IMMUNOGENICITY OF *ESCHERICHIA COLI* ROUGH MUTANT BACTERIN IN CHICKENS

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SUMMARY: Avian pathogenic *Escherichia coli* (APEC) cause a wide range of economically significant infections in chickens. Control of these infections by antimicrobial drug is no longer possible due to high prevalence of multidrug resistance strains. Involvement of large number of serotypes in these infections left none serotype specific vaccine as the only option. It has been well established that the Lipopolysaccharide (LP) core specific antibodies are broadly cross protective.

Present study determined the LPS core specific antibody titers of chickens immunized with a single dose of heat killed rough mutant *E. coli* strains comprising of LPS core types R1, R2, R3 and R4. Mean anti-LPS core antibody titres for all four core types increased significantly ($p < 0.05$) in immunized group than in control group. Thus, the heat killed mixture of rough mutant *E. coli* strains can be used as a vaccine to enhance LPS core specific antibodies in chickens.

INTRODUCTION

Colibacillosis caused by Avian Pathogenic *Escherichia coli* (APEC) is one of the principal diseases that induce various extra intestinal disease manifestations in poultry including septicaemia, enteritis, perihepatitis, pericarditis, peritonitis, synovitis, omphalitis, salpingitis and coligranuloma (Dho-Moulin and Fairbrother, 1999). These infections cause severe economic losses to the farmer through high mortality, growth depression, cost of antimicrobial therapy and carcass downgrading causing an immense threat to the advancement of the poultry industry (Janßen et al., 2001). Therefore, control of this infection has become a prime concern over the last few decades. At present antimicrobial therapy remains as the major control measure of this infection. However, further use of this treatment is limited by the emergence of antimicrobial resistance of *E. coli* (Blanco et al., 1997; Johnson et al., 2007; Yang et al., 2004). Apart from antimicrobial therapy becoming less

efficient, it is important to be aware of the potential serious public health consequences entails by the presence of antimicrobial residues in chicken meat (Warren et al., 2008). Hence development and use of a potent vaccine appears as the most logical approach to control *E. coli* infection in poultry.

It is well established that the core oligosaccharide region of bacterial lipopolysaccharide is well conserved and highly immunogenic (Bennett-Guerrero et al., 2000; Skidmore et al., 1975). The specificity of antibodies generated in response to immunization with live or killed, wild type, bacterial strains is determined by the O-polysaccharide of bacterial lipopolysaccharide (LPS) (Erridge et al., 2002; Hämmerling et al., 1971). Rough mutant bacteria have a defect in the biosynthesis of O-polysaccharide and give rise to LPS core specific antibodies.

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The antibodies to LPS core do not elicit serotype specificity and hence broadly cross reactive (Baumgartner et al., 1985; Baumgartner et al., 1990; Davis et al., 1978; Ziegler, 1988; Ziegler et al., 1982). For more than 170 O-specificities, only five different core specificities designated as R1, R2, R3, R4 and K12 were encountered so far (Amor et al., 2000; Currie and Poxton, 1999; Dissanayake et al., 2008; Gibbs et al., 2004).

Present study determined the efficacy of heat killed mixture of *E. coli* rough mutants representing LPS core types R1, R2, R3 and R4 to trigger LPS core specific antibody titers in chickens. As the *E. coli* rough mutants are scarcely distributed naturally, LPS of 193 avian *E. coli* isolates collected from diseased and healthy chicks from commercial poultry farms in Sri Lanka were analyzed by polyacrylamide gel electrophoresis (PAGE) to select four rough mutants representing four common LPS core types identified in APEC.

MATERIALS AND METHODS

Bacterial isolates

E. coli isolates used for the study comprised of 143 clinical and 50 commensal isolates. These isolates were collected from commercial broiler and layer chicken farms in Sri Lanka over a period of two years. Isolates were confirmed as *E. coli* biochemically and LPS core types identified by polymerase chain reaction in our previous study (Dissanayake et al., 2008).

