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In vitro neutralization assay for estimating immunogenicity of recombinant human Rotavirus VP6 protein via electroporation

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Rotaviruses (RVs) represent the principal causative agents of severe gastroenteritis leading to high mortality rates, especially in children < 5 years old in both developed and developing countries. Although the first generation of live attenuated RV vaccines such as RotaTeq and Rotarix achieved partial success in reducing the number of RV deaths worldwide, several concerns, such as low efficacy especially in developing countries, safety, and cost, imply a dire need to develop these vaccines. Also, sensitive methods to estimate the immunogenicity of the candidate recombinant subunit VP6 vaccines *in vitro* are of great need. In the present study, 1232 bp of the most frequent full-length VP6 in clinical and environmental isolates in Egypt with 98% nucleotide identity and 98% amino acid identity in comparison to human RoV Wa reference strain was expressed in *E. coli*. Examination of the sensitivity of antibodies produced in the male rabbits which were immunized intramuscularly with 20µg of the purified VP6 protein indicated a sensitivity up to 1/24000 dilution of antibodies against the expressed protein using ELISA. Introducing antibodies into the MA104 cell line was performed using electroporation to neutralize the human rotavirus Wa strain VP6 when exposed after viral uncoating. The efficiency of the antibodies which act intracellularly to neutralize the infectious human rotavirus Wa strain used in challenge was 1.4, 1.6, and 1.8 $\log_{10}$ for high, moderate, and low viral titres at 1/10 dilution of the antibodies and the efficiency decreased gradually by increasing the dilution.

Keywords: ELISA, Human rotaviruses, Neutralization test, Recombinant protein, Subunit vaccine, VP6 whole gene

INTRODUCTION

Group A rotavirus (RVA) is a major etiologic agent of acute viral gastroenteritis in children under the age of 5 years old worldwide; reports estimated that rotavirus infection is responsible for 235,331 deaths among children annually, which represents about 19.11% of all diarrheal deaths globally (Du et al., 2022). Developing countries such as Sub-

Saharan Africa and South Asia have more than 80% of these RV-related deaths (Tate et al., 2016).

In 2020, the World Health Organization (WHO) reported that 109 countries around the world introduced RV vaccines into their pediatric immunization programs (WHO, 2020); however, other countries did not introduce the vaccines in their mandatory vaccination program. There are two live, attenuated, RV vaccines which are available either commercially or in the national pediatric immunization programs. The first one is RotaTeq® (Merck and Co., Inc., PA, USA, and Sanofi Pasteur MSD SNC, Lyon, France) which is a pentavalent vaccine composed of four expressing human RV genotypes G1, G2, G3, and G4 (the most frequent G types worldwide) and P[8] in the genetic background of a bovine RV (G6P[5]) (Heaton & Ciarlet, 2007). The second one is Rotarix™ (GSK Biologicals, Rixensart, Belgium) which is an attenuated monovalent vaccine, derived from the most common human G1P[8] strain (O'Ryan, 2007). Although the burden of RV gastroenteritis has substantially declined following the introduction of these vaccines in many countries (Pietsch & Liebert, 2019; WHO, 2020), the clinical effectiveness of both commercial vaccines has been found to be consistently lower in low-income and lower middle-income countries, in Africa, Asia, and Central America, where vaccines are needed the most because of the high prevalence percentages of human rotaviruses in clinical specimens and environmental samples especially in the peak of the rotavirus in autumn and winter (Clark et al., 2019). Also, RV infection remains the leading cause of diarrheal death in children under the age of five years old and among infants in these countries (Bhuinya et al., 2022). Moreover, a reduction in childhood vaccination coverage was observed during the COVID-19 pandemic in 2020 due to social distance measures and lockdown (Kiely et al., 2022).

Lower efficacy and poor performance of commercial live attenuated, oral vaccines in developing countries are multifactorial (Glass et al., 2006), including the fact that many children in these countries are often suffering from medical conditions that are not common among children in developed countries, such as tuberculosis, malaria, malnutrition, and immunodeficiencies which can adversely affect the vaccine efficiency (Gruber et al., 2017), the vitamin D and zinc deficiencies which affect the immune system response and are proved to compromise the performance of RV vaccines (Colgate et al., 2016), the condition of the gut microbiota, with the existence of other microorganisms in the intestine including other enteric viruses, parasites, and bacterial coinfections which may compromise the efficiency of the vaccines (Harris et al., 2017), and the maternal antibodies that are transported through the placenta from the mother to her infant which may inhibit the vaccines (Moon et al., 2016). RV vaccine neutralizing activity of breast milk in developing countries is usually higher than its activity in developed countries and may adversely affect the vaccine by reducing its titre (Rongsen-Chandola et al., 2014), the administration time of RV vaccines, which may be given together with other oral vaccines (Patel et al., 2012), and the high diversity of circulating strains in Africa and Asia and the difference in epidemiology compared to the Americas and Europe (Villena et al., 2003; Zhao et al., 2022; Chan-It et al., 2023; Mathew et al., 2023). For all the previous reasons, in addition to concerns about the safety of live attenuated oral vaccines for immunocompromised children (Baek et al., 2010), the risk of adverse effects of intussusception associated with them (CDC, 2007), and the growing fear of reassortment events that might occur between the commercial vaccine strains and circulating wild-type strains in the environment, leading to the emergence of potentially more virulent new strains (Kaneko et al., 2017), the search for safe and more effective alternative nonliving vaccines led to the discovery that a systemic immune response could be induced by parental administration of a subunit vaccine (Wen et al., 2012).

Among many subunit vaccine candidates, the VP6 subunit vaccine seems to be one of the suitable alternative vaccines to solve the problems associated with live attenuated commercial vaccines, mainly because it is the most conserved, immunogenic, and abundant rotavirus protein (Choi et al., 2004). Also, VP6 protein is the only rotavirus' structural protein that carries antigen determinants that are common to all rotavirus group A (high sequence homology), and it also contains epitopes that have been used to classify group A rotaviruses into subgroups (Lopez-Guerrero et al., 2018). VP6 protein has the ability, alone, to elicit protection against all human rotavirus strains belonging to multiple serotypes (Choi et al., 2004). Although VP6 protein does not induce neutralizing antibodies, different VP6 preparations were found to induce homologous and crossreactive immune responses with partial

