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VeroNectin-4 is a highly sensitive cell line that can be used for the isolation and titration of *Peste des Petits Ruminants* virus

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Peste des Petits Ruminants virus (PPRV) is a member of the *Morbillivirus* subgroup of the family *Paramyxoviridae*, and is one of the most contagious diseases of small ruminants throughout Africa and the rest of the world. Different cell lines have previously been used to isolate PPRV but with limited success. Thus, to improve the isolation of *Morbilliviruses*, human, canine, and goat homologues of the lymphocyte receptor signaling lymphocyte activation molecule (SLAM) have been introduced into cells that can support virus replication. However, the amino acid sequence of SLAM varies between species, and often requires adaptation of a particular virus to different versions of the receptor. The protein sequence of Nectin-4 is highly conserved between different mammals, which eliminate the need for receptor adaptation by the virus. Cell lines expressing Nectin-4 have previously been used to propagate measles and canine distemper viruses. In this study, we compared infections in Vero cells expressing canine SLAM (*VeroDogSLAM*) to those in Vero cells expressing Nectin-4 (*VeroNectin-4*), following inoculations with wild-type strains of PPRV. Virus isolation using *VeroNectin-4* cells was successful with 23% of swabbed samples obtained from live infected animals, and was 89% effective using post-mortem tissues of infected sheep. By contrast, only 4.5% efficiency was observed from swab samples and 67% efficiency was obtained in virus isolation from post-mortem tissues using *VeroDogSLAM* cells. The average incubation period for virus recovery from post-mortem tissues was 3.4 days using *VeroNectin-4* cells, compared with 5.5 days when using *VeroDogSLAM* cells. The virus titers of PPRV obtained from *VeroNectin-4* cells were also higher than those derived from *VeroDogSLAM* cells. A comparison of the growth kinetics for PPRV in the two cell lines confirmed the superiority of *VeroNectin-4* cells for PPR diagnostic purposes and vaccine virus titration.

1. Introduction

Peste des Petits Ruminants (PPR) is a serious disease of sheep and goats that causes huge economic losses worldwide. To prevent food shortages, the Food and Agriculture Organization of the United Nations (FAO) and the World Organization for Animal Health (OIE) have implemented a global eradication program, by prioritizing early diagnostic surveillance and instituting a large vaccination campaign. The success of this program will depend on the establishment of sensitive diagnostic techniques and the development of cheap and effective vaccines (Diallo et al., 2007).

Although molecular and serological testing methods are available for diagnosis, the tools to isolate PPRV are limited and require further improvement. To date, very few PPR field strains have been isolated from around the world (Diallo et al., 1989), due to the lack of sensitive cell lines, the quality of isolates, and transport of samples without refrigeration (Bhuiyan et al., 2014). In addition, many outbreaks still occur in spite of attempted vaccination programs (Banyard et al., 2010; OIE, 2014a,b), due to the use of ineffective vaccines that have poor thermostability, and are low in titer.

Thus, available cell lines that support the growth of PPRV are quite rare, and many attempts to propagate the virus in

Abbreviations: SLAM, signaling lymphocyte activation molecule; PPR, Peste des Petits Ruminants.

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conventional cell lines or primary cells have failed (Benazet, 1973; Diallo, 2003; Gilbert and Monnier, 1962).

In an attempt to overcome this problem, researchers have developed specific cell lines for *Morbillivirus* isolation. These laboratory cells could support the replication of PPRV after they were engineered to express known receptors for other morbilliviruses (Hsu et al., 2001; Muhlebach et al., 2011; Noyce et al., 2011; Tatsuo et al., 2000) . For example, Vero/signaling lymphocyte activation molecule (SLAM) and marmoset B95a cells have been used for canine distemper virus isolation (Seki et al., 2003). Another study demonstrated that CHS-20 cells, monkey CV-1 cells expressing goat SLAM, could be used to isolate PPRV (Adombi et al., 2011). Nectin-4 (also known as PVRL4) was recently reported to be the epithelial cell receptor for measles and canine distemper viruses (Noyce et al., 2011; Noyce and Richardson, 2012; Noyce et al., 2013). Birch et al. (2013) confirmed that the ovine Nectin-4 protein was expressed in epithelial cells, and coincided with efficient replication of PPRV. Nectin-4 has the advantage of having a highly conserved amino acid sequence across several mammalian species, unlike that of SLAM. This conservation would facilitate the isolation of wild-type PPRV in Nectin-4-positive cell lines, without the need for the virus to adopt and use a non-natural receptor (Bieringer et al., 2013; Birch et al., 2013).

