Vaccinia virus: shedding and horizontal transmission in a murine model

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Vaccinia virus (VACV) has been associated with several bovine vaccinia outbreaks in Brazil, affecting cattle and humans. There are no available data about VACV environmental circulation or the role of wildlife in the emergence of an outbreak. Since VACV was isolated from rodents in Brazil, we investigated shedding and transmission of VACV strains in mice. The VACV excretion profile was assessed by PCR and chicken chorioallantoic membrane infection, revealing viral DNA and infectious virus in the faeces and urine of intranasally infected mice. Horizontal transmission was assessed by exposure of sentinel mice to wood shavings contaminated with excrement, to mimic a natural infection. Sentinel mice showed orthopoxvirus antibodies, and VACV DNA and infectious virus were detected in their faeces and intestines, even after six rounds of natural transmission. Together, these data suggest that murine excrement could play a relevant role in VACV spread and transmission, perhaps helping to explain how these viruses circulate between their natural hosts.

Following the end of the smallpox vaccination campaign, an increase in zoonotic diseases caused by poxviruses has been observed. These zoonotic diseases are mainly caused by orthopoxvirus (OPV) species, such as cowpox virus (CPXV) in Europe (Haenssle et al., 2006), monkeypox virus (MPXV), which occurs naturally in Africa and has recently been found in the USA (Reynolds et al., 2006), and vaccinia virus (VACV) in India and Brazil (Damaso et al., 2000; Trindade et al., 2006; Singh et al., 2007). In addition to the widespread distribution of OPV species around the world, they have also been detected in many naturally infected animal species. Serological screening for OPV antibodies has shown that rodents, shrews, red foxes, cats and other carnivores have antibodies against OPV (Tryland et al., 1998; Pelkonen et al., 2003; Laakkonen et al., 2006). With regard to the natural hosts of OPV species, CPXV persists in bank voles, field voles and wood and house mice, while squirrels seem to be the main natural reservoir of MPXV (Hutson et al., 2007). VACV was used as a vaccine during the smallpox eradication campaign, but to date, there are few data about the origins, natural reservoirs and mechanisms through which the virus persists in nature (Fenner et al., 1989; Trindade et al., 2007; Drumond et al., 2008).

VACV is the aetiological agent of bovine vaccinia disease in Brazil and has been described in this country since 1999. It can be characterized as an emerging zoonotic disease with economic, veterinary and human health impacts (Trindade et al., 2006). Many of the factors involved in the establishment of outbreaks on farms are unknown. As observed for other OPVs, the natural reservoirs of Brazilian VACV might consist of domestic and wild rodents (Pelkonen et al., 2003). Indeed, four VACV strains were isolated from rodents in Brazil (Lopes et al., 1965; Fonseca et al., 1998; da Fonseca et al., 2002; Trindade et al., 2004). Since rodents have already been associated with VACV infection, they might be involved in the spread or transmission of VACV in nature.

Some studies have described shedding by OPVs, including CPXV, variola virus and ectromelia virus, via host excrement or secretions, such as faeces, urine and conjunctival secretions (Gledhill, 1962; Sarkar et al., 1973; Maiboroda, 1982).
Despite the particularities of pathogenesis for these OPV species, it is possible that excrement and oropharyngeal fluids could play a relevant role in OPV shedding and transmission, since infectious virus has been detected in these clinical specimens (Sarkar et al., 1973). Due to the recent increase in the number of VACV outbreaks in Brazil (Damaso et al., 2000; Lobato et al., 2005) and the lack of information about the possible role of rodents in VACV transmission, we were encouraged to carry out a study of the shedding of VACV in excrement using a murine model. We also propose a possible relationship between viral shedding and horizontal transmission via excrement.

In order to characterize viral excretion and horizontal transmission, the VACV strains Western Reserve (VACV-WR; kindly provided by Dr C. Jungwirth, Universität Würzburg, Germany) and Guarani P2 virus (GP2V) (Trindade et al., 2006) were used as a control strain and a Brazilian low virulence strain (J. M. S. Ferreira and others, unpublished data), respectively. Viral strains were grown in Vero cells or chicken embryonic fibroblast (CEF) cells and purified on sucrose gradients, as described previously (Joklik, 1962).

