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Purification, crystallization and X-ray analysis of swine vesicular disease virus

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Swine vesicular disease virus (SVDV) is the etiological agent of swine vesicular disease, a highly contagious disease in pigs, and is related to coxsackie B virus. Crystalline arrays of SVDV can be observed in the cytoplasm of cells 4.5 h after inoculation to porcine kidney cells (IBRS-2 cells). Crystals of the JX/78 strain of SVDV were obtained from virus in two wells of crystallization conditions and present preliminary X-ray data to 3.6 Å resolution.

1. Introduction

Swine vesicular disease (SVD) is a highly contagious disease in pigs which was first recognized in 1966 in Italy (Nardelli *et al.*, 1968). Outbreaks have since been observed in Hong Kong between 1970 and 1991, Japan from 1973 to 1975 and Europe between 1972 and 1997 (Knowles & Sellers, 1994; Brocchi *et al.*, 1997). Symptoms of SDV are indistinguishable from those caused by foot-and-mouth disease virus (FMDV), with the major pathological symptoms being vesicular lesions in the mouth, foot and hock. The causative agent of SDV is swine vesicular disease virus (SVDV), an enterovirus and member of the Picornaviridae family. As with other picornaviruses, the genome is a single-stranded polyadenylated RNA (ssRNA) molecule which acts as a messenger for the virus polypeptides.

The complete sequence of the genome of swine vesicular disease virus (SVDV; H/3 '76) isolated from a healthy pig has been determined using molecular-cloning and DNA-sequencing techniques (Inoue *et al.*, 1989, 1993; Seechurn *et al.*, 1990). The RNA genome was 7400 nucleotides long, excluding the poly(A) tract, and appeared to encode a single polyprotein of 2185 amino acids. The predicted amino-acid sequence of the polyprotein showed around 90% homology to those of the previously sequenced coxsackieviruses B1, B3 and B4 and also showed around 60% homology to that of poliovirus. Coxsackievirus B5 (CB5) has been shown to share antigenic properties with SVDV (Graves, 1973; Brown *et al.*, 1973) and, although SVDV can be neutralized by anti-CB5 antisera, cross-neutralization and immunodiffusion tests have concluded that the two viruses are immunologically distinct. Direct size comparisons between CB5 and SVDV show obvious similarities in terms of peptide mapping and electrophoretic mobilities and it has long been thought that CB5 gave rise to the agent of SDV (Graves, 1973). While there is no direct evidence to show that direct infection of pigs with CB5 produces a vesicular disease typical of SVDV, a study of 45 SVDV isolates from Asia and Europe showed that they form a single monophyletic group distinct from coxsackieviruses (Zhang *et al.*, 1999). Despite the differences in host range and pathology between SVDV and CB5, the results were consistent with the view that SVDV is a recent genetic descendent of CB5 and it was proposed by the authors that SVDV be considered a subspecies of CB5.

The basic component of picornaviridae is a subunit made up of four structural coat proteins, namely VP1, VP2, VP3 and VP4. The SVDV virion is spherical, with a diameter of between 28 and 32 nm and a molecular weight of 10.4×10^6 Da. In 1995, the crystal structure of coxsackie B3 virus (CB3) was solved to 3.5 Å resolution (Muckelbauer, Kremer, Minor, Diana *et al.*, 1995; Muckelbauer, Kremer, Minor, Tong *et al.*, 1995). Crystalline arrays of SVDV can be observed in the cytoplasm of cells 4.5 h after inoculation of porcine kidney (IBRS-2) cells (Kubo *et al.*, 1990), indicating the high likelihood of crystallization of SVDV *in vitro*. In this paper, we report the crystallization of SVDV and the preliminary diffraction analysis of this virus.

2. Methods

2.1. Purification

SVDV (JX/78 strain) provided by Lanzhou Veterinary Research Institute was isolated from a clinic sample from an infected pig in

Hong Kong, China in 1978. The virus was grown in IBRS-2 cell monolayers in a bottle and infected cells were harvested 10–12 h post-infection when cells exhibited above 95% cytopathic effect (CPE). The virus was purified by a modification of the procedure described by Ferris *et al.* (1984). Briefly, the infected culture medium was collected and frozen at 253 K and centrifugation was used to separate out the broken cell walls. The virus in the supernatant was concentrated with 70%(*w*/*v*) PEG 6000 and 22.2%(*w*/*v*) NaCl with stirring at 277 K for 4 h and then left overnight. The supernatant solution was subjected to centrifugation, resuspended in a small volume of Tris buffer pH 7.6, diluted with trichloroethylene (TCE) and then subjected to further centrifugation to precipitate out the virus. The pellet was agitated to resuspend it in Tris buffer (containing 1% NP40). The resuspended solution was carefully placed on the upper layer of a 15–45% sucrose gradient in the tube. The solution was subjected to ultracentrifugation at 35 000 rev min^{-1} and the virus peak was collected and quantified at 259 nm. The purified virus was precipitated, resuspended in a small volume of TNE buffer (0.01 *M* Tris–HCl, 0.1 *M* NaCl and 0.01 *M* EDTA pH 7.8) and stored at 277 K.