protection from RVA replication *in vivo* (Afchangi et al., 2019). Rotavirus replication cycle could explain the ability of VP6 protein to induce these reactivity and immune responses. During the rotavirus replication cycle, virions attach to host cells as triple-layered particles (TLPs) and subsequently enter the cytoplasm by either plasma membrane or endosomal membrane penetration. As a result of cell entry, the outer layer which contains VP4 (VP8 and VP5) and VP7 is lost and the resulting double-layered particles (DLPs) become transcriptionally active, releasing mRNA transcripts through a system of channels that penetrate the VP6 and inner VP2 capsid layers at each of the icosahedral vertices (Feng et al., 2002). On the other hand, several studies have demonstrated that VP6 subunit vaccine alone can be very effective in inducing protection against the RV infection in animal models (Esteban et al., 2013; Pastor et al., 2014; Lopez-Guerrero et al., 2018). Mice experimentally infected with rotavirus are protected by passive transfer of anti-VP6 antibodies (Burns et al., 1996; Feng et al., 2002) or anti-VP6 nanobodies (Maffey et al., 2016). So, to examine the efficiency of VP6 proteins of the whole gene or epitopes as recombinant subunit vaccines *in vitro,* introducing antibodies produced in experimental animals into specific cell lines such as MA104 must be performed to be able to react with the VP6 exposed after viral uncoating and penetration into the cells. *In vitro* examination of the vaccine efficiency is a necessary step to determine the promising vaccines before transfer to the *in vivo* step to save time, money, efforts, and not use laboratory animals unnecessarily. In a previous study of El-Senousy and coworkers (2013), they used a recombinant VP6 protein expressed from an epitope of partial human rotavirus Wa strain VP6 gene (155 bp) from the most frequent Egyptian rotavirus isolated from sewage which represented 99% homology with rotavirus sequences with the accession numbers HQ392389, HQ738601, and HQ738599 and the target sequence codes for 51 amino acids resembles amino acids from 282 to 332 of human rotavirus A inner capsid protein VP6 with accession number ADO78525. A percentage of 90% reduction of infectious human rotavirus Wa strain was achieved *in vitro*. Low efficiency of this epitope when examined *in vivo* using guinea pigs with no neutralization *in vivo* was observed in the ten examined animals when exposed to 10^5 infectious viruses; however, neutralization was observed in only four animals of ten exposed animals to 10^3 infectious viruses (data not shown) which suggested the use of the whole recombinant VP6 gene structural protein in our present study instead of the short fragment epitope in the previous study of El-Senousy and coworkers (2013) as a candidate subunit vaccine. VP6 protein carries antigen determinants that are common to all group A RoV (Lopez-Guerrero et al., 2018). These antigen determinants definitely exist in the whole VP6 gene and not all of them are necessarily present in the epitope.

In a previous study for the Environmental Virology and Food-Borne Viruses Group in NRC, high similarity percentages between the whole VP6 gene of the same human rotavirus genotypes and also the different human rotavirus genotypes were observed in isolates from clinical specimens and sewage samples of Greater Cairo (Kamel et al., 2020). This indicates that recombinant subunit VP6 vaccine may be effective if used within the Egyptian community. In this present study, our objectives were to generate a recombinant rotavirus protein of the 1232 bp VP6 gene that represents the most frequent VP6 sequence in Egyptian clinical and environmental rotavirus isolates with codon optimization and to estimate the immunogenicity of the recombinant human rotavirus VP6 protein *in vitro* using neutralization after insertion of the antibodies into MA104 cells using electroporation.

MATERIALS AND METHODS

Construction of expression plasmid with recombinant VP6 (rVP6) gene

The most frequent full-length VP6 in clinical and environmental isolates in Egypt was identified in a previous study (Kamel et al., 2020) with 98% nucleotide identity and 98% amino acid identity in comparison to human RoV Wa reference strain, GenBank nucleotides accession number: K02086.1 and amino acid accession number: P03530.1 with 7 amino acid changes (arginine changed to proline, lysine changed to phenylalanine, arginine changed to serine, alanine changed to serine, threonine changed to leucine, glutamic acid changed to glutamine, leucine changed to valine, at positions: 102, 125, 126, 241, 279 and 327, and 382, respectively) (Figure 1). Gene with codon optimization (1232 bp) (Figure 2) was synthesized and cloned in expression plasmid pJ 404 (3966 bp) by (ATUM, Inc., Menlo Park, CA, USA). The VP6 sequence was optimized for expression in *E. coli* by reassigning the native codons to codons frequently used in *E. coli.* Expression

plasmid pJ 404 contains inducible promoter site T5 for induction of protein expression by IPTG, strong ribosome binding site for tight expression, ampicillin resistance gene for selection of transformed cells, and high copy number origin pUC (Figure 3). The cloned gene was tagged by a codon of six histidine residues at 5` end to facilitate the purification of the interesting protein. Also, the gene was flanked by two restriction sites NdeI and PstI at 5` and 3` ends, respectively. The constructed plasmid was delivered on dry filter paper and accessed in ultrapure water as described in the manufacture's instruction. One microlitre of 18pg/mL constructed plasmid was gently mixed with *E. coli* BL21 electrocompetent cells and electroplated in ice-cold gene pulser-cuvette with 0.1 cm electrode gap (Bio-Rad) with 2.5kV, 25mF, and 200 ohms for time constant 3.5ms (Wang et al., 2014). The plasmid was purified using the QIAprep Spin Miniprep kit as described in the manufacture's instruction. The purified plasmid was analysed by 1% agarose gel electrophoresis and stored at -20°C.

MEVLYSLSKTLKDARDKIVEGTLYSNVSDLIQQFNQMIVTMNGNDFQTGGIGNLPVRNWTFDFGLLGTTLL
NLDANYVENARTIIEYFIDFIDNVCMDEMAPESQRNGVAPQSEALRKLAGIKFFSINFDNSSEYIENWNLQN
RRQRTGFVFHKPNIFPYSASFTLNRSQPMHDNLMGTMWLNAGSEIQVAGFDYSCAINAPANIQQFEHIVQL
RRALTTATITLLPDAERFSFPRVINSSDGATTWFFNPVILRPNNVEVEFLLNGQIINTYQARFGLIIARNFDAIR
LLFQLMRPPNMTPAVNALFPQAQPFQHHATVGLTLRIQSAVCESVLADANETLLANVTAVRQEYAIPVGPV
FPPGMNWTELITNYSPSREDNVQRVFTVASIRSMLIK

Figure 1. Amino acid sequence of the full rotavirus VP6 gene (marked amino acids are the 7 amino acid changes frequent in Egyptian clinical and environmental samples from the human rotavirus Wa strain amino acids)

