STUDY *IN OVO* IMMUNISATION WITH FLAGELLIN AND WHOLE CELL PROTEIN ANTIGENS OF *CAMPYLOBACTER JEJUNI* IN CHICKENS

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ABSTRAK

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Telah dilakukan imunisasi secara *in ovo* pada ayam dengan antigen *Campylobacter jejuni* untuk mengetahui respon antibodi tingkat awal dan proteksi terhadap uji tantang dengan galur homolog. Empat kelompok telur ayam berembrio (10 butir per kelompok) diimunisasi secara *in ovo* pada hari ke-17 masa inkubasi dan booster pada hari ke-7 setelah menetas. Kelompok I diimunisasi *in ovo* dan booster peroral dengan protein *whole cell* dari *Campylobacter jejuni,* kelompok II diimunisasi *in ovo* dan booster peroral dengan protein *flagellar*, kelompok III diimunisasi *in ovo* dan booster intraperitoneal dengan protein *whole cell* dan kelompok IV sebagai kontrol, diimunisasi dengan PBS. Tanggap kebal sistemik diukur secara *enzyme-linked immunosorbent assay* (ELISA) dan tanggap kebal mukosal diukur dengan teknik histologi fluoresen. Hasil pengukuran tanggap kebal sistemik dan mukosal setelah vaksinasi pada kelompok ayam perlakuan (I, II dan III) terlihat lebih tinggi dibandingkan dengan kontrol (IV). Hasil titer anti-campylobacter dalam serum, cairan empedu dan kerokan usus setelah uji tantang tidak berbeda nyata antara kelompok perlakuan dan kontrol. Hasil serupa pada uji tantang dengan isolat hidup *C. jejuni* menunjukkan tidak ada perbedaan penurunan kolonisasi bakteri secara nyata dalam usus ayam perlakuan dan kontrol. Hasil-hasil tersebut menunjukkan bahwa walaupun respon tanggap kebal mukosal dan sistemik setelah vaksinasi *in ovo* pada ayam cukup tinggi, namun belum cukup memberikan proteksi terhadap kolonisasi *C. jejuni* pada saluran pencernaan.

Kata kunci : Imunisasi *in ovo*, *Campylobacter jejuni*, whole cell, flagellar, ayam

ABSTRACT

NOOR, S.M. and A.J. HUSBAND. 2000. Study *in ovo* immunisation with flagellin and whole cell protein antigens of *Campylobacter jejuni* in chickens. *Jurnal Ilmu Ternak dan Veteriner* 5(2)

In ovo immunisation of chickens with flagellin and whole cell protein antigens of *Campylobacter jejuni* was examined to determine *Campylobacter* infection. Four groups of embryonated chicken eggs (10 eggs per group) were immunised *in ovo* at day 17 of incubation and booster was given at 7 days post-hatch. Group I was immunised *in ovo* and oral booster with whole cell protein of *C. jejuni*, group II was immunised *in ovo* and oral booster with *C. jejuni* flagellin protein, group III was immunised *in ovo* and intraperitoneal booster with whole cell, and group IV was treated as control. The humoral immune responses were determined by enzyme-linked immunosorbent assay (ELISA) and the mucosal immune responses were examined by a direct fluorescent histology antibody technique. Immunised chickens of Group I, II, and III shown to have higher antibody titers than those of control chickens (group IV). The titres of anti-campylobacter antibodies of all isotypes in serum, bile, and intestinal scrapping after challenge were not significantly different in all groups. In addition, when immunised chickens were orally challenged with a homologous strain of viable *C. jejuni* organism, the chickens remained infected throughout the experiment based on cloacal swabs and caecal contents. These findings indicated that although *in ovo* immunisation resulted in increasing of the mucosal and humoral immune responses in chickens, it is not strong enough to protect the *Campylobacter* colonisation in the intestinal tract.

Key words : *In ovo* immunisation, *Campylobacter jejuni*, whole cell, flagellar, chickens

INTRODUCTION

The mucosal surfaces of the gastrointestinal tract of chickens are common sites of entry for enteric pathogens, such as *Campylobacter jejuni* which may contribute to intestinal infection. A number of studies have shown that controlling the intestinal infection can be achieved by stimulating local immunity with specific immunogent (PORTER *et al*., 1976). However, attempting to achieve early intestinal immunity by vaccination after hatching is unlikely to be an effective method to control *Campylobacter* infection because of

the lack of maturity of the immune system of newly-hatched chicks (KAZWALA *et al.,* 1990), so that active bacterial colonisation can occure before effective immunity has become established.

