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Oral immunization of chickens with avirulent *Salmonella* vaccine strain carrying *C. jejuni* 72Dz/92 *cjaA* gene elicits specific humoral immune response associated with protection against challenge with wild-type *Campylobacter*

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Abstract

It is well documented that poultry and poultry products are the major source of human campylobacteriosis and salmonellosis. This study examined the general efficacy of avirulent *Salmonella* vaccine strains expressing *Campylobacter* antigen as a bivalent chicken vaccine prototype. Three *C. jejuni* genes: *cjaA* (*cj0982c*), *cjaC* (*cj0734c*) and *cjaD* (*cj0113*) encoding highly immunogenic proteins which are conserved among different *Campylobacter* serotypes, were introduced into avirulent *Salmonella enterica* sv. Typhimurium (χ4550 and χ3987) strains of two different serotypes (UK-1 and SR). The high copy number plasmid pYA3341 Asd$^+$ was used as a cloning vector. The constitutive expression of all analysed genes as measured by Western immunoblot technique was independent of the particular host strain. Specific rabbit anti-rCjaA antibody reacted not only with CjaA but also with other solute-binding protein (family 3), component of the ABC transport system (CjaC protein), was chosen as the protective antigen for animal experiments. Chickens orally immunized with *Salmonella* expressing *Campylobacter cjaA* gene developed serum IgG and mucosal IgA antibody responses against *Campylobacter* membrane proteins and *Salmonella* OMPs, as measured by an ELISA test. Protection experiment showed that chicken immunization with avirulent *Salmonella* carrying *Campylobacter cjaA* gene greatly reduced the ability of heterologous wild type *C. jejuni* strain to colonize the bird cecum.

Keywords: Chicken bivalent vaccine; *Campylobacter* gene expression in avirulent *Salmonella*; Protection

1. Introduction

Campylobacter spp., gram-negative microorganisms, are actually among the world's most common enteropathogens. The clinical spectrum of enteric disease due to *Campylobacter* infection ranges from generally mild non-inflammatory diarrhoea to severe inflammatory diarrhoea with faecal blood and leukocytes [1–3]. In addition to acute gastrointestinal disease, infection with *C. jejuni* has been shown to be associated with GBS (Guillain-Barré syndrome), a neurological disease that may lead to respiratory muscle compromise and death. It has been documented that about 30% of GBS cases is preceded by *C. jejuni* infection [1,4].

C. jejuni was first isolated from human diarrhoeal stools in 1972 by a filtration technique. Despite more than 25 years of investigations, the molecular mechanisms involved in *Campylobacter* virulence and pathogenesis are far from being understood. Also, relatively little is known about immune responses during *Campylobacter* infection. Epidemiological and human challenge studies show that protective immunity develops after infection; thus, the prevention of *Campylobacter* disease seems to be difficult but feasible [1,3,5].

Campylobacteriosis is, at least in the developed countries, a food born disease. Outbreaks of *Campylobacter* enteritis are frequently traced to contaminated milk or water, whereas the most common cause of sporadic cases is eating of undercooked meat. The contaminated chickens are, by far, the principal vehicles of infection [2,6,7]. The epidemiology of *C. jejuni* in broiler flocks is still unclear. Generally, birds become infected about 3 weeks of age, but the sources and

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routes of transmission of the microorganism to the broilers on the farms remain undetermined. Recently obtained data have indicated several sources of infection, including water, wild birds and farm's personnel [6]. Once the microorganism is introduced into the flock it spreads very rapidly leading to infection of almost all birds in a very short time. Although, the reported level of *Campylobacter* organisms in the chicken intestine, especially in ceca, varies between 10^5 and 10^{10}/g of cecal contents, the massive colonization does not induce any signs of the disease. The high amount of *C. jejuni* in the bird faeces causes further cross-contamination of *Campylobacter*-negative chicken carcasses in the processing plants. As a result, *Campylobacter* contaminates 50–80% of the raw chicken carcasses, depending on the geographical region where the study was conducted out and the method used. This fact, in combination with the relatively low human infection dose can explain why eating undercooked poultry causes the majority of sporadic cases of the campylobacteriosis.

Efforts to reduce the level of contamination by a variety of intervention programs such as improvement of the biosecurity in the hatchery, a competitive exclusion technology or using chlorinated water have given, thus far, variable results when introduce at the farm level and in most cases have been unsuccessful [6,7]. An alternative, more realistic approach for the control of *Campylobacter* contamination is active immunization of the birds. To date, there is limited information about the function of the chicken immune system. Furthermore, the relationship between the host and microorganism is comensal, so that the elimination of *Campylobacter* from the bird intestinal tract cannot be easily achieved. The development of a vaccine is also complicated by factors such as a tremendous diversity of the microorganism [8–11], highly inconsistent data for the molecular mechanism of pathogenesis [12,13], as well as a poorly understood mechanism involved in GBS development [4,8]. The recent *C. jejuni* genome sequencing [14] unquestionably facilitates analysis of the *C. jejuni* genetic diversity [8,15] and enables identification of the genes involved in bird's gut colonization [16]. Stern et al. [17] were the first who demonstrated that chicken immunoprotection against *Campylobacter* infection can be achieved. In recent years, some attempts have been undertaken to develop an effective chicken vaccine against *Campylobacter.* The immunogenicity and efficacy of several vaccine regimens have been evaluated in a chicken model. Rice et al. [18] demonstrated some but not significant reduction of *Campylobacter* colonization of chicks orally vaccinated with formalin-killed whole bacterial cells including *Escherichia coli* heat-labile toxin when compared to non-vaccinated control. In contrast, Baqar et al. [19,20] immunized non-human primates and mice using the same vaccine prototype and observed stimulation of the immune response by LT. A different approach involves a subunit vaccine, which requires the choice of an appropriate protective antigen and a way to deliver it to the host immune system. Hitherto, mainly two *Campylobacter* immunogenic proteins (flagellin and OMPs) have been considered as candidates for subunit vaccines [1,21,22]. Of these, only flagellin has been evaluated in the chicken model [22,23]. Variability of the surface-exposed domains of the FlaA and its glycosylation complicate the use of flagellin for vaccination [24,25]. Live and genetically engineered bacterial strains are considered to be most effective as vaccines against many enteropathogens. To be used as chicken vaccine a *Campylobacter* strain needs not only to be attenuated for humans but also to be immunogenic for birds. This means that it needs to persist long enough in the birds' gut-associated tissues to induce the protective immune responses. Despite many efforts such a strain has not yet been developed. Ziprin et al. [26,27] have shown that genetic knockout of four *C. jejuni* genes (*ciaB*, *dnaJ*, *pldA* and *cadF*), which code for proteins involved in different stage of pathogenesis renders strains incapable of colonizing the chicken intestinal tract, although they are able to colonize the crop [28].

