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Use of chicken egg yolk antibody as an alternative to antibiotic treatment for control of ulcer producing *Helicobacter pylori*

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ABSTRACT

Helicobacter pylori multiply and cause infection in human gastric mucosal layer. New approaches have focused on using specific treatments, such as chicken antibody. The present study evaluated the potential use of immunoglobulin prepared from the egg yolk of chicken immunized with in the treatment of H. pylori infections. Between January and June 2011, a total of 107 H. pylori isolates were obtained from gastric biopsies of patients with upper gastrointestinal tract symptoms in endoscopy section at tertiary care hospitals in Coimbatore, South India. Genetic immunization of laying chicken with a plasmid expressing Helicobacter pylori UreB (large subunit of urease), IgY against UreB were obtained from egg yolks. These polyclonal and mono specific 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 IgY antibodies directed against antigens of H. pylori by DNA-based immunization.

Kew words: H. pylori. Chicken egg. ELISA kit. Urease activity.

INTRODUCTION

Helicobacter pylori are a gram-negative, microaerophilic bacterium that colonizes in the mucus layer associated with gastric-type epithelium in humans. *H. pylori* are the major pathologic agent in the development of gastric and duodenal ulcers, and its eradication is known to reduce recurrence of peptic ulcers [1]. Furthermore, epidemiological and statistical studies associated the infection with a higher risk of gastric malignancy leading the World Health Organization (WHO) International Agency for Research in Cancer to categorize *H. pylori* as a class I carcinogen [2]. Infection with *H. pylori* can often be treated with antibiotics. However, increase in antibiotic resistance is starting to affect the efficacy of treatment [3]. Consequently, it is important to seek new therapies and a broader based means of treating *H. pylori* infection which

do not invoke drug resistance problems. It has been shown that oral administration of antimicrobial immunoglobulin is an effective way to establish protective immunity against a variety of microbial pathogens [4].

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 [5].

The antibody therapy has an increasing number of applications in which it is possible to use inexpensive sources of animal antibodies, such as bovine colostral antibodies and egg yolk antibodies. With the exception of the systemic administration of purified egg yolk antibodies against snake venoms [6,10], all these applications involve peroral administration of antibodies for the treatment of enteric infections, such as enterotoxicogenic *E. coli*, *Helicobacter pylori*, the dental caries causing *Streptococcus mutans*, and rotaviral infections. Basically, these are almost the identical types of diseases that are treated with antibodies in the context of animal agriculture, as discussed in the section below [11].

Chicken egg yolk was recognized as an inexpensive, antibody source whereas hyper immunized hens could provide a convenient and economic source of immunoglobulin in their egg yolk [12]. The efficiency of egg yolk immunoglobulin (IgY) has been assessed for therapeutic application by passive immunization therapy through oral ingestion of IgY. This can be applied in fortified food products for prevention or control of intestinal infections, such as those caused by enterotoxigenic bacteria including *H. pylori* [13, 14]. As reported by Shin et al. [14] egg yolk IgY against *H. pylori* whole-cell lysates inhibited the growth of *H. pylori* and reduced gastric inflammation. These findings suggest that IgY could be used as a novel modality against *H. pylo*ri-associated gastric mucosal diseases.

These studies, taken together, provide the potential advantage of using IgY with specificity to *H. pylori* (IgY-Hp) for controlling *H. pylori* associated gastric disease and subsequently prevent disease resulting from chronic infection. Nevertheless, there has been no report so far on the use of IgY in the prevention and treatment of *H. pylori* infections.

METERLALS AND METHODS

Collection of samples.

Between January and June 2011, a total of 107 *H. pylori* isolates were obtained from gastric biopsies of patients with upper gastrointestinal tract symptoms in endoscopy section at tertiary care hospitals in Coimbatore, South India. Identification of *H. pylori* was confirmed by gram staining and tests for urease, catalase and Oxidase activity. *H. pylori* 26695 were used as a control in our study.

DNA manipulation

All manipulations were performed according to standard techniques [15]. The gene of *H. pylori* was amplified using the oligonucleotides UREBPCI1 (5’gcgaattccgccatggaaaagatta3’) and UREBPCI2 (5’gcgctctagactagaaaatgctaaag 3’) as primers, and pAR3 [16] as a template. The 1.7-kb DNA fragment was cloned into the NcoI and XbaI sites of pCI vector (Promega). The resulting plasmid pAR72 was purified from DH5α using commercial plasmid purification systems (Qiagen).

Immunization of Chicken

Immunization of chicken was performed in animal facilities of INSERM U271 (Lyon, France) with a total of 500 µg of plasmid DNA per animal by intramuscular injection in multiple sites, followed by two subsequent boosts [17]. Eggs collection and IgY purification from the eggs were performed as previously described [17].

Dot-blot.

Aliquots of 0.5 µl of partially purified recombinant antigens of *H. pylori* or bovine serum albumin (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-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.

ELISA (Enzyme Linked Immunosorbent Assay)

Immunization of hens was evaluated, using enzyme-linked immunosorbent assay (ELISA). Recombinant proteins were coated onto microtiter plates (2µg per well) in coating buffer (20mM Na2CO3, 35mM NaHCO3, pH=7.2) for overnight in 4°C. After washing the plates four times with PBST and blocking non-specific sites with blocking buffer (3% BSA in PBST) test sera were serially diluted (in PBST) on the plate. The total Ig titer was determined using anti hen HRP conjugates (Sigma, US) at a dilution of 1:30000. The colorimetric detection was carried out using ophenylenediamin (OPD, Sigma) as a chromogenic substrate of HRP, after washing the plate with PBST. The absorbance of each well was measured at 492nm. Furthermore, IgY preparations were also used in above protocol instead of sera in order to titrate its active immunoglobulin content. The cross activity of antisera, anti-UreCn and anti-UreCc sera were used against recombinant UreCt-coated wells as described above.

