THE MUCOSAL AND SYSTEMIC IMMUNE RESPONSES IN CHICKENS ORALLY IMMUNISED WITH *CAMPYLOBACTER JEJUNI* ANTIGEN ENTRAPPED IN *POLY-LACTIDE-CO-GLYCOLIDE* MICROPARTICLES

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ABSTRAK

NOOR, S.M. 1998. Respon kekebalan mukosal dan sistemik pada ayam setelah imunisasi oral dengan antigen *Campylobacter jejuni* dalam *poly-lactide-co-glycolide* mikropartikel. *Jurnal Ilmu Ternak dan Veteriner* 3 (4): 264-269.

Untuk mengimunisasi ayam secara peroral diperlukan teknik penyediaan imunogen yang sesuai agar tanggap-kebal yang diperoleh menjadi optimal. Teknik penyediaan antigen dengan mikropartikel merupakan suatu cara untuk meningkatkan efikasi kerja antigen vaksin peroral. Penelitian ini menguji sifat antigenik *Campylobacter jejuni* untuk menaikkan tanggap-kebal mukosal dan sistemik (humoral) pada ayam. Kelompok A (5 buah telur ayam berembrio) diimunisasi dengan formulasi antigen *C. jejuni* dalam mikropartikel *poly-lactide-co-glycolide* (PLG) pada hari ke-17 masa inkubasi ke dalam cairan amnion dan *booster* oral diberikan pada hari ke-7 setelah menetas. Kelompok B, telur berembrio diimunisasi dengan antigen campylobacter dilarutkan dalam PBS, dengan perlakuan seperti pada Kelompok A. Tanggap-kebal vaksinasi diamati pada hari ke-14 dengan mendeteksi kekebalan humoral dengan teknik ELISA dan mendeteksi kekebalan mukosal dengan teknik histologi fluoresensi. Titer antibodi (anti-campylobacter) IgG dan IgA dalam serum dan juga titer antibodi IgA dalam cairan empedu dan kerokan usus secara nyata lebih tinggi (P<0,05) pada ayam Kelompok A dibandingkan dengan Kelompok B. Jumlah imunoglobulin total yang terkandung di dalam sel untuk isotipe IgG, IgM dan IgA dalam ileum ayam Kelompok A juga meningkat secara nyata (P<0,05) dibandingkan dengan Kelompok B, tetapi dalam duodenum dan limpa peningkatan sel itu tidak nyata.

Kata kunci : *Campylobacter jejuni*, mikropartikel *poly-lactide-co-glycolide*, imunisasi oral, ayam

ABSTRACT

NOOR, S.M. 1998. The mucosal and systemic immune responses in chickens orally immunised with *Campylobacter jejuni* antigen entrapped in *poly-lactide-co-glycolide* microparticles. *Jurnal Ilmu Ternak dan Veteriner* 3 (4): 264-269.

An oral immunisation in chickens with antigen entrapping in biodegradable microparticles was evaluated in order to achieve optimal antibody responses following oral vaccination. This study was adapted to *Campylobacter jejuni* antigen in chickens to observe its stimulation both mucosal and systemic immune responses. A group of 5 embryonated chicken eggs was immunised with heat-killed *C. jejuni* entrapped in *poly-lactide-co-glycolide* (PLG) microparticles at day 17 of incubation deposited into the amniotic fluid. Seven days after hatching the chickens were oral boostered, these was design to as Group A. In the Group B, each embryonated egg was immunised with soluble *C. jejuni* as in the same as Group A. Immune responses of post vaccination were observed at day-14, the humoral immunity was evaluated with an ELISA and whereas mucosal antibody response was detected by fluorescent histology. The serum IgG and IgA antibody responses, and also the bile and intestinal scrapping IgA antibody responses to campylobacter in Group A were significantly higher than those of the soluble antigen of Group B (P<0.05). Total number of immunoglobulin-containing cells for IgG, IgM, and IgA isotypes in the ileum in Group A chickens were also significantly higher than those of Group B (P<0.05) but was not in the duodenum and spleen.

Key words : *Campylobacter jejuni*, *poly-lactide-co-glycolide* microparticles, oral immunisation, chickens

INTRODUCTION

In chickens, vaccination by means of intramuscular or subcutaneous injection has some disadvantages, these are expensive, labour intensive, and requires qualified personnel. Vaccines containing synthetic recombinant protein were reported to be poorly immunogenic for stimulating mucosal immune responses (MOWAT *et al*., 1991). Most adjuvants are not suitable to all immunogens and to stimulate humoral immune responses (ADIBERT *et al*., 1993).

Moreover, the available adjuvants, such as Freund's incomplete adjuvant, have negative effects, such as local granulomas in the site of injection, fever, pain and possibly malignancies (BEEBE *et al*., 1972).