2.2. Electron microscopy

The virus was dissolved in TNE buffer to a concentration of 0.5 mg ml^{-1}. The solution was diluted 50-fold with TNE in preparation for electron-microscopic observation. Grids were soaked in the diluted sample for 90 s and then negative-stained with uranium acetate at 1–2%(*w*/*v*) for 180 s. The sample was viewed using a Philips CM120 electron microscope at Tsinghua University, Beijing.

2.3. Protein crystallization

The vapour-diffusion method was employed and SVDV crystals were obtained from two wells with differing crystallization conditions. Crystals from well 1 were grown in a buffer consisting of 0.01 *M* Tris–HCl, 0.1 *M* NaCl pH 7.8. The precipitant used was 3% PEG 2000 and the maximum dimension of the crystals was 0.5 mm. Crystals from well 2 were also grown in a buffer consisting of 0.01 *M* Tris–HCl, 0.1 *M* NaCl pH 7.8. The precipitant used was 4% PEG 2000 and the maximum dimension of these crystals was 0.2 mm. The crystallization conditions of the virus are summarized in Table 1.

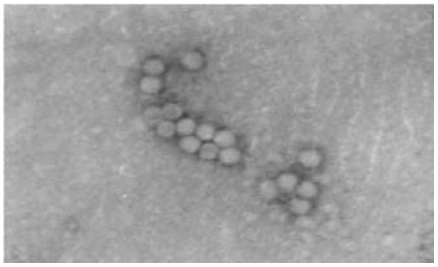

Figure 1
Crystalline array of SVDV from electron microscopy.

Table 1
Summary of crystallization results.

	Well 1	Well 2
Buffer	0.01 *M* Tris–HCl, 0.1 *M* NaCl, 1 m*M* EDTA pH 7.8	0.01 *M* Tris–HCl, 0.1 *M* NaCl, 1 m*M* EDTA pH 7.8
Precipitant	3%(*w*/*w*) PEG 20 000	4%(*w*/*w*) PEG 4000
Additives	4 *M* NH_4Cl	4 *M* NH_4Cl
Method	Vapour diffusion	Vapour diffusion
Max. dimension (mm)	0.5	0.2

2.4. Data collection

Virus crystals from well 2 were found to be too small for diffraction using in-house equipment. Data from well 1 crystals were collected in-house using a Rigaku 2000 rotating-anode X-ray generator with a MAR Research image-plate detector. The radiation wavelength was 1.5418 Å. A maximum resolution of 3.6 Å was achieved. However, a significant decline in diffraction was observed after two images of exposure time of 1.5 h per image. As a result, a cryomethod was implemented to extend the diffraction lifetime of the crystal, with a consequent fall-off in resolution to 4.0 Å. We used an Oxford Cryosystems Cryostream, which provided an ambient temperature of 100.5 K. The optimum cryoprotectant was determined to be 15% glycerol and a series of 12 diffraction images were collected.

All data analysis was performed with *DENZO*/*SCALEPACK* (Otwinowski & Minor, 1997). The SVDV crystals were found to be rhombohedral, with space group *R*3 or *R*32 and unit-cell parameters $a = b = 295.6$, $c = 1456.1$ Å, $\alpha = \beta = 90$, $\gamma = 12^\circ$. Data were collected to a maximum resolution of 3.6 Å, free of cryoprotectant.

3. Results

Under the electron microscope (Fig. 1), the SVDV particles in the cytoplasm of cells appear to form regular compact arrays, suggesting the high possibility of crystallization of SVDV *in vitro*. Crystals were obtained from two crystallization wells after 20 d. During the crystallization period in well 1, we observed an initial phase change from flower-like crystalline sheets to large regular-shaped crystals (Fig. 2*a*). This indicated a high potential for SVDV crystal formation in such a well solution. We optimized the crystallization procedure of well 1 in order to achieve larger and morphologically better crystals. We repeatedly achieved crystals with a maximum dimension of 0.5 mm, as shown in Fig. 2(*b*). Crystals from well 2, as shown in Fig. 2(*c*), with a maximum size of 0.2 mm, were too small for diffraction in-house using the Rigaku rotating-anode generator. In spite of repeated attempts to increase the size of the crystals, they could not be improved (Fig. 2*c*). Preliminary processing of the SVDV data (Fig. 3) suggests that the crystal belongs to space group *R*3 or *R*32 and has unit-cell parameters $a = b = 295.6$, $c = 1456.1$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$; data are summarized in Table 2.

