In Vitro study on comparative for chicken egg antibody and ducks egg antibody production against Helicobacter pylori

Venkitasamy Kalaigandhi*, Ponnusamykonar Poovendran, Eliyaperumal Poongunran

Department of Microbiology, Dr. G. R. Damodaran College of Science, Coimbatore – 641 014, Tamil Nadu, India

ABSTRACT

Helicobacter pylori are the most common cause of gastritis and gastric ulcers and play a pivotal role in the development of gastric carcinomas. Antibodies presently available for research, diagnostic and therapies are mostly mammalian monoclonal or polyclonal antibodies. The present study evaluated the potential use of immunoglobulin prepared from the chicken egg yolk and ducks egg yolk of immunized with the treatment of H. pylori infections. The standard strains of Helicobacter pylori (ATCC 43504) and Helicobacter pylori (MTCC 41653) were obtained from Institute of Microbiology Technology, Chandigarh. Immunization of chicken and duck production of large quantity on antibody with Helicobacter pylori infection. These polyclonal and monospecific IgY antibodies are of higher-titer method such as Enzyme linked immunosorbent assay (ELISA), Dot-blot and finally, urease neutralizing ability of the antibodies was evaluated in presence of the purified IgY. To our knowledge this is the first report describing generation of comparison on chicken and duck antibody IgY directed against antigens of gastric ulcer H. pylori by DNA-based immunization.

Key words: Helicobacter pylori. Chicken and Duck antibody IgY. Passive immunotherapy.

INTRODUCTION

Helicobacter pylori is a Gram-negative, spiral, microaerophilic bacterium that infects the stomach of more than 50% of the human population worldwide. It is associated with gastritis, peptic ulcer and gastric cancer and it has been classified as a category 1 carcinogen by WHO [1, 2]. Several existing detection methods such as Western blots or ELISA are useful to identify antibodies against H. pylori in blood, saliva or urine of the infected individuals. Other tests consist of the revealing of the urease activity (urea breath test) or detection of H. pylori antigens in biopsies or stools. Current therapies are not effective in 100% and fail due to antibiotic resistance. No effective vaccine against H. pylori exists, although several vaccine candidates
including an oral whole-cell vaccine (Helivax, Antex Biologicals) and an intramuscular trivalent acellular vaccine (Chiron Vaccines) have been tested in clinical trials (for a recent review: Ruggiero et al., [1]). Antibodies directed against specific *H. pylori* targets could be used either for diagnosis or as therapeutic agents.

Only few recent reports exist about generation of antibodies against *H. pylori* antigens. These reports include monoclonal antibodies isolated from an immune phage display library constructed from peripheral blood lymphocytes of infected patients [3], bovine colostral antibodies isolated from colostrums of cows immunized with killed *H. pylori* bacteria [4, 5] and chicken yolk-derived antibodies obtained, as in the previous case, against the whole-cell lysate of *H. pylori* [6-8].

Antibodies available for research, diagnostic and therapies are mostly monoclonal or polyclonal antibodies. Traditionally, bigger animals such as horses, sheep, pigs, rabbits and guinea pigs were used for the production of polyclonal antibodies, while mice and rats were used for the production of monoclonal antibodies. Nowadays, most frequently chosen mammals for polyclonal and monoclonal antibody production are rabbits and mice respectively. Both technologies have their advantages but also disadvantages. Major problem of monoclonal antibody production is that some antigens are weakly or not at all immunogenic for mice. In polyclonal antibody production purification of antibodies from mammalian blood has been found to be low yielding and laborious in many cases. Both technologies also involve some steps each of which causes distress to the animals involved: i) the immunization itself, ii) collecting of blood samples and iii) bleeding, which are a prerequisite for antibody preparation [9]. During the past 20 years, the use of chickens instead of mammals for antibody production has increased. A major advantage of using birds is that the antibodies can be harvested from the egg yolk instead of serum, thus making blood sampling obsolete. In addition, the antibody productivity of an egg-laying hen is much greater than that of a similar sized mammal [10].

Purification of immunoglobulin from mammalian blood is time-consuming and expensive. Today, chicken are recognized as a convenient and inexpensive source of antibodies. It has been reported that the amount of immunoglobulin that can be yielded from one egg of an immunized hen is as much as that can be obtained from 300 ml of rabbit blood. Chicken egg yolk antibodies (IgY) have been applied successfully for scientific, diagnostic, prophylactic and therapeutic purposes. Because of the phylogenetic distance between birds and mammals, mammalian proteins are often more immunogenic in birds than in other mammals and antibody synthesis readily stimulated in hens [11]. However, use of chickens for the purpose offers two advantages. The use of chicken egg and duck egg for the antibody production, as opposed to mammals, represents both a refinement and a reduction in animal use. It is a refinement in that the second painful step, the collection of blood is replaced by antibody extraction from egg yolk. It enables the reduction in the number of animals used because chickens produce higher amounts of antibodies than laboratory animals. Larsson et al. [12] found that IgY is more highly concentrated in egg yolk than it is in serum. The chicken is an excellent producer of antibodies, but despite this, is still an underused resource. This may be due to lack of information concerning the different methods and applications where IgY is more advantageous compared to the traditional mammalian IgG antibodies. The aim of this work was to use the above approach for generation and preliminary characterization of polyclonal, monospecific antibodies against the gastric ulcer producing *Helicobacter pylori*. 

