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Immunoperoxidase and neutralization/RT-PCR to estimate the efficiency of the human rotavirus VP8 structural protein candidate subunit vaccine

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Abstract

Improving the efficiency of the methods to estimate the sensitivity and specificity of vaccines is of great need and value. Immunoperoxidase and neutralization/RT-PCR were accomplished to estimate the efficiency of the human rotavirus VP8 structural protein candidate subunit vaccine previously examined using a neutralization test in another study of our group. This neutralization test was performed for expressed proteins of common P genotypes (P8, P4, and P6) of human rotaviruses either separately or as a mix. In our present study, results showed that 1.4, 1.6, and 2 $\log_{10}$ reduction of initial viral titre (10^5 infectious viral units) were achieved with 1/32 dilution of the rabbit anti-sera which produced against the tested P8 protein using immunoperoxidase, neutralization followed by RT-PCR, and neutralization followed by nested RT-PCR respectively. Also, 0.9 $\log_{10}$, 1.1 $\log_{10}$, and 1.5 $\log_{10}$ reductions of initial viral titre were achieved with 1/32 dilution of the rabbit anti-sera which produced against the tested P4 and P6 proteins using immunoperoxidase, neutralization followed by RT-PCR, and neutralization followed by nested RT-PCR respectively. The capacity of neutralization decreased with increasing the dilution of rabbit anti-sera. No significant difference was observed between the results of immunoperoxidase in relation to the neutralization test. Neutralization followed by RT-PCR for rotaviruses in MA104 cells infected with viruses showed higher sensitivity in the estimation of the human rotavirus Wa strain titre directly or after neutralization with antibodies of P8, P4, and P6 expressed proteins. Neutralization followed by nested RT-PCR may be recommended to estimate the efficiency of the human rotavirus VP8 candidate recombinant subunit vaccine.

Keywords: Human rotavirus, VP8, Recombinant subunit vaccine, Immunoperoxidase, Neutralization/RT-PCR

1. Introduction

Rotaviruses belong to the *Reoviridae* family and contain genomes consisting of eleven segments of dsRNA. The International Committee on Taxonomy of Viruses (ICTV) recognized eight species within the rotavirus genus: *Rotavirus* A, B, C, D, E, F, G, and H [1]. Viruses of the rotavirus group A (RVA) species had been the most widely studied, owing to their significance as the prime cause of severe diarrhea in infants and young children [2]. The World Health Organization (WHO) global rotavirus surveillance network estimated that the annual rotavirus-associated mortality is approximately 215,000 worldwide in children <5 years of age [3].

Rotavirus infections are widespread and by the age of five most children have had more than one rotavirus infection. Though children three months of age and younger, may not develop diarrhea symptoms when they are infected with rotavirus since they have transplacental maternal antibodies to protect them in the first few months of life [4]. The first infections are usually the most severe and take place when circulating maternal rotavirus-specific immunoglobulin G is declining. Recovery from a first rotavirus infection does not lead to permanent immunity, generally. Subsequent to several rotavirus infections, children develop natural antibodies that shield them from symptoms of diarrhea when they are reinfected. Consequent or recurrent rotavirus infections affect people of all ages and confer

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gradually more protection against severe disease [5-6].

Vaccination is considered the most effective public health strategy to prevent rotavirus disease and reduce the global burden of rotavirus gastroenteritis [7]. Vaccines take advantage of using relatively harmless foreign agents to evoke protective immunity for protection against several important pathogens. Vaccines can be divided according to route of administration into orally and parentally administrated vaccines. Nowadays, many types of vaccines have been proposed and used in vaccine development such as live attenuated whole virus vaccines and non-living viral vaccines [8].

Although clinical trials of both RV1 (Rotarix®) and RV5 (RotaTeq®) vaccines demonstrated high efficacy (generally >85%) against severe rotavirus disease in high-income countries, trials performed in low-income countries of Asia and Africa have reported lower vaccine efficacy [9-12]. Clinical trials using RV5 conducted in Vietnam and Bangladesh demonstrated an efficacy of 51% [9]. Clinical trials using RV1 conducted in South Africa found the vaccine to be 40–64% effective [11].

Reasons for lower vaccine efficacy in developing settings are multifactorial. The presence of pre-existing maternal antibodies in the infant has been shown to neutralize vaccine antigen in the gut and also reduce the infant's immune response to vaccine antigen [13-15].

