Journal of Virological Methods 182 (2012) 56–61

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Prokaryote expression of HPeV-1 VP1 protein, production of VP1 polyclonal antibody and the development of an ELISA

Ying Yu[a,1], Tongling Shan[a,b,1], Shaofeng Zhu[c], Caixia Zhu[a], Xiuguo Hua[a,*], Chunmei Wang[b,d]

[a] Zoonosis and Comparative Medicine Group, Department of Animal Science, School of Agriculture and Biology, Shanghai Jiaotong University, Shanghai 200240, China
[b] Shanghai Veterinary Research Institute, The Chinese Academy of Agricultural Sciences, Shanghai 200241, China
[c] School of Environment and Energy, Peking University Shenzhen Graduate School, Shenzhen, Guangdong 518055, China
[d] State Key Laboratory of Agricultural Microbiology, Division of Animal Infectious Disease, Huazhong Agricultural University, Wuhan, Hubei, China

Article history:
Received 3 July 2011
Received in revised form 8 March 2012
Accepted 12 March 2012
Available online 20 March 2012

Keywords:
Human parechovirus
VP1 protein
Expression
Hyper-immune serum
Indirect ELISA
Serological survey

A B S T R A C T

The VP1 protein of human parechovirus (HPeV) plays a critical role in receptor binding based on its functional arginine–glycine–aspartic acid (RGD) motif region. Currently, only the neutralisation assay is used for seroepidemiological surveys of HPeVs. In the present study, the *VP1* gene of HPeV-1 was cloned into the vector pET28a(+) to express the His-tagged VP1 protein in the bacterium *Escherichia coli* Rosetta. The recombinant protein was purified from inclusion bodies by Ni^{+}–NTA affinity chromatography under denaturing conditions, followed by a refolding process in gradient urea. The identity and antigenicity of the His-tagged protein was confirmed by Western blotting using an anti-His monoclonal antibody and human HPeV-1-positive serum respectively. Polyclonal antibodies against the His-tagged VP1 protein were raised in rabbits by standard procedures, and the reactivity and specificity were tested by enzyme-linked immunosorbent assay (ELISA) and Western blot analysis. An indirect ELISA was developed based on the fusion protein VP1, and evaluated in order to facilitate the detection of antibodies in persons who had been infected naturally with HPeVs. A serological survey was performed using the assay amongst children in the Shanghai region of China; the seropositivity rate was found to be about 73%.

1. Introduction

Human parechoviruses (HPeVs) are non-enveloped, positive-sense, single-stranded RNA viruses that belong to the family *Picornaviridae*, of the genus *Parechovirus* (Harvala and Simmonds, 2009), and comprise two known species, *Human parechovirus* (HPeV) and *Ljungan virus* (LV) (Niklasson et al., 1999; Nix et al., 2010). Two distinct viruses, HPeV-1 and HPeV-2, described originally as echoviruses 22 and 23 respectively, were first isolated from children in the USA during a summer outbreak of diarrhoea in 1956 (Wigand and Sabin, 1961). These viruses were found to be distinct from other picornaviruses in genome structure, proliferation, biological and molecular properties, therefore they were reclassified into a new genus named *Parechovirus*. A total of 14 distinct types (HPeV-1 to HPeV-14) have been identified, and the complete genome sequences of eight types (HPeV-1 to HPeV-8) are available currently from the GenBank database (Benschop et al., 2006a, 2010; Drexler et al., 2009; Ghazi et al., 1998; Harvala and Simmonds, 2009; Hyypia et al., 1992; Ito et al., 2004; Kim Pham et al., 2010; Li et al., 2009; Shan et al., 2010; Watanabe et al., 2007). Epidemiological surveys have shown that distinct HPeV types circulate widely throughout the world, however HPeV-1 is the dominant strain that affects mainly young children who have mild respiratory and gastrointestinal infections (Al-Sunaidi et al., 2007; Benschop et al., 2008; Ito et al., 2004; Watanabe et al., 2007). Seroepidemiological studies have indicated that children are susceptible to HPeVs infection in early life, and they are found to have a high titre of antibodies against the virus (>90%) by the time that they enter primary school (Joki-Korpela and Hyypia, 1998; Takao et al., 2001).