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MATERIALS AND METHODS

Bacterial strains and Culture media
The standard strains of *Helicobacter pylori* (ATCC 43504) and *Helicobacter pylori* (MTCC 41653) were obtained from Institute of Microbiology Technology, Chandigarh. Bacteria were cultured with Brucellar broth (5% FBS) and BAP (blood agar plate) at 37°C, 5% CO₂.

Experimental Animals
Five to Six months old, laying white leghorn chicken in good health, weighing approximately one kilogram were obtained from Venketeshwara Hatcheries Ltd., Namakkal Dist Tamil nadu similarly 6 to 7 months old, laying ducks were collected from Koopode panchayod, Palakkad Disct, Kerala.

Mass Culture of *H. pylori* and preparation of antigen
To prepare the *H. pylori* antigens, *H. pylori* ATCC 43504 and MTCC 41653 was cultured in a Jar fermentor (2 liter) by repeated batch culture using brucella broth containing 5% fetal bovine serum. The antigens for the immunization was the whole cell of *H. pylori* or outer membrane protein (OMP) prepared by the previous method [13]. Whole *H. pylori* antigens were prepared by 20 mM formalin fixation and *H. pylori* outer membrane protein (OMP) were prepared as follows. *H. pylori* cell mass were harvested from culture ture broth by centrifugation (6000 rpm, 20 min) and washed 3 times with 20 mM Tris-HCl buffer (pH 7.5). Resuspended *H.pylori* (in 20 mL, 20Mm Tris HCl buffer, Ph 7.5) were sonicated (30 sec, 4 times), treated with DNase (0.1 mg) and RNase (0.5 mg) at room temperature 30 min, cell debris were removed by centrifugation (12000 rpm,20 min) and supernatant was ultra centrifuged (100 000 g, 30 min). Precipitated total membranes were treated with 20 mL of 20 mM Tris-HCl buffer (pH 7.5) containing 2% sodium laurylsarcosine 30 min to remove membrane lipids and ultra centrifuged (100 000 g, 60 min).The precipitated OMP were resu spended in water and stored at -20°C.

Immunization to Chicken and Duck
The duck and chicken were immunized separately with whole cell antigens of *Helicobacter pylori*. Antigen prepared were diluted using sterile saline and adjusted to McFarland Barium sulphate standard tube number 1.The ‘insue’ suspension with adjusted opacity shows a final cell concentration of 3×10⁸ cell/ml. The 25 weeks old white leghorn and ducks was immunized with *Helicobacter pylori* antigen for development of duck and chicken egg yolk antibodies. Chicken were immunized with 0.5 ml of antigen, immunized intramuscularly at four different sites of breast muscles (two site per left and right) of chicken and ducks. One week after first injection chicken and duck received booster shots on every 7th day after first of same dose and route of administration. The bleeding was made frequently and chicken and duck anti *Helicobacter pylori* serum antibodies by slide agglutination. The quantitative estimation of antibodies was estimated by doing Widal test. The titre value of antibodies was found to be gradually increasing so that loose cottony woolly clumps were found till 1:1280 dilution showing elevated leaves of serum antibodies against *H. pylori* antigens.

Isolation and purification of IgY
The isolation of IgY was carried out as described by Akita and Nakai [14, 15], with some modifications. The egg yolk was first mixed with one of nine volumes of cold distilled water (acidified with 0.1N HCl to pH 5.0) and stored overnight at 4°C. The mixture was then centrifuged at 3125×g at 4°C for 40 min to obtain the water soluble fraction (WSF). The WSF was collected and filtered to remove solids. The resulting IgY-containing WSF was further
purified by ultra filtration using Amicon Ultra-15 filter (PL-100, Millipore), condensing the sample to 1/30 to 1/40 of its original volume. These WSF concentrates were then subjected to the urease neutralization test and chicken challenge experiments.

