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Characterization of the *Salmonella typhi* Outer Membrane Protein C

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Salmonella enterica serovar typhi, a Gram-negative food-borne pathogen, causes typhoid fever in humans. OmpC is an outer membrane porin of *S. typhi* expressed throughout the infection period. OmpC is potentially an attractive antigen for multivalent vaccines and diagnostic kit designs. In this study we combined *in silico*, *in vitro* and *in vivo* approaches to analyze various aspects of OmpC's antigenic properties. The conserved region, in addition to secondary and tertiary structures, and linear B cell epitopes, were predicted. A number of results obtained from *in silico* analyses were validated by experimental studies. *OmpC* was amplified, cloned and then expressed, with the recombinant protein then being purified. BALB/c mice were immunized by purified denatured OmpC. The titer of antibody was raised. Results of challenges with the pathogen revealed that the immunity is non-protective. Most of the theoretical and experimental results were in consensus. Introduced linear B cell epitopes can be employed for the design of diagnostic kits based on antigen-antibody interactions.

Keywords: Typhoid, OmpC, *Salmonella typhi*; *in silico*, vaccine, diagnosis

Introduction

Salmonella enterica serovar typhi is a Gram-negative food-borne pathogen belonging to the family of Enterobacteriaceae. It is the causative agent of a severe systemic disease in humans, namely typhoid fever [4, 23]. Typhoid fever remains a major public health problem in developing countries [10]. A diagnosis of typhoid fever is carried out by haemoculture or by the detection of antibodies in the patient's serum [6, 11, 23]. Isolation and biochemical characterization are lengthy processes; hence detection through *S. typhi* antibodies produced in the patient's serum is quicker and more reliable. Several reports, employing a variety of methods, have appeared concerning the detection of these antibodies [6, 11]. However, studies of the antibody response to *S. typhi* during typhoid infection in humans have frequently been restricted to the detection of antibodies induced by a small number of bacterial antigens. Little information exists about the microbial factors determining pathogenicity and those eliciting a protective immune response in humans with typhoid infections [3, 9]. Porins are outer membrane proteins of Gram-negative bacteria, including *S. typhi*, which act through the diffusion of small solute molecules through their channels [2, 13, 21]. They can also be phage receptors and B-cell mitogens [12]. In bacterial membranes, they are stable at high temperatures, and have a high resistance to denaturing and proteolytic reagents such as SDS and urea. These properties are essential for the survival of Gram-negative bacteria in the harsh environments of the intestinal tract [2, 18]. *Salmonella* infections result in the stimulation of both humoral and cell-mediated immunity in animal models [1]. OmpC, with a molecular weight of 39 kDa (357 amino acids), is an outer membrane porin in *S. typhi*, and many other Gram-

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negative bacteria, which induces immune system responses in mice and humans [19, 23]. Since OmpC is a surface antigen with exposed epitopes, it can potentially be an antigen of interest for use in diagnostic kits and multivalent vaccine designs. Furthermore, it is expressed throughout the infection period. In the present study, the specificity, antigenicity and immunogenicity of the porin protein OmpC was experimentally evaluated.

Materials and Methods

Bacterial Strains and Patient Sera

The bacterial strain used in this study was *S. typhi* PTCC 1609. *Escherichia coli BL 21* cells were used for the expression of the recombinant protein, OmpC. Other bacteria used were; *S. enteric serovar typhi, S. enteric serovar paratyphi A, S. enteric serovar paratyphi B, S. enteric serovar paratyphi C, S. enteric serovar tyhimurium, S. enteric serovar enteraiteis, shigella flexneri, E. coli O157, Acinetobacter baumannii, Pseudomonas aeruginosa, Staphylococcus aureus* (control)*, Klebsiella pneumoniae, Citrobacter koseri* and *Vibrio cholerae.*

Sera from individuals with acute typhoid fever, exhibiting positive Widal test results, were referred to the Razi laboratory in Andimeshk (Iran) to act as our test samples. Sera from 10 normal healthy individuals served as the control. BALB/c mice, 4-6 weeks old (16-22 g), were procured from the Razi Institute, Tehran, Iran.

