNA expressing the major outer membrane protein (MOMP) of a *Cp. psittaci* genotype A strain to raise protective immunity in budgerigars (*Melopsittacus undulatus*) experimentally infected with the homologous *Cp. psittaci* strain. Regarding our previous DNA vaccination results in SPF turkeys, we

tried to increase the immunogenicity of the vaccine further by optimising the codon-usage.

2. MATERIALS AND METHODS

2.1. *Cp. psittaci* strain

Cp. psittaci strain 89/1051, isolated from the liver of a blue-fronted Amazon parrot (*Amazona aestiva aestiva*) [16], was used. The strain was previously characterised using both serovar-specific monoclonal antibodies, *ompA* restriction fragment length polymorphism analysis and sequencing of the *ompA* gene and was classified as an avian serovar/genotype A strain [5, 16, 18]. The bacteria were grown in buffalo green monkey (BGM) cells as previously described [15] and the 50% tissue culture infective dose (TCID₅₀) was determined on BGM cells by the method of Spearman and Kaerber [8].

2.2. DNA vaccine

Plasmid pcDNA1::MOMP A was constructed by sticky-end ligation of the *ompA* gene of *Cp. psittaci* genotype A 89/1051 into the *EcoRI* and *BamHI* site of pcDNA1 [19]. The *ompA* gene was obtained by PCR amplification from genomic DNA. The amplified gene, with a total length of 1 098 bp, includes the startcodon ATG and the complete last conserved region. The *ompA* gene was codon optimised for expression in avian cells and restriction sites were added at both ends of the *ompA* gene (GenScript Corporation, Piscataway, NJ, USA). The gene now has a total length of 1 110 bp.

Previously, the expression of codon-optimised *ompA* of *Cp. psittaci* strain 92/1293 was evaluated in DF-1 cells (Chicken Embryo Fibroblasts; ATCC: CRL-12203) using a Polyfect[®] Transfection Kit (Qiagen, Venlo, The Netherlands). Successful codon-optimisation was shown by increased fluorescence for MOMP_{opt} when compared to MOMP and confirmed by an increased CAI 124 from 0.698 for *ompA* to 0.981 for *ompA_{opt}* in the chicken and from 0.606 for *ompA* to 0.948 for *ompA_{opt}* in turkeys (unpublished results).

E. coli MC1061/P3 cells were transfected by electroporation (Gene Pulser, Bio-Rad, Nazareth, Belgium), and clones were selected on medium containing ampicillin and tetracycline and grown in microtitre plates. The presence of inserts was confirmed by *EcoRI* and *BamHI* restriction enzyme

¹ Dovic A., Slavec D., Lindtner-Knific R., et al., The study of a *Chlamydophila psittaci* outbreak in budgerigars, Proceedings of the 5th Annual Workshop of COST Action 855 Animal Chlamydioses and Zoonotic Implications, 2007, 24 p.

analysis of plasmid mini preparations (Qiagen) and by PCR clone analysis using SP6 and T7 primers, which flanked the multiple cloning site. PCR clone analysis was performed in microtitre plates with the BioMek Thermal Cycler (Perkin-Elmer Cetus, Zaventem, Belgium). First, 5 µL of each clone was subjected to PCR in a 50 µL final reaction mixture containing 50 mM KCl, 20 mM Tris-HCl (pH 8.3), 2 mM MgCl₂, 0.1% Tween 20, 200 mM of each deoxynucleoside triphosphate, 20 mM each primer, and 0.1 U of SuperTaq (15 U/mL) polymerase. Samples were subjected to 25 cycles of amplification. Cycling conditions were as follows: denaturation for 30 s at 95 °C, primer annealing for 1 min at 55 °C, and primer extension for 2 min at 72 °C. The sequence of the codon-optimised *ompA* insert was confirmed by the VIB Genetic Service Facility (University of Antwerp, Antwerp, Belgium) using vector associated T7 and SP6 priming sites.

Finally, pcDNA1 control (placebo) and pcDNA1::MOMP A were grown in MC1061/P3 and purified using the Endo Free Giga plasmid preparation method (Qiagen). DNA concentration was determined by measuring the optical density at 260 nm and was confirmed by comparing intensities of ethidium bromide-stained *EcoRI/BamHI* restriction endonuclease fragments with standards of known concentrations. DNA was stored at -20 °C in 1 mM Tris (pH 7.8)-0.1 mM EDTA.

Expression of the *ompA* gene was confirmed by indirect immunofluorescence staining of DEAE dextran transfected COS-7 cells, transfected either with pcDNA1::MOMP A or pcDNA1 control plasmid, as described previously [19]. For each immunisation, 100 µg pcDNA1::MOMP A, diluted in sterile saline (0.9% NaCl) was used.