On the other hand, oral immunisation by drinking water in chickens has advantage of being fast, in inducing a mucosal immunity and reducing costs. However, oral route immunisation produced a poor antigen-specific secretory IgA response unless prolonged feeding or high dose of antigen was used (PROWSE *et al*., 1993). To overcome that problem recent techniques for controlling poultry diseases have been developed by improving vaccines efficacy and antigen delivery technique. One of the alternatives method for antigen delivery technique at mucosal sites in chickens is embryonal vaccination (PROWSE *et al*., 1993). Another method that has been developed in mammalian is a biodegradable microparticle of *poly-lactide-co-glycolide* (PLG) (ELDRIDGE *et al*., 1989; MORRIS *et al*., 1994; O'HAGAN, 1994).

The biodegradable microparticles have been considered as a potential method for releasing antigen in long-term to stimulate immune responses (MORRIS *et al*., 1994). This method has several benefits for oral immunisation, for enhancing the immune response of poorly immunogenic antigen (GILLEY *et al*., 1992; O'HAGAN *et al*., 1989), reducing total antigen dose required to achieve adequate immunity, protecting antigen from the acid and enzymatic digestion in the gastrointestinal tract (MORRIS *et al*., 1994), and enhancing antigen absorbtion by Peyer patch's (PP) and stimulating secretory immune responses (O'HAGAN, 1990; ELDRIDGE *et al*., 1990).

The mechanism of antigen released from microparticles is supposed by diffusion through matrix pores and matrix degradations. The antigen released depends on polymer composition and molecular weight (MORRIS *et al*., 1994; O'HAGAN *et al*., 1991). Enhancement of antibody response is affected by size of microparticles mixtured (MORRIS *et al*., 1994). Based on a study reported by ELDRIDGE *et al*. (1990), microparticles larger than 5 µm in diameter will remain in the PP from 1 to 35 days after administration, with the high peak at 1 to 4 days.

Previous study indicated that biodegradable microparticles containing antigen used in oral vaccination might enhance mucosal and systemic immune responses (ELDRIDGE *et al*., 1989; O'HAGAN *et al*., 1991; O'HAGAN *et al*., 1993), but it has not used in chickens. The present study reported here was the efficacy of PLG biodegradable microparticles as an oral delivery vehicle of *Campylobacter jejuni* antigen in inducing mucosal and systemic immune responses in chickens.

MATERIALS AND METHODS

Animals

Embryonated broiler eggs obtained from Ingham poultry hatchery (Casula, Australia) were used in this experiment. They were placed in an incubator at 37.4°C with humidity of 59 to 76%. Eggs were incubated at one time were used for treatment and control groups. These embryonated eggs were divided into two groups (Group A and B), each consist of 5 eggs.

Microparticles preparation

The PLG (Polyscientific, USA) microparticles entrapping *C. jejuni* antigens was prepared by solvent evaporation from an oil-in-water emulsion as previously described (JEFFERY *et al*., 1993). Briefly, 200 ml of 0.1% PVA (Polyvinyl alcohol) (Sigma 69F0692, MW 30,000-70,000) was placed in a 500 ml beaker and homogenised with ultra Turrax T 25 blender at 450 RPM (Solution 1). One ml of heat-killed *C. jejuni* was added to 20 ml PLG and homogenised at 13,500 RPM for 1 min, the 15 ml of 5% PVA was then added and homogenised for 30 seconds at 450 RPM (Solution 2). The Solution 2 was added to Solution 1 and mixed by magnetic stirrer at room temperature (RT) overnight to allow solvent evaporation and microparticle formation. Microparticles were collected the next day and washed with phosphate-buffered saline (PBS) (pH 7.3), non-entrapped antigens was removed by centrifuging at 2,500 RPM for 10 min at 21°C. The microsphere fractions were collected and rediluted in PBS, and then measured by laser diffractometry (Malvern Laser Sizer 2600 D).

Immunisation protocol

Each egg of Group A was injected at day 17 of incubation with 10^8 colony-forming-units (cfu) of heat-killed *C. jejuni* entrapped in PLG microparticles, 0.5ml of antigen was deposited into the amniotic fluid. Seven days after hatching, each chicken was given oral booster of 1 ml of the same antigen as used in the primary of egg injection. Similar procedure was done to Group B with soluble *C. jejuni* antigen.

Sample collections

Serum, bile, and intestinal scrapping (IS) samples were collected at day 14 to determine antibody responses. Under anaesthesia treatment with ether inhalation just before killing chicken blood sample was

collected by cardiac puncture for each animal and then chickens were euthanised. Bile was aspirated from the gall bladder after opened the chicken abdomen. The jejunum was removed immediately and rinsed with PBS and mucosal scrapping samples were collected by opening segments of intestine on to moistened filter paper. While, tissue section samples of ileum and duodenum were fixed in cold ethanol.