The excretion profile of VACV strains was assessed by intranasal (i.n.) infection of groups of 4-week-old male BALB/c mice (four per group). These animals were intranasally infected with 10<sup>6</sup> p.f.u. VACV-WR or GP2V in 10 μl PBS (Brandt & Jacobs, 2001). Mice from the negative control group were inoculated intranasally with 10 μl PBS. Mouse faeces and urine pools were collected daily from each group until 30 days post-infection (p.i.) using a microtube positioned directly in a mouse’s anus or penis. Faeces were macerated in PBS (100 mg ml<sup>−1</sup>) and the final mixture was clarified by centrifugation at 2000 g for 3 min. Saliva samples were collected using cotton swabs and these were soaked in 100 μl PBS. Faeces and saliva supernatants and unprocessed urine were diluted in PBS (1/50) and inoculated into the CAM of 9-day-old chicken embryonated eggs in order to check for the presence of infectious virus (Sarkar et al., 1973). Vero cells and CEF monolayers were used to determine virus titres in excrement using a plaque assay (Campos & Kroon, 1993).

Viral DNA was detected in faeces throughout the entire course of infection in mice infected with VACV-WR (6 days) or GP2V (30 days) (Fig. 1), suggesting a persistent infection. Viral DNA was also amplified from urine samples; however, this was restricted to the acute phase of infection caused by GP2V (1–7 days p.i.) or VACV-WR (1–6 days p.i.) (Fig. 1). On the other hand, viral DNA was detected in saliva from mice infected with VACV-WR only on days 5–6 p.i. (Fig. 1). No viral DNA was detected in clinical samples collected from the negative control group. Infectious virus was detected sporadically throughout the entire course of infection in the faeces of mice intranasally infected with VACV-WR (days 1–6) and GP2V (days 1–30), based on the appearance of typical white pocks on CAM. Infectious particles were also detected in urine from mice infected with both strains. No infectious virus was detected in saliva. Although Vero and CEF were inoculated with clinical samples for viral titration, no cytopathological effects were observed, even following three consecutive cell passages. This is probably because the sensitivity of the cell monolayer is lower than that of CAM for infectious virus detection in clinical samples (data not shown). Similarly, due to the high sensitivity of PCR, the frequency of viral detection by CAM infection was comparatively lower than that observed in the semi-nested PCR method (Fig. 1).

For viral DNA detection, a semi-nested PCR for vaccinia growth factor (vgf) gene amplification was standardized. vgf is a conserved OPV gene that is widely used as a PCR target in association with other genes, in diagnostic and phylogenetic analyses of Brazilian poxvirus outbreaks (Fonseca et al., 1998; Trindade et al., 2007). We used a semi-nested PCR targeting vgf (J. S. Abrahão and others, unpublished data) to detect VACV DNA from clinical samples without previous DNA extraction. The final PCR product of approximately 381 bp was amplified using primers described by Fonseca et al. (1998) as follows: VGF F, 5′-GGCGATTCAATGCTGAC-3′ and VGF R, 5′-ATAATGGATTTGCAGTG-3′. In parallel, urine and processed faeces and saliva were diluted in PBS (1/50) and inoculated into the CAM of 9-day-old chicken embryonated eggs in order to check for the presence of infectious virus (Sarkar et al., 1973). Vero cells and CEF monolayers were used to determine virus titres in excrement using a plaque assay (Campos & Kroon, 1993).

Horizontal transmission of vaccinia virus strains

Intranasal infection of mice with VACV-WR induced clinical signs in 100% of the animals within 2–3 days p.i., leading to weight loss, ruffling fur and back arching. VACV-WR induced an acute lethal infection, leading to death of 100% of animals on day 6 p.i. On the other hand, none of the mice infected with the GP2V strain presented any of these clinical signs during the entire 30 day period over which they were observed. Meanwhile, neutralizing antibodies against OPV were detected in serum of 100% of GP2V-infected mice, with titres ranging from 160 to 320 neutralizing antibody units (NU) per ml. Since mice infected with VACV-WR died shortly after infection (6 days p.i.), neutralizing antibodies could not be detected in their sera. Neutralizing antibodies were also not detected in mice inoculated with PBS.