AAGGAGGTAAACATATG*CATCATCACCACCACCAC*GAAGTCCTGTACAGCCTGTCGAAAACCTTAAAAG
ACGCACGCGATAAGATTGTTGAAGGCACGCTGTACAGCAATGTGTCCGATCTGATTCAACAGTTTAAT
CAGATGATCGTTACCATGAACGGCAATGATTTCCAGACGGGTGGCATCGGCAATCTGCCGGTCCGTAA
CTGGACCTTTGACTTCGGTCTGCTGGGTACCACGCTGTTGAACCTGGATGCTAACTATGTGGAGAATGC
GCGCACCATTATCGAATACTTTATCGATTTCATCGACAACGTTTGTATGGACGAAATGGCGCCTGAAA
GCCAACGCAATGGTGTTGCACCGCAGAGCGAAGCCCTGCGTAAACTGGCCGGTATTAAGTTTTTCAGT
ATCAACTTCGATAACTCTAGCGAGTATATTGAGAACTGGAATCTGCAAAACCGTCGTCAACGTACCGG
CTTCGTGTTCCACAAGCCGAACATTTTTCCTTATAGCGCCAGCTTTACTCTGAATAGAAGCCAGCCAAT
GCACGACAACCTCATGGGTACTATGTGGCTGAACGCCGGTTCTGAGATCCAAGTCGCGGGTTTCGATT
ACAGCTGCGCTATTAACGCACCGGCGAACATTCAGCAATTCGAGCATATCGTTCAACTGCGCCGTGCG
CTGACGACGGCAACCATTACGCTGTTGCCGGATGCGGAGCGTTTTTCCTTTCCACGCGTGATCAACTCC
AGCGACGGCGCGACCACCTGGTTTTTCAATCCGGTCATCTTGCGTCCGAATAACGTTGAAGTTGAGTTC
CTTCTGAATGGCCAAATCATTAATACCTATCAGGCACGTTTCGGTCTGATCATTGCGCGTAACTTTGAC
GCGATTCGCCTGTTGTTCCAGCTGATGCGTCCGCCGAACATGACTCCGGCTGTCAATGCATTGTTCCCG
CAGGCGCAGCCGTTTCAGCATCACGCGACCGTGGGTCTGACGCTGCGTATCCAGTCAGCCGTGTGCGA
AAGCGTACTGGCTGACGCAAATGAAACCCTGCTGGCGAATGTTACGGCCGTCCGCCAAGAGTACGCA
ATTCCGGTGGGTCCGGTCTTTCCGCCTGGTATGAATTGGACCGAGCTGATTACCAACTACAGCCCGAG
CCGCGAGGACAATGTGCAACGTGTGTTCACCGTTGCGAGCATCCGCAGCATGCTGATCAAGTAACTGC
AG

Figure 2. DNA sequence of full VP6 gene with NdeI and PstI restriction sites (underline codes), start and stop codons (bold codons), and histidine tag codons (italic codons) (marked nucleotides are the changes of the frequent sequences in Egyptian clinical and environmental samples in comparison to the human rotavirus Wa strain in the positions of the 7 amino acid changes)

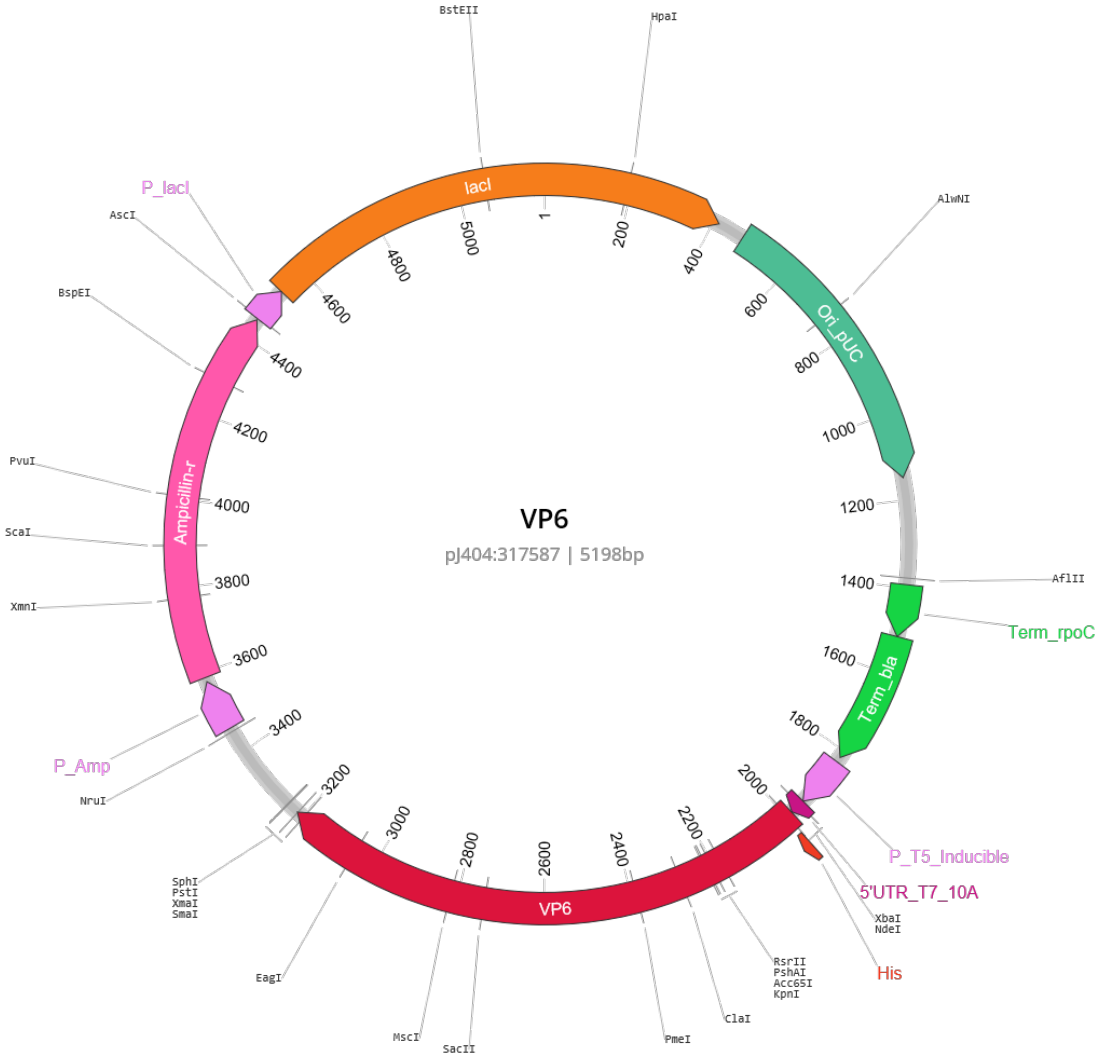

Figure 3. Physical map of recombinant expression plasmid pJ 404 and viral protein gene VP6 showing the position of His_6-tag and the two restriction sites NdeI and PstI (The physical map of recombinant expression plasmid was generated by CLC bio version 6.9.2)

Expression and purification of rVP6 protein

The expression of VP6 protein in BL21 bacterial cells was performed according to Sriyapai and coworkers (2011). Expressed VP6 protein was purified using Ni–IMAC affinity chromatography according to Rubiyana et al. (2015) and the eluted protein was recorded according to Warburg & Cristian (1942).