Cloning, expression, and purification of OmpC

The amplification of the *OmpC* gene was carried out using the following primers:

Forward primer: 5'-GCAGCGAATCATATGGAAATTT-ATAATAAAGAC-3'

Reverse primer: 5'-AACATCTTTGGATCCTTAGAAC-TGGTAAAC-3'

*Nde*I and *Bam*HI sites were introduced through primers at the 5' and 3' ends of the amplicon, respectively. After extraction *S. typhi* DNA, PCR reactions for OmpC containing 1 μl DNA (50 ng/ml), 250 μM each dNTP, 2 μl of each primer (20 pM), 2 μl Mgso4 (50 mM), and 0.5 μl of pfu DNA polymerase in a final volume of 25 μl, were prepared. A thermal cycler (Techne Gradient, Staffordshire, UK) was then employed with 30 PCR-cycles of initial denaturation at 95°C for 5 min, 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C, followed by an additional 20 min at 72°C. The PCR products were then purified with PCR preparation kits, digested with NdeI and BamHI, and cloned into pET28a(+) vector (Novagen, Darmstadt, Germany). This was then digested with the same endonucleases and ligated in the pET28a(+) vector. The new construct was named pET28a-OmpC. The ligated product was transformed into chemically competent *E. coli* BL21 cells. The recombinant clones were selected on LB plates containing 50 μg/ml kanamycin. The pET28a-OmpC was purified using a plasmid extraction kit, and was then sequenced to verify the cloned insert's integrity. The insert was confirmed in a right orientation by restriction digestion with Pst1 endonuclease. The expression vector had a six-histidine tag at the protein C-terminus allowing for detection of the recombinant protein. The selected transformants were inoculated into 200 ml of LB medium at an optical density of 0.6 at 600 nm. The culture was induced with 1 mM isopropyl β-D-thiogalactoside (IPTG) and kept overnight at 37°C for expression. The cells were then harvested by centrifugation at 14000 × g for 10 min at 4°C. The cell pellets were resuspended in lysis buffer (100 mM NaH_2PO_4, 10 mM Tris.Cl, 8 M urea), followed by the addition of lysozyme at a 1 mg/ml concentration, and was sonicated 5 times for 1 min at 1 minute intervals. The protein was purified from the supernatant by affinity chromatography using Ni^{2+}-NTA agarose (Qiagen, Hilden, Germany). The supernatant was separated from the cellular debris by centrifugation at 12,000 rpm for 30 min at 4°C. The protein was then eluted from the Ni-NTA matrix by the use of an elution buffer (100 mM NaH_2PO_4, 10 mM Tris, pH 4.5). The fractions were individually collected and analyzed by 12% SDS-PAGE. Densely purified protein was estimated by the Bradford method [2]. Western Blot with an Anti-His-Tag antibody was carried out in order to assess the specificity of the recombinant protein (OmpC). Protein samples were electrophoresed on a 12% SDS-PAGE gel and were electroblotted onto a nitro cellulose membrane at a constant current of 300 mA at 48°C for 1.5 h. The membrane was blocked with 5% skim milk in PBS–Tween (0.05%) with gentle shaking for 1 h at room temperature. The membrane was then washed 3 times with PBS-T (PBS + 0.05% Tween-20, pH 7.4) and incubated with a mouse anti-His-tag monoclonal antibody as the primary antibody at a dilution of 1:1,000 in blocking solution (1% BSA) for 1 h at 37°C. After washings with PBS-T, the membranes were incubated with rabbit anti-mouse IgG horseradish peroxidase (HRP) conjugated as the secondary

antibody at a dilution of 1:5,000 for 1 h at 37°C. The (HRP)-coupled anti-polyhistidine antibodies could fuse to the his-tag in the recombinant protein's C-terminal and could react with TMB (3,3', 5,5''-tetramethylbenzidine) substrate.