Prenatal immunisation could overcome this early unresponsiveness in mammals and created early development of local immune function (HUSBAND and MCDOWELL, 1978 and HUSBAND (1980). In chickens, prenatal (*in ovo)* immunisation has succeeded in evoking humoral immune response providing good protection after challenge against Marek's disease (SHARMA and BURMESTER, 1982), infectious bronchitis (WAKENELL and SHARMA, 1986), and coccidiosis (FREDERICKSON *et al.,* 1989).

The previous study showed that *in ovo* immunisation with heat-killed *C. jejuni* established early development of intestinal immunity in chickens (NOOR *et al.,* 1995). The objective of this studies were to observe the antibody responses of early vaccination, development of early intestinal protection following vaccination with two type protein antigens (*Campylobacter jejuni* flagellin protein and whole cell protein) againsted oral challenge of homologous strain.

MATERIALS AND METHODS

Bacterial strain

C. jejuni strains were isolated from carcass washings of fresh commercially-processed chickens, or from the faeces of laying birds maintained at Victorian Institute of Animal Science, Victoria, Australia. Isolates have been stored in 60% glycerol in phosphate-buffered saline (PBS) at -80^{o}C. Bacteria were grown on *Campylobacter* selective solid media (STERN *et al.,* 1990a) at 42^{0}C, under microaerobic atmosphere (10% CO_2, 5% O_2, 85% N_2). Selected colony was grown in liquid medium of brain heart infusion (BHI, Difco laboratories, USA) containing 5% horse serum (BHI/HS) and selective supplements of SR 69 and SR 155E (Oxoid), or in BHI containing 10% lysed sheep red blood cells and selective supplements.

Antigen preparation

For preparation of whole cell protein, an isolate of *C. jejuni* was grown on sheep blood agar plates (Oxoid) at 42^{o}C for 24 h. After overnight incubation, surface growth cells were harvested and dissolved into saline and heat-killed at 56^{o}C for 30 min. *C. jejuni* flagellin antigen suspension was prepared by the method of NACHAMKIN and HART (1985), the bacteria were disrupted in a blender (2x30s periods) and centrifuged at 10,000 g. Supernatant was separated and filtered through a 45 micron filter (Whatman International, England) and stored frozen at –20^{o}C. The protein content of flagellin antigen was measured using the BioRad protein assay and confirmed by performing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) by the standard procedure of LAEMMLI (1970). Gels were stained with coomassie blue stain (Bio-Rad) to visualize the protein bands.

Immunisation procedure

Forty embryonated chickens eggs (Casula, Australia) were divided into 4 groups (10 eggs per group). Group I was immunised *in ovo* with 0.5 ml of *C. jejuni* whole cell (10^6 cfu) and oral booster with 1 ml of whole cell, Group II was immunised *in ovo* with 0.5 ml of *C. jejuni* flagellin (2mg/ml) and oral booster with 1 ml of flagellin, Group III was immunised *in ovo* with 0.5 ml of whole cell (10^6 cfu) and intraperitoneal booster with 1 ml of whole cell protein, and Group IV as control was immunised with PBS.

At day 7 after booster vaccination, 5 chickens of each group were determined their immune responses (the mucosal and humoral immune responses), and remaining chickens were used for challenge trials.

Challenge trials

The remaining chickens of Group I to IV were challenged with 2 ml (10^{10} cfu) of bacterial suspension (the homologous isolate of *C. jejuni*) by oral at 14 days-of-age. The chickens were maintained with *ad libitum* access to feed and water for an additional 6 days. The mucosal and humoral immune responses after challenge trial were measured at 7 days after challenge.