Determining the purified VP6 protein contents and purity

The purified protein content was determined using Bradford method (Bradford, 1976). The purity of VP6 protein was examined by polyacrylamide gel electrophoresis (SDS-PAGE) using Bio-Rad Mini-Protean apparatus (USA) (Laemmli, 1970), and the electrophoretic analysis was performed in the Mini-Protean II Dual-Slab Cell (BioRad, USA).

Preparation of rabbit anti-rVP6 antisera

Four *Chinchilla Bastard* male rabbits (~3kg each) were used for the production of rabbit anti-VP6 antibodies. They were divided into two groups (two rabbits were used for purified rVP6 protein, and two rabbits were used as controls). Immunization of the rabbits intramuscularly was performed using 20µg of the purified VP6 proteins, in 0.5mL of 0.9% saline, and mixed with an equal volume of Freund's complete adjuvant (Sigma Chemical

Co., St. Louis, MO, USA) on day 0. Following the first intramuscular immunization, the rabbits were boosted using other doses on days 14 and 28. The rabbits were bled from the marginal ear vein two weeks after the last booster dose. After serum collection and pooling, the antibodies production was confirmed by indirect ELISA according to Kim et al. (2002).

Detection of the total antibodies developed against rVP6 protein using immunoblotting

Immunoblot analysis was performed using a NovaBlot semidry blotter (LKB, Bromma, Sweden). Preparation of buffers and samples and the transfer procedure were carried out according to Towbin et al. (1979). The rabbit anti-rVP6 protein was used at 1:500 dilution in 0.01M Tris-HCl buffered saline, pH 7.5 containing 0.5% bovine serum albumin. The protein A peroxidase conjugate (PIRCE Chemical, USA) was used at 1:1000 dilution in the same buffer and 4-chloro-1-naphthol was used as a substrate.

Detection of total antibodies developed against recombinant VP6 protein using ELISA

ELISA was performed according to Kim et al. (2002). The absorbance values were determined at 490nm with ELISA reader Bio-Tek ELX 800. All measurements were made in duplicates, the results were expressed as the mean of absorbance, and the dilution that gives 0.5 O.D. was taken as the ELISA titre.

***In vitro* examination of the sensitivity of human Rotavirus VP6 antibodies through neutralization**

1. Titration of human rotavirus Wa strain using $TCID_{50}$

It was performed as described previously (Arnold et al., 2009). Twelve multiwall plates with 80% confluent monolayers were selected for rotavirus infection. Tenfold serial dilution for the activated human rotavirus Wa strain with trypsin (Sigma-Aldrich) to a final concentration of 10µg/mL was performed. Addition of post-infection DMEM with trypsin to a final concentration of < 2µg/mL was performed. Bi-fold dilutions were performed between the last two positive tenfold dilutions to have accurate results. Cytopathogenic effect (CPE) was observed during 7-day maintenance of the cells at 37°C to determine the end point titration of the virus whereas the last well with cell CPE was determined and multiplied by the dilution factor of the virus for determination of the virus titre.

2. Neutralization test with antibody mediated intracellularly

Introducing antibodies into cells was performed according to Caddy and coworkers (2020). Briefly, ten-fold dilutions and bi-fold dilutions between the last ten-fold dilutions of the antibodies were performed to examine the efficacy of the antibodies against human rotavirus Wa strain. Then, 2µL total volume of antibody or serum after serial dilutions was electroporated using 2 pulses of 1400V, 20-pulse width, into 3 x 10^5 cells suspended in 13µL Neon® Resuspension buffer R using the Neon® Transfection System. Cells were resuspended in 330µL antibiotic-free media with 10% fetal bovine serum before being added to the wells of a 96-well plate using 100µL per well and eight wells for each test. Cells were incubated at 37°C for 4h to become adherent to the plate and then washed once with PBS to remove any extracellular antibody. Then, 100µL of 2x 10^7 $TCID_{50}$/mL, 1 x 10^4 $TCID_{50}$/mL, and 1x 10^2 $TCID_{50}$/mL as high, moderate, and low viral titres of human rotavirus Wa strain kindly provided by Prof. Dr. Javier Buesa (University of Valencia, Spain) and activated with trypsin (Sigma-Aldrich) to a final concentration of 10µg/mL were added to each well. After 1h, removal of viral inoculum and washing the monolayer twice with serum-free DMEM medium were performed. Then, 100µL of post-infection serum-free DMEM were added to each well with 1µg/mL trypsin. CPE was observed during 7-day maintenance of the cells at 37°C (Ruggeri & Greenberg, 1991). Positivity was estimated using the neutralization index (Du et al., 2016). The neutralizing capability of the sera was measured by determining the presence or absence of virus-induced CPE. Neutralizing antibody titres were expressed as the reciprocal of the highest dilution of sera that completely inhibited virus-induced CPE in at least 50% of the wells (NT50) (Corthier, 1976).

Statistics

Percentages of reduction and neutralization index were estimated. To compare the sensitivity of the same concentrations of antibodies originated from expressed proteins of VP6 whole gene inoculated in rabbits against different titres of human rotavirus Wa strain infectious units, the kappa test was used according to http://epitools.ausvet.com.au/content.php?page=Compare2Tests.

RESULTS

Expression of VP6 protein

The analysis of SDS-PAGE showed the presence of a band with molecular weight of around 45kDa, which represents the VP6 protein. On the other hand, the protein band which represents the expressed VP6 protein was absent in the crude lysate of noninduced transformed cells (Figure 4).

Concentration of the expressed rVP6 protein

The protein concentration of lysate was approximately 1.877 ± 0.033 mg/mL of culture, while the concentration of the purified VP6 was 162 ± 0.005mg/L of culture.

Immunoblot analysis

The immunoblot analysis using the rVP6 protein and their homologous rabbit antisera showed that the homologous rabbit antisera were able to detect the rVP6 protein band at molecular weights of around 45kDa of the recombinant proteins, while the negative control serum did not stain any protein band **(**Figure 5).

Enzyme linked immunosorbent assay (ELISA)

The immunogenicity of the rVP6 protein was confirmed by ELISA, where the sensitivity and specificity of the antibodies produced in male rabbits against the tested VP6 protein indicated sensitivity up to 1/24000 dilution of antibodies against the expressed protein, and the results showed the production of high amount of homologous rabbit antisera **(**Figure 6**)**.