OmpC seroreactivity

OmpC was coated (0.1 μg/well) on the microtiter plates. Sera samples from typhoid patients were tested at 1:100 through to 1:2,800 dilutions.

Immunization of mice with three different routes of inoculation and determination of anti-OmpC antibody titers

Male inbred BALB/c mice, of 3-4 weeks of age (15-20 g), procured from the Razi Institute (Tehran, Iran) were kept under standard conditions. They were divided into 4 groups (5 mice per group), with one further group serving as a negative control. The 20 mice were immunized on days 0, 15 and 30 via intraperitoneal, subcutaneous and interamuscular routes with the OmpC dissolved in complete Freund's adjuvant for the first dose. Incomplete Freund's adjuvant was used in the subsequent doses. The negative control was injected with 100 μl of PBS. Quantitation of antibodies was performed by ELISA [7]. All of the experiments with the animals were carried out in accordance with standard practice and the legal requirements of Iran. In order to comparatively analyze anti-OmpC antibody titers, the sera from BALB/c immunized mice were assayed by ELISA. 5 μg of recombinant OmpC protein per well was used to coat 96-well microtiter plates, followed by incubation at 4°C overnight. Serial dilutions of sera ranging from 1:400 to 1:51,200 were added to the wells. The wells were blocked with 5% skimmed milk in PBS–Tween (0.05%) with gentle shaking for 1 h at 37°C. They were then washed three times with PBS-T. 100 μl of mouse IgG conjugated with horseradish peroxidase (HRP) diluted to 1:3,000 was added to each well. The immune reaction was detected by adding 100 μl TMB (3,3', 5,5''-tetramethylbenzidine) as a substrate for 15 min. The reaction was stopped by adding 100 μl H_2SO_4 (2 M).

Determination of Bacterial lethal dose (LD_{50}) and animal challenge with bacteria

S. typhi suspensions at 3×10^5 to 3×10^{10} CFU/ml were administered intraperitoneally to five groups of BALB/c mice. Mortality in the mice was recorded for three consecutive post-challenge days. LD_{50} was defined as the volume (CFU/ml) of bacterial load that brought about death in half of the population size. The immunized and control groups were challenged intraperitoneally [14, 15].

Antibody titer raised against OmpC in mice infected with various enterobacteriaceae and immunoreactivity similarities in enterobacteriaceae

In a blast search, *OmpC* was found in *E. coli O157*, *Shigella flexneri*, *S. paratyphi A, S. paratyphi B, S. paratyphi C, S. enteraitedis, S. typhimurium, S. typhi*, *Acinetobacter baumannii,* and *Pseudomonas aeruginosa*. Mice groups were inoculated with $<LD_{50}$ of the above bacteria. Sera collected after a period of one week were assayed by ELISA against OmpC.

10^7 CFU/ml suspensions from gram negative bacteria were coated individually in 96-well microtiter plates followed by overnight incubation at 4°C. The wells were blocked with 5% skimmed milk in PBS–T, with gentle shaking for 1 h at 37°C. Serial serum dilutions from 1:100 to 1:12,800 of mice immunized with OmpC were added to the wells. The plates were then washed three times with PBS-T. 100 μl of peroxidase conjugated anti-mouse IgG secondary antibody diluted to 1:3,000 was added to each well. The immunoreactions were detected by adding 100 μl of TMB as a substrate for 15 min. The reactions were stopped by adding 100 μl H_2SO_4 (2 M). The optical density at 492 nm was read on an ELISA plate reader.

Results

Cloning, expression, and purification of OmpC

The DNA fragment of 1080 bp encoding OmpC was amplified successfully (Fig. 1) and the PCR product was ligated into pET28a(+) by incorporating restriction sites for *NdeI* and *BamHI* in the forward and reverse primers respectively.