2.3. Vaccination trial

The experimental design was evaluated and approved by the Ethical Commission for Animal Experiments of Ghent University. Sixteen one-year-old budgerigars were purchased from a private breeding facility in East-Flanders. We used conventional budgerigars, since SPF *Psittaciformes* are unavailable. Pharyngeal swabs of all birds of this breeding facility were negative in a *Cp. psittaci* species-specific nested PCR/EIA and in culture on BGM cells [23]. Eight pairs of male and female budgerigars were housed in 8 separate bird cages placed in 2 negative pressure isolators (IM 1500, Montair, Sevenum, The Netherlands), 4 cages per unit. In this way, 2 groups of 4 bird pairs each were created. Food (seed mixture for budgerigars, food

sticks, sepia shells) and water were provided ad libitum.

All budgerigars were immunised intramuscularly (*m. quadriceps*) with 100 µg plasmid DNA in 100 µL sterile saline (0.9% NaCl). Both groups received a primary DNA inoculation at day one and one booster inoculation 3 weeks later. Group 1 received pcDNA::MOMP A, while group 2 received the placebo vaccine pcDNA1. Budgerigars were challenged by aerosol (Cirrus Nebulizer, Intersurgical, Wokingham, Berkshire, UK) 2 weeks following the booster vaccination. The challenge consisted of 10⁸ TCID₅₀ of the *Cp. psittaci* genotype A strain 89/1051.

2.4. Samples

All budgerigars were observed daily for clinical signs. Pharyngeal swabs were collected at the beginning of the experiment, at day 1 and at euthanasia, 21 days post challenge infection. Faecal excretion was evaluated at the day of challenge and subsequently every day until 20 days post challenge by taking swabs from fresh faecal droppings on the cage floor. All swabs were stored at -80 °C in *Cp. psittaci* transport medium until tested. Blood samples for the quantification of MOMP-specific serum antibody titres were collected immediately prior to each DNA inoculation, immediately prior to the experimental infection and at the day of euthanasia. Blood samples were stored overnight at room temperature, centrifuged (325 × g, 10 min, 4 °C) and afterwards serum was collected and frozen at -20 °C until tested. At the time of euthanasia, 21 days post challenge, proliferative responses in spleen cells were determined. All budgerigars were examined for macroscopic lesions. Macroscopic lesions were scored (Tab. 1) as previously described [17]. Cryostat tissue sections of the lungs, conchae, abdominal and thoracic airsacs, pericardium, kidney, liver and gut (caecum) were examined for the presence of chlamydial antigen.

2.5. *Cp. psittaci* excretion

Pharyngeal and cloacal swabs were examined for the presence of viable *Cp. psittaci* by culture in BGM cells, as previously described [17]. The number of BGM cells with chlamydial inclusions was counted in five randomly selected microscopy fields (Radiance 2000MP, Bio-Rad; 600×). For each cage or bird pair, the excretion was scored from 0 to 5. Score 0 indicated no chlamydiae present; score 1 was given when a mean of 1–5 elementary bodies were

Table 1. Score system for macroscopic lesions.

Tissue	Score		
	+	++	+++
Conchae	Congestion	Necrosis	Necrosis and granulomata
Lungs	Congestion bilateral	Grey foci unilateral	Grey foci bilateral
Airsacs	Opacity	Focal fibrinous airsacculitis	Diffuse fibrinous airsacculitis
Pericard	Serous pericarditis	Fibrinous pericarditis	Fibrinous adhesive pericarditis
Liver	Congestion	Pale	Pale and soft
Spleen	Slightly enlarged	Moderately enlarged	Severely enlarged
Kidney	Slightly enlarged	Moderately enlarged	Severely enlarged
Gut	Slightly congested	Severely congested	Enteritis

present in the absence of replicating reticulate bodies; scores 2, 3, 4 and 5 were given when a mean of respectively 1–5, 6–10, 11–20 and more than 20 inclusion positive cells were present. Pharyngeal and cloacal shedding is presented as a mean score per bird pair \pm standard deviation (S.D.).

2.6. *Cp. psittaci* detection in the tissue organs

Cryostat tissue sections of different organs were examined by the ImagenTM direct immunofluorescence staining (Novo Nordisk Diagnostics, Cambridge, UK), as previously described [17]. The presence of *Chlamydomphila* antigen in tissues of the placebo-vaccinated control group and the pcDNA1::MOMP A vaccinated group was scored as mentioned above for swab cultures.

2.7. Antibody responses

Enzyme-linked immunosorbent assays (ELISA) were performed on sera being pre-treated with kaolin to remove background activity [9]. MOMP-specific antibody titres were determined using standard protocols with rMOMP as antigen directly coated on the plates. Recombinant MOMP was produced in COS-7 cells, transiently transfected with pcDNA1::MOMP A, as described previously [19].

