CLONING AND EXPRESSION OF HUMAN ROTAVIRUS VP6 GENE IN BACULOVIRUS EXPRESSION SYSTEM FOR ANTIBODY GENERATION IN CHICKENS

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ABSTRACT

Rotavirus is the most common cause of severe diarrhoea among infants and children. Group A rotavirus causes > 90% of infections in humans with 6, 00,000 children being infected worldwide every year. Quick diagnosis of rotavirus infection is required to avoid the unnecessary usage of antibiotics and generation of antibiotic resistance. IgY antibodies have gained considerable attention in recent years for their use in immunotherapeutics and diagnostics. The present research involves generation and purification of IgY antibodies against recombinant VP6 protein from chicken Egg Yolk and development of biosensor using gold nano particles coated with IgY for the diagnosis of rotavirus infection.

Key words: Rotavirus, VP6 gene, Cloning, IgY Antibodies, ELISA, Biosensor.

INTRODUCTION

Rotaviruses belonging to the family Reoviridae are the leading cause of acute infectious diarrhea in infants and young children, worldwide accounting for half a million deaths annually in children <5 years of age, with majority (82%) of the deaths occurring in developing countries mainly due to Group A Rotavirus [1, 2]. Rotavirus surveillance studies indicate that all young children experience at least one RVA infection by 5 years of age [3, 4]. Moreover, RV infection is responsible for about 30% of all hospital admissions for diarrheal disease and causes an estimated of 400,000–600,000 deaths per year among children worldwide [5, 6].

Rotaviruses are icosahedral viruses, with 3 concentric protein layers containing the viral genome composed of 11 segments of dsRNA with the total genome containing approximately 18,522 base pairs [7]. Each genomic segment encodes for a single protein, with the exception of the smallest genomic segment, which encodes for 2 proteins (NSP5 and NSP6), for a total of 6 structural or viral proteins (VP1 to VP7) and 6 non-structural (NSP1 to NSP6) proteins. The virion outer layer is composed of proteins VP7 and VP4, the intermediate layer of protein by VP6, and VP2 being the predominant inner core protein. Proteins VP1 and VP3 are part of the replication complex located within the inner core [8]. Non-structural proteins are synthesized in the infected cells and carry on functions during virus replication cycle and morphogenesis [9]. They also participate in the modulation of the innate immune response and pathogenesis.

The five most prevalent G (VP7) and P (VP4) genotypes worldwide include G1, G3, G4, and G9 with P[8] and G2 P[4]. The virus infects the mature villus epithelial cells of the small intestine and infection often leads to fever, vomiting and diarrhea in children. Dehydration and electrolyte disturbances are the major sequelae of HRV infection. A specific, effective and affordable therapy is currently not available [10]. However, two RVA vaccines were licensed in 2006. Rotarix® is a monovalent live attenuated vaccine derived from the most common circulating wild-type strain G1P[8] (Glaxo SmithKline Biologicals, Belgium) and RotaTeq® is composed of 5 RV strains, each of which is a single-gene reassortant based on a parent bovine strain WC3- that contains an outer capsid gene from a human strain that induces immunity to the most common genotypes of HRV in circulation 2G1 to G42 and P1A [8] (Merck & Co., USA). Both vaccines were efficacious in preventing severe gastroenteritis, predominantly due to the G1 HRV serotype [11], in developed countries.
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However in children from developing countries, the efficacy rates were lower [12].

Passive treatments against gastrointestinal pathogens such as RVA need to be safe, economical and highly specific. It has been reported that IgY Abs from hens are an excellent source of polyclonal Abs against RVA and other pathogens [13]. Studies including randomized double- blind clinical trial testing the therapeutic ability of IgY in patients with severe diarrhoea due to rotavirus which showed a positive effect of IgY in controlling the rotavirus diarrhoea in patients admitted in hospitals [14]. This shows the potential application of IgY for passive treatment for rotaviral diarrhoea as well as for diagnostic purposes including biosensor development for rapid detection of infection.

The present research work involves the cloning of VP6 gene from Human Group A rotavirus and its expression in Baculovirus expression system using insect cell lines and the VP6 protein purification using Ni NTA Purification system. The purified recombinant protein will be used as antigen to immunize White leghorn chickens for IgY Antibody generation and purification from Egg Yolk of Immunized chicken.

**Materials & Methods**

**Specimen:** Faecal samples were collected from patients hospitalized for acute gastroenteritis in Pune, western India. The specimen was collected within 24 h of hospitalization with prior informed consent from the parents. The severity of infection was found out by calculating Vesikari’s Scoring Method. A 10% faecal suspension was prepared in 0.01 M PBS (pH 7.4) and rotavirus positivity was identified by antigen-capture ELISA (IDEIA Rotavirus; Dako). The specimen was stored in aliquots at –20°C until analyzed by National Institute of Virology (NIV), Pune. This positive sample was collected from NIV and used for our further research works.