Sensitivity of neutralization for estimation of antibodies against purified VP6 expressed proteins of human Rotavirus Wa strain

Reduction of 1.4 log_{10}, 1.6 log_{10}, and 1.8 log_{10} of initial viral titres 2 x 10^7 $TCID_{50}$/mL, 1 x 10^4 $TCID_{50}$/mL, and 1 x 10^2 $TCID_{50}$/mL, respectively, was achieved with 1/10 dilution of the rabbit antisera produced against the tested VP6 protein using neutralization which depends on entrance of antibodies inside MA104 cells using electroporation. The reduction was less with the dilution of the antisera which are 1.2 log_{10}, 1.4 log_{10}, and 1.7 log_{10}; 0.9 log_{10}, 1 log_{10}, and 1.4 log_{10}; 0.8 log_{10}, 0.9 log_{10}, and 1.2 log_{10}; 0.8 log_{10}, 0.9 log_{10}, and 1.1 log_{10}; 0.6 log_{10}, 0.8 log_{10}, and 1 log_{10}; 0.6 log_{10}, 0.7 log_{10}, and 1 log_{10}; 0.4 log_{10}, 0.6 log_{10}, and 0.8 log_{10}; and 0.3 log_{10}, 0.4 log_{10}, and 0.7 log_{10} at dilutions 1/100, 1/1000, 1/2000, 1/4000, 1/6000, 1/8000, 1/10000, and 1/12000. At dilution 1/14000, 0.2 log_{10} was observed with an initial viral titre of 2 x 10^7 $TCID_{50}$/mL and no reduction in virus infectious units was observed with rabbit antisera dilutions of 1/16000 and above. On the other hand, and in case of the initial viral titre of 1x 10^4 $TCID_{50}$/mL, 0.3 log_{10} and 0.2 log_{10} reductions were achieved at antisera dilutions of 1/14000 and 1/16000 and no reduction in virus infectious units was observed with rabbit antisera dilutions of 1/18000 and above. At the lowest viral titre studied of 1 x 10^2 $TCID_{50}$/mL, 0.6 log_{10}, 0.4 log_{10}, and 0.2 log_{10} at dilutions of 1/14000, 1/16000, and 1/18000, respectively, were observed, while no reduction in virus infectious units was observed with rabbit antisera dilutions of 1/20000 and above (Figure 7).

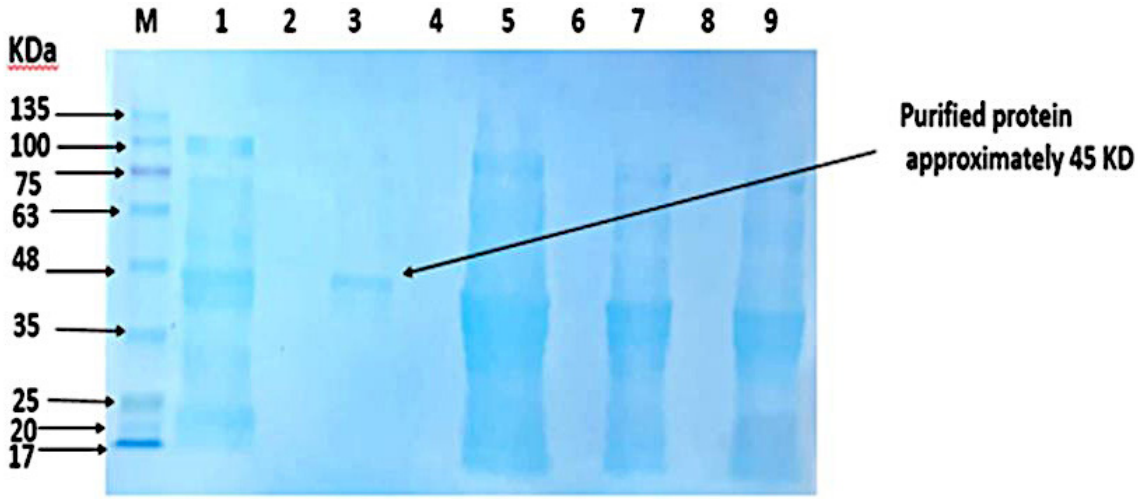

Figure 4. SDS-PAGE analysis of VP6 purification (Lane 1: cell lysate, lane 3: purified VP6 protein 45kDa, lanes 5, 7 and 9: wash fraction,lanes 2, 4, 6, and 8: empty, and M: protein molecular weight marker)

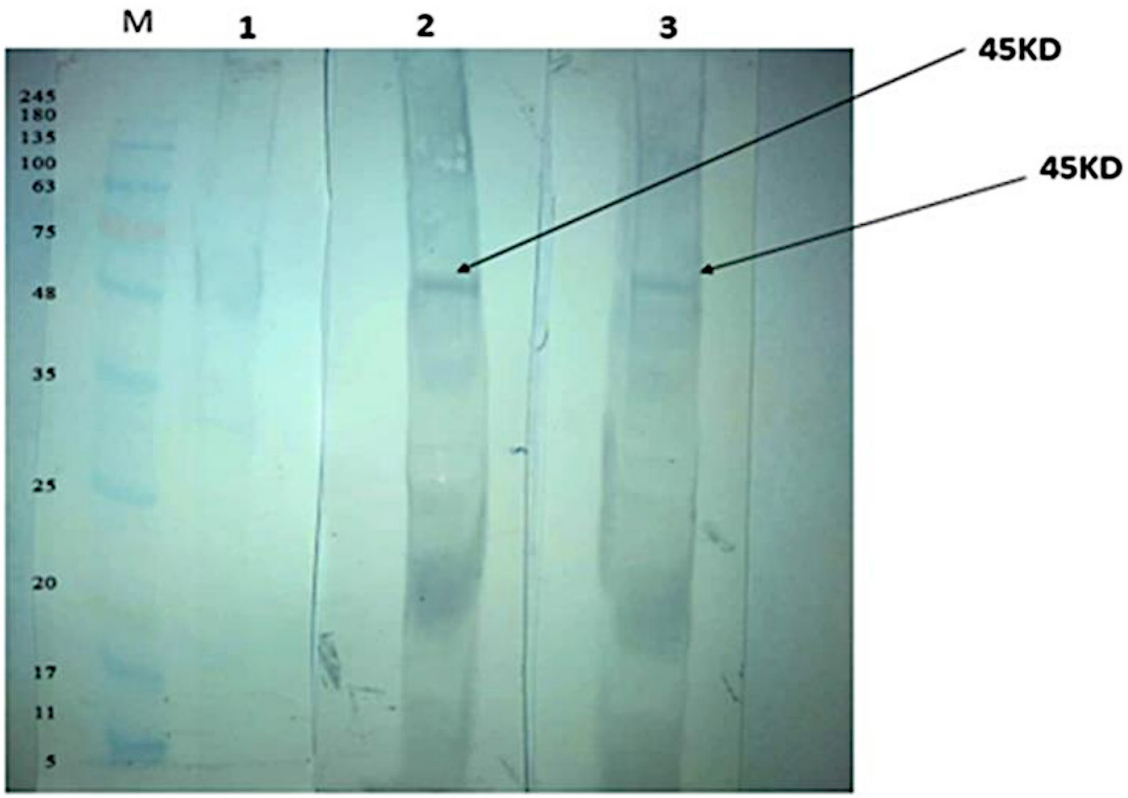

Figure 5. Immunoblot analysis of 15% continuous SDS-PAGE of the VP6 protein (Lanes 2 and 3: protein samples with homologous rabbit antisera, Lane 1: negative control, and M: molecular weight marker)