For the determination of antibody titres in serum, 1/1000 and 1/2000 dilutions of cross-reactive biotinylated anti-chicken/turkey IgG (H+L) antibody (Invitrogen) and peroxidase-conjugated streptavidin (Invitrogen) were used, respectively. Anti-MOMP immunoglobulin titres were presented as the reciprocal of the highest serum dilution that gave an optical density (OD₄₀₅) above the cut-off value. The cut-off value was the mean OD of a negative African grey parrot serum \pm twice S.D.

2.8. Lymphocyte proliferative responses

Leukocytes were isolated from the spleen of each budgerigar, at 21 days post challenge. Lymphocyte proliferative tests were performed as previously described [21]. Briefly, non-adherent cells were grown in duplicate in 96-well tissue culture plates at 6×10^5 cells in 150 μ L of Dulbecco modified Eagle medium (DMEM) supplemented with 20% heat-inactivated foetal calf serum (FCS; Integro, Zaandam, The Netherlands), 1% non-essential amino acids (Life Technologies), 1% sodium pyruvate (Life Technologies), 1% L-glutamine (Life Technologies), 1% gentamycin (Life Technologies) and 5×10^{-5} M β -mercaptoethanol (β -ME; Life Technologies). For antigen proliferation, 20 μ g of rMOMP was added to individual wells. Negative and positive controls included cells stimulated with either plain medium or with 10 μ g Concanavalin A (Con A), respectively. The cells were incubated at 39.5 °C in a humidified incubator with 5% CO₂. Con A or antigen-induced proliferation was measured by incorporation of ³H-thymidine (1 μ Ci/well) during the last 16 h of culture, at days 2 (Con A) and 6 (DNA primed groups), respectively. Cultures were harvested onto glass fibre filter strips with a cell harvester (Skatron, Liers, Norway). The radioactivity incorporated into the DNA was measured with a β -scintillation counter (Perkin-Elmer, Life Science, Brussels, Belgium) and was expressed as a stimulation index (SI: mean counts per minute in the presence of antigen divided by the mean counts per minute in the absence of antigen).

2.9. Statistics

The two-tailed Student *t*-test, Mann–Whitney and Fisher Exact test were employed for statistical

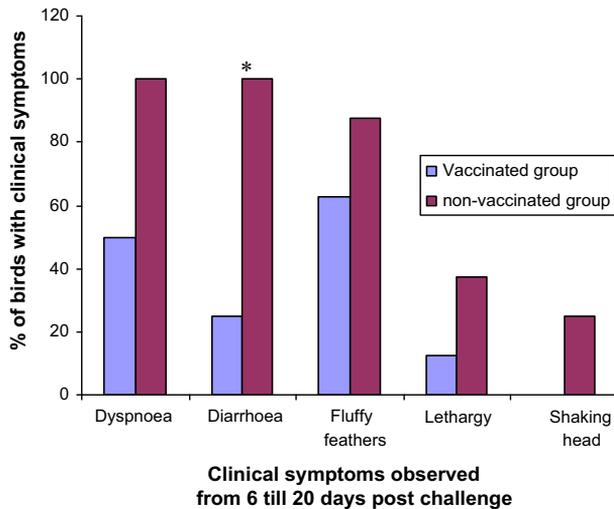


Figure 1. Percentage of budgerigars with clinical signs observed in vaccinated and placebo-vaccinated groups from 6 to 20 days post challenge. Two groups were compared using the Fisher Exact test. Vaccinated birds suffered significantly ($* p < 0.05$) less from diarrhea than the birds who received the placebo vaccine. But, the number of the birds was too small for this test to compare the two groups fairly for each clinical sign.

analyses. The results were considered significantly different at the level of $p < 0.05$.

3. RESULTS

3.1. Clinical signs

Clinical signs were first observed at five days post challenge. At that time, two placebo-vaccinated birds showed mild dyspnoea. Another placebo-vaccinated bird had diarrhoea (green watery droppings) and was shaking his head showing a sign of rhinitis. Vaccinated birds appeared healthy. From day 6 onwards till the end of the experiment, all placebo-vaccinated birds showed severe dyspnoea and diarrhoea; all but one (87.5%) had fluffy feathers. Three of eight birds (37.5%) showed lethargy and 2 of 8 birds (25%) were shaking their heads regularly (Fig. 1). Clinical signs in the placebo-vaccinated group were most severe from post inoculation day 6 till 15. In the vaccinated group, 2 of 8 birds (25%) had diarrhoea at post inoculation day 11, 4 of 8 birds (50%) showed dyspnoea

from post inoculation day 10 till 13 and at the same time 5 of 8 (62.5%) birds had fluffy feathers and 1 of 8 birds (12.5%) were lethargic. At euthanasia, at 21 days post challenge, only 1 of 8 (12.5%) vaccinated birds showed fluffy feathers and was less active. All other vaccinated birds were more active as compared to the placebo-vaccinated control group since 3 of 4 male birds were flaunting and some birds even played with the toys (mirror and bell) inside the cage. During the experiment, 2 of 8 (25%) vaccinated birds lost 7 and 7.7% of their body weight, respectively. In the placebo-vaccinated group, 6 of 8 (75%) birds lost 2.5, 2.6, 3, 5, 8.1 and 15% of their body weight, respectively.

