Research Journal of Animal, Veterinary and Fishery Sciences _____ ISSN 2320 – 6535
Vol. **2(2),** 9-12, February **(2014)** *Res. J. Animal, Veterinary and Fishery Sci.*

Generation and Characterization of specific Chicken Egg Yolk Antibodies (IgY) against Microbial Bio-terroristic Agent (*Vibrio cholerae*)

Megha P.U., Sentila R. and Michael A.
Department of Microbiology, PSG College of Arts and Science, Coimbatore, TN, INDIA

Available online at: www.isca.in, www.isca.me
Received 2nd December 2013, revised 17th January 2014, accepted 12th February 2014

Abstract

Bioterrorism, the deliberate release of microbial agents or other agents which can cause various illness or death in living things. Egg yolk immunoglobulin (IgY) was prepared against cholera, by immunizing hens with formalin killed V. cholera serotype ogawa. Booster injections of the antigen were subsequently given to the chicken. Using polyethylene glycol and ammonium sulfate precipitation method the antibodies produced were purified. Further Diethylaminoethyl (DEAE) cellulose ion-exchange column chromatography was also carried out. From the egg yolk the IgY fraction was separated and each step of the separation was viewed on Sodium Dodecyl Sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The specificity and reactivity of V. cholera was confirmed by ELISA method. The highest titer of antibody at the dilution of 1:10000 was observed at the 21st week of vaccination and the serum agglutination test also showed a positive result. So it can be suggested that anti- V. cholera produced had a high specificity towards V. cholera O1. As a result anti- V. cholerae IgY may be utilized as preventive agent against Cholera infection.

Keywords: Bioterrorism, V. cholera, ELISA, IgY, SDS-PAGE.

Introduction

Bacterial pathogens can be used as agents for biological terrorism. As they are easy to obtain prepare and disperse, it has an important role in limited terrorist attack Greenfield[1]. Cholera an intestinal infection is caused by *Vibrio cholera*, a gram negative bacterium which is also motile in nature. They are halo tolerant, oxidase positive curved bacteria which normally colonize the small intestine. Symptoms are limited to mild diarrhea but the severity of the disease can even cause death of the patient due to profuse diarrhea and vomiting. Cholera is generally regarded as an incapacitating agent, but in the face of large numbers of casualties, a large number of deaths are possible because of the overwhelming requirements for supportive therapy. Presently vaccination and antibiotic treatment is the most widely accepted method for preventing anthrax and cholera. Therefore attention is being paid to find alternative approaches. The antibiotic treatment may be helpful in minimizing the losses but simultaneously it may lead to drug resistance. Chicken antibodies are attractive for per oral immunotherapy in livestock especially for combating clinical and sub clinical infections. Therefore, IgY as a passive, inexpensive and easy producing immunoglobulin has attracted much attention and been recognized to be efficient in therapy and prevention reported in oral administration of polyclonal IgY. *Vibrio cholerae* which is a potential bacterial bio terroristic agent causing cholera, leading to high mortality rate in both animals and humans was selected in this study. The present investigation was focused on generating antibodies against *Vibrio cholerae* which in turn can be used for passive therapy, diagnosis and in detection kits.

Material and Methods

Growth, maintenance and preparation of V. holera antigen: The slant culture of *Vibrio cholerae* was obtained from King's Institute Chennai. The culture was sub cultured on TCBS plate and disc shaped yellow colored colonies were observed on the plates. The colonies were grown in 5ml broth and incubated at 37^0C overnight which was then transferred to 250ml of BHI broth for 15 hours at 37^0C in shaker at 100 rpm. The turbid broth was centrifuged at 11000 rpm for 15 minutes and the suspension was removed and the pellet was washed with sterile PBS saline at pH 7.2. Finally the pellet was suspended in PBS and the culture was inactivated by 0.5% formalin and incubated for overnight. After 24 hours the cells were adjusted to $1.5x10^9$ cells using McFarland's standard solution.

Dilution of Antigen Suspension: Antigens was diluted using saline which gave the 'in use' dilution i.e., an opacity equal to McFarland's Barium Sulphate tube no.1.The 'in use' suspension with adjusted opacity shows final cell concentration of 3×10^8 cells. 0.5mL of antigen was diluted with 0.5mL of 0.5% physiological saline and the suspension was adjusted to McFarland's tube no 1 to get a concentration of 1.5×10^{10} cells.