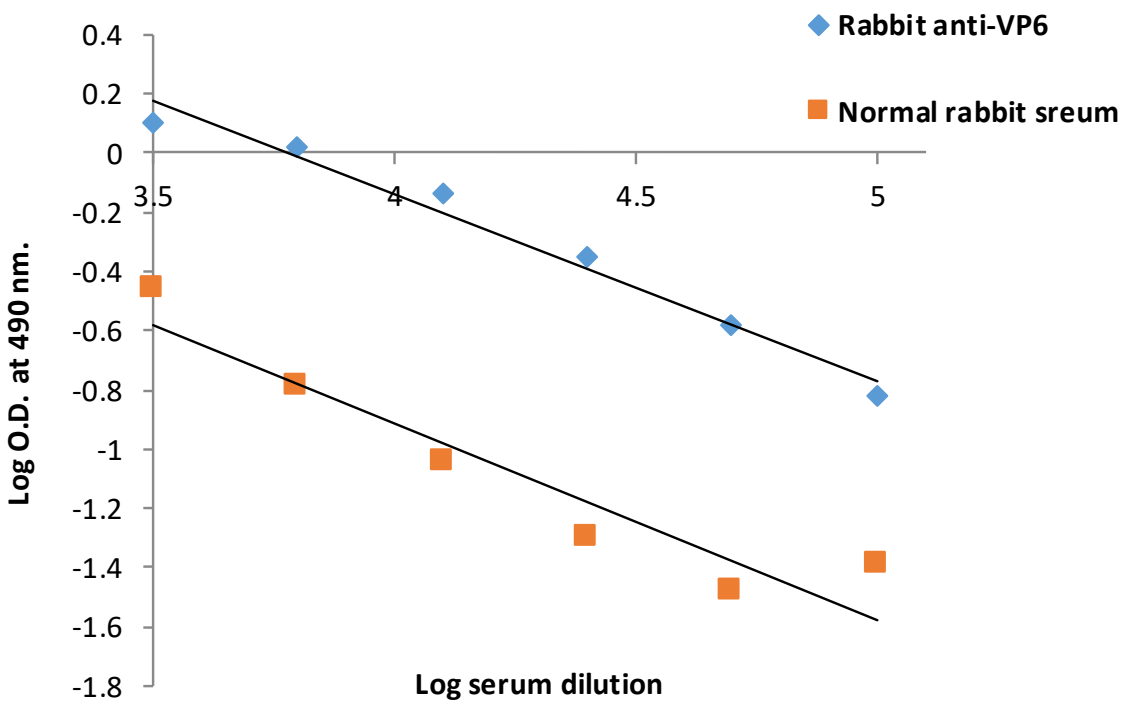

Figure 6. Relationship between the serum dilutions reciprocal and the corresponding OD values

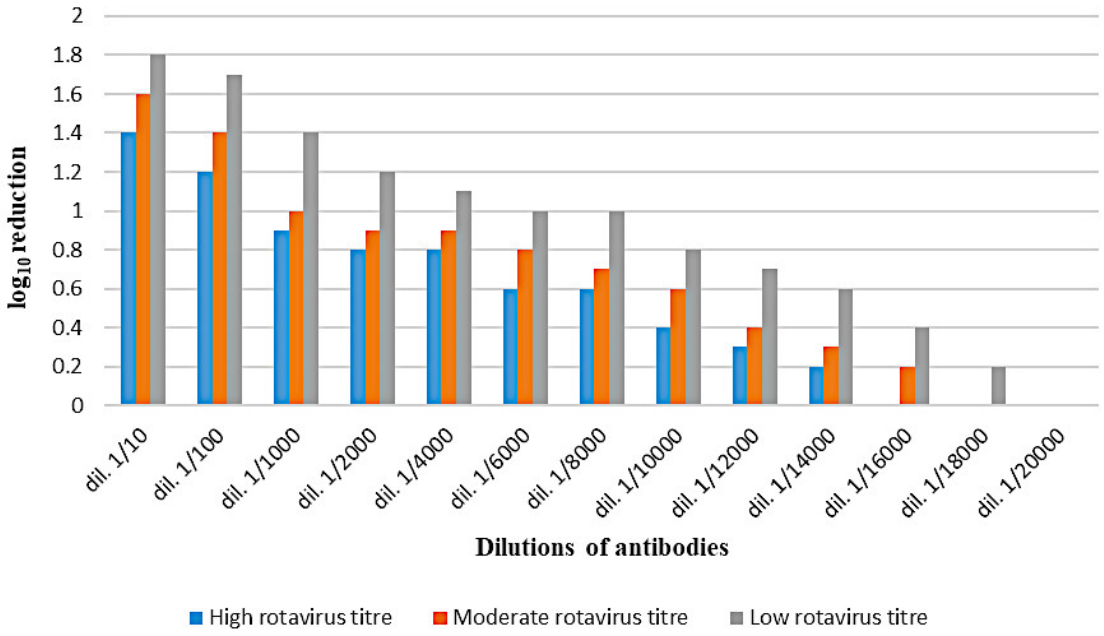

Figure 7. The relationship between the dilutions of VP6 protein antibodies and the $\log_{10}$ reduction of different titres of infectious human rotavirus Wa strain using neutralization depends on entrance of antibodies into MA104 cell line using electroporation

DISCUSSION

Vaccine efficiency and its immune response vary according to the type of vaccine (Jonesteller et al., 2017; Schmoele-Thoma et al., 2022). In our present study, the 1232 bp of VP6 gene represents the most frequent VP6 sequence in Egyptian clinical and environmental rotavirus isolates [with 98% nucleotide identity and 98% amino acid identity in comparison to human RoV Wa reference strain, GenBank nucleotides accession number: K02086.1 and amino acid accession number: P03530.1 with 7 amino acid changes (arginine changed to proline, lysine changed to phenylalanine, arginine changed to serine, alanine changed to serine, threonine changed to leucine, glutamic acid changed to glutamine, leucine changed to valine, at positions 102, 125, 126, 241, 279, 327, and 382, respectively)] with codon optimization expressed in *E. coli* by reassigning the native codons to codons frequently used in *E. coli.* Expression plasmid pJ 404 contains the inducible promoter site T5 (1232 bp) for induction of protein expression by IPTG, while the strong ribosome binding site for tight expression was used to have a recombinant protein as a subunit vaccine for human RVs. Rabbits' immunization with 20µg of rVP6 resulted in specific antibodies for the expressed protein which achieved positive results with ELISA up to a serum dilution of 1:24,000. This titre of antibodies is higher than the titre of antibodies obtained by Pastor et al. (2014) employing the same protein concentration. Codon optimization in our present study may be a reason for the elevation of the titre of antibodies. Another reason may be the difference in amino acids between our strain and the reference rotavirus Wa strain.