LPS extraction by rapid phenol micro method

To screen the collection of 193 *E. coli* isolates for rough mutants, LPS extracted by rapid phenol micro method (Currie and Poxton, 1999) was used. Briefly; bacteria were grown overnight in 20ml nutrient broth at 37°C and centrifuged at 3980g for 20 minutes. The pellet was re-suspended in 500µl of pyrogen free water to which an equal volume of 90% (w/w) phenol solution added. The suspension was mixed in a vortex for 10 seconds and incubated at 70°C in a water bath for 10 minutes. After cooling in an ice bath for 5 minutes, the samples were centrifuged at 3000g for 5 minutes at 4°C. The clear upper phase was collected and the LPS was precipitated by adding 1ml of acetone. The LPS pellet was obtained by centrifugation at 10,000g for 10 minutes. The supernatant was removed and the pellet was air dried. The dried LPS pellet

was re-suspended in 100µl of pyrogen free water and stored at -20°C until analysis.

Polyacrylamide gel electrophoresis (PAGE)

LPS samples were thawed and 10µl (5mg/ml) were mixed with equal volumes of double strength sample buffer (0.125M tris-HCl, pH 6.8, 4% w/v SDS 20%, v/v glycerol, 2% v/v 2-mercapto ethanol, 0.002% v/v bromophenol blue). Samples were heated at 100°C for 3 minutes and separated on 12% polyacrylamide gels (omitting SDS from both separating and stacking buffer) using Laemmli buffer system and LPS was visualized by silver staining. Briefly, gels were fixed in 25% propan-2-ol and 7% acetic acid overnight and oxidized in freshly prepared periodic acid (1.05g) in 150 ml of distilled water containing 4 ml of propan-2-ol acetic acid fixative. After washing over 4 hours in 4 changes of distilled water, gels were stained for 15 minutes in ammonical silver nitrate (1.4 ml ammonia, 21 ml of 0.36% NaOH, 4 ml of 19.4% AgNO₃ and 74.6 ml of distilled water). Silver solution was discarded and gels were washed in 4 changes of distilled water over 40 minutes. Colour was developed when gels were transferred into 200 ml of 0.019% formaldehyde containing fresh 0.005% citric acid.

Preparation of heat killed bacterin

Four rough *E. coli* strains (selected by analysing LPS profiles) representing R1, R2, R3 and R4 core types were grown separately in 20 ml nutrient broth for 6 hours. Equal volumes of nutrient broth from each culture (100 ml) were mixed together and centrifuged at 400g for 10 min. The resultant pellet was washed in PBS and re-suspended again in PBS to obtain a suspension of 0.5 MacFarland standards (approximately 1x10⁶ bacterial cells). The suspension was kept in a boiling water bath for 1 hour and a loopful was plated on blood agar to ensure that no viable cells remain in the suspension.

Immunization and collection of blood samples

Forty broiler chicks (day old) purchased from a local hatchery were housed in the poultry house, Veterinary Research Institute, Gannoruwa, Sri Lanka. All animal experiment procedures described below were performed in accordance with the standard ethical guidelines of the institute. Birds were separated

into two groups, (20 birds in each group) housed in two different pens and fed according to standard broiler feeding schedule using commercial broiler ration. On day 15, twenty birds were injected subcutaneously with the mixture of heat killed bacterin and the remaining 20 were injected subcutaneously with an equal volume of PBS. Blood samples from all these birds were collected from the brachial vein on day 4, day 14 and day 22 of age for serum separation. All blood samples were allowed to clot at room temperature for 30 minutes and immediately centrifuged at 4000g for 15 minutes at 4°C. The serum was separated and stored at -20°C until analyzed.

