بیش
Protective Immune Responses Induced in Chickens by Outer Membrane Proteins Extracted from Different Strains of *Escherichia coli*

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ABSTRACT

Different strains of *Escherichia coli* (*E. coli*) from human, chickens, and the common strain between human and chickens were isolated and typed with monospecific antibody. The *E. coli* strains from each group of human, chicken and common between human and chicken were selected. The polypeptide patterns of selected strains were analyzed and compared with each other by Sodium dodecyle sulphate polyacrylamide gel electrophoresis (SDS - PAGE). The SDS - polyacrylamide gel electrophoresis patterns between the strains were very similar, although the densities of some of the peptide bands were different, and some were missing in some strains. A similar comparison was made on extracted outer membrane proteins (OMP) using Triton X-100 and sodium dodecyle sulphate on the above-mentioned strains. The polypeptide patterns between the strains 078 (chicken strain), 06 (human strain) and O2 (the common strain between human and chicken) were also very similar and two major bands with molecular weights of 44 KD and 25 KD were very distinctive, and seen between all the strains.

The passive haemoagglutination tests (PHA) in chickens injected OMP from the common strain (*O*₂), showed increased level of antibody after the second injection which remained constant after repeated injections.

In the challenge study, with LD100 from homologous strain of *O*₂, a significant protection was observed in the groups injected the OMP (extracted from *O*₂ strain), compared with controls injected saline.

The results of this study indicate that purified *E. coli* outer membrane protein, can induce a significant protection immunity against colibacillus diseases in chickens, and probably in immunocompromised hosts with repeated urinary infections.

**Keywords:** Active Immunity, Bacterial Outer Membrane Proteins, *Escherichia coli*, Vaccines

INTRODUCTION

*E. coli* is the predominant nonpathogenic facultative flora of the human intestine. Some *E. coli* strains, however, have developed the ability to cause disease of the gastrointestinal, urinary, or central nervous system in even the most robust human hosts. *E. coli* have also been recognized as a major cause of morbidity and mortality, in chickens and turkeys. Infection may result in respiratory disease form air sac infection, followed by a more general infection of the internal organs, often leading to death. Through
Immune Responses to *E. coli* Protein Antigens

this way *E. coli* is responsible for considerable economic losses in the poultry industry.1-5

The outer membrane of *E. coli* contains 60% of the envelope protein. Using polyacrylamide gel electrophoresis, six protein bands were found in the outer membrane fraction, of which one band (mol. wt 44000) accounted for 70% of the total outer membrane protein. This protein was referred to as the major outer membrane protein.6,7 The outer membrane protein is a continuous structure on the surface of gram-negative bacteria and, in bacterial pathogens, has particular significance as a potential target for protective immunity. Outer membrane vaccines have been used with considerable success to induce protection against a number of organisms.8,9 The aim of this study was to determine the protective immunity of purified *E. coli* outer membrane proteins against colibacillus infections in chickens. The research was carried out as a joint project between Tehran Medical Sciences University and Razi Institute.

MATERIALS AND METHODS

**Bacterial Strains**

A total of 10 strains of *E. coli* (5 from human urine and 5 from different organs of chicken), were isolated and identified by biochemical tests according to standard methods.

The biochemical tests were performed on isolated colonies from pure culture for complete identification. *E. coli* ATCC 8739 was used as a positive control strain, the media and reagents were prepared or sterilized according to the manufacturer's instructions. The serotypes of the strains were identified by monospecific antibody. Four serotypes of isolated *E. coli*, 018, 02 (human strains), 078 (chicken strain), and 02 (common between human and chicken) were chosen for the purpose of this study. Strains were lyophilized and were kept in Razi Institute. The chickens used in this study were broiler chickens, which are reared for meat rather than eggs.

**Preparation of OMP by SDS Method**

The OMP was prepared with a few modifications as described previously.10 The bacteria were grown in tripticase soy agar (BBL) overnight, washed in 10 mM Hepes buffer and finally 2g w/w of bacterial suspension was transferred into sonicating tube. The cells were sonicated for 10 min at 4°C. The suspension was centrifuged at 10000g for 30 min at 4°C and the supernatant was collected and centrifuged again for 1h. The clear pellet was suspended in 4ml of 10 mM Hepes buffer containing 2% Triton X-100. 2ml of 2% SDS was added and left at 56°C for 15 min, and the suspension was centrifuged in the same manner for 1h. The pellet (mixture of protein complex and peptidoglycan) was suspended in 1 ml of distilled water and dialyzed against 2ml of 5M NaCl containing 2% SDS and left overnight at 37°C. The dialysate was centrifuged at 5000 g at 4°C for 10min; the supernatant was dialyzed three times against distilled water for 48h at room temperature, which separated SDS and NaCl from protein complex.

**Preparation of OMP by Sarciosyl Method**

4 gr w/w of bacteria was suspended in phosphate buffer saline (PBS) equivalent to 2 x 10^10 CFU/ml and OMP was prepared according to the method described previously.10

**Protein Estimation**

Protein concentrations of various preparations were estimated in a manner similar to that described previously11 with bovine serum albumin as the standard and folinicoalteus phenol reagent.

**Electrophoresis**

Sodiumdodecyle sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method described previously.12, 13 To assay the purity and determine the molecular weight of protein components high and low molecular weight markers were used.

**Determination of *E. coli* LD100 for Chickens**

For the determination of LD100 of *E. coli*, the standard method of up and down was used as described previously.14 Twenty-seven chickens were set in three groups of nine and injected with 0.5 ml suspension of *E. coli* (contained between 10^7 to 10^9 *E. coli* cells). The injected chickens were observed for 3 days.