It is well documented that poultry is a major source of human *Salmonella* as well as *Campylobacter* infection and that alive attenuated *Salmonella* strains are highly effective chicken anti-*Salmonella* vaccines [29,30]. Thus, in the present work we have determined whether chicken immunization with *Salmonella* χ3987 carrying immunodominant *C. jejuni* CjaA antigen could provide chicken protection against subsequent challenge with a heterologous broiler-isolated *Campylobacter* strain.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* χ6097, all *Salmonella* strains and Asd^+ plasmids were kindly provided by Roy Curtiss III (Washington University, St. Louis, USA). The Δ*crp* Δ*cya* *Salmonella* χ3985 (UK-1 serotype) strain is a derivative of highly virulent *Salmonella* χ3761 with a 50% oral lethal dose of 3×10^3 CFU for 1-day-old chicks. *Salmonella* χ3985 is avirulent, with a 50% oral lethal dose of $> 4 \times 10^9$ CFU for 1-day-old chicks [31,32]. *Salmonella* χ3987 has an extra deletion in the gene encoding aspartate β-semialdehyde dehydrogenase (Δ*asd* A1), which renders it deficient in diaminopimelic acid (DAP), an obligate component of the cell wall peptidoglican. The *Salmonella asd* gene cloned on Asd^+ plasmid complements the deletion. *Salmonella* χ4550 (SR-11 serotype) Δ*asd* Δ*crp* Δ*cya* is another avirulent vaccine strain used as a carrier for heterologous antigens, derivative of *Salmonella* χ4064 [33]. Both *Salmonella* strains contain the indigenous virulence plasmid. *Salmonella* χ4172, also derivative of χ3761, was the source of OMPs used for the ELISA test [31]. *E. coli* χ6097 and *S. enterica* sv. Typhimurium containing Asd^+ recombinant plasmids were grown in LB medium. The medium was supplemented with DAP (100 μg/ml) for the growth of Δ*asd* recipient

Table 1
Bacterial strains and plasmids used in this study

Bacterial strains and plasmids	Relevant genotype or phenotype	Source
E. coli strain		
χ6097	F$^-$ *ara* Δ(*pro-lac*) *ϕ80dlacZ*M15 *rpsL* Δ*asdA*4 *thi*	R. Curtiss III
XL1-Blue	*recA1 endA1 gyrA96 thi1 hsdR17 supE44 relA1lac* [*F' proAB lacIqZ* Δ*M15* Tn*10* (*tet*^{r})]	Stratagene
Salmonella enterica sv. Typhimurium strains		
χ4550	*gyrA*1816 Δ*asdA*1 Δ[*zhf-4*:Tn*10*] Δ*crp-1* Δ*cya-1*	R. Curtiss III
χ3987	Δ*crp-11* Δ[*zhc-1431*::Tn*10*] Δ*cya-12* Δ[*zid-62*::Tn*10*] Δ*asdA1* Δ[*zhf*::Tn*10*]	R. Curtiss III
χ4172	*fli-8007*::Tn*10*] (Fla$^-$ Mot$^-$ Tc^r) Δ(*galE-uvrB*)-*1005* (Bio$^-$ Gal$^-$UV^s)	R. Curtiss III
Campylobacter jejuni strain		
72 Dz/92	Serotype Lior71, biotype 1	Child Health Centre, Warsaw, Poland
Plasmids		
pYA3341	Asd$^+$ LacZ*α*	R. Curtiss III
pUOA18	Cm^r	[55]
pUWM10A	pBluescript II KS containing 3 kb *Eco*RI-*Eco*RI DNA fragment carrying *cjaA* gene transcribed from own promoter	[36]
pUWM97	pBGS18 containing 1.2 kb *Pvu*II-*Pvu*II DNA fragment carrying *cjaD* gene transcribed from own promoter	[37]
pUWM80	pBGS18 containing 1.1 kb *Hind*III-*Bam*HI DNA fragment carrying *cjaC* gene transcribed from own promoter	[40]
pUWM251	Asd$^+$, CjaA	This work
pUWM82	Asd$^+$, CjaC	This work
pUWM208	Asd$^+$, CjaD	This work

strains. *C. jejuni* 72Dz/92, the source of all examined genes, was obtained from a Warsaw Child Health Centre patient. *C. jejuni* strain employed in the protection experiment was a broiler-isolated strain labeled with the pUOA18 plasmid containing *cat* gene [34]. *C. jejuni* strains were grown at 42 °C on the Müller-Hinton (MH) agar supplemented with 7.5% horse blood and *Campylobacter*-selective supplement (consisting of five antimicrobial agents: vancomycin, polymyxin B, trimethoprim, amphotericin B and cephalotin) in a microaerobic atmosphere (CampyPak Plus, Becton Dickinson). The medium was supplemented with chloramphenicol (25 μg/ml), if necessary.