Purity testing of antigen: Complete killing of the bacteria was tested by responding an aliquot of the cell pellet in PBS saline and plating 199µl of this suspension in to TCBS agar medium. The plates were incubated over night at 37^0C and examined for the presence of bacterial growth.

Experimental animal: Twenty one week old egg laying white leghorn chickens in good health was obtained from LK Poultry farm, Iyampalayam. The birds were maintained free from specific pathogen and were fed with layer mesh.

Immunization of chickens: First immunization was done using white leghorn chickens which were of five month old. 0.5 ml of antigen was injected at multiple sites of the breast muscles. Booster doses were also given within a period of two weeks. Test bleedings were done to check for anti-*V.cholerae* serum antibodies. Individual cages were provided for the hens and their eggs were collected, properly marked and stored at 4^0C until further studies. The isolated egg yolk was mixed using a glass rods and it was stored at 4^0C.

Separation of egg yolk from white: Separation of egg yolk was done by washing with distilled water, thereby removing most of the albumin in it. Further it was rolled on paper towel to remove adhering egg white. The yolk without the membrane was allowed to flow in to a graduated cylinder by puncturing the membrane. The egg yolk for purification was also collected in a conical flask and mixed well. This separated volume of egg yolk was measured and subjected for further purification.

Purification of antibodies from egg yolk: Polyethylene glycol (PEG) was used to extract antibodies from egg yolk and precipitation was done by ammonium sulphate. Dialysis of the partially purified antibody was also done. The IgY was further purified using DEAE cellulose ion-exchange column chromatography. The IgY fraction was then concentrated with polyvinylpyrrolidone (PVP) at room temperature. Lowry's method was used to estimate the concentration of total protein and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyse the result[2]. The lipid content extracted from the protein purified from egg yolk was determined gravimetrically.

Protein profile of IgY: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) as described by Laemmli were used to analyze the protein profile of egg yolk antibodies[3]. According to the procedure proteins are resolved with 10% (w/v) Polyacrylamide separating gel and 4% (w/v) polyacrylamide stacking gel at 250V and 10mM. Equal ratio of prepared bacterial antigens (30ul) and sample treating buffer (30µl) were mixed well and loaded into sample wells. A wide range molecular weight (65-205 KDa) marker was also run along with the proteins[4]. The sample was run until they reach the bottom of the gel. The characteristic protein pattern for the IgY antibodies can be visualized after Coomassie Brilliant Blue staining.

Titration of antibodies by ELISA: The antibodies titer was assayed by an ELISA procedure as described by Sunwoo *et al* with modifications[5]. A micro titer plate was coated with 100µl *Vibrio cholerae* (1.11mg of cells/ml; 10µg of protein/ml) whole cells in carbonate-bicarbonate buffer (0.05M, pH 9.6). The (diluted 1:1,000 in PBS, 100ul per well) specific egg yolk antibodies (IgY) was reacted with coated antigens. The same volume (100ul) of Rabbit anti-chicken IgG conjugated with horse radish peroxidise (diluted 1:1,000 in PBS) and freshly prepared substrate solution, 2-2-azinobis (3-ethylbenzthiazoline-6-sulponic acid) in 0.05M phosphate citrate buffer (pH 5.0) containing 30% Hydrogen peroxide were used for secondary antibody and substrate, respectively. Absorbance of the mixture was read at 490nm by a kinetic micro plate reader.

Results and Discussion

Vibrio cholerae (Ogawa) the standard strain causing Cholera were characterized and identified. The Gram stained smear of *Vibrio cholerae* showed gram negative, curved (vibrio-shaped), rods and formed large, disc shaped, smooth, opaque and yellowish colonies in TCBS agar. As a part of this study specific poly clonal antibodies were raised in chicken against the predominant bacterial pathogen *Vibrio cholerae*. The antigen was used to immunize the 21 week old white leghorn chickens to generate IgY. Subsequent booster doses of increasing concentrations were given at weekly intervals to raise the antibody titer. The serum samples were first analyzed to detect the presence of antibodies specific to the antigen used. Serum of chicken immunized with *Vibrio cholerae* showed positive serum agglutination test (figure 1). Later the eggs were collected stored and purified from the chicken egg yolk by Polson *et al* method and by DEAE cellulose Ion column chromatography[6]. The molecular weight of the purified IgY were confirmed as 180KDa through SDS-PAGE (figure 2). The antibody titer of egg yolk antibodies determined by ELISA showed the presence of antigen specific antibodies for the specific bacterial pathogen[7]. The antibody titer of egg yolk antibodies increased after every booster dose and reached a highest at day 56 after which it remained stable till the 90th day of immunization (figure 3). Regarding the antibody titer potency of egg yolk sample a high peak at the dilution of 1:10000 was obtained at the 21st week it was observed that unimmunized chickens normal antibodies did not show any specific activity against *V. cholerae* antigen (figure 4).