Over expression of the recombinant protein by pET28a vector led to the formation of inclusion bodies. The fractions individually collected and analyzed by SDS-PAGE revealed a single band of approximately 39 kDa (Fig. 2). The minor increase in molecular weight was due to the 12 histidine tags in the C and N terminals. Western blotting with anti-His-tag antibody detected a 39 kDa protein in the induced bacterial lysates representing the recombinant OmpC (Fig. 3).

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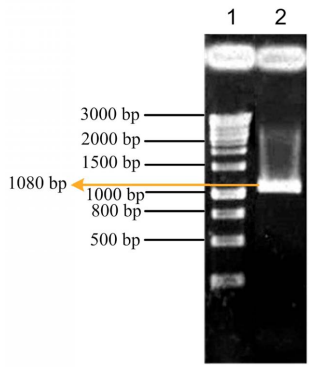

Fig. 1. PCR products (OmpC:1080 bp).

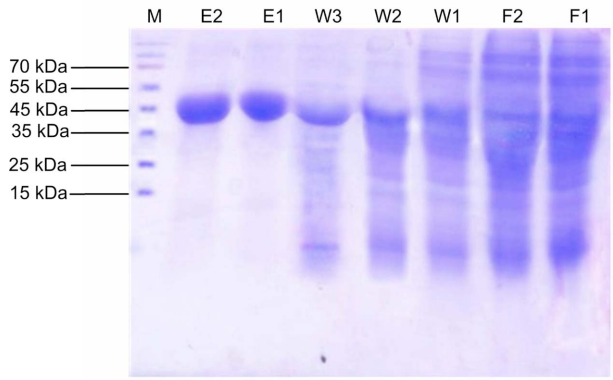

Fig. 2. Expression and purification of OmpC protein.
SDS-PAGE analyses of periplasmic extract showing a single band of OmpC. Lane 1, Molecular marker; Lanes F1, F2, first unbound protein flow; Lanes W1-W3, column washed with buffer; Lanes E1, E2, column washed with elution buffer.

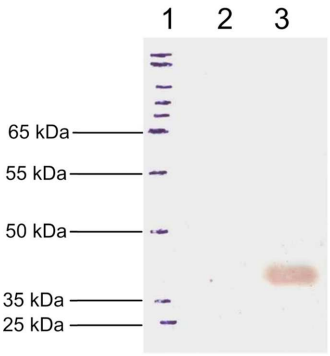

Fig. 3. Western blot analyses of recombinant OmpC with the anti-His-tag monoclonal antibody.
Lane 1, protein molecular mass markers (kDa); Lane 2, uninduced *E. coli* lysate; Lane 3, *E. coli* lysate induced with IPTG.

Seroreactivity with typhoid patients' sera

The reactivity of the recombinant OmpC assayed in the 10 typhoid patients, and in normal healthy sera by indirect ELISA, revealed a significant difference ($p < 0.01$) between the OD values (Fig. 4).

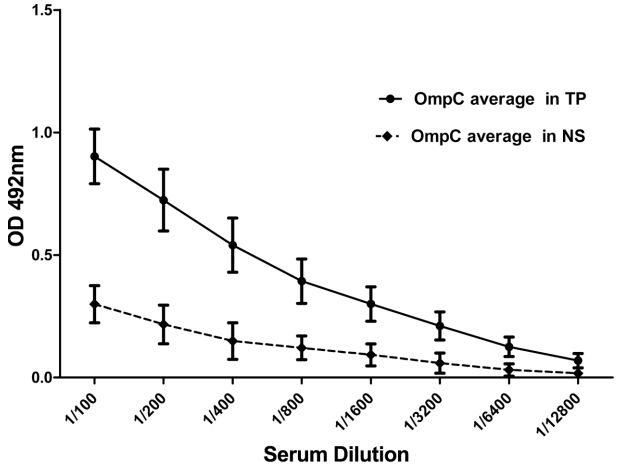

Fig. 4. Determination of IgG against *S. typhi* OmpC in normal (NS) and typhoid infected human (TP) patients.