2.2. DNA manipulations

DNA techniques including plasmid mini preparations, ligations and transformations into *E. coli* were according to standard procedures [35]. Restriction endonucleaeses and DNA-modifying enzymes were obtained from Promega and used according to the manufacturer's instruction. Polymerase chain reactions (PCR) were performed with Taq polymerase (Qiagen) on Mastercycler Personal (Eppendorf) under standard conditions. Recombinant plasmids were introduced into *Salmonella* strains by electroporation and into *E. coli* strains by transformation.

2.3. Construction of the Salmonella strains containing C. jejuni genes

Recombinant plasmids [pUWM10A, pUWM80, pUWM97 [36,37]] carrying *C. jejuni* genes were cut with appropriate endonucleases and subjected to agarose electrophoresis. Restriction DNA fragments containing *C. jejuni* genes (*cjaA*, *cjaC* or *cjaD*) were isolated from agarose gels using a commercial kit purchased from A&A Biotechnology, and ligated into pYA3341 (derivative of pYA292 [38]), which had been cut with an appropriate restriction enzyme and dephosphorylated. After ligation the DNA was transformed into *E. coli* χ6097 competent cells. Asd$^+$ transformants were selected using LB agar. All constructs were verified by restriction enzyme analysis and checked by Western blot analysis using rabbit anti-*Campylobacter* antibodies. Upon characterization, recombinant plasmids were isolated and introduced by electroporation into two *S. enterica* sv. Typhimurium (χ3987 and χ4550) strains. Transformants selected on LB agar were examined by PAGE and silver staining technique to ensure that they had completely smooth lipopolysaccharide [39].

2.4. In vitro plasmid stability

PUOA18 [34] was introduced into *C. jejuni* by electroporation. The chloramphenicol resistance marker present on the plasmid was used as an indicator of plasmid stability. *C. jejuni* pUOA18 was grown in MH at 42 °C for up to 100 generations. Every 20 generations the number of chloramphenicol resistant colony forming units (CFU) among the total number of *C. jejuni* cells was determined by replica plating. A 0.1 ml volume of the appropriate dilution of *C. jejuni* cells growing in MH broth was plated on MH agar plates and the resulting colonies (about 200) were replicated onto MH plates supplemented with chloramphenicol.

2.5. Protein analysis—Western blots (immunoblots)

The total proteins expressed by *E. coli* and *S. enterica* sv. Typhimurium containing *C. jejuni* DNA were analyzed by standard SDS–PAGE and Western immunoblot techniques. Proteins were resolved by electrophoresis in 12% polyacrylamide gels containing SDS and were electrotransferred onto nitrocellulose filters and the blots were developed using rabbit anti-*Campylobacter* antibodies. To eliminate nonspecific reactivity, rabbit serum was absorbed first with heat-killed *E. coli* cells, and then by an *E. coli* cell lysate obtained by sonication. Thereafter the serum was sterilized by filtration and kept frozen at −20 °C. Sera against rCjaA and rCjaC were raised in rabbits as described earlier [40].

2.6. Growth of carrier strain (Salmonella χ3987 containing C. jejuni cjaA gene) for chicken immunization; immunization procedure

To prepare bacterial suspensions for chicken infection, an overnight culture of bacteria was diluted 1:50 in fresh pre-warmed LB broth and grown at 37 °C under aeration to an optical density $A_{600} = 0.6–0.8$ (∼2×10^9 CFU/ml). The cells were sedimented by centrifugation at 8000 × g for 10 min at 4 °C and then suspended in buffered saline with gelatin (BSG). CFU were determined by plating serial dilutions of culture on LB agar plates. Cells were also plated on Difco McConkey supplemented with 1% maltose to verify Crp^- Cya^- phenotype.

Commercial broiler chickens were obtained from the local hatchery on the day of hatch. Birds were immunized according to a protocol worked out for the avirulent *Salmonella* vaccine strains. Briefly, chickens deprived of food and water for 4 h were immunized orally with 100 μl of 10^9 CFU/ml of *Salmonella* χ3987 carrying pUWM251 (*cjaA*). Booster doses were administrated 2 weeks after primary immunization. Following vaccination, chickens were observed for the development of diarrhoea and other potential adverse side effects.

2.7. Antigen preparation

S. enterica sv. Typhimurium χ4172 OMPs used as a test antigen for ELISA assay were prepared using the sodium-lauryl sarcosinate method described by Newell et al. [41] and modified by Hassan and Curtiss [30]. *C. jejuni* membrane fraction was obtained according to the same protocol, except that the procedure was stopped before adding the detergent (sarcosyl).

2.8. Sample collection

Blood samples were collected from a wing vein at 0, 2, 4, 6 and 8 weeks of the experiment to monitor the development of circulating *Salmonella* and *Campylobacter* specific IgG; gut secretion samples were collected concurrently with serum samples from euthanized chickens. The intestinal antibodies were extracted using a PBS solution containing Tween 20 (0.05%), soybean trypsin inhibitor (0.1 mg/ml), EDTA (0.05 mg/ml) and phenylmethylsulfonyl fluoride PMSF (0.35 mg/ml). Intestinal lavages were mixed with extraction solution and shaken for 2 h at 4 °C. After centrifugation 20,000 × g for 30 min at 4 °C the supernatant was collected. Bovine serum albumin was added to the final concentration of 0.1% and samples were preserved by freezing in −20 °C.