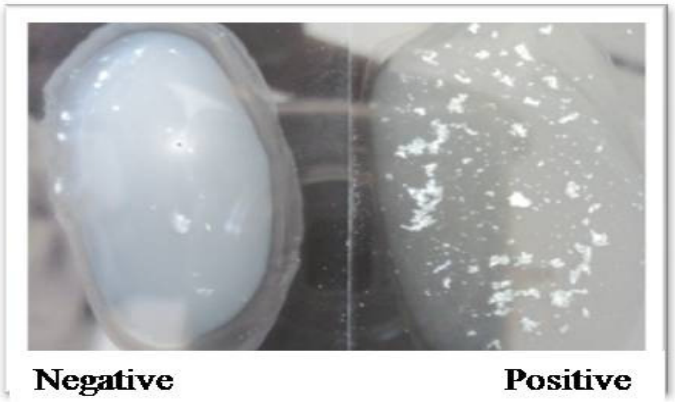

Figure-1
Serum Agglutination test for Vibrio cholerae

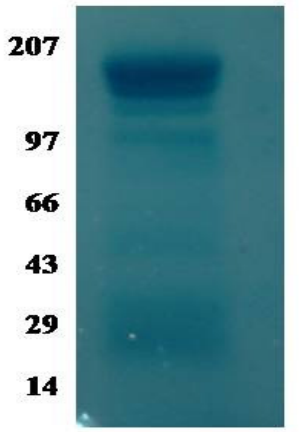

Figure-2
Protein Profiling of antibody using SDS-PAGE

In conclusion, specific IgY antibodies were generated against *Vibrio cholerae* and they were known to have convincing reactivity and specificity against the antigen. Further work has to be done in order to justify the use of IgY in passive therapy and diagnosis against such microbial bio-terroristic agents. This study is the preliminary work for the application of IgY technology against microbial infections, with special reference to *Vibrio cholerae*. This chicken egg yolk IgY produced can be applied for passive immunotherapy, diagnosis and in detection kits during a microbial bio terroristic attack. Science, medicine and also the whole society can gain new solutions from the IgY technology. The findings of the present study was in accordance with those of Kazuyuki Hirai *et al* who recorded that the anti-O1 and O139 IgYs and the mixture of either IgY with anti-CTB IgY significantly protected the occurrence of Cholera caused by both O1 and O139 infection. Since large amount of IgY can be prepared very easily and at low cost, this seems to be a useful procedure for preventing and treating Cholera[8].

Conclusion

In conclusion, specific IgY antibodies were generated against *V. cholerae* and they were known to have convincing reactivity and specificity against the antigen. Further work has to be done in order to justify the use of IgY in passive therapy and diagnosis against such microbial bio-terroristic agents. This study is the preliminary work for the application of IgY technology against microbial infections, with special reference to *V. cholerae*. This chicken egg yolk IgY produced can be applied for passive immunotherapy, diagnosis and in detection kits during a microbial bio terroristic attack. Science, medicine and also the whole society can gain new solutions from the IgY technology.

Acknowledgments

The authors would like to thank the management of PSG CAS, Department of Microbiology, Coimbatore for providing laboratory facilities and for their constant support to carry out this publication.