Recently, environmental enteropathy (EE) is recognized as an important factor in lowering oral vaccine efficacy in the developing world [16]. EE can be defined as chronic intestinal inflammation and dysfunction as a result of frequent intestinal infections. Villous blunting, chronic inflammation, and increased intestinal permeability are major characteristics of EE [17]. In developing countries, water and food are highly contaminated with a variety of microbes. Dweller Person from these regions has high oral intake of this type of contaminated food and water. This results in a high microbiota load in the intestine. It leads to chronic activation of the mucosal immune system and an altered intestinal immune system [18-19]. Under this circumstance, intestinal immune cells are constantly engaged in preventing infection by the microbiota. For this reason, the preoccupied cells show less affinity for oral vaccine antigens and thereby dampen immunity [20-21].

So, looking for safe and efficient vaccines is of great importance. Our group (Food-Borne Viruses Group) in the National Research Centre (Giza, Egypt) developed a recombinant subunit human rotavirus vaccine based on the common P types from Egyptian human rotavirus isolates (Seham F. Hasan et al., manuscript in preparation). The sensitivity of this vaccine was examined using a neutralization assay. In this present work, our objective is to improve the efficiency of the methods used to determine the sensitivity of the examined vaccine using immunoperoxidase and neutralization followed by nested RT-PCR.

2. Materials and Methods

2.1. Human rotavirus Wa strain propagation

It was performed according to Arnold et al. [22]. Human rotavirus Wa strain (kindly provided by Prof. Dr. Javier Buesa, University of Valencia, Spain) was activated with trypsin to a final concentration of 10 µg/ml for 30 minutes. MA104 cell line of 80% confluent monolayer was used for viral propagation. Growth media was discarded and the cells were washed twice with serum-free media (Eagle's minimum essential medium). Activated human rotavirus Wa strain was inoculated on MA104 cell line and kept at 37 °C for 1 h to facilitate virus adsorption onto the cells. Following adsorption, virus inoculum was removed and washing the confluent monolayer once using serum-free media was performed. Serum-free maintenance medium containing trypsin (1 µg/ml) was added. Tissue culture flasks containing the monolayers which were incubated at 37°C were observed at 24 h intervals, under an inverted microscope for any alteration of cell morphology up to 4–5 days post infection. Thereafter, the plates were kept at -20 °C. For lysis of the rotavirus infected cells, 3 cycles of freezing-thawing were done. The inoculum obtained was clarified by centrifugation at 1,000 g for 15 min. The fluid containing the virus was aliquoted in small screw capped vials and stored at -20 C until further use.

2.2. Titration of human rotavirus Wa strain

Twelve multiwall plates with 80% confluent monolayer were selected for rotavirus infection. Ten-fold serial dilution for the activated human rotavirus Wa strain was performed as described above and virus and dilutions were inoculated separately on MA104 cell line and kept at 37 °C for 1 h to facilitate virus adsorption onto the cells. The inoculated cells were washed with serum-free media (Eagle's minimum essential medium). Serum-free maintenance medium containing trypsin (1 µg/ml) was added. Plates containing the monolayers were incubated at 37 °C and were observed at 24 h intervals, under an inverted microscope for any alteration of cell morphology up to 4–5 days post-infection to observe the cytopathogenic effect (CPE). Thereafter, the plates were kept at -20 C. For lysis of the rotavirus infected cells, 3 cycles of freezing-thawing were done. The inoculum obtained was clarified by centrifugation at 1,000 g for 15 min. The fluid containing the virus (if

present) was aliquoted in small screw capped vials and stored at -20 C until further use [22].

2.3. Neutralization/RT-PCR assay

This was performed according to Ruggeri and Greenberg [23], using 10^5 PFU of human rotavirus Wa strain kindly provided by Prof. Dr. Javier Buesa (University of Valencia, Spain) and with some modifications using Median Tissue Culture Infectious Dose (TCID50) instead of plaque assay to calculate the reduction of viral titer using the antibodies of expressed proteins of three sequences of rotavirus VP8 partial gene fragment of the VP4 from the most frequent Egyptian human rotavirus P genotypes (P[8], P[4] and P[6]) of our previous study [24] with codon optimized. These produced antibodies previously performed (Seham F. Hasan et al., manuscript in preparation) were used separately and in combination with an equal serum volume of each genotype. Ten-fold dilutions, followed by bi-fold dilutions, were performed to examine the efficacy of the antibodies against the human rotavirus Wa strain. Each experiment was repeated three times. In the normal neutralization, only observation of CPE is usually performed and the last well containing CPE usually determines the titre of the virus either directly or neutralized with monoclonal or polyclonal antibodies. In our study, RT-PCR was performed for MA104 cells (after freezing, thawing, and centrifugation) of all wells previously inoculated with serial dilutions of the human rotavirus Wa strain, either directly or neutralized with antibodies produced in rabbits for the common P8, P4, and P6 genotypes of human rotavirus Egyptian isolates. Nested RT-PCR was performed. Results were estimated according to the neutralization index for the eight wells for direct virus (without dilution) and for each dilution [25].