2.9. Serological analysis—ELISA test

The level of the antibodies against *Salmonella* OMP and *Campylobacter* membrane proteins in intestinal secretions and sera was measured by an enzyme-linked immunoabsorbent assay (ELISA). Generally, 96-well plates were coated with either *Salmonella* OMPs or *Campylobacter* membrane proteins in 0.05 M carbonate–bicarbonate buffer (pH 9.6) and incubated over night at 4 °C. After tapping out the liquid contents from the wells, plates were blocked with 300 μl volume of skim milk for 1 h at 37 °C. Subsequently, the plates were washed and incubated with 100 μl volume of dilutions of either serum or intestinal extract. Serum samples were diluted 1:100 and intestinal secretion samples 1:5. Goat anti-chicken IgA Fc HRP conjugate (AAI28P Serotec) was employed to detect chicken IgA that bound to *Salmonella* or *Campylobacter* antigens. The plates were developed using the chromogenic substrate TMB according to the manufacturer's directions. The reaction was stopped with 0.5 M H_2SO_4 and optical density was determined at *A* 450 using an ELISA reader (Labsystem Multiscan Plus). The level of specific serum IgG was measured using rabbit anti-chicken IgG alkaline phosphatase conjugate (A-9171 Sigma). The reaction was run with *p*-nitrophenyl phosphate (1 mg/ml) in diethanolamine buffer (pH 9.8) as substrate and was stopped after 30 min incubation (room temperature) with 3 N sodium hydroxide. Optical density was determined at 405 nm using an ELISA reader (Labsystem Multiscan Plus).

2.10. Assessment of protection

Protection experiments were carried out to test the ability of CjaA delivered by *Salmonella* to protect chicken gut colonization by *C. jejuni*. Twenty commercial broiler chickens, obtained from local hatchery on day of hatch, were vaccinated with *S. enterica* χ3987/pUWM251 strain according to the above-described protocol and another group of 20 birds were untreated. Two weeks after the booster all chicks received a dose containing approximately 2×10^9 CFU/ml of the wild type *C. jejuni*/pUOA18 strain in 0.1 ml PBS with gelatine. *Campylobacter* colonization was confirmed on days 3, 6, 9 and 12 after the challenge. In each instance, four chicks were euthanized, the intestine was excised and the *C. jejuni* present in chicken cecal contents

were enumerated by plating. Samples were weighed and nine times their weight of buffered saline (PBS) was added. Samples were homogenised and serially diluted. The 0.1 ml of each dilution was plated on selective plates supplemented with chloramphenicol. Plates were incubated at 42 °C for 48 h. Plates that were culture negative at 48 h were reincubated for additional 48 h. This procedure permits detection of 10^3 CFU/g of cecal contents.

3. Results

3.1. Expression of the C. jejuni genes in avirulent Salmonella strains

Several *C. jejuni* genes coding immunodominant proteins have been identified in our laboratory. Three of them have been cloned, sequenced and characterized. CjaA (Cj0982c: 30 kDa) and CjaC (Cj0734c: 28 kDa) proteins exhibit relevant overall homology to several prokaryotic solute-binding proteins (family 3) components of the ABC transport system [36,42–44]. CjaD (Cj0113: 18 kDa) protein exhibits homology to PAL (peptidoglican-associated lipoprotein) of gram-negative bacteria [40,45,46]. In our initial series of experiments, it was of interest to examine the influence of the genetic background of the host on *C. jejuni* gene expression. Three genes, *cjaA*, *cjaC* and *cjaD* were cloned into Asd^+ cloning vector and introduced by transformation into *E. coli* χ6097 and by electroporation into two avirulent *S. enterica* sv. Typhimurium Δ*asd* Δ*crp* Δ*cya* strains (χ3987 and χ4550) of two different serotypes (UK-1 and SR-11). PYA3341, Asd^+ high-copy number plasmid (pUC origin of replication), was used as a cloning vector in this experiment. Three recombinant plasmids were constructed: pUWM251 (3.0 kb insert), pUWM82 (1.1 kb insert) and pUWM208 (1.2 kb insert) which carry *cjaA*, *cjaC* and *cjaD* genes, respectively. Fig. 1 demonstrate the results of a Western blot analysis comparing the expression of *C. jejuni* genes cloned in *E. coli* and in two *Salmonella* strains. *C. jejuni* gene products present in *E. coli* and in two *Salmonella* strains reacted strongly with the antiserum raised against whole-killed *Campylobacter* cells. No significant differences were found when the antigenicity of a particular gene product produced in different genetic backgrounds was compared. However, the individual antibody–antigen reactions differ in their intensity. The most intense band was observed for *cjaD* gene product. Besides *C. jejuni* gene products, two extra proteins of a molecular weight approximately 39 and 36 kDa expressed in *E. coli* and *Salmonella* strains, respectively, were detected by anti-*Campylobacter* antibodies (Fig. 1). The reaction with the *E. coli* 39 kDa protein was much weaker than that with the *Salmonella* 36 kDa protein. This effect what may have been caused by the initial adsorption of the anti-*Campylobacter* serum against *E. coli* proteins. Both proteins reacting with anti-*Campylobacter* antibodies were found in total protein extracts of all examined strains indicating that they were chromosomally encoded. Although the nature of the *Salmonella* 36 kDa protein has not been further investigated, the immunological similarity between *Campylobacter* and *Salmonella* antigens may enhance the efficacy of the avirulent *Salmonella* carrying *Campylobacter* genes as an anti-*Campylobacter* vaccine.