Sample collections

The samples were collected under anasthesia treatment by ether. Serum, bile, and intestinal scrappings (IS) samples were collected at day 7 after booster vaccination and 7 days after challenge trial, whereas tissue sections from duodenum, ileum and spleen were collected only at seven days after booster vaccination. The tissue sections were fixed in cold ethanol and embedded in paraffin as previously described by SAINTE-MARIE (1962). Cloacal swab samples were taken immediately before challenge, and then daily for 6 days period. assays.

Bacteriological examination of caecal contents

Caecal contents were collected and weighed to get the same weight of sample before culturing. Then, it was diluted with volume (w/v) of brain heart infusion broth (Difco laboratories, USA) was added. The suspension was vortexed, and 10-fold serial dilutions

were made in 0.1% buffered peptone. 100 µl aliquots were put and spread on *Campylobacter* selective agar plate (Oxoid) containing *Campylobacter* selective supplement (SR 117, Oxoid) and cefoperazone selective supplement (SR 125, Oxoid). Plates were incubated under microaerophilic conditions (10% CO2, 5% O2, 85% N2) at 42^{o}C for 48 h. After incubation *Campylobacter* colonies were counted, the colony forming units of each plate were converted into the number of bacterial cells per gram of caecal contents.

Antibody assays

Campylobacter-specific IgA, IgG and IgM antibodies were measured in serum, bile, and IS by an indirect *enzyme-linked immunosorbent assay* (ELISA) using flagella, whole cell extract antigen as an antibody captured (NOOR *et al*., 1995). The reactions were determined on ELISA reader (Biorad model 3550-UV). The results of optical density (OD) reading were expressed as a percentage of the OD of a high positive standard obtained from pooled sera from hyperimmunised chickens. For intestinal scrappings, the sample was homogenised with buffer before diluting (1 g scrapping samples in 5 ml 0.1M NaCl, pH 8.0 with sodium azide added to 0.02%) and centrifuged at 44.000 g, 4^{o}C for 75 min (DUNCAN *et al.,* 1978).

Immunofluorescent procedure

The immunoglobulin-containing cells in spleen, duodenum and ileum were examined using a direct fluorescent antibody technique. The tissue sections were dewaxed, rehydrated and stained for 20 min with FITC-labelled goat anti-chicken immunoglobulin reagents specific either for IgG, IgM or IgA isotype reagent (Bethyl) as previously described (NOOR *et al*., 1995).

The immunoglobulin-containing cells were counted by fluorescence microscopy using an Axioskop 20 microscope (Zeiss, Germany) with incident light illumination. A minimum of 30 high power fields were observed for spleen tissues and counts expressed as cells per field. In intestinal tissues, cells were counted in a minimum of 30 scans each of one field diameter in width (540 µm) from the muscularis mucosae to the tips of the villi. The mean number of cells per scan was then converted to cells/cm of intestine in the plane of the section as previously described (HUSBAND, 1982).

Statistical analysis

A single factor analysis of variance (ANOVA) was used to determine the statistical significance of differences between and within groups (STEEL and TORRIE, 1980).

RESULTS AND DISCUSSION

The results of anti-campylobacter antibody responses in serum, bile and IS before and after challenge trial are presented in Table 1. Before challenge trial, the specific IgA antibodies in serum of chickens received oral booster with whole cell or flagellar protein were significantly higher ($P<0.05$) than controls. IgA antibody in the intestinal scrappings had increased significantly ($P<0.05$) only in the chickens of group I but in bile, all immunised chickens had significantly ($P<0.05$) elevated the levels of IgA antibody. This finding is consistent with the previous results that *in ovo* oral immunisation (NOOR *et al*., 1995; NOOR, 1998) elicited an early mucosal antibody responses in chickens.

Surprisingly, chickens that had been received intraperitoneal (i.p.) booster with whole cells (Group 3) failed to elevate IgA antibodies specific for *C. jejuni* in serum compared with controls. However, the mucosal antibodies in the spleen, and duodenum increased dramatically, especially IgA-containing cells, in response to immunisation. This indicates that using the intraperitoneal route for secondary immunisation seems to stimulate the mucosal immune response. This finding is consistent with a previous finding by PIERCE and GOWANS (1975) that intraperitoneal immunisation with cholera toxoid stimulated the IgA antibody response in the rat intestine. Intraperitoneal immunisation has also been reported to stimulate antibody production in intestinal secretion of chickens (ELFAKI *et al*., 1992). The failure to increase humoral antibody in this study might not be explained at this time. However, from the previous experiment of intraperitoneal delivery of antigen in mammalian species, and in chickens demonstrated a priming inoculum for subsequent oral delivery of antigen induced both humoral and mucosal responses (MUIR *et al*., 1995). This study was reversed, whether the failure of induction of a humoral immune responses following intraperitoneal immunisation was due to the different sequence of administration or caused by other factors requires further investigation.