Viral DNA was detected in faeces throughout the entire course of infection in mice infected with VACV-WR (6 days) or GP2V (30 days) (Fig. 1), suggesting a persistent infection. Viral DNA was also amplified from urine samples; however, this was restricted to the acute phase of infection caused by GP2V (1–7 days p.i.) or VACV-WR (1–6 days p.i.) (Fig. 1). On the other hand, viral DNA was detected in saliva from mice infected with VACV-WR only on days 5–6 p.i. (Fig. 1). No viral DNA was detected in clinical samples collected from the negative control group. Infectious virus was detected sporadically throughout the entire course of infection in the faeces of mice intranasally infected with VACV-WR (days 1–6) and GP2V (days 1–30), based on the appearance of typical white pocks on CAM. Infectious particles were also detected in urine from mice infected with both strains. No infectious virus was detected in saliva. Although Vero and CEF were inoculated with clinical samples for viral titration, no cytopathological effects were observed, even following three consecutive cell passages. This is probably because the sensitivity of the cell monolayer is lower than that of CAM for infectious virus detection in clinical samples (data not shown). Similarly, due to the high sensitivity of PCR, the frequency of viral detection by CAM infection was comparatively lower than that observed in the semi-nested PCR method (Fig. 1).

The shedding of VACV in murine excrement, especially in faeces, raises an interesting question: could excrement represent a mechanism of VACV transmission between BALB/c mice? In order to answer this question, three groups (five in each) of 4-week-old male BALB/c mice were infected with VACV-WR or GP2V or inoculated with PBS (negative control), as described above. Mice were placed in
cages (isolators) containing sterilized wood shavings, where they were maintained for 10 days without bedding change. On day 10 post-exposure (p.e.), mice were removed from the cages and sacrificed and blood was collected for NT. Intestine biopsy samples were collected in order to perform histological analyses and viral titration. On the same day, groups (five in each) of 4-week-old male BALB/c uninfected mice (sentinel) were placed in the same cages, exposing them to the excrement-contaminated wood shavings; food and water were changed at this time. In order to analyse the maintenance of VACV infection over several generations of mice, five rounds of non-infected sentinel mice were exposed to contaminated wood shavings, using the infected bedding from the previous group of sentinel mice. To avoid direct transfer of virus from the excrement of the original infected mice to new groups, sentinel mice were placed in a clean cage with sterilized wood shavings, food and water after 5 days of exposure to contaminated faeces. All rounds of transmission followed the same protocol described above. All uninfected mice were previously tested by NT to confirm OPV seronegativity (Abdalrhman et al., 2006). Faecal samples from sentinel mice were collected for 10 days p.e. and analysed by PCR and CAM inoculation, as described previously. In this case, since mice did not present any clinical signs, it was possible to collect faeces individually. This was in contrast with the debilitated mice infected intranasally with VACV-WR that produced small amounts of excrement. On day 10 p.e., mice were euthanized and blood samples were collected to be tested by NT to check for the presence of antibodies against OPV. Intestine samples were also collected for titration assays (Campos & Kroon, 1993) and histological analyses (Crouch et al., 1995; Zaucha et al., 2001).

Despite the absence of clinical signs, VACV-WR and GP2V DNA and infectious virus were detected in faeces of sentinel mice from days 1–10 p.e. in all rounds of virus transmission (Table 1). Both VACV-WR and GP2V strains showed a high frequency of viral shedding in faeces, ranging from 40 to 100 % of the infected sentinels per round (Table 1). Neutralizing antibodies against OPV were detected only in mice in which virus was also detected in faeces at titres of 120 NU ml$^{-1}$ for VACV-WR and 93.3 NU ml$^{-1}$ for GP2V (Table 1).