In our present study, a neutralization assay was used to estimate the potency of the antibodies which were formed in male rabbits inoculated with 1232 bp of VP6 purified expressed protein when a challenge with different titres (high, moderate, and low) of infectious human RV Wa strain *in vitro* was performed. Muruato and coworkers (2020) reported that virus neutralization remains the gold standard for determining antibody efficacy. The neutralization assay was performed by entrance of the antibodies into MA104 cell line because of the internal position of the VP6 protein in the rotavirus particle. Our results indicated a significant effect of the viral titre in the efficiency of the antibodies inside the cells to neutralize infectious human rotavirus Wa strain after viral penetration into the cells. The lower the titre of the virus, the more effective the antibody is in neutralizing the virus. This may be due in turn to the suitability of the antibody concentration in relation to the viral titre; however, promising viral infectious units' reduction was achieved even with the high viral titre (2×10^7 $TCID_{50}$/mL).

The efficiency could be considered promising and the reduction percentages in high, moderate, and low viral titre may indicate success of the electroporation process in entrance of antibodies into MA104 cell line. On the other hand, the results showed lesser efficiency of the antibodies of the VP6 expressed protein to achieve $\log_{10}$ reduction of the rotavirus Wa strain infectious units than the efficiency of the antibodies of the P[8] genotype expressed protein in the study of El-Senousy and coworkers (2024). This difference in results may be attributed to the different mechanisms of VP6 and VP8 antibodies in dealing with the rotavirus. VP8-expressed proteins produce neutralizing antibodies against outer capsid protein upon challenge with RV which prevent viral entry to the cells, while VP6 protein does not induce neutralizing antibodies, because it is an internal protein in the RV particle (Afchangi et al., 2019). VP6 antibodies act intracellularly after rotavirus uncoating inside MA104 cells and the release of DLPs which causes exposure of viral VP6 layer to the antibodies. Then, interruption of the viral replication cycle occurred (Feng et al., 2002; Estes & Greenberg, 2013; Desselberger, 2014). So, intracellular induction of VP6 antibodies was necessary to be performed in our present study to give the opportunity to VP6 antibodies to act intracellularly after the exposure of the viral VP6. Caddy and coworkers (2020) developed an assay to examine how antibodies neutralize rotavirus intracellularly. They showed that neutralization by VP6-specific IgG was much more efficient than VP6-specific IgA using simian rotavirus for challenge. In our present study, promising results were achieved when using the method of Caddy and coworkers (2020) to insert the antibodies of the expressed protein of 1232 bp of the most frequent VP6 of clinical and environmental isolates in Egypt into MA104 cells *in vitro* by electroporation and using high, moderate, and low titres of infectious viral units of human rotavirus Wa strain for challenge. On the other hand, the antibodies of genotype P[8]-expressed protein had higher efficiency than the antibodies of genotypes P[4]- and P[6]-expressed proteins in the study of El-Senousy and coworkers (2024). This may be because of the specificity of the expressed P[8] protein against human rotavirus Wa strain G1P[8] and this may be the same reason for higher efficiency of the antibodies of VP6 expressed protein than that of the antibodies of either P[4]- or P[6]-expressed proteins. The 1232 bp VP6 gene has 98% nucleotide identity and 98% amino acid identity in comparison to human RoV Wa reference strain. Another important reason for the higher efficiency of the P[8]-expressed protein antibodies is that the method used in the study of El-Senousy and coworkers (2024) was neutralization followed by RT-PCR and neutralization followed by nested RT-PCR which showed higher sensitivity than the neutralization assay. In our present study, we could not use these methods to improve the efficiency of the neutralization test in the efficiency evaluation of the antibodies against VP6 protein because of the different mechanisms of the antibodies of VP8 and VP6 proteins. VP8 antibodies are neutralizing antibodies that prevent neutralized viruses from cell entry, so, using RT-PCR and nested RT-PCR after the neutralization assay increased the sensitivity of the assay. RT-PCR and nested RT-PCR could detect positivity in some wells which do not show CPE using an inverted microscope because they could detect infectious viruses which entered the cells, but they are low or slow enough to be able to show CPE. So, using RT-PCR and nested RT-PCR after the neutralization assay gives accurate results of viral titre before and after exposure to the specific neutralization antibodies. If using only the neutralization assay, some wells could be neglected or considered without CPE; however infectious viruses could enter the cells. In case of VP6, it is difficult to use RT-PCR and nested RT-PCR after the neutralization assay because the VP6 antibodies are not neutralizing antibodies. Introducing the antibodies into the cells is a necessary process to give the antibodies the opportunity to neutralize the exposed viral VP6 after the viral uncoating process. Further research is needed to examine the efficiency of the human rotavirus expressed proteins' antibodies against infectious human rotavirus Wa strain in *vivo*; however, using *in vitro* studies before doing challenge in animals is very important to choose the promising candidate vaccines to be examined *in vivo.* Using VP6 antibody mediated intracellularly may be a promising solution to overcome the problem of the internal position of the VP6 in the rotavirus in case of *in vitro* examination of the vaccine efficiency. Determination of the efficient candidate vaccines *in vitro* before examination *in vivo* gives a lot of advantages such as use of a smaller number of animals *in vivo* which is preferable from an ethical point of view in addition to saving money and time.