**Passive Haemagglutination (PHA) Assay**

The PHA was performed by utilizing the method described.15

**Chicken Immunization and Challenge Protocols**
To assess the immunogenicity of OMP, two groups each comprising of 15 chickens four weeks old were set.

Group 1, was injected twice with an aliquot of 0.5 ml of OMP in nine days interval.

Group 2, as a control received saline only.

Some of the chickens injected with OMP were bled after 11 and 16 days and both groups were challenged after 18 days.

RESULTS

The list of *E. coli* strains isolated from human and chickens is shown in table 1.

**Protein Estimation by Lowery Method**

The protein estimation of OMP prepared by SDS was 7 mg/ml, OMP prepared by sarcosine method using lysozyme was 4 mg/ml, and without it was 0.6 mg/ml. The results of the comparison between polypeptide patterns of 4 isolates of human, chickens and the common strains (078, 02, 06 and 018) are shown in figure 1. As shown in figure 1, six polypeptides with molecular weights of 14, 25, 44, 15, 58 and 68 KD were extracted. Most of the bands were common between all the strains, although the densities of some of the bands were different.

**Table 1. The list of *E. coli* strains isolated from human and chickens.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli (1)</td>
<td>O 18</td>
<td>Human</td>
</tr>
<tr>
<td>E. coli (2)</td>
<td>O 6</td>
<td>Human</td>
</tr>
<tr>
<td>E. coli (3)</td>
<td>O 78</td>
<td>Chicken</td>
</tr>
<tr>
<td>E. coli (4)</td>
<td>O 2</td>
<td>Common between human and chicken</td>
</tr>
</tbody>
</table>

*O18, O6: human strains
O78: chicken strain
O2: common between human and chicken

**Figure 1. Comparison between polypeptide patterns of different strains of *E. coli*.(O18, O6, O2, O78)**
The results of the OMP extraction of strains O6, O2 and O78 are shown in figure 2. As it is shown in figure 2, 6 peptide bands between 14 to 68 KD were detected between all the OMP, although 25 and 44 KD bands showed a higher density than the rest.

The PHA test in chickens injected with OMP extracted by SDS showed agglutination in 1/2, 1/4, titer after 11 days, and 1/2, 1/4, 1/8 after 16 days of first injection. The titer remained steady afterwards.

**Immunization and Challenge of Chickens**

The result of immunization and challenge of chickens are shown in table 2.

As shown in table 2, chickens injected OMP extracted by SDS showed 3 dead out of 15 after challenge with a lethal dose, while non-vaccinated chickens showed twelve dead out of 15 when they were challenged with LD100 homologous strain of O2. The lethal dose in injected chicken with *E. coli* O2 was $1 \times 10^8$ bacterial cells.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>No. of Chickens</th>
<th>No. of Injection</th>
<th>Dose of Injection (µl)</th>
<th>Days of Intervals</th>
<th>Amount of Challenge (ml)</th>
<th>Challenge Dose CFU/ml</th>
<th>No. of Dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMP SDS</td>
<td>15</td>
<td>2</td>
<td>50</td>
<td>9</td>
<td>0.2</td>
<td>$1 \times 10^4$</td>
<td>3/15</td>
</tr>
<tr>
<td>Control (A)</td>
<td>15</td>
<td>2</td>
<td>50</td>
<td>9</td>
<td>0.2</td>
<td>$1 \times 10^4$</td>
<td>12/15</td>
</tr>
</tbody>
</table>

**Figure 2. Comparison between OMPs extracted from different strains of *E. coli.* (O6, O78, O2)**

O6: human strain
O78: chicken strain
O2: common between human and chicken
DISCUSSION

Many scientists have used several methods for extraction of OMP. In this study, we compared two methods for OMP extraction and found that on using SDS and Triton X-100, seven clear polypeptide bands were observed using SDS-PAGE.

In this study, we prepared OMP from three strains isolated from human, chickens and a common strain between human and chicken. The polypeptide bands with 44 KD is known to be the major OMP band accounting for 70% of the total OMP. We also observed a band with 25 KD, which is also shown by others as a band with molecular weights between 29 KD to 33 KD. An interesting thing is the presence of 3 bands with molecular weights of 50 KD, 58 KD and 65 KD, which have not been observed by others.

The comparison between OMP extracted from all isolates of E. coli 06, 02 and 078, showed that these proteins were very similar with the presence of two major bands of 44 KD and 25 KD in all extracts. The protein estimation of OMP prepared by SDS and Triton X-100 showed a higher amount than that of sarcosyl method.

In passive haemagglutination (PHA) assay we observed an increased level of antibody after the second injection of OMP in chickens which remained constant after further injections.

In our protective study chickens immunized with OMP and challenged with LD 100 from homologous strain of O2, showed a very good level of protection with 3 dead out of 15, while the saline injected controls, showed 12 dead out of 15. Various strains of E. coli inactivated either by heat, formaline, or attenuation have been used for immunization of chickens to prevent colibacillus infections, but they have not been able to protect chickens during the outbreaks of infection. This may be due to the nature of this organism, being as part of normal flora or as a secondary cause of infections, as well as an increased level of antibiotic resistance. However due to repeated outbreaks of this organism in the field, there is a need for an effective vaccine. The results of this study have direct implication in considering further development of OMP as a vaccine option to protect chickens against E. coli infections.

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