VeroDogSLAM cells are most frequently used worldwide to isolate PPRV. However, to our knowledge, Vero cells expressing Nectin-4 have yet to be tested and used in this capacity. In this study, the ability of Vero cells, Vero cells expressing canine SLAM (VeroDogSLAM), and Vero cells expressing Nectin-4 (VeroNectin-4) to support PPRV replication and improve isolation of the virus from fresh and frozen samples of diseased animals were compared. Overall, we show that VeroNectin-4 cells can be used to complement current diagnostic techniques, and improve the quality and production of vaccine strains of PPRV.

2. Material and methods

2.1. Cell lines and media

In this study, VeroDogSLAM, Vero (African green monkey kidney cells, ATCC No.CCL-81), and VeroNectin-4 cells were used. VeroDogSLAM cells were provided by M.D. Baron, from IAH Pirbright, UK, and VeroNectin-4 cells were supplied by C.D. Richardson. Vero cells were engineered to express PVRL4 using a retrovirus expression vector pBMN-IRES-NeV, into which the PVRL4-coding sequence was inserted (Noyce et al., 2011; Noyce and Richardson, 2012; Noyce et al., 2013). VeroNectin-4 cells were originally selected after retrovirus transduction using G418 (neomycin).

All cell lines were grown on Dulbecco's-modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and 1% mixed antibiotic–antimycotic solution (gentamicin and fungizone), 2 mM L-glutamine, and 1 mM sodium pyruvate. VeroDogSLAM medium was supplemented with 100 μg/mL Zeocin and VeroNectin-4 culture medium contained 500 μg/mL G418.

2.2. Viral strains

Nigeria 75 PPRV strain (Diallo et al., 1989), a local isolated virulent strain (PPR MOR 2008), and an attenuated Moroccan strain (MO57) were used in this study. The PPR MOR 2008 strain was isolated, during the 2008 outbreak in Morocco from a sheep showing characteristic symptoms. Serial passages of MO57 virus were performed using Vero cells and the 57_{th} passage was used in this study. The attenuated strain was used as controls for virus titration, and the virulent strain was used as a control for virus isolation.

2.3. Origin of wild-type virus samples

The samples used in this study were from experimentally infected goats (*Capra aegagrus hircus*, Alpine breed) that are routinely housed in our laboratory for evaluating vaccine efficacy. The infection was established following the protocol defined by Elharrak et al. (2012). All goats were aged 6–8 months, and were verified to be initially negative for PPRV.

The animal experiments were performed under BSL3 containment animal housing in accordance with the guidelines described for the care and handling of experimental animals by the Laboratory Committee for Control and Supervision of Animal Experimentation, which is recognized by the national authorities.

Samples consisted of conjunctival, nasal, and rectal swabs taken in the acute phase of the disease (6–9 days post infection [dpi]). At necropsy, the lungs and lymph nodes from the mesenteric tissue and bronchial tissue were collected. Ten samples were stored frozen at -80 °C for about 6 months, and 32 samples were used directly for isolation on cells.

Swabs were collected in 2 mL of PBS supplemented with a 2% antibiotic–antimycotic solution. After centrifugation for 20 min at 2000 rpm (4 °C), the supernatant was retained for infection analysis. Post-mortem tissues were disrupted by grinding, and the homogenates were centrifuged in the same manner as described above, and these supernatants were used for inoculating cells.

2.4. Virus isolation

The supernatant samples (500 μL) described above were inoculated to confluent cells, left to adsorb for 45 min at 37 °C, and then incubated at 37 °C in the presence of 5% CO2, and observed daily. The virus was harvested after cytopathic effects (CPEs) were observed, or was harvested weekly following blind passages over 3 weeks. Virus detection was performed using qPCR as described by Batten et al. (2011). RNA extraction was accomplished using a genomic RNA kit (Bioline BIO-52075, isolate II RNA Mini kit). Amplification was carried out with the Invitrogen Superscript III Platinum R one step qRT-PCR system (Cat. no. 11745-100).