In order to investigate the source of VACV-WR and GP2V in faeces, intestines were collected for titration in CEF and histopathological analysis as described for OPV intestinal infections (Maiboroda, 1982; Goff et al., 2007). The most prominent histopathological finding observed in intranasally infected and sentinel mice was lymphoid hyperplasia of Peyer’s patches (Fig. 2a). Lymphoid hyperplasia was characterized by highly reactive germinal centres and numerous cell types. Infectious virus was detected by titration in CEF cells in the intestines of mice infected intranasally with VACV-WR or GP2V and in sentinel mice (Fig. 2b). Viral titres in intranasally infected mice were higher than in sentinel mice, independent of the transmission round. Additionally, VACV-WR reached higher viral titres than GP2V (Fig. 2b).

Taken together, our results indicate that VACV strains can be shed and transmitted among BALB/c mice via excrement until the sixth round of infection. Despite the observation that VACV-WR and GP2V showed different virulence profiles in intranasally infected mice, both strains induced long-lasting faecal viral shedding in naturally infected sentinel mice without any associated clinical signals, suggesting a subclinical infection. Although pathogenesis models differ substantially,
our results are in agreement with previous studies that
demonstrate the role of excrement in the shedding of other
OPV, including ectromelia virus and variola virus (Gledhill,
1962; Sarkar et al., 1963). Previous studies suggested
horizontal transmission of VACV among mice (Gaertner
et al., 2003), but the present work is the first to clearly
establish a link between excrement and VACV transmission.

Table 1. Detection of neutralizing antibodies against OPV and detection of VACV DNA and infectious virus in faeces of mice
naturally infected by exposure to contaminated wood shavings after six transmission rounds

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<th>Sentinels (round of transmission)</th>
<th>VACV-WR</th>
<th>GP2V</th>
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<td>Viral detection in faeces</td>
<td>Mean NU ml⁻¹</td>
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<td></td>
<td>PCR positive</td>
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<td>1st</td>
<td>80 (4)</td>
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<tr>
<td>Mean</td>
<td>76.7 (23/30)</td>
<td>70 (21/30)</td>
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Given the possible shedding and transmission of VACV via
excrement in a mouse model, we speculate that this kind of
event could also take place in nature. To make that feasible,
it is necessary to consider the characteristics of possible
hosts (e.g. rodents), the environment and the VACV strain
itself. The biological and behavioural characteristics of
rodents, such as living in superpopulated communities and

Fig. 2. (a) Light micrographs of Peyer’s patch tissue sections. Intranasally inoculated and sentinel mice (6th round) show
lymphoid hyperplasia in the Peyer’s patches 10 days p.i. Control groups (PBS) presented normal appearance of colonic
mucosa with Peyer’s patches (arrows). Tissue sections were stained with haematoxylin–eosin. Magnification, ×100.
(b) Intestinal viral titres of intranasally inoculated and sentinel mice (6th round) infected with VACV strains. Viruses were not
detected in intestines of control group mice. Means ± SEM are shown.
coprophagy, could favour virus circulation among rodents via ingestion or inhalation of contaminated victuals or faeces, as has been observed for other zoonoses (Hjelle et al., 1995). Nevertheless, since OPV infections are usually associated with more than one transmission mechanism, other routes like respiratory, skin or mucosal contact – or even vertical transmission – should not be overlooked (Fenner et al., 1989). With regard to the environment, VACV outbreaks usually take place in small properties, generally those with precarious infrastructures (Leite et al., 2005; Lobato et al., 2005).

The spread and maintenance of viruses in nature and their host spectrum are important ecological issues associated with the circulation of viruses among different animal species (Maiboroda, 1982). The intrinsic environmental resistance of VACV (Essbauer et al., 2007) associated with the variety of chemical micro-niches present in faeces could contribute to maintenance of the viability of infectious virus, even for some time after excretion. Wild host reservoirs in contact with that excrement could become infected, leading to the establishment of a subclinical infection with low viral titres and without acute or lethal disease, as observed in this study for the sentinel mice. The ability of OPV to replicate in the intestine, as previously observed in other studies (Goff et al., 2007), could be a permanent source of faecal contamination by VACV (Maiboroda, 1982). Thus, those infections could contribute to viral maintenance and continuous transfer among its host. This work provides important information about VACV shedding and transmission patterns in a murine model, which could contribute to the establishment of a model of circulation and transmission of VACV in nature.

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