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A DNA prime/protein boost vaccine protocol developed against *Campylobacter jejuni* for poultry

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ABSTRACT

Vaccination of broilers is one of the potential ways to decrease *Campylobacter* intestinal loads and therefore may reduce human disease incidence. Despite many studies, no efficient vaccine is available yet. Using the reverse vaccinology strategy, we recently identified new vaccine candidates whose immune and protective capacities need to be evaluated *in vivo*. Therefore, the goal of the present study was to develop and evaluate an avian subunit vaccine protocol for poultry against *Campylobacter jejuni*. For this, flagellin was used as vaccine antigen candidate. A DNA prime/protein boost regimen was effective in inducing a massive protective immune response against *C. jejuni* in specific pathogen free Leghorn chickens. Contrastingly, the same vaccine regimen stimulated the production of antibodies against *Campylobacter* in conventional Ross broiler chickens harbouring maternally derived antibodies against *Campylobacter*, but not the control of *C. jejuni* colonization. These results highlight the strength of the vaccine protocol in inducing protective immunity and the significance of the avian strain and/or immune status in the induction of this response. Nevertheless, as such the vaccine protocol is not efficient in broilers to induce protection and has to be adapted; this has been done in one of our recent published work.

1. Introduction

Campylobacter, a Gram-negative micro-organism which grows under microaerophilic conditions, is the leading cause of human bacterial gastroenteritis, the campylobacteriosis, in the European Union [1], affecting approximately nine million people each year [2]. Domestic and wild birds constitute the main reservoir of the bacteria, carrying them as intestinal commensals. In Europe, the prevalence of *Campylobacter* is up to 70% and 75% in poultry primary production of broiler batches and at the slaughterhouse, respectively [3]. Human contaminations are mainly associated with handling and/or consumption of raw or undercooked poultry meat [1]. *Campylobacter jejuni* is mainly responsible for human diseases causing approximately 90% of cases, followed by *Campylobacter coli* (<10% of cases) [4]. Quantitative microbiological risk assessment estimated that a reduction in caecal poultry colonization of about 2–3 $\log_{10}$ colonies forming units (CFU)/g of faeces may reduce the risk of human campylobacteriosis by 76–100% [5,6]. Different *Campylobacter* control strategies were evaluated at the poultry production level, including hygiene, biosecurity, nutritional and immune strategies [7]. However, despite promising results, there is actually no efficient measure to impact *Campylobacter* avian gut colonization.

Among these strategies, poultry vaccination is one of the most relevant ones. Many studies have been conducted in this field using whole-cell [8–12], subunit [13–18] or microorganism-vectored vaccines [19–23]. However, despite all these efforts and some promising results, there is currently no vaccine on the market to control *Campylobacter* colonization in chickens. Recently we identified 14 potential vaccine antigens against *Campylobacter*

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[24] that must be now assessed for their protective potentials. For this we need a vaccine protocol that can be easily implemented to evaluate various antigens.

This paper aims to develop a DNA based vaccine protocol against *Campylobacter* using *C. jejuni* strain 81-176, known to be highly virulent in humans. Flagellin, the immune-dominant antigen of *Campylobacter*, was used as vaccine antigen [8,25]. Specifically, a heterologous DNA prime/recombinant protein boost vaccine regimen was assessed into 2 avian breeds: specific pathogens free (SPF) Leghorn chickens and commercial Ross broilers.

2. Materials and methods

2.1. Bacterial strains and growth

Campylobacter jejuni strain 81-176 was cultured under microaerophilic conditions (85% N_2, 10% CO_2 and 5% O_2) at 41.5 °C on Columbia plates (Oxoid, Dardilly, France) or in Brucella broth (Becton Dickinson, Le Pont-de-Claix, France). *Escherichia coli* (*E. coli*) XL-1 Blue and Top 10 strains were maintained on Luria-Bertani (LB) agar plates or in LB broth (Sigma-Aldrich, St. Quentin Fallavier, France) aerobically at 37 °C.

2.2. Construction of recombinant plasmids

Campylobacter 81-176 genomic DNA was extracted using the QIAamp DNA Mini kit (Qiagen, Courtaboeuf, France) according to the manufacturer instructions. Gene encoding the flagellin A (*flaA*) was amplified by Polymerase Chain Reaction (PCR) using the primers listed in Table 1. Amplification was conducted in a volume of 50 μL, containing 0.2 μM of each primer, 0.2 mM of dNTP (Clontech, Saint-Germain-en-Laye, France), 1× ThermoPol Reaction Buffer (NEB) and 1 U of Vent DNA polymerase (NEB, Evry, France). Reactions were performed with a thermal cycler (Eppendorf, Montesson, France) using the following parameters: 5 min at 95 °C followed by 30 cycles of 30 s at 95 °C, 30 s at 54 °C, 2 min at 72 °C and a final extension step of 5 min at 72 °C.