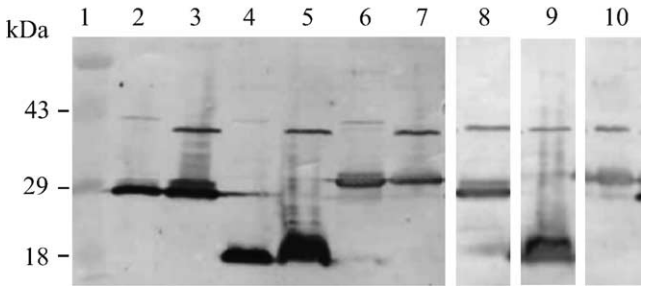

Fig. 1. Expression of the *C. jejuni* 72Dz/92 genes introduced into *E. coli* and *Salmonella enterica* sv. Typhimurium of two different serotypes. Protein extracts were resolved by SDS–PAGE (30 μg of the proteins per lane), transferred to nitrocellulose and reacted with rabbit anti-*Campylobacter* serum. Lanes: (1) Molecular weight standards, (2) *E.c.** χ6097/CjaC; (3) *S.*** χ4550/CjaC; (4) *E.c.* χ6097/CjaD; (5) *S.* χ4550/CjaD; (6) *E.c.* χ6097/CjaA; (7) *S.* χ4550/CjaA; (8) *S.* χ3987/CjaC; (9) *S.* χ3987/CjaD; (10) *S.* χ3987/CjaA. **E.c.*: *E. coli*; ***S.*: *Salmonella enterica* sv. Typhimurium.

3.2. Characterization of the CjaA, antigen chosen for bird immunization

It is widely accepted that to be effective as a vaccine a *Campylobacter* antigen needs to induce protection against multiple serotypes. We have previously demonstrated the presence of the CjaA protein in thirty human clinical isolates, members of two species: *C. jejuni* and *C. coli* [40]. A similar immunological analysis using Western immunoblots with rabbit anti-rCjaA antibodies was done for the present work using whole-cell lysates obtained from 10 chicken colonizing *C. jejuni* strains. The particular species of the *Campylobacter* strains that had been isolated from chicken carcasses in a slaughterhouse were determined by PCR with primers complementary to 23S rRNA [47]; serotypes of the isolates were not determined. All examined strains produced a 30 kDa protein that reacted strongly with rabbit antiserum raised against rCjaA [data not shown].

To enhance the immunogenicity of the CjaA produced by recombinant *Salmonella*, we tested the possibility of increasing the amount of *cjaA* gene product by cloning the gene under the control of *lac*Z gene promoter. Several attempts to generate a *lacZ–cjaA* transcriptional and translational fusions were unsuccessful, probably due to the difference in codon usage between two species (*E. coli* and *C. jejuni*).

We also analysed the immunological similarity between CjaA and CjaC, which are both substrate-binding proteins component of the ABC transport system (family 3). Both proteins were reacted with specific anti-rCjaA and anti-rCjaC antibodies. Because of similarity in molecular

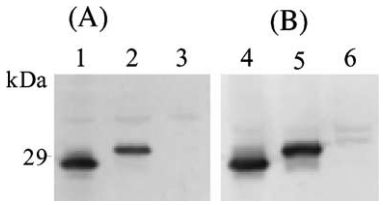

Fig. 2. Immunological similarity between *C. jejuni* solute binding CjaA and CjaC proteins. Whole-cell extracts were separated in 12.5% SDS–PAGE, transferred to nitrocellulose and reacted with anti-rCjaC (A), or anti-rCjaA (B) antibodies. Lanes: 1 and 4, *Salmonella enterica* sv. Typhimurium/pUWM82 (CjaC); 2 and 5, *Salmonella enterica* sv. Typhimurium/pUWM251 (CjaA); 3 and 6, *Salmonella enterica* sv. Typhimurium/pYA3341.

weight between CjaA (30 kDa) and CjaC (28 kDa), heterologous host *Salmonella* strains producing CjaA or CjaC were used as a source of *Campylobacter* proteins. The Western immunoblot data (Fig. 2) showed that anti-rCjaA and anti-rCjaC antibodies recognized both proteins. Therefore, it can be concluded that specific anti-CjaA antibodies induced by chick vaccination with *Salmonella* expressing whole mature CjaA would also react with *C. jejuni* CjaC protein.

3.3. Serum and intestinal antibody responses

Salmonella χ3987 strain was chosen as a delivery vector based on previous results by Covone et al. and Zhang et al. [32,48]. *Salmonella* OMPs, the most immunogenic surface antigen of *Salmonella*, isolated from rough, non-motile strain χ4172 were used as a test antigen to evaluate immune response against a carrier strain in an indirect ELISA assay. Localization of the CjaA in *C. jejuni* as well as in *S. enterica* cells is still unclear. In *C. jejuni* fractionation experiment CjaA was predominantly found in the inner membrane [data not shown]. Thus, the cell fraction containing both *C. jejuni* membranes was used as coating antigen to detect the specific antibody against cloned *C. jejuni* gene product.