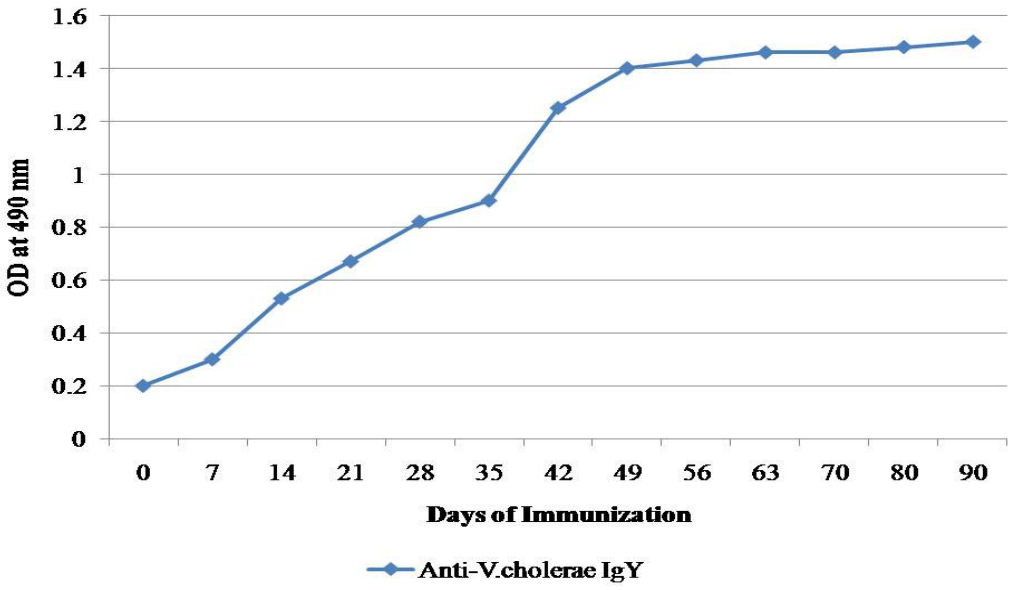

Figure-3
Quantification of Antibody titer in Chicken Eggyolk using Indirect KLISA

A - Blank
B - Positive control
C - Negative control
D - Neat
E - 1:10 Dilution
F - 1:100 Dilution
G - 1:1000 Dilution
H - 1:10000 Dilution

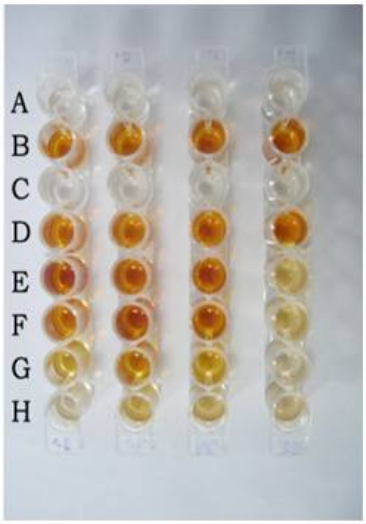

Figure-4
Determination of the specificity of IgY using Indirect ELISA

References

1. Greenfield Ronald A., Drevets M., Douglas A., Machado., Linda J., Voskuhl Gene W., Cornea, Pau., Bronze., Michael S., Bacterial Pathogens as Biological Weapons and Agents of bioterrorism, *American Journal of the Medical Sciences,* **323(6)**, 299-315 **(2002)**

2. Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J., Protein measurement with the Folin phenol reagent, *J Biol Chem*, **193(1)**, 265–75 **(1951)**

3. Laemmli U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature,* **227**, 680–5 **(1970)**

4. Akita E.M. and Nakai S., Comparison of four purification methods for the production of immunoglobulins from eggs laid by hens immunized with an enterotoxigenic E.coli strain, *Journal of Immunological Methods,* **160**, 207-214 **(1993)**

5. Sunwoo H.H., Lee E.N., Mennien K., Suresh M.R. and Sim J.S., Growth inhibitory effect of Chicken egg yolk antibody (IgY) on Escherichia coliO157:H7*, J Food Sci,* **67**, 1486-1494 **(2002)**

6. Polson A., Von Wechmar M.B., Van Regenmortel M.H., Isolation of viral IgY antibodies from yolks of immunized hens, *Immunol Commun.*, **9(5)**, 475–493 (**1980)**

7. Voller A., Bartlett D.E., Bidwell D.E., Clark M.F., Adams A.N., The detection of viruses by enzyme-linked immunosorbent assay (ELISA), *J Gen Virol,* **33(1)**, 165–167 **(1976)**

8. Kazuyuk, Hirai, Hideyuki Arimitsu, Koji Umeda, Kenji Yokota, Lianhua Shen, Kiyoshi Ayada, Yoshikatsu Kodama, Takao Tsuji, Yoshikazu Hirai, Keiji Oguma, Passive Oral immunization by Egg Yolk Immunoglobulin (IgY) to Vibrio cholera effectively prevents Cholera, *Acta Medica Okayama,* **64(3)**, 163-170 **(2010)**