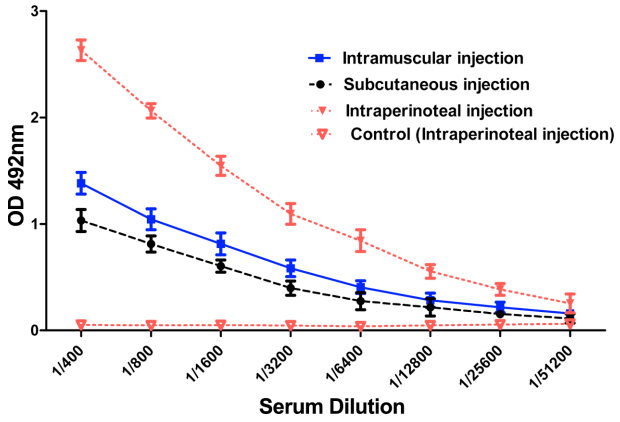

Fig. 5. Comparison of antibody titers raised by subcutaneous, intraperitoneal and interamuscular routes of immunization.

Immunization of mice with three different routes of inoculation and OmpC-specific IgG

Fig. 5 is a comparison of the titration process of anti_ OmpC IgG in the control, and the test mice groups by three routes; subcutaneous, intraperitoneal and interamuscular. The results for the control groups of each immunization route are shown as a single graph due to the similarity of the results. The antibody titer seen through the intraperitoneal route of immunization was higher than the other two routes.

Sera were collected from mice before immunization (day 0) and 7 days after the last dose. The IgG titer measured by ELISA was compared with the control group injected with PBS. Sera collected after immunization showed significantly (p<0.001) higher IgG titer than the control (Fig. 6).

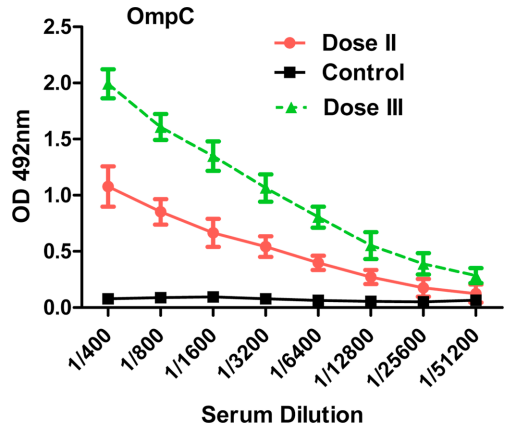

Fig. 6. Mean antibody titers at increasing times following immunization.

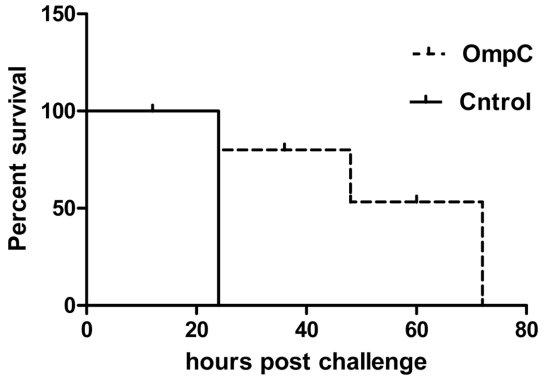

Fig. 7. Percent survivals of the immunized mice challenged with *S. typhi*.

Determination of LD_{50} and challenge studies in mice

LD_{50} was determined as 3×10^7 CFU/ml/Mouse at intraperitoneal injection and mortality in the mice was recorded for two consecutive post-challenge days. Percent survival for immunized mice in this challenge is summarized in Fig. 7. Despite the induction of a strong immune response, OmpC conferred no significant protection against *S. typhi* in BALB/c mice.