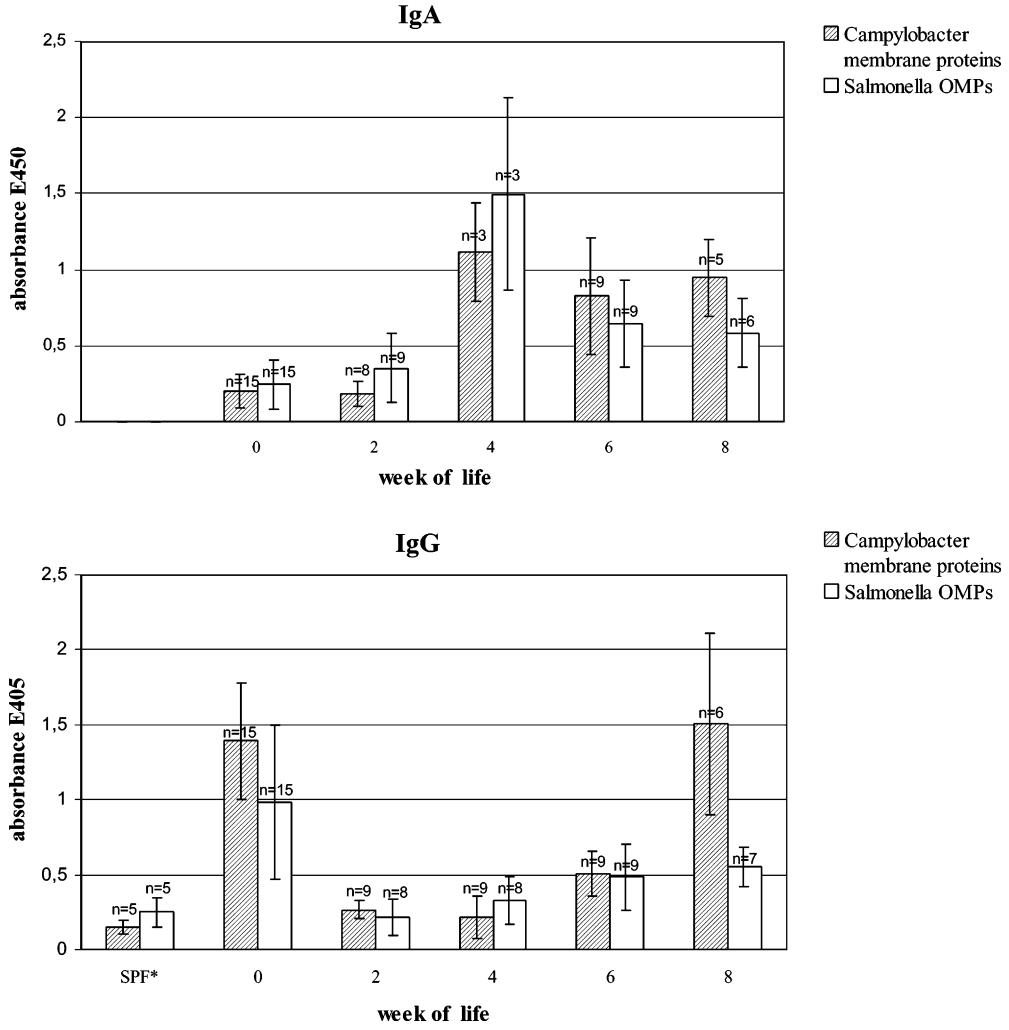

Fig. 3. ELISA analysis of the immune responses of chickens immunized with avirulent *S. enterica* χ3987 producing *C. jejuni* CjaA protein. The two doses were orally given to chickens on days 0 and 14. Serum and intestinal samples (ceca) were collected at 0, 2, 4 and 6 weeks after the booster. *Salmonella* OMPs and *Campylobacter* membrane proteins were used as coating antigens. Serum samples were diluted 1:100 and intestinal secretion samples 1:5. Error bars indicate standard deviation, numbers on *X*-axis indicate week of life. *The blood was taken from 2-week-old chickens.

An experiment was carried out with 50 chickens orally immunized at the age of one day with ~10^8 CFU of *Salmonella*/pUWM251 (CjaA). Birds were given identical booster doses at week 2. Weeks 0, 2, 4, 6 and 8, serum and intestinal samples were collected and analyzed by ELISA assay for specific anti-*Salmonella* OMP and anti-*Campylobacter* (both membrane proteins) IgG and IgA antibodies, respectively (Fig. 3). The kinetics of the IgG responses against both antigens were different. There was high anti-*Campylobacter* IgG titer present at the first time point (week 0) dropping at second and third time points (weeks 2 and 4). A moderate increase of *Campylobacter* specific IgG level was observed at week 6 followed by significant increase of anti-*Campylobacter* IgG level at week 8. As with anti-*Campylobacter* IgG, there was also a decline of anti-*Salmonella* IgG titer at week 2. However, in contrast to anti-*Campylobacter* IgG, the level of anti-*Salmonella* IgG increased at week 4. During the next 4 weeks only slight elevation of anti-*Salmonella* IgG was detected. Serum IgG titers to *Campylobacter* and *Salmonella* whole cell lysates were also determined [data not showed]. They followed the similar pattern, as those measured with membrane antigens, with respect to time, although the increase of anti-*Campylobacter* IgG titer at week 8 was not so significant. The drop in serum IgG titers at 2 and 4 weeks was especially noticeable for anti-*Campylobacter* IgG. The results suggest that high IgG titer observed at the 0 week reflects maternally derived immunity while increasing of serum IgG titers at 6 and 8 weeks is a consequence of vaccination. The ability of sera from chickens inoculated with *Salmonella* carrying pUWM251 to detect *C. jejuni* CjaA protein was also demonstrated by Western immunoblot. Serum samples collected 4 weeks post-vaccination were immunoblotted with proteins isolated from recombinant *Salmonella* cells containing pUWM251 or pYA3341 and with total *C. jejuni* proteins. The results are shown in Fig. 4. The serum consistently recognized antigens of approximately 18, 29 and 35 kDa molecular weight among *Salmonella* proteins. However, the ~30 kDa immunoreactive protein was detected only when the protein extract

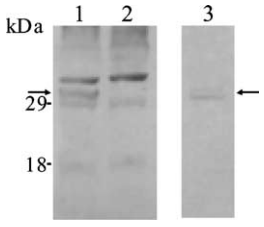

Fig. 4. Induction of the specific anti-*Campylobacter* serum IgG antibodies. Protein extracts isolated from avirulent *Salmonella* χ3987 carrying *C. jejuni* gene *cjaA* and *C. jejuni* 72Dz/92 were separated in SDS–PAGE, transferred to nitocellulose and reacted with diluted 1:10 serum from chickens inoculated with *S. enterica* χ3987 /pUWM251 (CjaA). Serum samples were collected 4 weeks after the booster. Lanes: (1) *S. enterica* χ3987/pUWM251 (CjaA); (2) *S. enterica* χ3987/pYA3341; (3) *C. jejuni* 72Dz/92. The extra immunoreactive protein is indicated by arrow.

obtained from *Salmonella*/pUWM251 (CjaA) cells was analysed. Moreover, serum immunoblotted with *C. jejuni* proteins also reacted with the CjaA (30 kDa) present in the extract. These observations indicate that vaccination with *Salmonella*/pUWM251 stimulates specific anti-CjaA IgG antibodies.