2.5. Comparative growth curves of PPRV in cell culture

The growth kinetics of PPRV was tested with the Nigeria 75 strain cultivated in Vero and VeroNectin-4 cells. The virus was inoculated with a multiplicity of infection (M.O.I.) of 0.01 in 25 cm^2 flasks containing cell suspensions, and then incubated for 6 days at 37 °C. Every 24 h, one pool of supernatant (representing extracellular virus) was removed from the flasks and titrated for virus infectivity. Every 24 h, one flask was frozen at -80 °C and titrated after freeze-thawing the cells to obtain the supernatant (representing the total virus).

2.6. Virus titration

Different batches of vaccine viruses were titrated and compared using Vero, VeroNectin-4, and VeroDogSLAM cells. Virus titration was performed using the method described in the OIE Terrestrial Manual (Chapter 2.7.14).

3. Results

Only one out of 10 frozen isolated virus samples (10% efficiency) yielded viral replication with an observed CPE, following three passages in VeroNectin-4 cells (Table 1). No virus isolation was observed using Vero or VeroDogSLAM cells. These latter two cell lines seemed less sensitive to infection than VeroNectin-4 cells. The

Table 1
Virus isolation attempt from frozen and fresh specimen on two different cell lines.

Sample conservation	Sample origin	Original threshold cycle (Ct)	Cells used for isolation			
			VeroNectin-4	Days post infection	VeroDogSLAM	Days post infection
Frozen	Pulmonary node	33.5	–		–	
Frozen	Mesenteric node	25.5	+	6	–	
			CPE at third passage			
Frozen	Mesenteric node	22.3	–		–	
Frozen	Lung	27.9	–		–	
Frozen	Lung	24.3	–		–	
Frozen	Nasal swab	33.0	–		–	
Frozen	Nasal swab	32.1	–		–	
Frozen	Rectal swab	28.9	–		–	
Frozen	Rectal swab	33.7	–		–	
Frozen	Ocular swab	32.7	–		–	
Fresh	Pulmonary node	21.3	+	3	–	
Fresh	Pulmonary node	21.0	+	3	+	6
Fresh	Pulmonary node	42.0	–		–	
Fresh	Mesenteric node	30.3	+	3	+	5
Fresh	Mesenteric node	17.9	+	5	–	
Fresh	Mesenteric node	15.6	+	3	+	3
Fresh	Lung	18.5	+	3	+	5
Fresh	Lung	29.1	+	4	+	9
Fresh	Lung	16.6	+	3	+	5
Fresh	Ocular swab	23.7	+	8	–	
Fresh	Ocular swab	19.6	–		–	
Fresh	Ocular swab	23.9	+	10	–	
Fresh	Ocular swab	24.5	+	10	–	
Fresh	Ocular swab	35.8	–		–	
Fresh	Ocular swab	35.0	–		–	
Fresh	Ocular swab	35.4	–		–	
Fresh	Ocular swab	36.7	–		–	
Fresh	Ocular swab	36.5	–		–	
Fresh	Rectal swab	26.2	+	8	+	8
Fresh	Rectal swab	14.7	+	10	–	
Fresh	Rectal swab	24.9	–		–	
Fresh	Rectal swab	22.7	–		–	
Fresh	Rectal swab	35.3	–		–	
Fresh	Rectal swab	29.2	–		–	
Fresh	Rectal swab	32.8	–		–	
Fresh	Rectal swab	28.9	–		–	
Fresh	Rectal swab	36.2	–		–	
Fresh	Rectal swab	35.1	–		–	
Fresh	Rectal swab	34.8	–		–	
Fresh	Rectal swab	35.6	–		–	
Fresh	Rectal swab	33.8	–		–	