Immunoreaction of gram negative bacteria with mice sera immunized with OmpC and IgG titer in mice inoculated with some enterobacteriaceae

A comparative graph (Fig. 8) illustrates antibody titer raised against OmpC in mice infected by various *Enterobacteriaceae*. The immunoreactivity of sera from immunized mice with various bacteria was observed in the order of; *K. pneumoniae* > *S. flexneri* > *E. coliO157* > *C. freundii* > *P. aeruginosa* > *A. baumannii* > *V. cholerae* (Fig. 8A). The immunoreactivity of sera from immunized mice with various *Salmonella* strains was observed in the order of; *S. typhi* > *S. tyhimurium* > *S. paratyphi A* > *S. enteraiteis* > *S. paratyphi B* > *S. paractyphi C* (Fig. 8B). Sera from mice inoculated with *Enterobacteriaceae* reacted with sera from immunized mice in the following manner; *S. typhi* > *E. coli* > *S. flexneri* > *S. tyhimurium* > *S. paratyphi A* > *S. enteraiteis* > *S. paractyphi B* > *S. paratyphi C* > *P. aeruginosa* > *A. baumannii* > *S. aureus* (control) (Fig. 9).

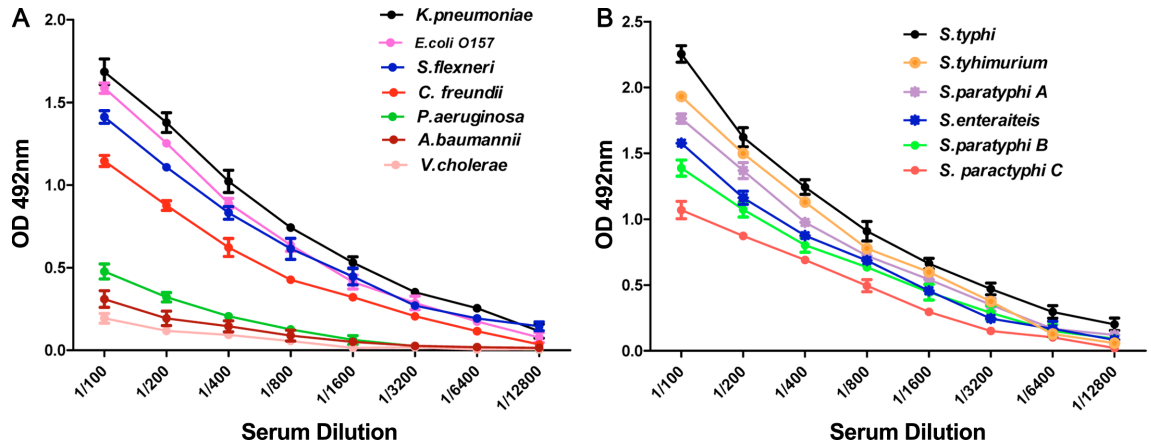

Fig. 8. Immunoreaction of gram negative bacteria with mice sera immunized with OmpC.

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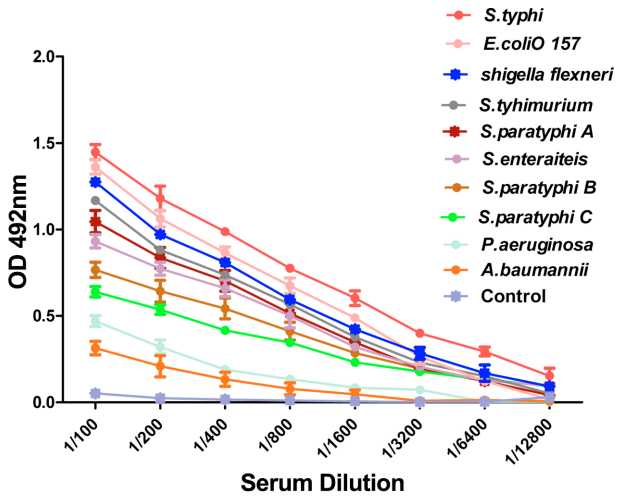

Fig. 9. Antibody titer raised against OmpC in mice infected by various Enterobacteriaceae.