The ability of the recombinant *Salmonella*/pUWM251 strain to induce *Campylobacter* specific local IgA response at the mucosal surface was also assessed. Mucosal anti-*Salmonella* and anti-*Campylobacter* IgA antibodies were present in samples, taken from chickens inoculated with *Salmonella*/pUWM251, at every tested time point. In contrast to serum IgG, the kinetics of IgA responses to both antigens were similar. In intestinal secretions the level of the antibodies dropped at week 2 and then peaked 2 weeks after the booster. During the next 2 weeks, IgA titers decreased and maintained at almost the same level during the remainder of the experiment. Since IgA antibodies directed towards both antigens were present in the intestinal fluids of 1-day-old birds we concluded that, similarly to IgG antibodies, IgA present at 0 week were maternally derived. In intestinal secretions, taken from different parts of chicken gut (rectum and jejunum), levels of IgA antibodies to *Campylobacter* and *Salmonella* antigens also peaked 2 weeks after the secondary immunization [data not showed]. Intestinal IgA titers to *Campylobacter* and *Salmonella* whole cell lysates followed the similar patterns as those measured with membrane antigens (Fig. 5).

3.4. Protection analysis

PUOA18 plasmid in vitro stability was checked prior to initiation of the experiment. To examine plasmid stability *C. jejuni* strain carrying pUOA18 (*cat* gene) was grown without selection for up to 100 generations. It was observed that at the beginning and at the end of the experiment 100% of colonies were chloramphenicol resistant. In total, 20 vaccinated and 20 non-vaccinated 4-week-old chickens were challenged with a high dose (2×10^8 bacterial cells) of a broiler-isolated *C. jejuni* strain carrying pUOA18 and examined for *Campylobacter* colonization. Only three out of 20 vaccinated birds were colonized above the 10^3 CFU/g of cecal contents (level of detection). *C. jejuni* concentration reached ~3×10^4 CFU in one bird, ~3×10^7 CFU in the second and ~5×10^3 CFU in the third one (Table 2). In contrast, all of the 20 non-immunized birds became colonized, with *C. jejuni* concentration in their ceca reaching mean concentrations of about 1×10^9/g of cecal contents. Therefore, these experiments showed that the above used immunization strategy greatly reduced the ability of *C. jejuni* to colonize chicken intestinal tracts even at a challenge dose of 2×10^8 CFU, which greatly exceeds the doses faced by birds under field conditions. The experiment also showed that the *cat*-containing plasmid (pUOA18) was stably maintained in vivo.

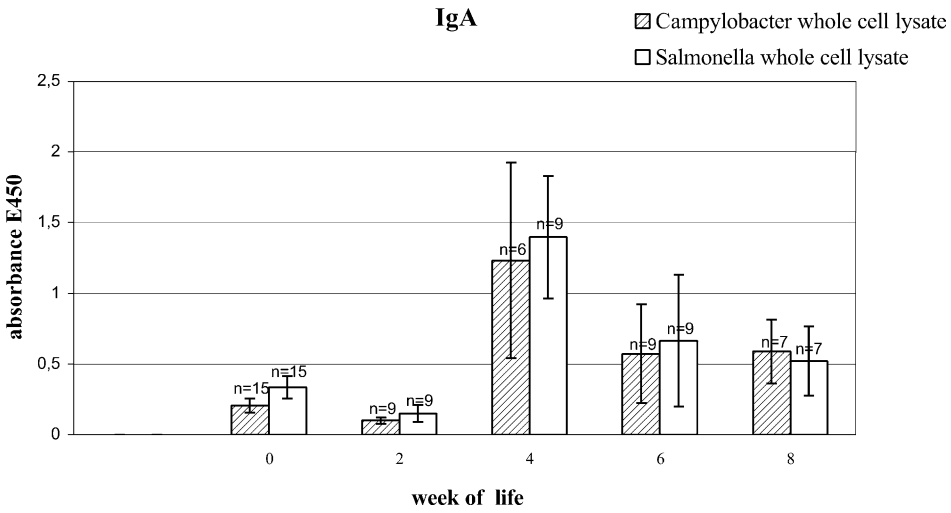

Fig. 5. ELISA analysis of the immune responses of chickens immunized with avirulent *S. enterica* χ3987 producing *C. jejuni* CjaA protein. The two doses were orally given to chickens on days 0 and 14. Intestinal samples (ceca) were collected at 0, 2, 4 and 6 weeks after the booster. *Salmonella* and *Campylobacter* whole cell lysates were used as coating antigens. Intestinal secretion samples were diluted 1:5. Error bars indicate standard deviation, numbers on *X*-axis indicate week of life.

Table 2
Colonization of vaccinated and non-vaccinated chickens after inoculation with broiler-isolated *C. jejuni* strain carrying pUOA18 (Cm^r) (inoculation dose: 2×10^8 bacterial cells)

Vaccinated chickens		Non-vaccinated chickens	
Day after challenge with *C. jejuni* (no. of chicks)	CFU/g of cecal content	Day after challenge with *C. jejuni* (no. of chicks)	CFU/g of cecal content
3 (4)	$<1 \times 10^3$[a]	3 (4)	1.6×10^8
	$<1 \times 10^3$		3.6×10^7
	$<1 \times 10^3$		3.2×10^9
	$<1 \times 10^3$		2.7×10^9
6 (4)	$<1 \times 10^3$	6 (4)	1.2×10^9
	3.0×10^4		2.2×10^9
	$<1 \times 10^3$		3.7×10^9
	$<1 \times 10^3$		8×10^6
9 (4)	2.9×10^7	9 (4)	1.8×10^8
	$<1 \times 10^3$		7.7×10^8
	5.0×10^3		2.1×10^9
	$<1 \times 10^3$		8.3×10^8
12 (4)	$<1 \times 10^3$	12 (3)	1×10^9
	$<1 \times 10^3$		1×10^9
	$<1 \times 10^3$		1.6×10^9
	$<1 \times 10^3$		

The difference in colonization level between vaccinated and non-vaccinated chickens is obviously highly significant ($P \leq 0.0002$ Student's *t*-test).
[a] $< 1 \times 10^3$-level of detection.