**ELISA (enzyme-linked immunosorbent assay)**
The antibody activity of anti-EV71 IgY was determined using the ELISA method described by Lee et al. [16], with some modifications. Briefly, microtiter plates were coated with 100µl of inactivated EV71 4643 antigen (10µg/well), while control wells were coated with rabbit anti-chicken IgY antibody (10µg/ml, Sigma C2288). The plate was then incubated overnight at 4°C. After washing with PBS–TWEEN 20 buffer, 2% BSA blocking was conducted overnight at 4°C. The wells were then washed with PBS–TWEEN 20 buffer. Next, 100-fold diluted WSF was added to the sample wells (100µl/well) for testing. WSF from the same chicken before immunization was used as a control. To generate the standard curve, wells were filled with 100µl serial-diluted pure chicken IgY at a concentration from 0.015µg/ml to 1µg/ml (Promega, G116A) and incubated at 4°C for overnight. After washing with PBS–TWEEN 20 buffer, 100 µl of alkaline phosphate-conjugated goat anti-chicken IgY (Promega, G115A) was added to the wells and incubated at 37°C for 2 h. After washing with PBS–TWEEN 20 buffer, 100µl of disodium p-nitrophenyl phosphate was added to each well as a substrate (Sigma, N9389) and allowed to react at 37°C for 10 min. The absorbance was then measured at 405nm using microplate reader (Multiskan MS: Thermo Labsystems). The resulting absorbance of standard curves provided a relative measurement of anti-EV71 IgY concentration. For the total IgY determination, each well on the micro titer plate was first coated with 100µl of rabbit anti-chicken IgY antibody (10µg/ml, Sigma C2288), to which 100µl of 10,000-fold diluted WSF was then added. The following experiments were performed following the same protocol described above.

**Urease- neutralization assay**
*H. pylori* were cultured overnight in BHI at 37°C and 10% CO₂. A fresh 10ml BHI media were incubated with 50µl of the overnight culture and incubated at the same condition (37°C, 10% CO₂). When the optical density of these cultures at 600nm (OD₆₀₀) reached 0.5, several dilutions of the IgY preparations were added to them, followed by 6 hours of additional incubation (37°C, 10% CO₂) for IgY Urease interaction. Urease activity then was assayed with addition of 25µl of urea-phenol red solution (2% urea, 0.03% phenol red) followed by reading the optical absorption in 550nm (OD₅₅₀).

**Dot-blot**
Aliquots of 0.5 µl of partially purified recombinant antigens of *H. pylori* or bovine serum albumins (BSA) were spotted on Nylon membranes. Each dot contained approximately 125 ng of protein. The spots were allowed to dry and the membranes were blocked for 1 h in phosphate-buffered saline (PBS) with 5% skim milk, incubated for 1 h with IgY diluted to 10 µg/ml, washed (3 ×10 min) and incubated with anti chicken and duck-IgG, AP conjugated (Kirkegaard & Perry Laboratories) used as secondary antibodies (5000 fold diluted, 1 h). After four washes of 5 min each in PBS the NBT/BCIP detection system (Promega) was applied.

**RESULTS**

**Antibody Titre in Chicken and Duck Egg Yolk**
Anti *H. pylori* ATCC 43504 and MTCC 41653 were observed in serum from 2nd weeks after first immunization. It reached a highest titre value of 1: 1280 from the 4th week onwards and continued to be same. However the appearance of specific IgY in egg yolk showed some
The appearance of specific IgY started from 4\textsuperscript{th} week after 1\textsuperscript{st} immunization with \textit{H. pylori} ATCC 43504 and MTCC 41653 antigens. A highest titre value of 1:1280 was reached after 6\textsuperscript{th} week after 1\textsuperscript{st} immunization with \textit{H. pylori} ATCC 43504 and MTCC 41653 antigens respectively. These results were presented in Table 1 and 2.

**Production of IgY against \textit{Helicobacter pylori}**

The concentration of total IgY and specific anti \textit{H. pylori} IgY was analyzed by ELISA. The content of IgY in the WSF of egg yolk was investigated for chicken and duck egg yolk. The average content of total IgY in chicken yolk was 180 ± 12mg and ducks yolk 160 ± 13, which was 28 ± 4 and 20 ± 4 purity of the chicken and duck egg yolk. After condensation by ultrafiltration, the chicken and duck egg yolk was 140±18 and 130 ± 17 mg and purity of the duck yolk 29 ± 2 and chicken yolk was 32 ± 4 % on the purity of the total IgY. These results were presented in Table 3. Isolated from egg yolks, were also verified by ELISA assay to titrate the amount of active specific IgY in each sample.

**Urease-neutralization activity**

Antiserum samples were used to determine the ability of IgY in chicken and duck antibodies to interact with the antigen by ELISA technique. The urease-neutralization ability of each IgY was assessed in a separated assay in which neutralization of urease activity in a \textit{H. pylori} culture was monitored by color alteration and therefore changing the optical absorption of phenol red as a pH indicator. OD550 values of \textit{H. pylori} cultures, co incubated with IgY urea-phenol red containing solution, illustrated in the activity of urease in presence of each IgY. Results revealed that although all two IgYs significantly decreased urease activity (p<0.05), there was a significant difference (p<0.05) between neutralization activity.