Determination of serum anti-LPS core antibody titers by indirect enzyme-linked immunosorbent assay (ELISA)

Extraction of LPS by aqueous phenol method to coat wells

Four rough mutant *E. coli* strains representing LPS core types R1, R2, R3 and R4 were selected to extract LPS by aqueous phenol method (Galanos et al., 1969) in order to coat the micro titre plates (antigen for ELISA). Overnight nutrient broth culture (60 ml) was dispensed to 6 L of nutrient broth and incubated at 37°C for 18 hours in a shaking incubator. Cultures were centrifuged at 10,000g for 15 minutes at 4°C in an enclosed rotar (Sorval SS34). The pellet was washed 3 times in phosphate buffered saline (PBS) under the same conditions and freeze dried. The freeze dried bacterial pellet was ground up using a glass rod and suspended in 5% (w/v) in pyrogen free water. Then it was mixed with an equal volume of 90% phenol (both pre-heated to 67°C) and kept in a water bath at 67°C for 15 minutes with stirring every 2-3 minutes. The suspension was transferred to an ice bath to separate phases and centrifuged at 10,000g for 15 minutes at 4°C. Using a glass pipette, the clear upper phase containing LPS was transferred to dialysis tube (Viking 32/32), dialyzed under running tap water over night and transferred to ultra centrifuge tubes to centrifuge at 100,000g for 3 hours at 4°C. The supernatant was discarded and the pellet was re-suspended in pyrogen-free water and ultra-centrifuged again under the same conditions. The pellet was dissolved in small volumes of pyrogen free water and transferred to a pre-weighed glass container for freeze drying. Endotoxin proteins present in the freeze dried samples were removed as

described by Manthey and Vogel (Manthey and Vogel, 1994).

Preparation of antigen (LPS core-polymyxin B sulphate)

LPS core-polymyxin B sulphate complexes were prepared as described previously (Scott and Barclay, 1987). Aqueous phenol extracted LPS of core types R1, R2, R3 and R4 (Ref. 3.1.2) were prepared as 1mg/ml in pyrogen free water and sonicated at 5µm for 30 seconds (Sonyprep sonicator). Equal volumes of the LPS solution and 1mg/ml polymyxin B sulphate (Sigma) were mixed and sonicated as above. Polymyxin B sulphate LPS core suspensions were transferred to MWCO 2000 dialysis tubing (Spectrapore) and dialyzed overnight against 2L of pyrogen free water.

Coating micro-titre plates with antigen

The dialyzed mixtures were transferred to glass bijou bottles and sonicated again under the same conditions to dissolve floccular masses. Mixtures were diluted in coating buffer (0.05M sodium carbonate buffer, pH 9.6) 1:50 and mixed thoroughly in a vortex. Plates were coated by adding 100µl of LPS core-polymyxin B complex to each well (Costar® coming incorporated, high binding micro-titre plate). To avoid coating large complexes, the suspensions were mixed intermittently. Plates were kept at 4°C overnight for coating. The plates were washed four times with PBS- Tween 20 (0.05% Tween in PBS pH 7.4), blocked with 3% fish gelatin overnight at 4°C, washed again, dried and stored at -20°C until use.

Preparation of antiserum

Serum samples stored in -20°C thawed and diluted in 1:200 in PBS dilution buffer containing 4% polyethylene glycol 8000 w/v and 0.05% Tween v/v, pH 7.4.

ELISA

A volume of 100µl from each serum sample was added in duplicate into four micro- titre plates, each coated with a different core type. Only the dilution buffer was added to the wells of negative controls. A serum sample which gave the colour near to the mean in a preliminary titration was used as the known positive and included in each plate. Plates were incubated for 90 minutes at 37°C, washed four times with PBS-tween 20 and incubated with horseradish peroxidase (HRP) conjugated goat anti-chicken antibodies (Particell Ltd, UK for 90 minutes

at 37°C.

HRP colour development

One tablet of tetramethylbenzidine dihydrochloride (Sigma-Aldrich) and 2 µl of hydrogen peroxide were added per 10 ml of 0.05M phosphate citrate buffer (pH 5.0) and 100 µl was added to each well. Plates were incubated at room temperature until the colour of the known positive serum reached close to that of the mean. The reaction was stopped by adding 50µl of 2M sulphuric acid and plates were read at 450nm. Results were normalized to the standard before analyzing.