2.4. Viral Nucleic Acid Extraction

Viral nucleic acid was extracted from 100 µl of the supernatant by BIOZOL Total RNA Extraction reagent (BioFlux, Japan) according to the manufacturer's instructions.

2.5. Nested RT PCR of a VP6 Coding Gene Fragment of Rotaviruses Group A

The primers used for RT-PCR to amplify a 379-bp region were the forward VP6-F 5′-GACGGVGCRACTACATGG T-3′ and the reverse VP6-R 5′- GTCCAATTCATNCCTGGT GG-3′ primers (1 µM of each) and according to Iturriza Gomara et al. [26] using 100 units of M-MLV reverse transcriptase enzyme (Thermo Fisher) in a total volume of 10 µl and 2.5 units of Taq DNA polymerase (Thermo Fisher) in a total volume of 50 µl. Nested PCR amplifcation of the target RT-PCR products was performed using the forward VP6-NF 5′-GCWAGAAATTTTGATACA-3′ and the reverse VP6-NR 5′-GATTCACAAACTGCAGA-3′primers 1 µM of each and according to Gallimore et al. [27] to amplify a 155 bp fragment. Ten µl of PCR products were analyzed by 3% agarose gels (Panreac-Spain). Nested RT-PCR was performed separately to the 8 wells for each dilution, which was examined for CPE using the neutralization test, and positivity was estimated using the neutralization index.

2.6. Indirect immunoperoxidase test (I-IPT)

Indirect IPT was performed as per the method described in the European Union diagnostic manual [28] with slight modification. MA104 cell monolayer that attaining approximately 80 % cell growth in 96 well tissue culture plate was infected with human rotavirus Wa strain as described above for 4–5 days. For fixation of cells, acetone/methanol (1:1) solution was added at a volume of 100 µl/well and left for 10 min at room temperature. The cell monolayer was treated with 0.4 % Triton X-100 (in PBS-T) for 5 min. The non-specific binding sites were blocked by 2 % BSA (in PBS-T) for 1 h at room temperature. Thereafter, 50 µl of polyclonal pig anti-rotavirus antibody (1:100 in 1 % BSA) was added to the wells. The plate was incubated for 1 h at 37 ºC. After that, the plate was washed twice with PBS-T for 5 min. Then, rabbit anti-swine HRPO conjugate (DAKO, Denmark) diluted 1:1000 in 1 % BSA was added at the rate of 50 µl/well. The plate was incubated again at 37 ºC for 1 h. After washing with PBS-T three times, the wells were filled with 50 µl of chromogen-substrate solution and stained for 15–30 min at room temperature. After discarding the substrate, the wells were washed with deionized water to stop the colour reaction. The plate was observed under an inverted microscope (Zeiss, Primo Vert, Germany) for evaluation of the results. Cells showing dark red staining of the cytoplasm were considered to be infected by rotavirus.

2.7. Statistics

Percentages of reduction and neutralization index were estimated for neutralization followed by RT-PCR and neutralization followed by nested RT-PCR. To compare the performance of used tests that were evaluated on the same antibodies originated from expressed proteins of P8, P4, and P6 partial genes inoculated in rabbits, the kappa test was used accordingto http://epitools.ausvet.com.au/content.php?page=Compare2Tests [29].

3. Results

3.1. Cytopathogenic effect (CPE) of human rotavirus Wa strain

A cytopathogenic effect was observed on MA104 cells after three days of infection with the human rotavirus Wa strain. The cytopathogenic effect was observed with different intensity according to the viral titre from virus without dilution till virus diluted to 10^{-3}. The shape of the cytopathogenic effect on the MA104 cell line infected with the human rotavirus Wa strain in relation to control cells is shown in Fig. 1 (A, B, and C).