3.2. *Cp. psittaci* excretion

Faecal excretion was examined by bacterial culture in BGM cells. Faecal *Cp. psittaci* excretion was not observed before challenge and during the first four days post challenge. At five days post challenge, fresh faecal droppings present on the cage floor of 3 of 4 (75%) placebo-vaccinated bird pairs were

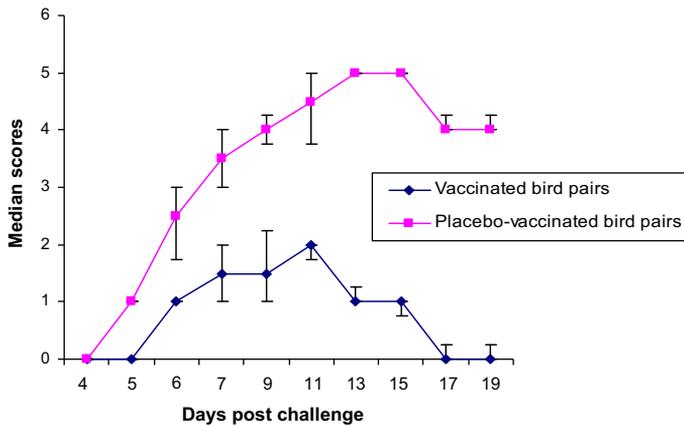


Figure 2. Presence of *Cp. psittaci* in fresh faecal droppings of vaccinated and placebo-vaccinated bird pairs following challenge. Two groups were compared using the Mann–Whitney U test. On all time points, except the day of the challenge, placebo-vaccinated bird pairs showed significantly ($p < 0.001$) higher median culture scores as compared to the vaccinated group. Bars indicate 25 and 75 percentiles around each median.

culture positive. Mean culture scores per bird pair are presented in Figure 2. One day later, all four (100%) placebo-vaccinated bird pairs excreted live bacteria and the faeces of all pairs remained positive until 20 days post challenge. In the four pcDNA1::MOMP A vaccinated bird pairs, bacterial excretion was first observed at 6 days post challenge. At that time, 2 of 4 (50%) vaccinated bird pairs were shedding *Cp. psittaci* and one day later, the faeces of all vaccinated bird pairs became culture positive. All four vaccinated bird pairs remained culture positive until 14 days post challenge. On day 15, 2 of 4 (50%) vaccinated bird pairs were culture positive and from day 17 to 20, 1 of 4 (25%) vaccinated bird pairs was culture positive. Overall, placebo-vaccinated bird pairs showed significantly ($p < 0.001$) higher mean culture scores as compared to the placebo-vaccinated control group (Fig. 2). At 21 days post challenge, only 2 of 8 (25%) cloacal swabs of the vaccinated birds were positive with a mean immunofluorescence score of 0.37. On the contrary, all (100%) cloacal swabs of the placebo-vaccinated budgerigars were culture positive with a mean immunofluorescence score of 3.25.

3.3. Macroscopic lesions

At euthanasia, budgerigars were examined for the presence of macroscopic lesions. The scoring system for macroscopic lesions is presented in Table I. Lesion scores in individual birds of vaccinated and placebo-vaccinated groups are presented in Table II. Macroscopic lesions were clearly more severe in the placebo-vaccinated group, with prominent lesions in especially the respiratory tract and the spleen since all budgerigars showed focal to diffuse fibrinous airsacculitis, bilateral congestion to bilateral pneumonia and a slightly to severely enlarged spleen. Additionally, all birds showed congestion of the gut, all but one (12.5%) had a congested liver, 5 of 8 (62.5%) showed serous to fibrinous adhesive pericarditis, 3 of 8 (37.5%) showed slightly to moderately enlarged kidneys and 2 of 8 (25%) showed necrosis of the conchae. Macroscopic lesions in the vaccinated group were less severe and as in the placebo-vaccinated group mainly located in the respiratory tract and the spleen. The two groups were significantly different for macroscopic lesions in all tissues with the exception of the conchae and spleen ($p < 0.05$). Actually, all vaccinated

Table II. Macroscopic lesion in tissues of budgerigars, 3 weeks post *Cp. psittaci* challenge. The scores for the macroscopic lesions of different tissues in vaccinated and placebo-vaccinated birds were compared. The median plus 25 and 75 percentiles and the level of significance with the Mann–Whitney *U* test is shown for each tissue. *p* values are indicated in the last column as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ and ns for non significant. The scores were significantly different for all tissues except conchae, spleen and kidney.