4. Discussion

A variety of attenuated and immunogenic *Salmonella* strains have been recently generated and tested as live vectors to deliver heterologous antigens into the immune system in both human and animal models [49,50]. The Δ*crp* Δ*cya* Δ*asd* *Salmonella*, a lethal-balance vector–host system, in which *asd* deletion introduced to the chromosome of the host is complemented by the *asd* gene present on plasmid, enables cloning of the foreign genes without antibiotic selection. *S. enterica* sv. Typhimurium χ3987 was chosen for our studies. It is a Δ*asd*A1 derivative of χ3985 which induced protective immunity, when orally administered in chickens and mice, and is a licensed chicken vaccine (Megan Health Inc., St. Louis, MO, USA) [29,30]. *Salmonella* χ3987 cells harbour the indigenous *Salmonella* virulence plasmid, which is involved in pathogen invasion after oral route administration. It has been shown that *Salmonella* χ4095, an isogenic virulence plasmid-cured mutant of χ3985, is more attenuated and less protective in a murine experimental model [32].

The three *Campylobacter* immunogenic proteins (CjaA, CjaC and CjaD) produced by recombinant *Salmonella* differed from each other with respect to their reactivity with rabbit anti-*Campylobacter* serum. The reactivity of the CjaD protein with anti-*Campylobacter* serum was stronger than those displayed by *cjaA* and *cjaC* gene products. The transcription of all the studied genes cloned into the same high-copy-number plasmid was driven from their native promoters. The obtained results reflect either differences in the affinity of the host RNA polymerase to different *C. jejuni* promoters or alternatively, differences in antigenicity of the proteins. In addition to cloned *Campylobacter* gene products, rabbit anti-*Campylobacter* antibodies react with chromosomally encoded proteins of *E. coli* and *S. enterica* sv. Typhimurium. Judging by their molecular weights, these proteins could be OMPs. These observations are consistent with those presented by Bolla et al. who documented the presence of common epitopes in *E. coli* OMPC and *C. jejuni* MOMP [51].

Campylobacter is an organism, which does not ferment carbohydrates and obtains energy mainly from amino acids metabolism. Thus, it is possible that the induction in the birds' intestine of the antibodies directed towards proteins involved in the amino acid transport potentially could reduce chicken intestinal tract colonization by the pathogen. Based on this assumption, we evaluated the usefulness of *C. jejuni* antigen (CjaA) delivered by recombinant *Salmonella* strain for chicken vaccination. A few other features attributed to CjaA antigen make it valuable for bird vaccination. Besides its high immunogenicity and conservation among multiple *Campylobacter* serotypes of different origin, there is also immunological similarity between CjaA and CjaC.

In the present studies, one plasmid–host system was employed to evaluate the chicken immune responses. As the immune response to the cloned gene product is dependent upon the amount of foreign antigen produced by recombinant *Salmonella*, the high-copy-number plasmid pYA3341 was chosen as a cloning vector. The cloned *cjaA* gene was expressed from its own promoter, which differs in its structure from *E. coli*/*Salmonella* promoters recognized by σ^{70} RNA polymerase ([52], unpublished data) and probably does not display high affinity for the host enzyme. Further attempts to increase the amount of the *cjaA* gene product produced by recombinant *Salmonella* were unsuccessful. Examination of *Campylobacter* codon usage bias indicated that achievement of higher yields of *Campylobacter* antigen production by recombinant *Salmonella* will require genetic manipulation of the coding sequence.

The immunogenicity of *Salmonella* is both dose and genotype dependent [53]. Thus, in our experiments, using *Salmonella* χ3987 strain, we followed the schedule of immunization that had been shown to be most effective for its parental *asd*$^{+}$ strain. This vaccination strategy induced the production of specific anti-*Campylobacter* antibodies present in the serum (IgG) and in the intestinal fluid (IgA) of the birds. The high titer of the anti-*Campylobacter* IgG antibodies observed at 0 week reflects the maternally derived immunity. The observation is consistent with epidemiological studies that indicate that *Campylobacter* seldom infects chickens under commercial conditions of growth before 2 weeks of age. On the other hand, experiments conducted by Hald et al. [54] show that under laboratory conditions, high level of anti-*Campylobacter* OMP IgG antibodies did not protect 1-day-old chicks from infection. Similarly to Hald et al. [54] we noticed the fluctuations of anti-*Campylobacter* IgG and IgA titers in samples taken from 1-day-old chickens [data not showed]. Thus, the influence of the maternal immunity on the chick protection against *Campylobacter* infection requires further investigation. The IgG anti-*Campylobacter* antibodies present after the booster was induced by vaccination as they were selectively directed towards CjaA protein (the antigen used for immunization).

In summary, the above presented experiments showed that immunization of chickens with recombinant *Salmonella* vaccine strain carrying *C. jejuni cjaA* gene may be an attractive and efficient approach for bird vaccination. To our knowledge this study is the first attempt to evaluate the chicken immune responses against *Campylobacter* antigens other than FlaA, delivered by avirulent *Salmonella*. The efficacy of this vaccine prototype may be enhanced by the immunological similarity between *Campylobacter* solute-binding proteins, members of the ABC transport system and also by the fact that *Salmonella* protein/s is/are recognized by anti-*Campylobacte*r antibodies.

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