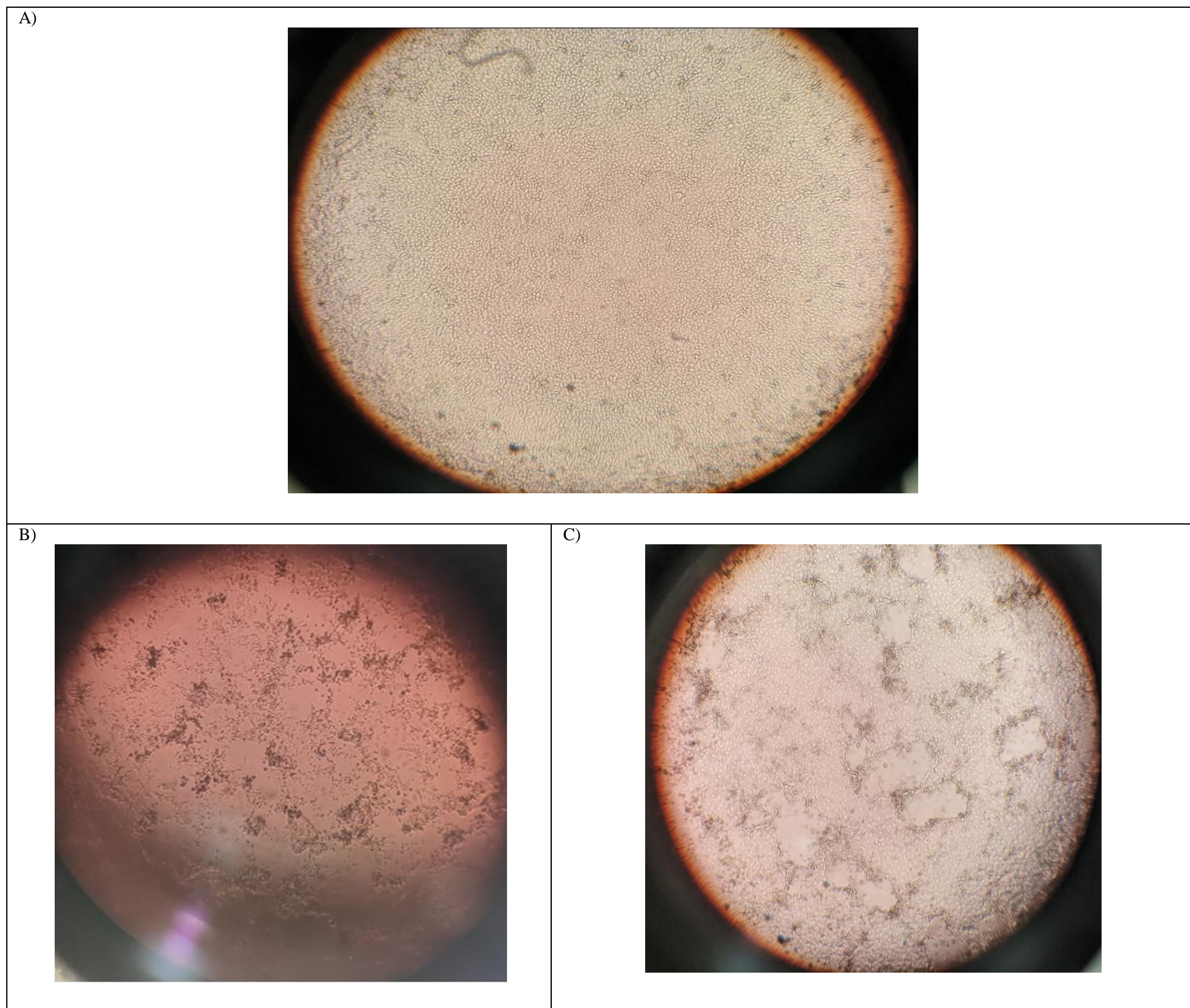

Fig. 1. The shape of cytopathogenic effect on MA104 cell line infected with human rotavirus Wa strain in relation to control cells. A) Control MA104 cell line which was not infected with human rotavirus Wa strain after 3 days of incubation at 37ºC. B) CPE on MA104 cell line infected with direct human rotavirus Wa strain without dilution after 3 days of incubation at 37ºC. C) CPE on MA104 cell line infected with direct human rotavirus Wa strain diluted to 10-3 after 3 days of incubation at 37ºC.