Tissue	Vaccinated birds	Placebo-vaccinated birds	Vaccinated versus placebo-vaccinated
	Median (Minimum–Maximum)	Median (Minimum–Maximum)	<i>p</i> value
Conchae	1 (0–1)	0 (0–5)	ns
Lungs	1 (1–1)	2 (1–2.25)	*
Airsacs	1 (1–1)	2 (2–3)	***
Pericard	0 (0–0)	1 (0–3)	*
Spleen	1 (0.75–1)	1 (1–2)	ns
Liver	0 (0–0)	1 (1–1)	***
Kidney	0 (0–0)	0 (0–1)	ns
Gut	0 (0–0)	1 (1–1)	***

budgerigars showed mild lesions in the lower respiratory tract, 6 of 8 (75%) had a slightly enlarged spleen and 5 of 8 (62.5%) showed mild lesions in the upper respiratory tract. No further lesions were observed in this group.

3.4. *Cp. psittaci* presence in different tissues

Immunofluorescence staining of frozen tissue sections of the placebo-vaccinated control group revealed strong chlamydial replication in the upper (conchae and trachea) and lower (lungs and airsacs) respiratory tract of all placebo-vaccinated budgerigars (Fig. 3). With the exception of the pericard of one bird, replication was less prominent in the pericard, liver, kidney and gut of placebo-vaccinated budgerigars. However, all placebo-vaccinated birds experienced a systemic infection since bacterial replication was clearly observed outside the respiratory tract resulting in faecal shedding in all birds until the end of the experiment at 21 days post challenge. The infection probably also spread throughout the body of immunised birds since faecal shedding occurred in all four vaccinated bird pairs from 7 until 14 days post challenge. However, the infection seems to be eliminated more efficiently as demonstrated

by diminished faecal shedding during subsequent days and by the absence of bacteria in 6 of 8 (75%) cloacal swabs, at 21 days post challenge. Notwithstanding this, replicating *Cp. psittaci* organisms were still present in the upper and especially lower respiratory tract of all but one (87.5%) vaccinated budgerigars at 21 days post challenge. The presence of *Cp. psittaci* were significantly ($p < 0.05$) different in all tissues of two groups except conchae.

3.5. MOMP antibody responses

All budgerigars had anti-MOMP antibodies (titre of 60 to 120) before being vaccinated with the *Cp. psittaci* genotype A vaccine pcDNA1::MOMP A or the placebo vaccine pcDNA1 (Fig. 4). MOMP antibody titres were elevated at least four-fold in all pcDNA1::MOMP A vaccinated birds, except for bird number 8, in which the MOMP antibody titre was raised only two-fold. The effect of the booster vaccination was apparently less spectacular since antibody titres following pcDNA1::MOMP A booster vaccination were only raised in 3 of 8 (37.5%) immunised budgerigars. Following challenge, antibody titres remained identical in 4 of 8 (50%), declined in 3 of 8 (37.5%) and rose in 1 of 8 (12.5%) immunised budgerigars.

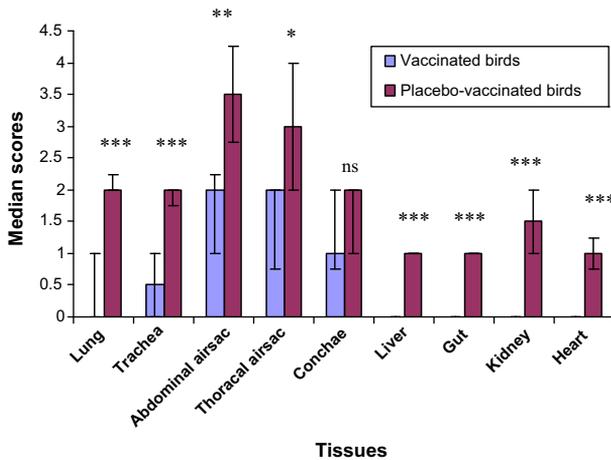


Figure 3. Immunofluorescence scores for the presence of *Cp. psittaci* antigen in tissues of vaccinated and placebo-vaccinated budgerigars. The scores were compared with the Mann–Whitney test. The level of significance is indicated with asterisks as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ and ns stands for non significant. Bars are indicating 25 and 75 percentiles around each median. The groups were significantly different for all tissues, except for the conchae.