Statistical analysis

Significance in differences of antibody titers for LPS core types between vaccinated and control groups were determined by two way ANOVA. Differences were considered statistically significant if $P < 0.05$.

RESULTS

LPS was extracted from 193 avian *E. coli* isolates by conventional rapid phenol-micro method and subjected to PAGE and silver staining to determine the heterogeneity of O-antigen chain length (Figure 1). Lipopolysaccharide which lack the heterogeneity of O-antigen chain length and characterized by zone of fast migrating low molecular weight bands were identified as 'rough' and those with heterogenic O-polysaccharide chains with characteristic laddering were identified as 'smooth'. Majority of isolates (92.3%) showed the typical ladder pattern of smooth LPS and only 15 isolates with rough LPS were identified (Figure 1). Most isolates varied widely in their LPS profiles and identical profiles were observed in a few isolates. LPS core types of these isolates were identified previously (Dissanayake et al., 2008) and *E. coli* isolates with same core types differed widely in their LPS profiles. The LPS extracted by the rapid phenol micro method

contained significant amounts of contaminants particularly, protein and capsular polysaccharides.

By examining the LPS profiles of these bacterial isolates, four rough strains representing the core types; R1, R2, R3 and R4 were selected to immunise chickens with the assumption that those rough mutants will give rise to LPS core specific antibodies. A group of 20 chickens were immunised with that mixture of heat killed bacterin and serum antibody titres were determined before and after immunisation. The results were compared with a group of un-immunised chickens.

At day four of the age before immunized with bacterins, chicks already had anti-LPS core antibodies which increased spontaneously with age. Antibody titres for four different core types varied widely. On the day four of the age the highest mean antibody titre was observed for R4 LPS core type and the lowest mean was for R3 core type. The R2 specific antibody titres varied within a narrow range when compared to core specific antibody titres of other core types (Figure 2).

Remarkable differences in the immunological responses were observed among individual birds. Some birds had optical density (OD) values (representing comparative antibody titres) of 1.881 and in certain other birds it was as low as 0.4. Individual antibody titres for different core types also did not exhibit constant pattern. In some instances, antibodies specific for one core type was very high while that for another core type was extremely low. It is interesting to note that the mean anti-LPS core antibody titres for all four core types increased significantly ($p < 0.05$) in immunized group when compared to un-immunized control group as shown in the Figure 2. However, this immune response was not uniform among the individuals within the group.

Figure 1 Heterogeneity of O-antigen length of Avian *E. coli*

Rough LPS are shown in lanes 9, 12 and 17. Smooth LPS are shown in lanes 2,3,,5,6,7,8,10,11,13,14,15 and 16. First lane contains the standard smooth LPS and the last lane (18) contains standard rough LPS. Lanes 2, 3, 4 and 5 contained LPS with R1 core type and 6, 7 , 8 and 9 contained LPS with R2 core type, 10,11,12 and 13 contained LPS with R3 core and 14,15,16,17 contained LPS with R4 core type

Figure 2: Serum Antibody titres (IgG) to each of the LPS core types, R1, R2 R3 and R4 of healthy chicks before and after immunization

Lines within the box plots represent the median and the top and bottom lines of the box represent the 25th and 75th percentile. Pink colour boxes represent the antibody titres to R1 core type and blue, green and yellow boxes represent anti body titres to R2, R3 and R4 respectively.

DISCUSSION

In the present study LPS extracts of 193 avian *E. coli* isolates were studied by PAGE and silver staining to identify naturally occurring

rough strains. In contrast to the wild type smooth strains, these mutants were less prevalent. We used LPS extracted by rapid phenol micro method to identify these mutants and the only limitation of the technique was the presence of

large amount of protein contaminants which interfered the identification of rough LPS. The LPS extracted by aqueous phenol method and decontaminated by the method described by Manthey and Vogel (1994) was relatively pure but the techniques were laborious.