3.2. Sensitivity of immunoperoxidase and neutralization followed by RT-PCR for estimation of antibodies against expressed proteins P8, P4, and P6 human rotavirus genotypes

1.4, 1.6, and 2 log_{10} reduction of initial viral titre (10^5 infectious viral units) were achieved with 1/32 dilution of the rabbit anti-sera which produced against the tested P8 protein using immunoperoxidase, neutralization followed by RT-PCR, and neutralization followed by nested RT-PCR respectively. The reduction was reduced with the dilution of the antisera which 1.1 log_{10},1 log_{10}, 0.7 log_{10}, 0.5 log_{10}, and 0.3 log_{10}, reductions were achieved with dilutions 1/100, 1/1000, 1/5000, 1/10000 and 1/15000 dilutions of the rabbit anti-sera using immunoperoxidase. Also, 1.3 log_{10}, 1.2 log_{10}, 0.8 log_{10}, 0.7 log_{10}, and 0.5 log_{10}, reductions were achieved with dilutions 1/100, 1/1000, 1/5000, 1/10000 and 1/15000 dilutions of the rabbit anti-sera using neutralization followed by RT-PCR. Using neutralization followed by nested RT-RT-PCR, 1.6 log_{10}, 1.5 log_{10}, 1 log_{10}, 0.8 log_{10}, and 0.7 log_{10}, reductions were achieved with dilutions 1/100, 1/1000, 1/5000, 1/10000 and 1/15000 dilutions of the rabbit anti-sera. No reduction was observed for viral infectious units with a dilution of 1/20000 of the rabbit anti-sera using immunoperoxidase and neutralization followed by RT-PCR, while a 0.3 log_{10} reduction was observed using neutralization followed by nested RT-PCR. No reduction was observed for all the methods used with a dilution of 1/25000 of the rabbit anti-sera of the expressed protein of human rotavirus P8 genotype. Also, 0.9 log_{10}, 1.1 log_{10} and 1.5 log_{10} reductions of initial viral titre (10^5 infectious viral units) were achieved with 1/32 dilution of the rabbit anti-sera which produced against the tested P4 and P6 proteins using immunoperoxidase, neutralization followed by RT-PCR, and neutralization followed by nested RT-PCR respectively. The reduction was reduced with the dilution of the antisera which 0.6 $log_{10,}$ 0.6 log_{10}, and 0.4 log_{10} reductions were achieved with dilutions 1/100, 1/1000, and 1/5000 of the rabbit anti-sera for both P4 and P6 proteins using immunoperoxidase. Also, 0.9 log_{10}, 0.8 $log_{10,}$ and 0.6 log_{10} reductions were observed using neutralization followed by RT-PCR and 1.1 log10, 1.1 log10, and 0.9 log10 were observed using neutralization followed by nested RT-PCR. No reduction was observed for viral infectious units with a dilution of 1/10000 of the rabbit anti-sera for P4 and P6 proteins using immunoperoxidase and neutralization followed by RT-PCR, while, 0.2 log_{10} reduction was observed using neutralization followed by nested RT-PCR. No reduction was observed for viral infectious units at dilution 1/15000. Mixed P8, P4, and P6 antibodies achieved 1.1 $log_{10,}$ reduction of initial viral titre (10^5 infectious viral units) with 1/32 of dilution of the rabbit anti-sera using immunoperoxidase.

Also, 0.9 log_{10}, 0.8 log_{10}, 0.6 log_{10}, and 0.4 log_{10}, reductions were achieved with dilutions 1/100, 1/1000, 1/5000, and 1/10000 of the rabbit anti-sera respectively. Using neutralization followed by RT-PCR, 1.4 $log_{10,}$ 1.2 $log_{10,}$ 1 $log_{10,}$ 0.7 log_{10}, and 0.5 log_{10} were achieved with dilutions of 1/32, 1/100, 1/1000, 1/5000, and 1/10000 respectively, while using neutralization followed by nested RT-PCR, 1.7 $log_{10,}$ 1.5 log_{10},1.3 log_{10}, 1 log_{10}, and 0.8 log_{10} were achieved with dilutions 1/32, 1/100, 1/1000, 1/5000, and 1/10000 respectively. No reduction was observed for viral infectious units with dilution 1/15000 of the rabbit anti-sera using immunoperoxidase and neutralization followed by RT-PCR, while a 0.2 log_{10} reduction was observed using neutralization followed by nested RT-PCR. No reduction was observed for viral infectious units at dilution 1/20000 (Fig. 2, 3, 4, and 5). Some wells with low CPE and other wells with no CPE observed on the MA104 cell line showed positive results using nested RT-PCR.