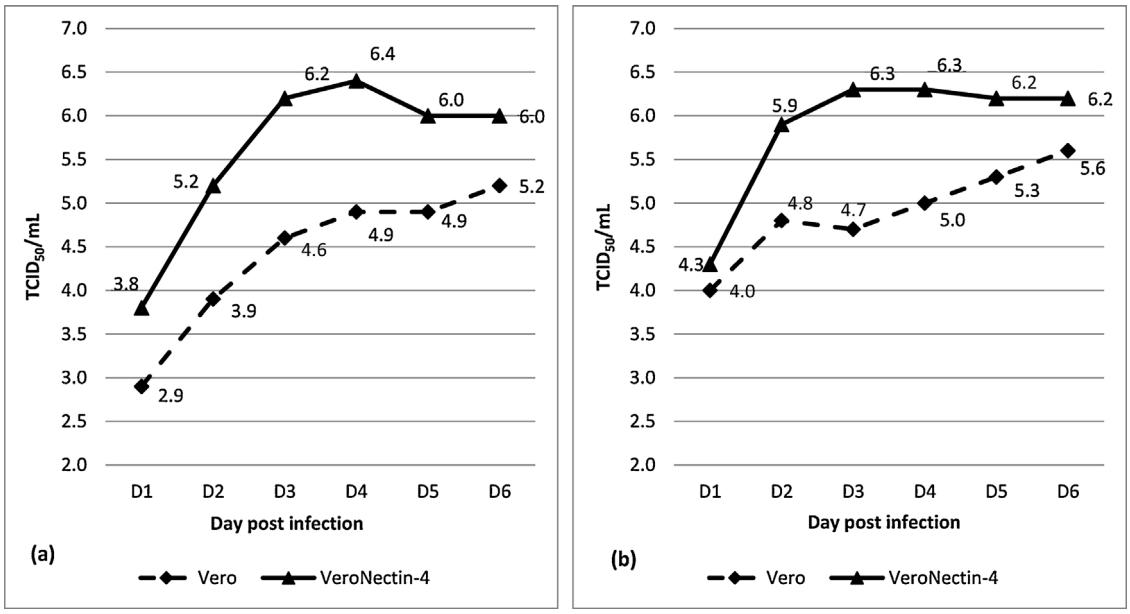

Fig. 1. Comparative viral kinetic of multiplication of PPR Nigeria 75 strain, (a) extracellular and (b) total virus. The virus growth on two cell lines: Vero and VeroNectin-4 titrated on VeroNectin-4.

mean original threshold cycle (Ct), a relative measure of the concentration of target in the PCR, was 29.8 for the negative samples and was 25.5 for the positive samples.

Table 1 also presents the comparative results of PPRV isolation from fresh samples. Virus isolation was successful from 13 fresh samples using VeroNectin-4 cells (42% efficiency) and from seven fresh samples using VeroDogSLAM cells (22% efficiency). A total of 31 freshly isolated samples were tested in each case using the two different cell lines.

Of the fresh swabs collected from infected animals, only five positive isolations were obtained from Nectin-4 cells among 22 samples (23% efficiency). When the different swabbed sites were considered, 33% of the positive swabs were of ocular origin and 15% were from the rectum. The average Ct value of positive swabs for virus isolation was 22.6 and that of negative samples was 32.2. Only one positive virus was isolated using VeroDogSLAM cells (4.5% efficiency). These results are also summarized in Table 1.

Using post-mortem organs as a virus source, isolation was successful in eight out of nine samples (89% efficiency) with VeroNectin-4 cells, compared with only six isolates with VeroDogSLAM cells (67% efficiency). A CPE was detectable at 3.4 days post-inoculation in VeroNectin-4 cells and at 5.5 days in VeroDogSLAM cells. Lung tissue and mesenteric lymph nodes were found to be the best sources of the virus.

PPRV isolation was successful after only one passage in VeroNectin-4 cells using virus inoculums from the fresh samples, in comparison with the three passages required when using frozen virus samples. The average incubation period for the isolation of virus from fresh swabs was 9.2 days, while that from post-mortem organs was only 3.4 days. Three blind passages were performed with no virus detection before deciding whether samples were negative.

The titrations for different PPRV preparations are reported in Table 2, comparing either Vero and VeroNectin-4 cells (a) or VeroNectin-4 and VeroDogSLAM cells (b). From 10 different vaccine batches, the average viral titer was 0.9 log and 1.7 log $TCID_{50}$ higher in VeroNectin-4 cells compared with Vero cells and VeroDogSLAM cells, respectively.

Fig. 1 shows the growth kinetics of PPRV on Vero and VeroNectin-4 cells. The maximum titers were obtained at 3 to 4 dpi with VeroNectin-4 cells (6.4 $TCID_{50}$/mL), and at 6 dpi with Vero cells (5.6 $TCID_{50}$/mL). Differences between extracellular and intracellular virus titers were only seen at the early phase of infection, during the first days following virus inoculation.

Table 2
Comparative virus titration on VeroNectin-4 and (a) Vero or (b) VeroDogSLAM.