Our results indicated that significantly higher

sensitivity for the antibodies of the 1232 bp represents the whole gene of the most frequent isolate of human rotavirus VP6 in clinical specimens (collected in 2015–2017) and sewage samples (collected in 2015–2018) from Greater Cairo, Egypt, than the sensitivity of the antibodies of the 155 bp epitope of the VP6 in the previous study of El-Senousy and coworkers (2013). The target in our present study represents 397 amino acids with 98% nucleotide identity and 98% amino acid identity in comparison to human RoV Wa reference strain, GenBank nucleotides accession number: K02086.1 and amino acid accession number: P03530.1 with 7 amino acid changes (arginine changed to proline, lysine changed to phenylalanine, arginine changed to serine, alanine changed to serine, threonine changed to leucine, glutamic acid changed to glutamine, leucine changed to valine, at positions 102, 125, 126, 241, 279, 327, and 382, respectively); however the 155 bp epitope represented 99% homology with rotavirus sequences with the accession numbers HQ392389, HQ738601, and HQ738599 with target sequence codes for 51 amino acids resembling amino acids from 282 to 332 of human rotavirus A inner capsid protein VP6 with accession number ADO78525. In our present study 1.4, 1.6, and 1.8 $\log_{10}$ reductions of infectious human rotavirus Wa strain were achieved using 1/10 dilution of the produced antibodies of the 1232 bp of human rotavirus VP6 when challenge with different titres (high, moderate, and low) of infectious human RV Wa strain *in vitro* was performed, while only 1 $\log_{10}$ reduction was achieved with undiluted antibodies of the 155 epitope of the human rotavirus VP6 in the previous study of El-Senousy and coworkers (2013). This significantly higher activity may be due to the higher efficiency of the antibodies of the whole gene to interrupt the viral replication cycle inside the MA104 cell line by neutralizing the exposed VP6 after viral uncoating than that of the antibodies of the short 155 bp fragment epitope in the study of El-Senousy and coworkers (2013). This short fragment epitope did not achieve successful prevention of rotavirus symptoms when challenge was performed in guinea pigs (data not shown), and this was the reason of using the whole VP6 fragment in our present study. Another important factor in our present study to achieve accurate results is the use of the method developed by Caddy and coworkers (2020) to insert the antibodies inside the MA104 cells using electroporation to be able to neutralize the exposed VP6 after viral uncoating. The amino acid changes in the whole VP6 gene with codon optimization of 1232 bp are 7 amino acid changes (arginine changed to proline, lysine changed to phenylalanine, arginine changed to serine, alanine changed to serine, threonine changed to leucine, glutamic acid changed to glutamine, leucine changed to valine, at positions 102, 125, 126, 241, 279, 327, and 382, respectively). The only amino acid change in the position of the 155 bp short fragment is the amino acid number 327 in which Glutamic acid changed to Glutamine. A large body of evidence indicates that antibodies targeting the VP6 protein of the middle capsid layer play a key role in protection against rotavirus infection. VP6-specific antibodies are produced to high titres in response to rotavirus infection or vaccination in animal models (Svensson et al., 1987a,b). So, promising results concerned with the efficiency of the VP6 expressed protein of the most frequent rotavirus VP6 whole gene sequence in Egypt could be added to this previous evidence especially for the pattern prevalent in Egypt and the same-model countries.

The problem if thinking of transferring this technology to industrial scale is the relatively low quantity of the protein concentration of lysate (1.877 ± 0.033mg/mL of culture) in relation to the concentration of the purified VP6 (162 ± 0.005mg/L of culture). It may indicate relatively low quantity and low solubility of the expressed VP6 protein. The quantity of immunogen is a critical factor in the process of vaccine production at industrial scale. The low quantity and low solubility are due to the insoluble nature of VP6 protein, and its native trimeric conformation assumed by the protein which might be too difficult for *E. coli* to process (Mathieu et al., 2001), causing the major disadvantage of this expression system. Another explanation is the overexpression of VP6 protein which leads to the accumulation of partially folded or misfolded protein. When protein translation occurs at a fast rate, protein folding is a challenge (Lorimer, 1996). The fast expression rate and misfolding of protein cause exposure of hydrophobic areas, which are hidden in the native form of the protein. These exposed hydrophobic areas have a very strong tendency to aggregate (Fink, 1998). This problem was suggested to be overcome by lowering the temperature used to induce the expression of rVP6 protein to slow down the expression rate to avoid misfolding. Also, optimizing IPTG concentration and induction time can help in obtaining a more efficient yield of soluble rVP6 protein (Masoudi

et al., 2021). In the present work, the effect of decreasing the temperature from 37°C to 20°C during the protein expression on the yield of soluble fraction of rVP6 protein was investigated and did not give a significant increase of soluble rVP6 protein fraction. Also, the effect of different IPTG concentrations (0.1, 0.25, 0.5, and 1mM) on the yield of soluble fraction of rVP6 protein was studied and did not enhance the yield of soluble rVP6 protein fraction (data not shown). Another proposed solution could be through the use of a molecular chaperone which will facilitate the proper folding of the bacterial expressed rVP6 proteins, through binding to and stabilizing unfolded or partially folded protein parts (Hartl et al., 2011; Liu et al., 2019). Notably, a large portion of the purified protein was lost during the washing process which was obvious on SDS-PAGE by the presence of VP6 protein in the wash fraction. Similar phenomena were reported by Afchangi et al. (2022), who observed a partial loss of VP6 protein trimer structures in western blot assays. This indicates the need to use a different purification method, like ion exchange or gel filtration, to minimize the loss of the protein during the purification process.

E. coli BL21 strain, which was used in this study, is the most commonly used expression system for rVP6 protein (Afchangi et al., 2019; Li et al., 2019), although several studies explored different expression systems including eukaryotic systems such as insect cells through a baculovirus expression vector (Gomez-Sebastian et al., 2012), yeast including *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Hansenula polymorpha* (Da Silva & Srikrishnan, 2012; Bredell et al., 2016), mammalian cells (Pourasgari et al., 2007), viruses such as herpes simplex virus 1 (HSV-1)-based vectors (Palacios et al., 2015), and plant cells (Castillo-Esparza & Gómez-Lim, 2021). The relatively low protein concentration of lysate obtained in this study may indicate the need to use another expression system in future studies as one of the suggested solutions to increase the amount of the expressed protein. Using a different expression system (*eukaryote* instead of *prokaryote*), such as yeast, plant, or mammalian cells, may offer new advantages and be a better expression system, avoiding *E. coli*-related problems (Da Silva & Srikrishnan, 2012; Bredell et al., 2016). On the other hand, *E. coli* has some advantages, making it still commonly used as an expression system for different proteins despite the problems associated with it. These advantages include its ability to produce high amounts of protein. It is time saving compared to other expression systems which require more time to grow, like yeast. Another advantage is its low cost, and most important is the *E. coli* ability to produce higher VP6 protein yield compared to yeast on volumetric specific productivity levels (Bredell et al., 2016). Therefore, optimizing the conditions for VP6 protein expression in bacterial cells, such as *E. coli* codon adaptation index, codon pair bias, and guanine-cytosine content, as well as redesigning ribosome binding site, can increase the yield of expressed rVP6 and solve the main problems associated with prokaryotic expression system (Masoudi et al., 2021).

Future research is needed to study the homotypic and heterotypic efficiency of the antibodies of this 1232 bp VP6 expressed protein *in vivo*.

CONCLUSIONS

First, antibodies against the expressed protein of the 1232 bp of the most frequent full-length VP6 in clinical and environmental isolates in Egypt have a promising sensitivity rate against the human RoV Wa reference strain (homotypic) *in vitro*. Second, using a VP6 antibody mediated intracellularly may be a promising solution to overcome the problem of the internal position of the VP6 in the rotavirus when examination of rotavirus candidate vaccine efficiency is performed *in vitro*. Third, the promising efficiency of VP6 antibodies after entrance into the MA104 cell line using electroporation to neutralize human rotavirus Wa strain even in high titre gives a chance for the research of VP6 recombinant subunit vaccine evaluation *in vitro*. Finally, the low amount of soluble expressed protein requires further investigation to increase the solubility of the expressed VP6 protein, which is a critical point when producing the vaccine at an industrial scale.

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