Antibody assay

The specific anti-campylobacter IgG, IgM, and IgA antibody responses of each sample were determined by ELISA. The ELISA antigen was obtained from Dr. Philip Widders (V.I.A.S., Australia). Briefly, a culture of *C. jejuni* was centrifuged at 10.000g and the supernatant filtered through a 0.22 μm filter. The protein content was confirmed by performing polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS) (Bacto, Liverpool, Auastralia) followed by staining with commasie blue.

The ELISA procedures for detecting those anti-campylobacter antibody responses had been previously validated for anti-campylobacter IgG, IgM, and IgA antibodies (Noor *et al.*, 1995), briefly: The wells of microtiter ELISA plates NUNC (Medos Company, Burwood, Australia) were coated with 100 μl per well of *C. jejuni* flagellin antigen (14 mg per ml) diluted in PBS (1 in 300) and incubated at 4°C for overnight. The wells were washed twice with PBS-Tween-20 (PBST) (Sigma-Aldrich, Castle Hill, Australia) and once with distilled water. Each well was blocked with 100 μl of PBST containing 0.01% ovalbumin (Sigma) at 37°C for 1 hr. After washing with PBST as previous step 100μl of appropiate diluted serum samples was added (serum and IS were diluted 1:5, bile was diluted 1:100 in PBST containing 0.01% ovalbumin). After incubation at 37°C as before, plates were washed 3 times with PBST, 100 μl of goat anti-chicken IgG horseradish peroxidase (HRP) conjugates (Bethyl Laboratories, Montgomery, USA) was added at working dilution 1:1000 in PBS. While, to detect IgM response, anti-chicken IgM HRP was diluted 1:500, whereas to detect IgA response, anti-chicken IgA HRP was diluted 1:100. Plates were incubated at 37°C for 1 hr and washed as before, enzyme substrate of 2,2'-azino-di-[3-ethyl-benzthiazoline sulphonate] (ABTS) was added into each well and incubated at 37 ^{o}C for 40 min. The reaction was stopped with 1% sodium dodecyl sulphate diluted with distilled water

The reactions were determined on ELISA reader of an automated plate reader (Biorad model 3550-UV). The optical density (OD) of each well was determined at 405 nm on each well after the instrument was zeroed with unreacted substrate. Results were expressed as a percentage of the OD of a high positive standard from hyperimmunised chickens.

Immunofluorescent procedures

The tissue section of spleen, duodenum and ileum were embedded in paraffin as previously described by Sainte-Marie (1962) to examine the immunoglobulin-containing cells using a direct fluorescent antibody technique. Briefly, the embedded tissue were dewaxed, rehydrated and stained for 20 min in the humid chamber with 40 μl of FITC-labelled goat anti-chicken IgA isotypes (Bethyl) at working dilution 1:50 in PBS. Slides were then washed in PBS for 1h, then mounted under a coverslip with phosphate-buffered glycerol. To examine the IgM-containing cells, the tissues were stained with FITC-labelled goat anti-chicken IgM isotypes (Bethyl) at working dilution 1:50, whereas for IgG-containing cells, FITC-labelled goat anti-chicken was diluted 1:20.

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

Statistics

Statistical analysis was carried out using a single factor Analysis of variance (Anova) to determine the statistical significance of differences between and within groups of experimental chickens (Steel and Torrie, 1980).

RESULTS

The ELISA results were expressed as a percentage of the optical density of a high positive standard prepared from pooled sera from hyperimmunised. The protein percentage of anti-campylobacter IgG, IgM and IgA antibody responses in serum, bile and IS of chickens each group are presented in Table 1. The result showed that the high percentage of anti-campylobacter antibody were detected in serum, bile and IS of chickens immunised with *C. jejuni* antigen entrapped in PLG (Group A).

Table 1. The means of protein percentage of IgG, IgM and IgA antibody responses in serum, bile and IS of chickens injected with *C. jejuni* antigen

Group of chicken	Serum			Bile	IS
	% IgG	% IgM	% IgA	% IgA	% IgA
A	84.75±8.93*	30.78±6.82ns	38.90±6.82*	5.43±1.10*	24.08±4.36*
B	53.59±6.13	10.19± 2.81	16.69±2.04	1.46±0.22	12.27±3.61

A = Vaccinated with *C. jejuni* antigen encapsulated in PLG microparticles
B = Vaccinated with soluble *C. jejuni* antigen
IS = Intestinal scrapping
Result shown as the means of % positive std. ± SE
Significance: * = P<0.05, ns = not significant