The appearance of immunoglobulin-containing cells before challenge trial in the duodenum, ileum, and spleen are shown in Table 2. The number of immunoglobulin-containing (Ig-containing) cells for IgG, IgM and IgA isotypes in the duodenum of all immunised chickens were significantly greater ($P<0.05$) than those of controls except for IgG isotype in the chickens of Group I.

Table 1. Antibodies against *Campylobacter jejuni* determined by ELISA in serum, bile, and intestinal scraping (IS) of chickens before and after challenge trials

	Groups	Before challenge			After challenge		
		IgG	IgM	IgA	IgG	IgM	IgA
Serum	I	35.48 ± 9.07*	6.46 ± 0.63*	21.18 ± 3.55*	82.16 ± 14.52	68.92 ± 11.48	90.41 ± 24.01
	II	13.63 ± 1.93 ns	5.38 ± 0.60 ns	10.89 ± 1.10*	85.09 ± 18.38	68.39 ± 15.26	90.81 ± 33.86
	III	29.96 ± 9.44 ns	5.63 ± 1.11 ns	19.02 ± 7.04 ns	95.01 ± 20.94	92.52 ± 19.61	109.03 ± 19.04
	IV	9.45 ± 5.37	4.15 ± 0.94	7.38 ± 0.98	77.09 ± 32.05	67.22 ± 8.20	83.85 ± 35.41
Bile	I	NA	NA	24.0 ± 4.81*	50.34 ± 22.70	31.50 ± 35.90	148.6 ± 65.50
	II	NA	NA	21.10 ± 2.30*	55.49 ± 26.33	14.10 ± 4.80	107.5 ± 82.80
	III	NA	NA	28.26 ± 17.38*	57.62 ± 45.80	34.70 ± 26.30	119.2 ± 62.30
	IV	NA	NA	7.07 ± 2.27	79.26 ± 21.16	43.40 ± 25.10	173.1 ± 62.10
IS	I	NA	NA	7.04 ± 0.63*	28.63 ± 11.54	4.21 ± 8.33	0.74 ± 18.81
	II	NA	NA	2.04 ± 1.18 ns	21.92 ± 8.65	4.60 ± 6.70	4.09 ± 17.64
	III	NA	NA	1.30 ± 0.60 ns	74.26± 33.39*	25.63 ± 17.61*	33.97 ± 10.24*
	IV	NA	NA	1.49 ± 0.79	39.86 ± 19.52	5.99 ± 7.03	2.33 ± 18.39

Data are presented as percentages of a positive standard serum ± standard error of observations. (NA = not assayed, significance of difference between groups: * P<0.05)

I = *In ovo* vaccination and oral booster with whole cell protein of *C. jejuni*
III = *In ovo* vaccination and intra peritoneal booster with whole cell protein
II = *In ovo* vaccination and oral booster with flagellar protein
IV = Control group

Table 2. Immunoglobulin-containing cells determined by fluorescent histology in sections obtained from duodenum, ileum, and spleen of chickens before challenge trial

Organs	Groups	IgG	IgM	IgA
		Average ± S.E	Average ± S.E	Average ± S.E
Duodenum	I	15.76 ± 5.45	44.48 ± 5.16*	124.65 ± 23.56*
	II	44.00 ± 15.17*	159.01 ± 56.49*	251.67 ± 61.32*
	III	11.94 ± 6.39*	79.57 ± 14.18*	108.41 ± 25.82*
	IV	4.85 ± 0.59	31.33 ± 10.90	29.90 ± 9.70
Ileum	I	14.95 ± 8.43	21.41 ± 8.68	34.36 ± 17.75
	II	27.27 ± 12.44	53.09 ± 9.51*	26.66 ± 4.91
	III	13.51 ± 6.08	40.72 ± 21.19	42.66 ± 11.93*
	IV	9.70 ± 4.64	4.85 ± 1.66	16.73 ± 4.46
Spleen	I	8.84 ± 3.29	24.34 ± 5.86	13.22 ± 2.68*
	II	26.18 ± 5.64*	48.63 ± 6.44*	5.72 ± 1.20*
	III	19.73 ± 7.26	27.78 ± 4.25*	7.81 ± 0.76*
	IV	5.11 ± 2.00	11.99 ± 2.50	1.92 ± 0.58