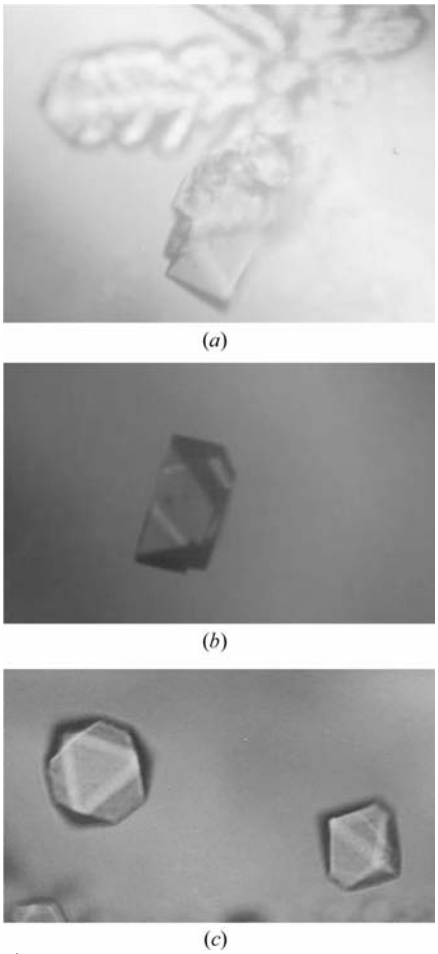

Figure 2
In (*a*), the regular-shaped crystal of well 1 can be seen among the flower-like crystalline sheets, indicating that a phase change occurred during the period of crystal growth. After optimizing the conditions by a very slight concentration change of PEG 2000 in well 1, we achieved very good crystals as shown in (*b*). (*c*) shows virus crystals grown from well 2. They are too small to be used for diffraction in-house.

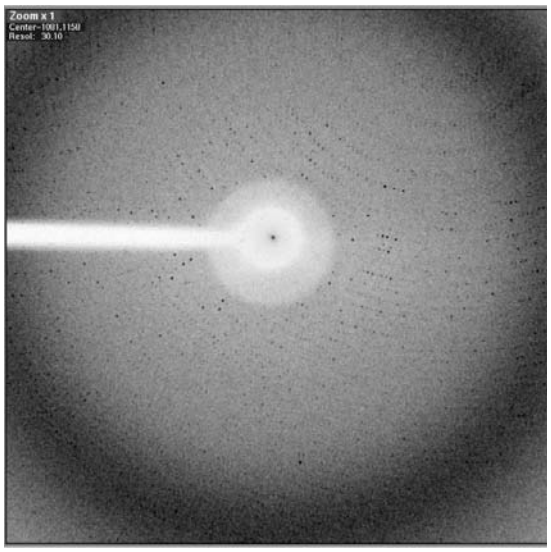

Figure 3
Oscillation photograph of a crystal of SVDV: the highest resolution at the edge of film was 3.6 Å. Current, 90 mA; wavelength, 1.5418 Å; crystal to image-plate distance, 370 mm.

4. Discussion

Muckelbauer and coworkers observed that CB3 crystallized in a pseudo-*R*32 space group, but reported that the true space group was $P2_1$ with unit-cell parameters $a = 574.6$, $b = 302.1$, $c = 521.6$ Å, $\beta = 107.7^\circ$ (Muckelbauer, Kremer, Minor, Diana *et al.*, 1995; Muckelbauer, Kremer, Minor, Tong *et al.*, 1995). In our work, the fact that the *c* axis of the SVDV crystals was measured to be almost five times that of the *a* and *b* axes was surprising assuming that the virion is spherical and suggests the possibility of a special packing arrangement of the virus particles. If the *c* axis was incorrectly determined, we would expect to observe absences in the indexed 00*l* reflections. Our failure to observe such absences implies that our unit cell is indeed correct. However, we cannot absolutely rule out the possibility of inaccurate measurement of the systematic absences by non-synchrotron sources, which has hindered the space-group determination of several crystal structures in our experience. Nevertheless, the same space group, *R*32, and similar unit-cell parameters ($a = b = 300.9$, $c = 1476.6$ Å) have also been observed for another member of the enterovirus family, Echovirus 11 (D. Stuart, personal communication).

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Table 2
Experimental parameters of the diffraction data collection.

X-ray generator	Rigaku 2000
Operating current (mA)	90
Operating voltage (kV)	46
Oscillation range (°)	0.5
Oscillation time per image	3
Crystal-to-film distance (mm)	370
Exposure time (s)	900
Resolution limit (Å)	3.6
Space group	*R*3/*R*32
Unit-cell parameters (Å, °)	$a = b = 295.6$, $c = 1456.1$, $\alpha = \beta = 90$, $\gamma = 120$

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