**Faecal suspension Preparation:** 20% faecal suspension was prepared using 1X of 0.01M PBS (pH 7.2-7.4). 200µl of liquid sample or 200mg of solid sample was suspended in 800µl and 1000µl of PBS respectively in a 1.5ml Eppendorf tube and vortexed for 40 seconds. The contents were centrifuged at 10,000rpm for 10 minutes at -4°C. Transfer the supernatant to a fresh Eppendorf tube without disturbing the pellet and discard the pellet. Store 20% faecal suspensions at -20°C for further analysis.

**RNA Extraction and Quantification:** Viral Total RNA from 20% faecal suspension was extracted using TRIzol Ls method (Invitrogen) according to manufacturer’s protocol. The reagent contains a mono-phasic solution of phenol and guanidinium thiocyanate. The protocol involves the following steps; Homogenization, Phase Separation, RNA Wash, RNA Precipitation and Re dissolving the RNA Pellet in DEPC Water. The Total RNA isolated was quantified using Nano Drop Software.

**RNA PAGE Gel Analysis:** Electropherotyping of viral RNA extracted from faecal sample was performed in 7.5% polyacrylamide gels and the gel was silver stained as previously described to visualize the 11 segment pattern of rotavirus RNA [15].

**RT-PCR:** RT PCR was carried out for full length VP6 gene (1356bp) using the gene specific primers VP6-R1F with primer sequence 5’→3’ GGTCACATCCTCAATAGCGTTCT and with primer sequence 5’→3’ VP6-1366R GGCTTTTAAACGAAGTCTTC [16, 17, 18] and random primer. cDNA was synthesized using BIORAD cDNA synthesis kit by following manufacturer’s instructions. cDNA for whole length VP6 gene was obtained with PCR conditions as initial denaturation of 65°C for 5 minutes and the cycle condition including 95°C for denaturation, 55°C for annealing and 80°C for extension and Holding the PCR product at 4°C. The results were analyzed using 1.5% Agarose Gel. The VP6 gene amplified by PCR will be used to clone in Baculovirus vector for expression in insect cell lines (Invitrogen) and purification of recombinant VP6 protein [19].

**RESULTS AND DISCUSSION**

About 20% faecal suspension was prepared initially using 1X of 0.01M PBS (pH 7.2) from the faecal sample collected from NIV, Pune.
Total RNA was isolated from the sample and the concentration of RNA in the sample was found to be 56ng/µl of suspension using Nano Drop software in Ultra small volume Spectrophotometer.

RNA PAGE was performed to the isolated RNA using 7.5% Polyacrylamide gel. The protocol was standardized to use only the resolving gel without stacking gel. The 11 segmented pattern of Rotavirus RNA was visualized after performing Silver staining technique by standard protocol mentioned above. 11 distinct bands were seen in PAGE gel representing the different segments of the rotaviral RNA (Fig.1).

**Fig.1 RNA PAGE gel Analysis (Silver staining) of RNA isolated from stool specimen.**

Lane 1: 100bp marker
Lane 2: RNA Sample 1
Lane 3: RNA Sample 2

RT PCR was done and cDNA was prepared for whole length VP6 gene (1.3kb) using BioRad kit by manufacturer’s protocol. The PCR product was analyzed using 1.5% Agarose gel and it was found to be 1.3kb in size which indicates the amplified VP6 gene.

Hence from the above results, the amplified product of VP6 gene will be used to clone in Baculovirus vector and expressed in insect cell lines sf9 & sf21 for the recombinant VP6 protein. This recombinant protein will further be used to immunize chickens for antibody generation.

**Figure.2. Agarose Gel Electrophoresis Images of PCR product (amplified VP6 gene – 1.3kb).**

Lane 1: 100bp marker
Lane 5: PCR Product

**Conclusion:** The present research work forms the basis for IgY antibody generation in chickens against Rotavirus which finds application in passive immunotherapy for children infected with rotavirus under 5 years of age. IgY antibodies can be given orally to treat the disease as in case of the reference mentioned above to reduce the disease burden in infant community. Treatment with anti rotavirus immunoglobulins from immunized chicken’s egg resulted in decrease in rotavirus particles in stool and diarrhoea frequency in children [19]. It can also be used for diagnosis of the infection in ELISA by replacing antibodies generated from rabbits nowadays and in developing a Biosensor using nano particles combined with IgY antibodies for rapid detection of rotavirus infection.
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REFERENCES