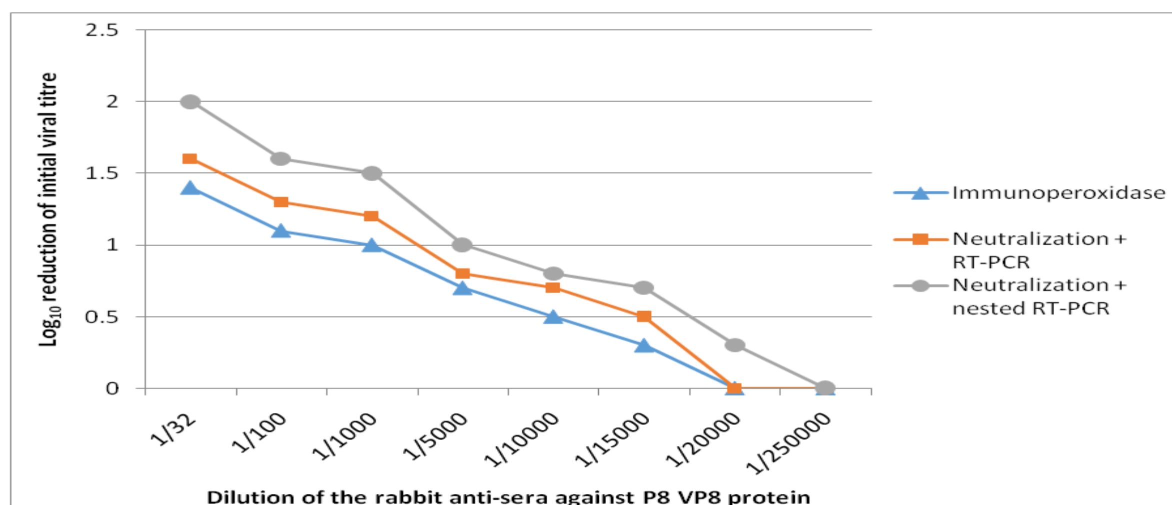

Fig. 2. Log_{10} reduction of initial human rotavirus Wa strain titre using antibodies of expressed protein of P8 genotype.

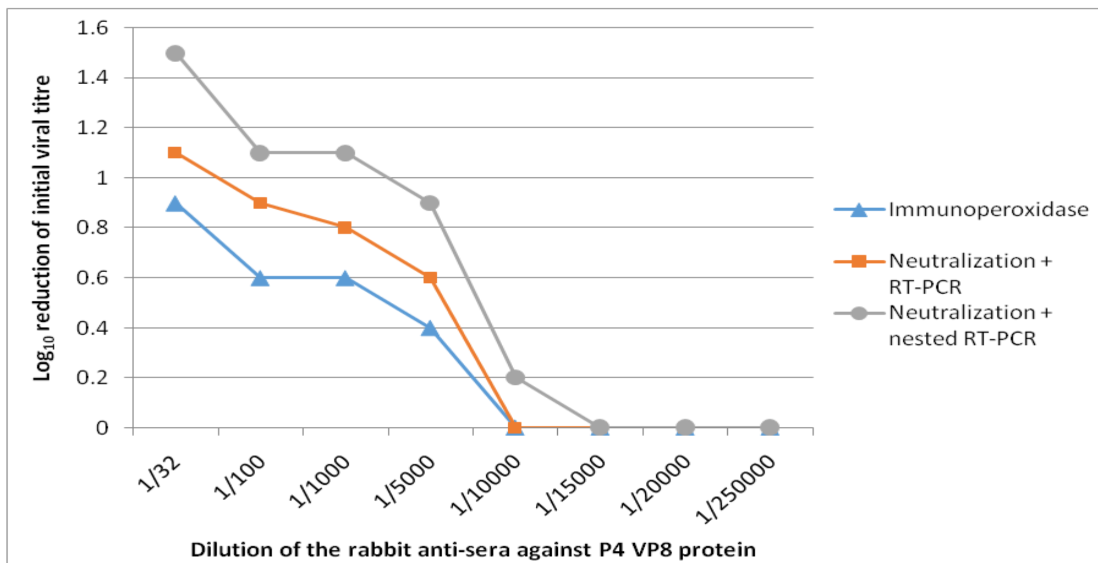

Fig. 3. Log_{10} reduction of initial human rotavirus Wa strain titre using antibodies of expressed protein of P4 genotype.