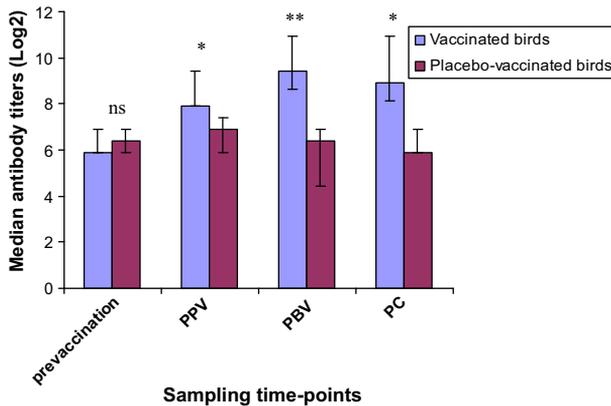


Figure 4. MOMP specific antibody titres. The vaccinated group was intramuscularly immunised with pcDNA1::MOMP. Budgerigars in the placebo-vaccinated group received an empty pcDNA1 plasmid (placebo). Sampling time-points: day of the primary vaccination (Prevaccination); 21 days post primary vaccination (PPV); 14 days post booster vaccination (PBV); 14 days post challenge (PC). Mann–Whitney test was used to perform statistics. Before vaccination, antibody titres were not statistically different. Antibody titres were significantly different on all other time points. The 25 and 75 percentiles are indicated using the bars. The p values for these points are indicated with asterisks as in [Figure 3](#).

Strangely, antibody titres were elevated four-fold in 2 of 8 (25%) pcDNA1-vaccinated controls at 3 weeks post primary vaccination. However, at the day of the experimental

infection, MOMP-antibodies were absent in 2 of 8 (25%) placebo-vaccinated control birds, and MOMP antibody titres were 60 and 120 in 2 of 8 (25%) and 4 of 8 (50%) pcDNA1

immunised birds, respectively. Antibody titres in placebo-vaccinated controls did not augment following challenge, except for bird numbers 10, 12 and 16. There was a significant ($p < 0.05$) difference in mean antibody titres between the vaccinated and placebo-vaccinated groups at 3 weeks post primary vaccination and at all subsequent time points during the experiment.

3.6. MOMP-specific lymphocyte proliferation test

Proliferative responses to rMOMP (genotype A strain 89/1051) of spleen lymphocytes of pcDNA1::MOMP A or pcDNA1 immunised control budgerigars were determined 21 days following challenge with the *Cp. psittaci* genotype A strain 89/1051. The mean SI and S.D. of the placebo-vaccinated group and the vaccinated groups were respectively 11.0 ± 2.25 and 25.45 ± 4.25 . The spleen lymphocytes of pcDNA1::MOMP A immunised birds displayed significantly ($p < 0.01$) higher proliferative responses than the spleen lymphocytes of the placebo-vaccinated budgerigars.

4. DISCUSSION

Reported human psittacosis cases are most often related to contact with *Cp. psittaci* infected poultry (ducks and turkeys) or *Psittaciformes*. *Psittaciformes* are kept in specialised breeding facilities, zoos and quarantine stations, but with respect to public health the increasing habit of keeping cockatoos, parrots, parakeets and lorries as pets in many households, needs even more attention. Vaccination of *Psittaciformes* against *Cp. psittaci* infections might diminish the zoonotic risk, but successful vaccination could also improve the breeding performances of captive *Psittaciformes* and their general health status. Commercial *Cp. psittaci* vaccines are non-existent.

In the present study, pcDNA1::MOMP A immunised budgerigars were significantly protected against severe clinical signs and lesions following challenge with a very high dose

(10^8 TCID₅₀) of the highly virulent homologous *Cp. psittaci* genotype A strain, 89/1051. Placebo-vaccinated budgerigars showed severe clinical signs and lesions and in contrast to the vaccinated birds, excreted high amounts of *Cp. psittaci* for at least 3 weeks post challenge. However, notwithstanding the severity of the disease, none of the placebo-vaccinated control budgerigars died. This might be due to the pre-existing anti-MOMP serum antibody titres detected in all budgerigars prior to the experimental infection. Indeed, pharyngeal and cloacal swabs taken prior to the experimental infection were negative in both nested PCR and culture, but nevertheless, all budgerigars must at least once have been infected, during their stay in the breeding facility since they all had pre-existing MOMP serum antibody titres. Indeed, the prevalence of *Cp. psittaci* infections is high among *Psittaciformes* [23] and therefore previous exposure to the organism is hardly surprising. Moreover, *Cp. psittaci* serum antibody titres in *Psittaciformes* can remain for months.

Interestingly, DNA vaccination could significantly reduce clinical signs, macroscopic lesions, bacterial excretion as well as chlamydial replication, even in the presence of pre-existing serum antibodies. Maybe not surprisingly, since MOMP antibody titres in pre-sera were rather low (maximum 120) and experimental DNA immunisation is known to be effective in turkeys having high maternal antibody titres [14]. Nevertheless, this could be an essential observation regarding future vaccination, since *Cp. psittaci* genotype A strains are endemic in *Psittaciformes* and most birds, if not all, will have antibodies against the organism.

As demonstrated before, serum antibodies did not protect against a respiratory *Cp. psittaci* infection, since all placebo-vaccinated controls got sick, even when having (low) pre-existing MOMP-serum antibody titres. We have no information on protective mucosal antibody titres in these birds prior to immunisation, but the experimental infective dose was extremely high, thus clinical disease in placebo-vaccinated budgerigars was probably inevitable. It might be interesting to determine the protective capacity of the DNA vaccine using lower infective doses.