The genome RNA of parechovirus is 7300 bases approximately in length, it encodes a single polyprotein that includes three structural proteins (VP0, VP3 and VP1) and seven non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D) (Harvala and Simmonds, 2009; Hyypia et al., 1992; Stanway et al., 1994). Sequence comparisons of the capsid region amongst HPeVs have revealed a high degree of amino acid sequence divergence, principally in the VP1 region (Harvala and Simmonds, 2009). Furthermore, HPeVs present frequent rapid sequence drift in the VP1 or in the whole P1 structural gene region over time (Harvala and Simmonds, 2009). Base mutations occur at synonymous sites, when measured either directly or indirectly, as well as at non-synonymous sites due to immune-driven sequence change over a long-term evolutionary period, and from which new HPeV serotypes emerge. Nevertheless, absolute genetic

* Corresponding author. Tel.: +86 21 34206149; fax: +86 21 34206263.
E-mail address: hxg@sjtu.edu.cn (X. Hua).
[1] These two authors contributed equally to this study.

0166-0934/$ – see front matter
doi:10.1016/j.jviromet.2012.03.009

diversification alone cannot account for the multiple serotyping found and based only on antigenic properties (Bailly et al., 2009; Simmonds, 2006).

The C-terminus region of VP1 contains a functional arginine–glycine–aspartic acid (RGD) motif that has been identified in all known HPeV genotypes, with the exception of HPeV-3, and that is involved in cell entry and interaction with cellular receptors (Abed and Boivin, 2005; Benschop et al., 2008; Ito et al., 2004; Seitsonen et al., 2010; Stanway et al., 1994). Studies have confirmed that the RGD motif-containing protein VP1 is immunogenic and is a target for neutralising antibodies against HPeVs (Joki-Korpela and Hyypia, 1998; Stanway et al., 2000). A prominent epitope was found also in VP0 by peptide scanning and this peptide has been used as the basis for seroepidemiological studies (Abed et al., 2007; Joki-Korpela et al., 2000). It is interesting to speculate that there might be additional immunological epitopes in VP1 and VP0 outside the epitopes that contribute to its antigenicity (Alho et al., 2003). VP1 would be a promising candidate as an agent for detection of antibodies against HPeVs on the basis of its antigenic properties. At the present time, only the neutralisation assay is used for seroepidemiological surveys of HPeVs (Faria et al., 2009; Harvala and Simmonds, 2009; Joki-Korpela et al., 2000; Shan et al., 2009; Takao et al., 2001). Enzyme immunoassays, as an alternative test, are of potential value for detection of HPeV-specific IgG or IgM antibodies when using an expressed antigen such as VP1.

In the present study, the VP1 protein of HPeV-1 was expressed, purified and the hyper-immune serum was raised in rabbits against VP1. A VP1 protein-based ELISA was also developed and then applied as a serological method to screen for the presence of HPeV-specific antibodies in serum samples.

2. Materials and methods

2.1. Virus and serum samples

HPeV-1 was isolated from stool specimens of hospitalised children who had symptoms of diarrhoea, and the complete genome of strain SH1 was sequenced (GenBank accession no. FJ840477) (Shan et al., 2009). Informed consent was obtained from the parents of children enrolled in the study and serum samples were all collected from clinical cases. HPeV-positive sera and HPeV-negative sera from 257 children (3 months to 6 years old) were characterised by microneutralisation test (Alho et al., 2003). Serum that was positive for *Streptococcus suis* 2 (*S. suis* 2), human astrovirus 1 (HAstV1), human bocavirus 1 (HBoV1) or hepatitis E virus (HEV) was obtained from the Zoonosis and Comparative Medicine Group laboratory and used as controls.