The IgY from the chicken and duck were not able to detect any of the 2 proteins tested Detailed characterization of IgY specificity was performed by ELISA. The IgY from duck and chicken specifically recognized urease in this test. Importantly, the titer of these IgY was high since a 8000-fold dilution was still able to recognize.

**DISCUSSION**

In recent years, pathogen-specific IgY has also been demonstrated to be effective in the passive protection of human’s diseases, such as \textit{Staphylococcus} for holotoxin [17], Rotavirus for diarrhea [18], dental caries caused by \textit{S. mutans} [19], and \textit{H. pylori} for gastric ulcers [20]. In this study, we tried to produce the specific IgY against \textit{H. pylori}. This bacterium was first reported in the India on plasmid profiling in Indian isolates of \textit{H. pylori} and has subsequently been reported worldwide [21]. \textit{H. pylori} Infection causes peptic ulcers, gastritis, duodenitis, and gastric cancers. Infection with the organism has been shown to follow geographic and socio-demographic distribution. The increased risk of infection is especially high among those living in Africa due to precarious hygiene standards, crowded households and deficient sanitation associated with this part of the world. Interestingly however; the infection rate in various populations does not parallel the incidence of morbidity caused by the infection. This has been termed by a number of authors as the ‘African enigma’ based on an apparently low incidence of gastric carcinoma and other \textit{H. pylori}-associated morbidities in the continent of Africa [22].

The specific IgY against \textit{H. pylori} in the egg yolk antibody increased in the several weeks after the first immunization, though there were obvious differences among individual chicken and duck. This variation in antibody production, based on the specific IgY production, might be the
result of differences in individual immune responses [23]. The origin of antigen also plays an important role in the immune response, as different outer membrane proteins or fimbrial adhesions from the bacteria affected the rate of IgY production [24,25]. When the antigen contained whole bacteria, or when the bacteria achieved better specific antibody production, the in vivo detection of antibody effect of the result was also better [26]. This study used the whole bacterial as the antigen source to obtain a better IgY titer. The total antigen obtained from an egg yolk by the water dilution method is around 595mg per egg. In this study, the purity of chicken antibody IgY in WSF was approximately 45–60% (Table 3. This demonstrates that the water dilution method can easily separate the IgY from other egg yolk components, as described previously [27, 28].

Table 1. Anti Helicobacter pylori (ATCC 43504) antibody titre in immunized chicken serum and their egg yolk

<table>
<thead>
<tr>
<th>Days</th>
<th>Titre Value of Antibody in Serum</th>
<th>Egg yolk</th>
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<tbody>
<tr>
<td>0</td>
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<tr>
<td>7</td>
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<td>1:80</td>
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<tr>
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<td>1:320</td>
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<tr>
<td>28</td>
<td>1:1280</td>
<td>1:640</td>
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<td>35</td>
<td>1:1280</td>
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<tr>
<td>42</td>
<td>1:1280</td>
<td>1:1280</td>
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</tbody>
</table>

Table 2. Anti Helicobacter pylori (MTCC 41653) antibody titre in immunized ducks serum and their egg yolk

<table>
<thead>
<tr>
<th>Days</th>
<th>Titre Value of Antibody in Serum</th>
<th>Egg yolk</th>
</tr>
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<tbody>
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<td>42</td>
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Table 3. Purity of antibody after procedure of purification on chicken and duck eggs

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Chicken IgY (mg/egg)</th>
<th>Purity (%)</th>
<th>Duck IgY (mg/egg)</th>
<th>Purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water soluble solution</td>
<td>180 ± 12</td>
<td>28 ± 6</td>
<td>160 ± 13</td>
<td>20 ± 4</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>140 ± 18</td>
<td>32 ± 4</td>
<td>130 ± 17</td>
<td>29 ± 2</td>
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</tbody>
</table>

CONCLUSION

Since it is possible to produce antibodies in chicken against a vast array of antigens and epitopes, there exists scope for raising antibodies against any number of bacterial, viral, or biological antigens. The significant potential of avian antibodies for further use in immunodiagnostics and identification of disease markers, immunotherapy and the treatment and prevention of disease is expected. Since lot of benefits of IgY technology and its universal application in both research
and medicine, it is expected that IgY will play an increasing role in research, diagnostics, and immunotherapy in the future.

Acknowledgements
The authors would like to thank everyone at the Dr. G. R. Damadaran College of Science Laboratory, Department of Microbiology, Department of Endoscopy; Purchase standard strain in Chandigarh India.

REFERENCES