Budgerigars 2 and 8, responding best to both primo and booster vaccinations and showing no secondary antibody responses upon challenge were best protected. This has also been observed following MOMP-based DNA immunisation in SPF turkeys. In turkeys, the best protection occurred in immunised turkeys that upon challenge did not demonstrate secondary antibody responses, since the bacteria were probably unable to replicate to boosting levels [14, 20, 22]. During former DNA vaccination studies in turkeys, placebo-vaccinated birds showed a primary antibody response upon challenge. In the present study, birds 13 and 16 showed a MOMP specific immune response following immunisation with the placebo vaccine, which could only mean that they experienced a *Cp. psittaci* infection during the first 3 weeks of the experiment. However, pharyngeal and faecal swabs of these 2 birds were PCR and culture negative at the day of the experimental infection, and at that time, 2 weeks post booster (placebo) vaccination, antibody titres were lower than after the primary vaccination. Placebo-vaccinated birds 13 and 16 were most severely infected. Maybe, these 2 birds were *Cp. psittaci* "carriers" and handling them reactivated the persistent infection. However, bacterial shedding by these 2 birds prior to the experimental infection must have been low, since their cage mates (birds number 12 and 15) did not become infected.

Currently, we have no real explanation for the absence of a significant antibody response in all but one (bird number 16) non-vaccinated controls following infection. Maybe, circulating antibodies remained longer in vaccinated birds and serum antibodies in placebo-vaccinated birds were more difficult to detect since most antibodies were "captured" by the extremely high amount of bacteria presenting in these heavily infected birds.

Evidence for the mobilisation of T cell memory in response to challenge was shown by the significantly increased spleen lymphocyte proliferative response upon challenge when compared to the placebo-vaccinated control group.

The essential observation of this report is that a codon-optimised, MOMP-based DNA vaccine is capable of circumventing the

influence of circulating antibody inhibition. Budgerigars in the present study showed relatively moderate levels of pre-existing circulating antibodies and a totally inhibited antibody response to the vaccine construct in the presence of higher circulating antibody titres cannot be excluded. However, circulating antibody titres affected neither the induction of vaccine-specific T cell response nor significant protection in budgerigars. Therefore, prime-boost DNA vaccination may be useful for preventing *Cp. psittaci* infections in *Psittaciformes*, a bird population in which *Cp. psittaci* infections are nearly endemic. In addition, research on DNA vaccination in persistently infected birds would be useful in order to study possible clearance of the infection by DNA immunisation.

Acknowledgements. The Zanjan University and Ministry of Science, Research and Technology of Iran are acknowledged for providing a doctoral grant to T. Harkinezhad. We gratefully thank K. Verminnen, C. Van Droogenbroeck and S. De Schynkel for technical assistance.

REFERENCES

- [1] Andersen A.A., Vanrompay D., Avian Chlamydioses (Psittacosis, ornithosis), in: Saif Y.M. (Ed.), Diseases of poultry, 11th ed., Ames, Iowa, 2003, pp. 863–879.
- [2] Bracewell C.D., Bevan B.J., Chlamydiosis in birds in Great-Britain. 1. Serological reactions to *Chlamydia* in birds sampled between 1974 and 1983, *J. Hyg.* (1986) 96:447–451.
- [3] Dorrestein G.M., Wiegman L.J., Inventory of the sedding of *Chlamydia psittaci* by parakeets in the Utrecht area using ELISA, *Tijdschr. Diergeneeskd.* (1989) 114:1227–1236 (in Dutch).
- [4] Dovc A., Dovc P., Kese D., Vlahovic K., Pavlak M., Zorman-Rojs O., Long-term study of chlamyophilosis in Slovenia, *Vet. Res. Commun.* (2005) 29: 23–36.
- [5] Geens T., Desplanques A., Van Loock M., Bönner B.M., Kaleta E.F., Magnino S., et al., Sequencing of the *Chlamydomphila psittaci ompA* gene reveals a new genotype, E/B, and the need for a rapid discriminatory genotyping method, *J. Clin. Microbiol.* (2005) 43:2456–2461.
- [6] Harkinezhad T., Verminnen K., Van Droogenbroeck C., Vanrompay D., *Chlamydomphila psittaci* genotype E/B transmission from African grey parrots to humans, *J. Med. Microbiol.* (2007) 56:1097–1100.