After purification using MinElute PCR purification kit (Qiagen), *flaA* amplicons were digested by *BamHI*/*NotI* for pcDNA3 (Invitrogen, Paris, France) cloning or *NcoI*/*NotI* for pQE-Trisystem (Qiagen) cloning and ligated with similarly digested vectors by the T4 DNA ligase (NEB) overnight at 16 °C.

E. coli Top10 (Invitrogen) was transformed with pcDNA3-*flaA* for DNA vaccine production while *E. coli* XL-1 Blue was transformed with pQE-TS-*flaA* for recombinant protein production. Clones carrying the correct insert were selected based on restriction patterns and sequencing (3130 Genetic Analyzer, Thermo Fischer Scientific).

Table 1
Primers used for the amplification by PCR of the *Campylobacter* gene encoding the flagellin A. For cloning in pcDNA3 and pQE-Trisystem plasmids, a restriction site was inserted in each primer.

Primer	Sequence
Cloning in pcDNA3	
FlaA_F.1	*Bam*HI 5′-GCCGCC***GGATCC***ATGGGATTTCGTATTAACACAAATG-3′
FlaA_R.1	*Not*I 5′-CCGCCG***GCGGCCGC***CTATTGTAATAATCTTAAAAC-3′
Cloning in pQE-Trisystem	
FlaA_F.2	*Nco*I 5′-CGGCCG***CCATGG***GATTTCGTATTAACAC-3′
FlaA_R.2	*Not*I 5′-CCGCCG***GCGGCCGC***TTGTAATAATCTTAAAACATTTTGC-3′

2.3. Production of DNA vaccine

The production of pcDNA3-*flaA* and pcDNA3 were performed by culturing transformed *E. coli* Top10 in LB broth + Amp and using the NucleoBond PC 10000 Endotoxin Free extraction kit (Macherey-Nagel, Hoerdt, France) according to the manufacturer recommendations.

2.4. Production of the recombinant flagellin (recFlaA) for the protein vaccine

The QIAexpressionist protocol (Qiagen) was used for the production of the recombinant flagellin according to the manufacturer recommendations. *E. coli* XL-1 Blue transformed by pQE TS flaA was used as starting material. As recFlaA was found to be insoluble, the cell pellet was resuspended in an 8 M urea buffer containing 100 mM NaH_2PO_4 and 10 mM Tris-Cl, pH 8. After stirring at room temperature (RT) until total lysis, the suspension was centrifuged at 10,000*g* for 30 min and the supernatant harvested. Thereafter, the protein solution was progressively dialysed using decreasing urea concentrations from 8 to 0 M and the final recFlaA concentration was measured using Qubit 2.0 (Invitrogen) according to manufacturer instructions.

2.5. Avian vaccine trials

A total of 117 day-of hatch Ross PM3 broiler chicks (male and female) were purchased from a commercial hatchery (Couvoir Perrot SA, Pommerit-Jaudy, France) and 36 day-of-hatch specific-pathogen-free (SPF) Leghorn chicks were obtained from ANSES facilities where no *Campylobacter* was detected. Ross chicks had been vaccinated against infectious bronchitis before their arrival, but not the SPF Leghorn ones. The birds were kept in floor pens with unused wood shavings as bedding material. Animals were randomly allocated in groups of 16–20 or 32–36 chickens, in order to have at least 16 chickens per group for each evaluation of *Campylobacter* caecal loads. At the beginning of each experiment, animals and environment were checked for *Campylobacter* status.

Chickens were vaccinated at the ages of 1 day and 8 or 12 days by the subcutaneous (sc, i.e. in the neck) or the intramuscular (IM, i.e. in the thigh) route using 26 G needles. Two types of vaccine regimens were evaluated: homologous DNA vaccine prime/DNA vaccine boost or heterologous DNA vaccine prime/protein vaccine boost. The DNA vaccine consisted of 100–300 μg of pcDNA3-*flaA* and 25 μg of unmethylated CpG ODN2007 (TCGTCGTTGTCGTTTTGTCGTT, with phosphorothioate backbone) (Sigma-Aldrich) used as an adjuvant [26]. MONTANIDE™ ISA70 VG [27] (Seppic, Puteaux, France) emulsified 100 μg recFlaA was used for the protein vaccination. Corresponding control groups consisted of chicks similarly injected but with empty pcDNA3 (with or without CpG-ODN) or MONTANIDE™ ISA70 VG emulsified PBS. Afterward, the chicks were orally challenged by 10^5 CFU of *C. jejuni* 81-176 at the ages of 15 or 21 days. Three days after challenge, caecal colonization by *C. jejuni* were assessed in 2–4 birds. Blood samples were taken from the occipital sinus or the wing vein every 7–10 days for the evaluation of the systemic immune response kinetic. At the ages of 28 and/or 42 days, bile and caeca were sampled for the assessment of mucosal immune response and *Campylobacter* enumeration, respectively.

2.6. Antibody level assessment by enzyme-linked immunosorbent assay (ELISA)

Campylobacter jejuni 81-176 outer membrane and periplasmic proteins were extracted as described by McCoy et al. [28]. Protein