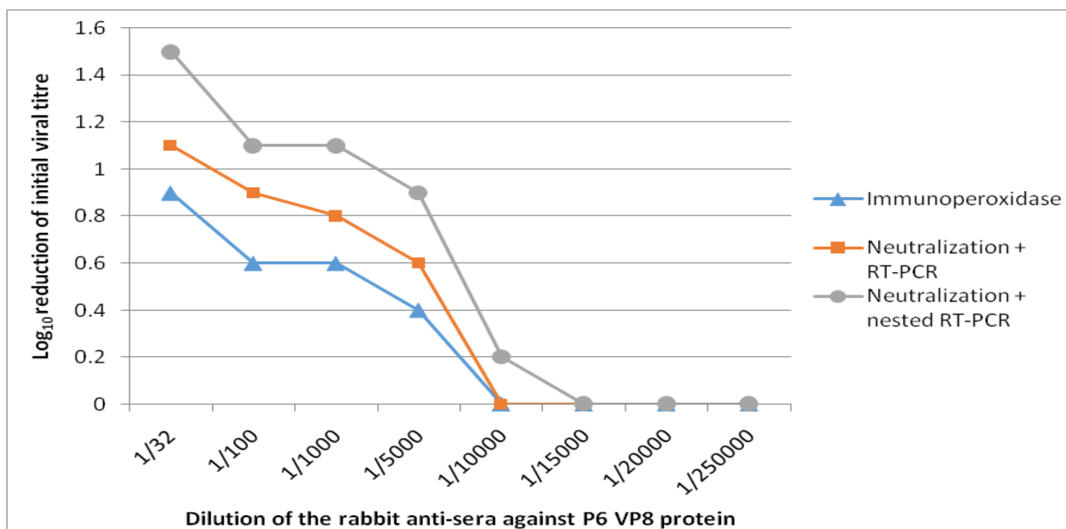

Fig. 4. Log_{10} reduction of initial human rotavirus Wa strain titre using antibodies of expressed protein of P6 genotype.

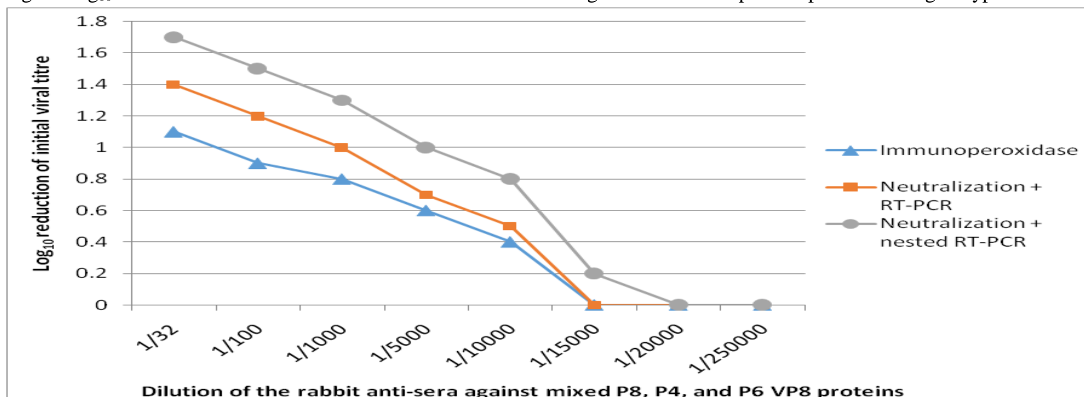

Fig. 5. Log_{10} reduction of initial human rotavirus Wa strain titre using antibodies of expressed protein of mixed P8, P4, and P6 genotypes.