Our previous study (Dissanayake et al., 2008) confirmed the distribution of R1, R2, R3 and R4 core types among chicken *E. coli* isolates. Therefore, in this study a mixture of heat killed bacterin prepared with four rough *E. coli* isolates representing the above mentioned core types were used to immunize chickens. Results indicated that the mean antibody response of immunized group was significantly higher than that of un-immunized group. It is clear that the immune system of chickens can recognize and respond to core oligosaccharide of LPS. This result further supported that the antibodies to the core oligosaccharide occur naturally as well as in response to immunization.

The potential of controlling *E. coli* infections in commercial poultry through vaccination has been explored widely. In those attempts, live or killed bacteria and various cellular components have been used to immunize chickens (Gyimah et al., 1986; Melamed et al., 1991) but the involvement of more than 60 serotypes in causing avian *E. coli* infection limited the use of these serotype specific vaccines in the field. An effective vaccine to avian colibacillosis should be directed against antigenic determinants shared by all serotypes involved in causing the disease. Serotype specificity of Gram-negative bacteria is usually determined by the O-polysaccharide side chains of bacterial LPS (Galanos and Lüderitz, 1984). In *E. coli* the antigenic diversity expressed by the O-polysaccharide chains is extensive, whereas that of LPS core structures and Lipid-A component is limited.

The potential of using rough mutants to induce anti-LPS core antibodies has been explored previously and most often heat killed bacterin of rough mutants with incomplete core structure has been tested; *E. coli* J5 (Gonzalez et al., 1989; Hogan et al., 1992a; Hogan et al., 1992b; McCutchan et al., 1982; Ziegler et al., 1982) (Rc-chemotype) or *S. minnesota* 595 (Re-chemotype) (Johns et al., 1977). The vaccine described here contained rough *E. coli* with the wide variations observed in individual response and to elicit better immunity it would be more appropriate to immunise chickens with two doses of a suitable preparation of pure LPS extracted from these rough mutant bacteria.

complete core structure. The LPS expressed on Gram-negative bacteria outer membrane consists of a mixture of rough complete-core LPS without O-polysaccharide chains (Ra chemotype) and smooth LPS with complete core structure and O-polysaccharide chains (Bennett-Guerrero et al., 2000; Johns et al., 1977). Therefore, the Ra chemotype is considered as a better vaccine candidate than the chemotypes with incomplete core structures as the complete core specific antibody binding sites are surface exposed in the intact cell.

The spontaneous rise of anti-LPS core antibody titres with age is understandable as it is possible to translocate certain amount of LPS through gut wall to the circulatory system (Allan et al., 1995). The anti-LPS core antibodies presented in chicks of four days old may have transmitted to them through yolk. Wide variations of anti-LPS core antibody titres observed in chicks probably may be due to variations in antibody titres of hens or due to differences in individual immune systems. In this experiment only a single dose of heat killed bacterin was given at day 15 of age. It would have been more effective if two doses of bacterin were given at least at two weeks interval. Further, the heat killed bacterin may contain many other factors which influence the development of anti LPS core antibodies. Heat killed bacterin used in this experiment is the simplest method of immunising with LPS. However, it would be more appropriate to immunise chickens with purified LPS to induce better immunity. Also it is important to carry out a challenge study in order to determine the protective role of anti LPS core antibodies in chickens. Early studies carried out in other host species demonstrated that the immunisation with rough mutant bacteria can protect the hosts from lethal challenge of heterologous species (McCabe, 1973). Several groups reported that immunization with rough mutant bacteria; J5 mutant of *E. coli* and Re mutant of *Salmonella minnesota* generate antisera which had protective effects against Gram-negative infections. Importantly, the protection was elicited against homologous strains as well as heterologous strains (Johns et al., 1977) There is no doubt about the protective role of anti-LPS core antibodies in chickens as well. However, to limit

CONCLUSION

A mixture of rough *E. coli* representing different LPS core types can be used to increase LPS core specific antibody titres in chickens.

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