(a)

PPR strain	Sample code	Titer on Vero Nectin-4 ($TCID_{50}$/mL)	Titer on Vero ($TCID_{50}$/mL)	Difference +log
Moroccan	MO01	6.0	5.7	0.3
Moroccan	MO02	6.0	5.9	0.1
Moroccan	MO03	6.1	5.4	0.7
Moroccan	MO04	5.5	5.1	0.4
Moroccan	MO05	6.6	5.3	1.4
Moroccan	MO06	6.4	5.0	1.4
Moroccan	MO07	6.0	5.3	0.7
Moroccan	MO08	5.6	5.0	0.6
Nigeria 75	N7501	5.4	4.6	0.8
Nigeria 75	N7502	4.7	3.8	0.9
Nigeria 75	N7503	6.6	5.0	1.6
Nigeria 75	N7504	6.2	4.9	1.3
Nigeria 75	N7505	6.0	4.5	1.5
Nigeria 75	N7506	6.0	4.6	1.4
Average value		5.9	5.0	0.9

(b)

PPR strain	Sample code	Titer on VeroNectin-4 ($TCID_{50}$/mL)	Titer on VeroDogSLAM ($TCID_{50}$/mL)	Difference +log
Moroccan	MO09	4.9	2.7	2.2
Moroccan	MO10	5.0	4.0	1.0
Nigeria 75	N7507	5.2	3.8	1.4
Nigeria 75	N7508	4.6	3.5	1.1
Nigeria 75	N7509	5.9	2.8	3.1
Nigeria 75	14PPRN75006	6.4	5.1	1.3
Average value		5.3	3.6	1.7

4. Discussion

Isolation of wild-type virus from freshly obtained swabs or post-mortem tissues using VeroNectin-4 cells was much more sensitive (42% efficient) than that obtained with VeroDogSLAM cells (22% efficient). In addition, VeroNectin-4 cells were also more effective for the titration of PPRV strains. Isolation of virus from fresh samples was more efficient than when deriving the virus from frozen samples. This difference could be explained by a decrease in virus infectivity during the freeze–thaw process. We found that isolation of wild-type PPRV was more efficient from post-mortem tissues than from swabs of infected animals. Subsequent infection of VeroNectin-4 cells produced similar cytopathic effects compared with those observed on VeroDogSLAM cells. Finally, kinetic growth studies using Vero and VeroNectin-4 cells also confirmed that PPRV replication, growth, and resulting viral titers were higher in VeroNectin-4 cells (Fig. 1).

In this study, we compared the ability of two cell receptors, Nectin-4 and SLAM, to increase the sensitivity of laboratory cell lines for PPRV infection and virus titration. Our results clearly show that Vero cells expressing the Nectin-4 receptor are much more suitable for PPRV isolation or vaccine titration than VeroDogSLAM cells. Expression of the canine SLAM receptor in a variety of cell lines has previously been used to isolate PPRV and other morbilliviruses by several researchers (Ono et al., 2001; Sato et al., 2012; Seki et al., 2003; Tatsuo et al., 2001a,b; Tatsuo and Yanagi, 2002). CHS-20 cells, which express SLAM derived from goats, were also recently developed (Adombi et al., 2011) and are expected to exhibit increased sensitivity to PPRV compared with cells that express human SLAM. It will be interesting in the future to compare the growth of PPRV in CH-20 and VeroNectin-4 cells. The B95a marmoset B cell line, which naturally expresses marmoset SLAM, has been tested for its ability to isolate and propagate PPRV. These cells were found to be less sensitive for both attenuated and wild-type PPRV strains (Nizamani et al., 2014; Sreenivasa et al., 2006). Coupled with the fact that B95a cells are semi-adherent and shed Epstein-Barr virus, they are less desirable for use in PPRV isolation than VeroNectin-4 cells.

The fact that Nectin-4 (unlike SLAM) is highly conserved between all mammalian species (Noyce et al., 2011), makes it an ideal receptor for isolating a wide variety of morbilliviruses in cells that support the replication of these viruses. Vero cells have a crippled innate immune/interferon system, which supports the growth of many different types of viruses (Chew et al., 2009). This study also confirmed the findings of a previous report indicating that ovine Nectin-4 is the epithelial cell receptor for PPRV (Birch et al., 2013). We have further shown that VeroNectin-4 cells are ideal for PPRV isolation from field samples as well as for the titration of PPRV.

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