Discussion

The disadvantages of traditional vaccines available against typhoid fever make the development of new vaccines which do not possess these hindrances an imperative [4]. Several researchers have suggested that OMPs are suitable candidates for both vaccines and diagnostic purposes [2, 11, 23]. OmpC is an immunodominant antigen of *S. typhi* [23], which exists in many Gram-negative bacteria. Linear B cell epitopes are appropriate for the detection of pathogens [16, 20, 22]. It has been observed that various strains could have different sequences of a single protein [5, 17, 22]. These differences may be the basis for their use in diagnostic kits.

In this study a sequence was cloned and expressed in *E. coli BL21* for further immunological analyses. Western blotting confirmed the expression of OmpC. The purification was performed by use of the denaturing method mentioned above. This suggests that epitopes introduced to antibodies, and host immune systems, are linear in probability.

Human typhoid patients' sera were analyzed employing the denatured OmpC protein coated on microtiter plates. The coated antigen was then detected by patient serum antibodies (Fig. 4). These results revealed that the antibodies raised against natural OmpC on the bacterial surface are able to identify Linear B cell epitopes of denatured OmpC. The immunization of mice with denatured OmpC was conducted to analyze antibody elicitation, antibody-antigen interactions, and to study the provocation of protective immune responses against the pathogen. In order to arrive at the best route for vaccinations, mice were immunized through three injection pathways. Intraperitoneal injections were found to be the best route (Fig. 5). This result is in agreement with research reports on other antigens [8]. Raised antibodies were validated by ELISA through OmpC coated on microtiter plates. ELISA results were correlated to epitope cross-reactivity analyses of the OmpC sequences (Figs. 8A & 8B). Sequences with more cross-reactivity to *S. enterica ser typhi* OmpC had a higher optical density in ELISA. This implies that differences in the linear epitopes of OmpC sequences are responsible for differences in optical densities. Results from bacterial inoculation of the experimental animals revealed that the antibodies produced thereby could react with the denatured recombinant OmpC protein (Fig. 9). Based on *in vitro* and *in vivo* analyses, exposed linear epitopes are suitable from a cross-reactivity point of view. These features are attractive for Enterobacteriacae detection purposes. The results are also applicable for diagnostic purposes in order to detect some pathogenic Eenterobacteriacae in a single step test. Interestingly, linear epitopes, and their corresponding antibodies, which are different in various bacteria, can be employed for the precise detection of *S. typhi*. Antibodies raised in infected mice with live bacteria, could detect denatured OmpC (Fig. 9). This phenomenon indicates that OmpC was exposed to the hosts' immune systems. Since OmpC is introduced to host immune systems as a natural form of the antigen, detection of the denatured protein by raised antibodies confirms the involvement of linear epitopes in this detection. We hypothesize that linear epitopes laid in the exposed loops can be attractive for our purposes. Conserved epitopes could have cross reactivity with other Enterobacteriacae pathogens. Variable epitopes are responsible for different antibodies triggered against OmpC. The variable epitopes can be exploited for the precise detection of corresponding pathogens.

Challenge results showed that despite the elicitation of high titer of antibodies against the antigen, no protection was developed against the pathogen. In contrast with other research suggesting OmpC is an immunodominant antigen that can be used as a vaccine candidate [1, 2, 23], we have demonstrated that Linear B cell epitopes are not protective. If this antigen is protective, conformational epitopes of the antigen are responsible this protection. Nowadays, PCR

and antigen-antibody based methods are used for the detection of pathogens [6]. The results obtained from this study are promising for the design of new antigen-antibody diagnostic kits to detect Enterobacteria.

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