- [7] Hirai K., Fukushi H., Iwata Y., Ogawa Y., Tsukumi K., Shimakura S., Prevalence of *Chlamydia psittaci* in imported psittacine birds from 1981 to 1983, *Nippon Juigaku Zasshi* (1984) 46:929–931.
- [8] Mayer A., Bachmann P.A., Bibrack B., Wittman G., *Virologische Arbeitsmethoden*, Bd I. Jena, Gustav Fischer Verlag, 1974, 35 p.
- [9] Novak M., Moldoveanu Z., Schafer D.P., Mestecky J., Compans R.W., BrkRules Murine model for evaluation of protective immunity to influenza virus, *Vaccine* (1993) 11:55–60.
- [10] Raso T.D., Berchieri A., Pinto A.A., Evidence of *Chlamydomphila psittaci* infection in captive Amazon parrots in Brazil, *J. Zoo Wildl. Med.* (2002) 33: 118–121.
- [11] Schachter J., Sugg N., Sung M., Psittacosis – the reservoir persists, *J. Infect. Dis.* (1978) 137: 44–49.
- [12] Schwartz J.C., Fraser W., *Chlamydia psittaci* infection in companion birds examined in Florida, *Avian Dis.* (1982) 26:211–213.
- [13] Smith K.A., Bradley K.K., Stobierski M.G., Tengelsen L.A., Compendium of measures to control *Chlamydomphila psittaci* (formerly *Chlamydia psittaci*) infection among humans (psittacosis) and pet birds, 2005, *J. Am. Vet. Med. Assoc.* (2005) 226: 532–539.
- [14] Van Loock M., Lambin S., Volckaert G., Goddeeris B.M., Vanrompay D., Influence of maternal antibodies on *Chlamydomphila psittaci*-specific immune responses in turkeys elicited by naked DNA, *Vaccine* (2004) 22:1616–1623.
- [15] Vanrompay D., Ducatelle R., Haesebrouck F., Diagnosis of avian chlamydiosis: specificity of the modified Giménez staining on smears and comparison of the sensitivity of isolation in eggs and three different cell cultures, *Zentralbl. Veterinarmed. B* (1992) 39:105–112.
- [16] Vanrompay D., Andersen A.A., Ducatelle R., Haesebrouck F., Serotyping of European isolates of *Chlamydia psittaci* from poultry and other birds, *J. Clin. Microbiol.* (1993) 31:134–137.
- [17] Vanrompay D., Van Nerom A., Ducatelle R., Haesebrouck F., Evaluation of five immunoassays for detection of *Chlamydia psittaci* in cloacal and conjunctival specimens from turkeys, *J. Clin. Microbiol.* (1994) 32:1470–1474.
- [18] Vanrompay D., Butaye P., Sayada C., Ducatelle R., Haesebrouck F., Characterization of avian *Chlamydia psittaci* strains using omp1 restriction mapping and serovar-specific monoclonal antibodies, *Res. Microbiol.* (1997) 148:327–333.
- [19] Vanrompay D., Cox E., Mast J., Goddeeris B., Volckaert G., High-level expression of *Chlamydia psittaci* major outer membrane protein in COS cells and in skeletal muscles of turkeys, *Infect. Immun.* (1998) 66:5494–5500.
- [20] Vanrompay D., Cox E., Vandebussche F., Volckaert G., Goddeeris B., Protection of turkeys against *Chlamydia psittaci* challenge by gene gun-based DNA immunizations, *Vaccine* (1999) 17: 2628–2635.
- [21] Vanrompay D., Cox E., Volckaert G., Goddeeris B., Turkeys are protected from infection with *Chlamydia psittaci* by plasmid DNA vaccination against the major outer membrane protein, *Clin. Exp. Immunol.* (1999) 118:49–55.
- [22] Vanrompay D., Cox E., Kaiser P., Lawson S., Van Loock M., Volckaert G., Goddeeris B., Protection of turkeys against *Chlamydomphila psittaci* challenge by parenteral and mucosal inoculations and the effect of turkey interferon-gamma on genetic immunization, *Immunology* (2001) 103:106–112.
- [23] Vanrompay D., Harkinezhad T., van de Walle M., Beeckman D., van Droogenbroeck C., Verminnen K., et al., *Chlamydomphila psittaci* transmission from pet birds to humans, *Emerg. Infect. Dis.* (2007) 13:1108–1110.
- [24] Verminnen K., Van Loock M., Cox E., Goddeeris B.M., Vanrompay D., Protection of turkeys against *Chlamydomphila psittaci* challenge by DNA and rMOMP vaccination and evaluation of the immunomodulating effect of 1 alpha,25-dihydroxyvitamin D-3, *Vaccine* (2005) 23:4509–4516.
- [25] Yamashita T., Hirai K., Isolation of *Chlamydia psittaci* from imported psittacine birds in Japan, *Nippon Juigaku Zasshi* (1981) 43:561–563.
- [26] Zhou J.Z., Qiu C.Q., Cao X.A., Lin G.Z., Construction and immunogenicity of recombinant adenovirus expressing the major outer membrane protein (MOMP) of *Chlamydomphila psittaci* in chicks, *Vaccine* (2007) 25:6367–6372.