Data of duodenum and ileum are the mean number of cells per cm of tissue, whereas data of spleen is cells per high power field (hpf) (HUSBAND and GOWANS, 1978) (Significance of difference between groups: *P<0.05)
I = *In ovo* vaccination and orally booster with whole cell protein of *C. jejuni*
II = *In ovo* vaccination and orally booster with flagellar protein
III = *In ovo* vaccination and intra-peritoneal booster with whole cell protein
IV = Control group

The immunisation with flagellin induced an increase in the number of IgM-containing cells ($P<0.05$) compared with those of controls. Oral immunisation with flagellin elicited an increase in the number of Ig-containing cells of all isotypes in the spleen ($P<0.05$). Thus, flagellin can be considered as an appropriate relevant subunit antigen for vaccination to control *Campylobacter* infection in chickens. Based on genetic studies using a PCR test to identify all strains of *C. jejuni* (WIDDERS, unpublished data), the flagellin protein has been recognised as a specific target antigen in the gastrointestinal tract in the development of immune response.

High numbers of Ig-containing cells either IgG, IgM or IgA occurred in the intestine following vaccination with flagellin, indicating the ability of flagellin protein to induce an immune response in the intestinal mucosae. It is interesting to note that comparison of systemic antibody response and mucosal antibody response of immunised birds either with whole cells or flagellin were not statistically different. It is suggested that both whole cell and flagellin are equally effective as *Campylobacter* antigen.

The bacteriological results (the cloacal shedding and caecal infection rates of *C. jejuni* after challenge trial) are shown in Table 3. Daily monitoring of cloacal swabs following oral challenge with viable *C. jejuni* showed that there was no significant difference in the onset of shedding of organisms from birds in all groups, both immunised and control. *C. jejuni* was first detected in cloacal swabs collected 2 days after oral inoculation with the organisms. There were no differences between groups in the detection of *Campylobacter* by cloacal swabs and in the reduction level of caecal colonisation for immunised groups.

Table 3. The onset of shedding and the number of *C. jejuni* colonising the caecum after challenge

Groups	Day first positive	cfu/gm caecal contents
I	3	4.7×10^7
II	3	2.9×10^7
III	2	5.2×10^7
IV	2	2.5×10^7

I = *In ovo* vaccination and oral booster with whole cell protein of *C. jejuni*
II = *In ovo* vaccination and oral booster with flagellar protein
III = *In ovo* vaccination and intra-peritoneal booster with whole cell protein
IV Control group

The possible reason for the failure to demonstrate greater differences between groups following challenge is probably due to the limited antibody responses in immunised chickens. In this experiment, even though *in ovo* immunisation with *Campylobacter* antigen resulted in elevated numbers of IgA cells in the intestinal tract, it is not clear whether those cells were specific to *Campylobacter*, since double labelling of those cells was not able to identify their antigen specific. Therefore, it is probably that not all of the IgA cells detected in the chicken intestine was IgA specific to *Campylobacter.*

Oral dosing with 10^{10} viable *C. jejuni,* as used in all trials might overwhelm an existing intestinal antibody response protection, with the result that all groups became colonised in this study. It needs subsequent trials to promote dissemination of *C. jejuni* among the experimental population, in order to more effectively mimic the level of exposure encountered under commercial conditions.

CONCLUSION

In ovo combined with oral immunisations using flagellin and whole cell protein antigens of *Campylobacter jejuni* induced early development of humoral and mucosal immunity in chickens. Both flagellin and whole cell protein were equally effective as immunogens in stimulating the precocious development of intestinal immunity of chickens but was not strong enough to protect the intestinal colonisation of homologous *C. jejuni* in chickens.

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