Table 2. The numbers of immunoglobulin-containing cells for IgG, IgM and IgA isotypes in the duodenum, ileum and spleen of chickens following oral vaccination with *C. jejuni* antigen

Organs	Group A			Group B		
	IgG	IgM	IgA	IgG	IgA	D
Duodenum #	1.65±1.65ns	42.17±10.41ns	132.31±61.53ns	3.03±3.03	18.43±2.93	32.32±2.70
Ileum #	11.82±1.22*	28.79±3.46*	37.88±4.07*	1.52±1.52	5.81±5.81	9.58±2.91
Spleen	3.09±1.38ns	13.04±1.13ns	7.78±2.77ns	1.28±1.28	12.83±3.66	2.34±0.58

A = Vaccinated with antigen encapsulated in PLG microparticles
B = Vaccinated with soluble antigen
Significance: *= P<0.05, ns= not significant, # =(cells/cm ± SE), ## =(cells/hpf ± SE)

The results obtained from chickens in Group A, indicate that oral immunisation with PLG micro-particles entrapping *C. jejuni* antigen induced significantly higher levels of serum IgG and IgA specific antibody to campylobacter than those of equivalent amounts of soluble antigen in control chickens (Group B) (P<0.05). However, the serum IgM levels in chickens immunised with and without microparticles entrapping antigen were not significantly different (P>0.05).

The IgA antibody response in bile and IS were also significantly higher (P<0.05) following immunisation with campylobacter entrapped in PLG microparticles compared with those of immunised chickens with soluble *C. jejuni* antigen.

The appearance of immunoglobulin-containing cells associated with IgM and IgA isotypes in the ileum, duodenum and spleen after oral immunisation with *C. jejuni* antigen encapsulated in PLG microparticles is shown in Table 2. There were significant increases in the number of Ig-containing cells for IgG, IgM and IgA isotypes in the ileum of immunised chickens with microparticles-encapsulated antigen (Group A) compared with those of immunised chickens with

soluble antigen (Group B) (P<0.05). However, in the duodenum and the spleen there was no significant difference for IgG, IgM or IgA-containing cells (P>0.05).

DISCUSSION

The previous studies reported that entrapment of antigen in the PLG microparticles led to the elevation of the production of IgG and secretory IgA antibody responses following oral immunisation in small animal experiments (CHALLACOMBE *et al*., 1992; O'HAGAN *et al*., 1993), similarly. The present study, immunised chickens with campylobacter entrapped in the PLG microparticles displayed elevated serum IgG and IgA antibody responses and as well as elevated the levels of IgA antibody secretion in bile and intestinal responses, confirming that PLG microparticles are potent inducers of local and systemic antibody responses when administered orally. These responses were reflected in a dramatic increase in total of immunoglobulin-containing cells detected by fluorescent histology, particularly those associated with IgG, IgM, and IgA isotypes in the ileum of chickens which were

immunised with microparticles-encapsulated *C. jejuni* antigen.

The ability of antigen entrapped in microparticles to induce systemic IgA antibody response and IgA antibody-containing cells response following oral administration suggested that it might be due to the uptake of microparticles containing antigen by the lymphoid tissue of Peyer's patches (PP) in the intestine. It was reported by O'HAGAN (1990), microparticles containing antigens were taken up by PP following oral administration in mice. The uptake of the antigen depended on the size of microparticles. The range of particle diameter between 5 to 10 μm was required to achieve the optimal stimulation of mucosal immunity. Microparticles which are greater than 10 μm in diameter will not be taken up by the PP, and less than 5μm will be passed directly to mesenteric lymph nodes and to the spleen (ELDRIDGE *et al.*, 1991). In this experiment, the average diameter size of 6.05 μm was assumed that this size was suitable for inducing systemic IgG and IgA antibody in serum, and in intestinal scrappings and also increase the number of immunoglobulin-containing cells of all isotypes in the ileum.

Although the number of immunoglobulin-containing cells for IgM and IgA isotypes in the duodenum of immunised chickens with microparticles-entrapped *C. jejuni* antigen were still higher than those of immunised chickens with soluble antigen, there was no significant difference. The failure of the stimulation of immunoglobulin-containing cells in the duodenum might be explained by the lack of lymphoid tissue in the duodenum (BEFUS *et al.*, 1980). Whereas, the absence of immunoglobulin-containing cells in the spleen is probably also associated with the particle size of the microparticles (JENKINS *et al.*, 1994).

The results described above demonstrated that biodegradable microparticles entrapping antigen preparation stimulates the humoral and mucosal immunity of chickens higher than those of the soluble antigen. The entrapping antigen in microparticles PLG technique has a great deal of potential used a vaccine preparation in future, particularly in the field of oral vaccination.

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