The 3-dimensional play of human parechovirus infection; Cell, virus and antibody
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Anatomy of a human parechovirus 3 at near-atomic resolution

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Abstract

Human Parechovirus 3 (HPeV3) is one of the main causative agents of meningitis and sepsis in neonates. Currently, there are no therapies available to combat its infection, but insight into its structure could help design of antiviral therapies. This virus is unique among the Parechovirus genus as it lacks the ‘arginine-glycine-aspartic acid’ motif in the C-terminus of the capsid protein VP1, used by the others to gain entry into cells using integrins. Here, we present a high-resolution structure of HPeV3 as determined by cryo-electron microscopy and image reconstruction. The HPeV3 structure has features similar to enteroviruses such as raised 5-fold and 3-fold vertices, an open channel though the capsid and an annulus below each 5-fold vertex. By fitting homology models of HPeV3 into the EM density we have identified loops, which could be utilized as epitopes for generation of therapeutic antibodies against the HPeV3 capsid. Overall, the HPeV3 structure gives the first instance of high-resolution structural information for the Parechovirus genus.
Introduction

The Picornaviridae family contains both a large group of enteroviruses (EVs) as well as a smaller group of parechoviruses (HPeVs) that are prevalent causes of viral infections in humans. HPeV infections are mainly mild, causing respiratory symptoms and gastrointestinal symptoms, but can also result in sepsis-like disease and CNS symptoms. HPeV genotype 3 is one of the main causative agents of meningitis and sepsis-like illness in neonates. Despite the importance of HPeV3 in CNS involvement no antiviral therapies are available to combat HPeV3 infections. HPeV3 was first isolated in 2004 from a stool specimen from a 1-year old child with transient paralysis (1). Since then, several studies have highlighted significantly different clinical and biological differences between HPeV3 and the other prevalent genotypes HPeV1, 2, 4, 5 and 6. Clinically, HPeV3 is most often associated with severe disease (1–6).

The genome of HPeVs consists of a ~7.5kb, single-stranded, positive-sense RNA, which is enclosed in a capsid containing 60 copies of each capsid protein (VP0, VP3 and VP1). HPeV3 lacks the arginine-glycine-aspartic acid (RGD) motif in the VP1 C-terminus VP1, which has been shown to be essential in viability and infection of HPeV1. HPeV1 utilises via the RGD motif the αvβ3, αvβ6 integrins as their receptors (7–10), for entry into the host cell by a clathrin-dependent endocytic pathway (8). Structural studies on HPeV1 confirmed the importance of the RGD motif and their interaction with the αvβ3, αvβ6 integrins (10). The missing RGD motif in HPeV3 implies different receptor use. Therefore, we generated three different HPeV3 mAbs which showed specific binding, but failed to neutralize HPeV3 replication in vitro (Manuscript in preparation). These mAbs most likely have non-linear epitopes. Further studies in the differences in receptor binding and neutralization are essential in understanding viral infection and pathogenesis, and in the design of antiviral therapies. Towards this end, the 3D reconstruction of HPEV3 from cryo-EM could be of significant benefit. In this study we generated present a high-resolution structure of HPeV3 as determined by cryo-electron microscopy, giving the first instance of high-resolution structural information for the Parechovirus genus.
Materials and Methods

Virus culture, purification and inactivation

HPeV3 isolate 152037 was and grown on the African green monkey, Vero cell line. The Vero cells were maintained in growth medium composed of Dulbecos Modified Eagles Medium (DMEM) with glutamax (1X), non-essential amino acid (1X), streptomycin (0.1 μg/ml), penicillin (0.1 μg/ml) and 10% heat-inactivated fetal bovine serum (FBS). A 90% confluent cell layers were inoculated with HPeV3 at a multiplicity of infection (MOI) of 0.01. At 100% infection of the cell monolayer evident by the cytopathic effect (CPE), the cells and spent media were collected and freeze-thawed thrice at -70°C and 37°C, centrifuged at 4000 rpm for 15 minutes in an Eppendorf A-4-62 swing bucket rotor at 4°C and the supernatant was filtered using a 0.22 µm filter. The virus was concentrated using 100kD cutoff membrane filters (Millipore). The concentrate was pellet was loaded on to a cesium chloride step gradient with a 5ml 40% (w/v) bottom layer and a 5ml 15% (w/v) top layer and centrifuged at 32000 rpm, for 16 h at 4 °C in a Beckman SW41Ti rotor. The virus band was buffer exchanged with 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl2 (1X TNM buffer) using a 100kD cutoff membrane filter (Millipore). The purified HPeV3 was inactivated by adding 0.1 mg/ml formaldehyde and keeping at 37°C for 72h. Inactivation was confirmed by infecting Vero cells with inactivated virus. No cytopathic effect was seen up to 7 days.