Urease- neutralization assay

H. pylori were cultured overnight in BHI at 37^0C and 10% CO2. A fresh 10ml BHI media were incubated with 50µl of the overnight culture and incubated at the same condition (37^0C, 10% CO2). When the optical density of these cultures at 600nm (OD600) reached 0.5, several dilutions of the three IgY preparations (IgY-UreCT, IgY-UreCn and IgY-UreCc) were added to them, followed by 6 hours of additional incubation (37^0C, 10% CO2) for IgY-Urease interaction. Urease activity then was assayed with addition of 25 µl of urea-phenol red solution (2% urea, 0.03% phenol red) followed by reading the optical absorption in 550nm (OD550).

RESULTS

In the present study, during January 2011 and June 2011,107 antral biopsies of patients with non ulcer dyspepsia, duodenal ulcer and gastric ulcer were isolated. The patients were between 15 and 75 years old. The samples were cultured on brucella agar medium. Prevalence of *H. pylori* was measured (34.65%) in these samples. Among the patients with positive culture for *H. pylori*, 56.28% were male, 43.71% were female. 23.71% of the patients had peptic ulcer and 77.28% had non ulcer dyspepsia. *H. pylori* isolated from different disease group were 5.1% in duodenal ulcer isolates, 10% in gastric ulcer, 3.4% in non-ulcer dyspepsias, and 16.6% in gastric cancers. None of the *H. pylori* isolates isolated from controls possessed plasmids reported [18].

H. pylori infections are prevalent in humans and although they can be cured with antibacterial therapy, the extensive use of antibiotics leads to the emergence of antibiotic-resistant strains [3]. The conceivable alternative is passive immunotherapy with *H. pylori*-specific antibodies. Eggs have been considered a convenient source for the production of polyclonal antibodies, known as IgY [19, 20]. Large amounts of IgYs can be obtained from the egg yolk by quick and economic purification procedures [21, 22]. For instance, approximately 1500mg of chicken IgY can be harvested each month, and between 2 and 10% is the specific IgY [23].

Plasmid pAR72, containing *H. pylori ureB* gene was used for DNA immunization of two chicken. Eggs were collected and large amounts of IgY antibodies (about 60 mg/egg) were purified from their yolks. Preliminary characterization of the obtained IgY using dot-blot technique indicated that IgY from one of the immunized chicken (No. J475) specifically recognized partially purified recombinant *H. pylori* UreB (Fig. 1). The same antibodies bound neither recombinant *H. pylori* HspB nor BSA, both used as controls. In addition, IgY purified from eggs of another chicken (chicken No. J521), which was immunized with a plasmid expressing *hspB* of *H. pylori* and which has not mounted a specific anti-HspB response, were used as an additional negative control. The IgY from the chicken No. J521 were not able to detect any of the three proteins tested.

DISCUSSION

Moreover, it has been shown that the egg yolk IgY against *H. pylori* whole-cell lysates inhibits the growth of *H. pylori* and reduces gastric inflammatory cell accumulation in *H. pylori* infected Mongolian gerbils [3] . However, IgY produced by whole-cell lysates can cross-react with other bacteria, naturally found in human intestinal flora, possibly decreasing its efficiency and specificity [24].

Rising of specific IgYs for each recombinant protein was detected with ELISA-titration of immunized chicken sera against three recombinant proteins separately. IgYs, isolated from egg yolks, were also verified by ELISA assay to titrate the amount of active specific IgY in each sample.

Table 1. Immunization scheme and isolation of chicken antibody by ELISA

Serial Number	Urease Serum, OD value 492nm	1/1000	1/2000	1/4000	1/8000	1/16000	1/32000	1/64000	1/128000
1	Cont-serum	0.4	0.3.5	0.3	0.2.5	0.2.0	0.1.5	0.1	0.0
2	Anti Ure Ct serm/UreCt	1.1	1.0	0.8	0.6	0.4	0.3	0.2	0.0
3	AntiUre Cn serm/UreCt	1.8	1.6	1.5	1.4	1.0	0.5	0.3	0.1
4	Anti Ure Cc serm/UreCt	2.4	2.0	1.9	1.8	1.7	1.6	1.4	0.2

Antisera samples were used to determine the ability of IgY-UreCc and IgY-UreCn antibodies to interact with the intact UreC protein by ELISA technique (Table 1). The urease-neutralization ability of each IgY was assessed in a separated assay in which neutralization of urease activity in a *H. pylori* culture was monitored by color alteration and therefore changing the optical absorption of phenol red as a pH indicator. OD550 values of *H. pylori* cultures, coincubated with IgY-UreCc, IgYUreCn and IgY-UreCt and urea-phenol red containing solution, illustrated in the

activity of urease in presence of each IgY. Results revealed that although all three IgYs significantly decreased urease activity.

Specific immunocompetence antigens from *H. pylori* proteins are therefore needed to decrease nonspecific reactions and increase specificity. Urease of *H. pylori* showed an acceptable potential as a vaccine in animal models [25, 26]. Therefore, we used the urease subunit, UreC, recombinant protein as a *H. pylori* specific antigen to elicit anti-*H. Pylori* IgY.

CONCLUSION

Concluded that 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 chicken 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.

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