2.2. Construction of the pET28a–VP1 expression plasmid

Vero cells were cultured with strain SH1 and total RNA was extracted using Trizol reagent (TransGen, Beijing, China). Primers were made based on the sequence of SH1 and were designed for nested reverse transcription polymerase chain reaction (RT-PCR) to obtain the initial templates. Primer sequences were as follows: first round sense 5′-TAGCTCCAGTCCTAATGAAG-3′; first round anti-sense 5′-ATCCAGTTCACATTCCTCCT-3′; second round sense 5′-AAATGGGACAAGATGCTAGG-3′ and second round anti-sense 5′-AGTGAACCTAGCTTTCCCTG-3′ (Shan et al., 2009). The VP1 fragment was amplified with specific primers that included the sense strand primer (5′-TCGGATCC**ATG**AATTCATGGGGTTCACAA-3′) and the anti-sense strand primer (5′-CACTCGAGCTGGTCTGAAAAATTTGCCAA-3′). The expected length of the amplicon VP1 was 696 bp, the fragment was then cloned into the expression plasmid pET28a(+).

2.3. Expression and purification of the His-tagged VP1 protein

Escherichia coli Transetta (DE3) (TransGen) transfected with the recombinant plasmid pET28a–VP1 was propagated at 37 °C in Luria Bertani (LB) medium supplemented with 100 μg/ml kanamycin until the bacterium reached logarithmic growth phase (at OD_{600} 0.6–0.7) and then induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG). The expression of the His-tagged VP1 protein was optimised at different IPTG concentrations and at different durations. Solubility analysis of the recombinant protein produced in *E. coli* Transetta was carried out in accordance with the method of Lin et al. (2010). Purification of the His-tagged VP1 protein was carried out in accordance with the manufacturer's procedures for Ni^+–NTA resin-packed columns (GE Healthcare, Chalfont St Giles, UK).

Renaturation of the purified protein was performed using urea gradient dialysis (Zhang et al., 2011). Centricons (Amicon Ultra-4, Millipore, Bedford, USA) were employed to concentrate the renatured protein. The final protein concentration was quantified using the Coomassie (Bradford) Protein Assay Kit (Pierce, Beijing, China) with bovine serum albumin as the standard (Bradford, 1976).

2.4. Western blot analysis of the His-tagged VP1 protein

Western blotting was carried out as described below to characterise the identity of the recombinant protein. The renatured VP1 protein was subjected to 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes using a semi-dry transfer cell (Bio-Rad, Shanghai, China) at 15 V for 1.5 h. The membrane was blocked overnight at 4 °C with 5% skimmed dried milk in Tris-buffered saline that contained 0.05% Tween-20 (TBST). Anti-His monoclonal antibody (1:1000 dilution; Novagen, Germany) or HPeV-1-positive human serum (1:2000 dilution) was added to the membrane as primary antibody and incubated for 2 h at room temperature. The membrane was washed with TBST and then incubated for 1 h at room temperature with either goat anti-mouse or goat anti-human antibody conjugated to peroxidase at a 1:1000 dilution or 1:3000 dilution respectively (Proteintech Group, USA) (Subramanian et al., 2009). The colour reaction was developed and visualised with 3,3′,5,5′-tetramethylbenzidine (TMB) as the substrate.

2.5. Production and characterisation of polyclonal antibody against the His-tagged VP1 protein

The renatured recombinant protein was used to prepare antibodies in New Zealand white rabbits. Rabbits were injected subcutaneously with 500 μg recombinant protein emulsified in an equal volume of complete Freund's adjuvant (Sigma, Shanghai, China) for the primary immunisation. Two booster injections were given with 250 μg recombinant protein each in incomplete Freund's adjuvant (Sigma) at 2-week intervals to achieve a prolonged stimulation. Immune response was monitored by indirect ELISA at each step in immunisation. The antiserum was harvested from rabbits at 10 days after the final boost. The study protocol was approved by the Animal Care and Use Committee (ACUC) of the Shanghai Research Center for Biomodel Organisms.

Antibody titre was determined by ELISA using the standard checkerboard titration procedure. The recombinant protein (1.9 μg/ml) was prepared by two-fold serial dilutions in carbonate–bicarbonate buffer (pH 9.6) and at a range from 1:64 to 1:256 dilutions; protein was coated onto plates at a 100 μl aliquot per well in 96-well immunoplates (Sangon, Shanghai, China), and incubated at 4 °C overnight. The plates were washed three times for 15 min with phosphate-buffered saline (PBS) plus 0.05% Tween-20 (PBST) and blocked with 300 μl blocking buffer (PBST plus