Electron cryo-microscopy

Aliquots of the purified HPeV3 were vitrified on Quantifoil R3.5/1 holey carbon nickel grids in a home-built guillotine by plunging into liquid ethane maintained in a liquid nitrogen bath. After vitrification, the grids were stored in liquid nitrogen until use. The grids were examined in a Cs corrected FEI Titan Krios transmission electron microscope at 300 keV. The images were recorded on a back-thinned Falcon II detector under low dose conditions at a nominal magnification of 59000X with a sampling size of 1.14 Å per pixel. Seven frames per image were collected in counting mode using FEI EPU automated single particles acquisition software. The integrated unaligned image was used for further image processing. The contrast transfer function of each micrograph was estimated using CTFFIND3 and images containing drift or astigmatism were discarded (11). Particles were picked using the program ETHAN (12) with a box size of 401 pixels and inspected by eye in the program suite EMAN (13). Random model generated from 150 particles (EMD-1690) was used as a starting model to initiate full orientation and origin determinations of the of full set of particles using AUTO3DEM ver4.04.1 (14). The final reconstructions calculated to the Nyquist frequency were used to estimate the B-factors with EM-Bfactor, and then the reconstructions were truncated to the resolution indicated by the Fourier shell correlation analysis with a threshold criterion of 0.5 (15, 16). The HPeV3 density map will be deposited
Homology modelling and fitting of models into cryoEM maps

The structures of the three HPeV3 capsid proteins were predicted by multiple-template comparative modeling using the I-TASSER server (17). The template structures for VP0 were foot and mouth disease virus (PDB ID: 1QQP and 1BBT) (18, 19), poliovirus 1 (PDB ID: 1POV) (20), bovine enterovirus (PDB ID: 1BEV) (21), Theiler’s murine encephalomyelitis virus (PDB ID: 1TME) (22), equine rhinitis A virus (PDB ID: 2WFF) (23) and Seneca Valley virus-001 (PDB ID: 3CJI) (24). For VP1, they were human enterovirus 71 (PDB ID: 3VBF) (25), triatoma virus (PDB ID: 3NAP) (26), human rhinovirus 14 (PDB ID: 1D3I and PDB ID: 1NCQ) (27, 28), human rhinovirus 16 (PDB ID: 1AYM) (29), echovirus 1 (PDB ID: 1EV1) (30), and staphylococcal enterotoxin H (PDB ID: 1EWC) (31). For VP3, they were poliovirus 1 (PDB ID: 1POV) (20), coxsackievirus A9 (PDB ID: 1D4M) (32), equine rhinitis A virus (PDB ID: 2XBO) (33), poliovirus Mahoney strain (PDB ID: 1AL2) (34), echovirus 1 (PDB ID: 1EV1) (30), Seneca Valley virus-001 (PDB ID: 3CJI) (24) and bovine enterovirus (PDB ID: 1BEV) (21). An atomic model of echovirus 1 capsid (PDB ID: 1EV1) (30) was placed into a 5.45 Å resolution HPeV3 map. The fitting was done using a modification of a protocol described elsewhere (35). The homology models were aligned with the echovirus 1 capsid, before being rigidly fitted into the HPeV3 map using the ‘fit in map’ feature in UCSF-Chimera (36). Using the ‘zoning’ feature in UCSF-Chimera (36), the HPeV3 capsid map was zoned to an asymmetric unit with a radius of 6 Å using the VP0-VP3-VP1 rigidly-fitted model. Residues 1-65 of VP0, 1-70 of VP3 and 1-36 of VP1 were truncated because these were the regions of lowest confidence in the homology models. The truncated VP0-VP3-VP1 model was flexibly fitted into the asymmetric unit using iMODfit with the default settings (37). All the visualization was carried out in UCSF-Chimera (36). The fitted model will be deposited in the Protein Databank in Europe.
Results

HPeV3 structure

Using a stable microscope Titan Krios with a back-thinned direct electron detector, we were able to reach a resolution of 5.45 Å with as few particles as 1276 (Figure 1A-C and Table 1). The micrographs had a high signal to noise ratio, which greatly aided in determining the orientation and origin centres correctly. Using a random model generated initially from 150 particles as a reference model, the full dataset converged within 207 iterations. The HPeV3 structure shows features similar to other picornavirus such as the presence of a canyon on the capsid surface and the presence of an annulus below the five-fold vertices (Figure 1 C and D). Similar to CVA7, the HPeV3 five-fold vertices also have an open channel which stays open even when viewed at a threshold close to the mean density of the map (38).