4. Discussion

Sensitivity and specificity are usually the most important factors to estimate the quality of test methods [30]. On the other hand, the accuracy of test methods to estimate the efficiency of vaccines is of great concern [31]. In a previous work of our group (Seham F. Hasan et al., manuscript in preparation), a neutralization test was used to estimate the efficiency of antibodies produced in rabbits inoculated with expressed proteins of the most frequent isolates of the P8, P4, and P6 genotypes of human rotaviruses in Egypt to the neutralize infectious human rotavirus Wa strain. 1.5 log_{10} reduction of initial viral titre (10^5 infectious viral units) were achieved with 1/32 dilution of the rabbit anti-sera which produced against the tested P8 protein. The reduction was reduced with the dilution of the antisera which 1.2 log_{10}, 1 log_{10}, 0.8 log_{10}, 0.6 log_{10}, and 0.3 log_{10} reductions were achieved with dilutions 1/100, 1/1000, 1/5000, 1/10000 and 1/15000 of the rabbit anti-sera. No reduction was observed for viral infectious units with a dilution of 1/20000 of the rabbit anti-sera. Also, 0.9 log_{10} reduction of initial viral titre (10^5 infectious viral units) was achieved with 1/32 dilution of the rabbit anti-sera which produced against the tested P4 and P6 proteins. The reduction was reduced with the dilution of the antisera which 0.7 log_{10}, 0.5 log_{10}, and 0.3 log_{10}, reductions were achieved with dilutions 1/100, 1/1000, and 1/5000 of the rabbit anti-sera for both P4 and P6 proteins. No reduction was observed for viral infectious units with dilution 1/10000 of the rabbit anti-sera for either P4 or P6 proteins respectively. Mixed P8, P4, and P6 antibodies achieved 1.2 log_{10} reduction of the initial viral titre (10^5 infectious viral units) with 1/32 dilution of the rabbit anti-sera. Also, 1 log_{10}, 0.8 log_{10}, 0.5 log_{10}, and 0.3 log_{10} reductions were achieved with dilutions 1/100, 1/1000, 1/5000, and 1/10000 of the rabbit anti-sera. No reduction was observed for viral infectious units with a dilution of 1/15000 of the rabbit anti-sera (Seham F. Hasan et al., manuscript in preparation). In our present study, different methods were used to compare their efficiencies in estimation of the potency of the antibodies to neutralize the human rotavirus Wa strain in relation to neutralization test. Our results indicated no significant difference in the efficiency of immunoperoxidase in relation to neutralization test for antibodies of all separate proteins of P8, P4, and P6 and also for P genotypes mix. Previous reports showed comparable sensitivity between immunoperoxidase and neutralization test in the detection of antibodies to chicken anaemia agent [32] and canine distemper virus [33]. Neutralization followed by RT-PCR or nested RT-PCR has significant higher efficiency in estimation of the capacity of antibodies to neutralize infectious virus. The values were higher in all dilutions examined. On the other hand, neutralization followed by nested RT-PCR is more sensitive than neutralization followed by RT-PCR. A lot of previous reports were concerned with the higher sensitivity of nested RT-PCR than RT-PCR [34-37]. This may return to the higher number of amplification cycles to amplify a shorter fragment in the nested RT-PCR. The end point dilution at which neutralization could be happened was higher with neutralization followed by nested RT-PCR than the other used methods. Not only nested RT-PCR but also other confirmatory methods could be used to confirm the specificity and increase the sensitivity of the test such as southern blot hybridization. Villena and co-workers [38] reported higher positivity of rotaviruses using southern blot hybridization as a confirmatory method in relation to the positivity using only RT-PCR in environmental samples collected from Cairo and Barcelona.

On the other hand, using RT-PCR after neutralization may have some other advantages. The cytopathogenic effect of the human rotavirus Wa strain is sometimes slow, especially for the high dilutions of the virus, which means a low viral titre. So, sometimes it is difficult to determine the end point of viral titre either as viral titration or viral titre after neutralization with specific antibodies. Using the neutralization index to estimate the viral titre or efficiency of antibodies to neutralize the virus, some wells or dilutions could be neglected, however they contain infectious virus. RT-PCR and nested RT-PCR may be a suitable solution to confirm the positivity of these neglected wells or dilutions. In our present study, some wells had very low CPE or almost no observable CPE on MA104 cells, although, positivity with nested RT-PCR was observed. Previous reports showed the higher efficiency of CC-RT-PCR to accurately quantify the infectious units of fastidious viruses or slow CPE-causing viruses [39-43].

The nature of the test methods used is more or less similar; however, viral cell interaction happens either in immunoperoxidase or in neutralization. The difference is in the mechanism of detection of viral titre in these methods. Serological mechanism (enzyme reaction) of using conjugate and substrate for detection of antibody reacted with the antigen on some cell sites could happen in immunoperoxidase, however, observation of CPE and calculation of the end point of viral positivity are the mechanisms used in neutralization test. Using nested RT-PCR increases the efficiency of estimating the potency of antibodies to neutralize infectious viruses. RT-PCR confirms the positivity of the virus in some wells because of its action on viral genome amplification which succeeded to adsorb/penetrate the specific cell lines. Although using nested RT-PCR after a neutralization test is more expensive, its importance to accurately

estimate the efficiency of antibodies to neutralize infectious viruses and consequently the efficiency of the vaccine *in vitro* may force us to neglect the cost factor.

5. Conclusions

The efficiency of neutralization followed by nested RT-PCR to estimate the effectiveness of antibodies to neutralize infectious viruses is significantly higher than the efficiency of the immunoperoxidase or neutralization test. Neutralization followed by nested RT-PCR could be considered as a recommended method to estimate the efficiency of the recombinant subunit vaccine *in vitro*.

6. Conflicts of interest

All authors declare that there are no conflicts of interest.

7. Acknowledgments

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