Table 1: Statistics of the reconstruction.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HPeV3 reconstruction</th>
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<tbody>
<tr>
<td>No. of micrographs</td>
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<tr>
<td>No. of particles used in the</td>
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<td>reconstruction</td>
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<td>B-factor applied</td>
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</tbody>
</table>

Pseudo-atomic model of HPeV3

The I-TASSER (17) based homology model of VP0 had a model confidence score (C-score) of -0.21, VP3 had a C-score of -0.48 and VP1 had the lowest C-score among the three capsid proteins of -2.06 (Figure 2A). Due to homology modelling errors, the N-termini of the capsid proteins were truncated before being flexible fitted into the HPeV3 asymmetric density. The β-barrels, helices and loops constrained by secondary structure elements fitted well into the EM density whereas long loops and the termini fitted with low confidence (Figure 2B). The pseudo-atomic model generated by applying symmetry operators on the VP0, VP3 and VP1 asymmetric fit agreed well with the HPeV3 capsid density, although there were a few clashes especially between neighbouring asymmetric units (Figure 2C).
Figure 1. HPeV3 structure. A) Raw micrograph showing the HPeV3 capsids at defocus of 2 µm. Scale bar 50nm. B) Cross-section view of HPeV3 with five-fold (5f), three-fold (3f) and two-fold (2f) symmetry axes marked. C) Radially depth-cued isosurface representation of HPeV3 at resolution of 5.45 Å with a bfactor of 50. D) Central-cross section of HPeV3 showing the open channel at five-fold vertex. E) A slab of thickness 30 Å showing an annulus below the five-fold symmetry vertex. The black arrow shows the open channel at five-fold vertices in B and D, the blue arrow shows the canyon on the HPeV3 surface in C and the red arrow shows the annulus.
Figure 2. Pseudo-atomic model of HPeV3. A) Homology models of HPeV3 capsid protein from I-TASSER. B) Fitting of homology models into an asymmetric unit of HPeV3 EM density. C) Pseudo-atomic model of HPeV3 full capsid. Model of VP1 is shown in red, VP0 in blue and VP3 in yellow. EM density is shown in a UCSF Chimera surface representation with transparency.
Discussion

HPeV3 are highly pathogenic in neonates, and the great differences shown compared to the well studies HPeV1 makes it essential to investigate HPeV3- structural properties for better understanding of HPeV3 pathogenesis. Here we determined a 5.45Å resolution reconstruction of HPeV3 showing features similar to other picornaviruses. Noticeable, is the open channel in the five-fold axis, which was not seen in the reconstruction of HPeV1, but has been frequently observed in other picornaviruses such as enterovirus CAV7 (38). As well as the two-fold channel, in case of CAV16 the conformational changes during uncoating results in an increase of the cross section area of the two fold axis channel (39). The channels in other picornaviruses, both on the five-folds and the two-folds have been implicated in the conformational changes in all the capsid proteins that are initiated during receptor binding, release of pocket factors, emergence of VP4 and the termini of VP1 that are required for RNA genome release. HPeVs do not contain the VP4 capsid protein per se, it remains as the uncleaved N-terminus of VP0, and the mechanism of viral uncoating remains unclear. Additional experiments are needed to look into the mechanism of viral uncoating and the role of the conformational changes in HPeV3 during RNA release.

Further analysis of this pseudo-atomic model of HPeV3 could be utilized to identify surface exposed residues on the capsid and comparison with other known picornaviral structures is ongoing. Thereby further comparative sequencing, I-TASSER, MODELLER, Robetta, and refined alignment techniques can lead to the prediction of the receptor binding site of HPeV3, as shown for HRVC, were modelling had led to insights in receptor preference an immunogenicity (40).

The HPeV3 structure can be used for the prediction and design of mAb targets in the fight against HPeV infection. Secondly the reconstruction of the HPeV3 labelled with the non-neutralizing mAbs is ongoing. This analysis will be of great value in the understanding of the HPeV3 genotype and the development in antivirals.

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