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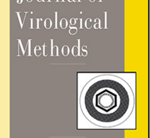

Short communication

Effects of carcase decomposition on rabies virus infectivity and detection

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ABSTRACT

Carcases received for rabies diagnosis are occasionally decomposed due to delays in finding, submitting or storing them. Positive diagnostic results from such samples are reliable but negative results may be invalid. Previous studies assessed the effect of decomposition on rabies detection using excised brains. To better reflect decomposition in the field, intact infected mouse carcases were stored at three temperatures for up to 70 days. The brains were then removed and tested using routine rabies diagnostic assays. Rabies virus was isolated using the Rabies Tissue Culture Inoculation Test (RTCIT) on days 18, 3 and 3 at 4 °C, 25 °C and 35 °C, respectively. The Fluorescent Antibody Test (FAT) detected viral antigen on days 36, 12 and 3, whilst a rabies specific Hemi-nested RT-PCR detected viral RNA on days 70, 48 and 48 at 4 °C, 25 °C and 35 °C, respectively. These findings suggest the persistence of infectious rabies virus in carcases left for 18 days at cold temperatures (4 °C) and up to 3 days in temperatures reaching 35 °C. The detection of viral RNA from a carcase decomposing at 35 °C for 48 days supports the use of molecular assays to accompany OIE-prescribed rabies diagnostic tests particularly when decomposed samples are likely to be submitted. Count = 199.

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Rabies is one of the most significant zoonotic diseases, with a case-fatality rate approaching 100% and a global distribution (Baxter, 2012; Fooks et al., 2014). The reported annual figure (50,000–70,000) for human rabies fatalities is considered an underestimate (Knobel et al., 2005; Fooks, 2005). Rabies diagnosis in animals using OIE-prescribed tests is essential to formulate control programmes, conduct epidemiological surveys and implement prophylactic measures (Fooks et al., 2014; OIE 2011). Rabies should always be confirmed by laboratory tests, conducted post mortem on central nervous system tissue removed from the cranium (McElhinney et al., 2008; Fooks et al., 2009). In the majority of resource-limited countries, however, the detection and confirmation of rabies virus in animal samples is unsatisfactory and tests are routinely not performed to international quality standards (Banyard et al., 2013). Moreover, the confirmation of rabies virus in human samples is rarely undertaken due to the high costs of testing and for cultural or religious reasons leading to misleading results and under-reporting of cases (Mallawa et al., 2007).

The Fluorescent Antibody Test (FAT) is the foundation assay for all rabies diagnostic laboratories and is recommended by both WHO (World Health Organisation) and OIE (World Health Organisation for Animal Health). It may be used to confirm the presence of the rabies virus nucleocapsid protein in the submitted sample (e.g. brain smear). The FAT gives reliable results on fresh samples within a few hours in 95–99% of cases (OIE, 2011). The Rabies Tissue Culture Inoculation Test (RTCIT) is recommended by WHO and OIE as a confirmatory test to detect live virus in infected tissues. The RTCIT involves the inoculation of the sample into a neuroblastoma cell line. Positive results are commonly obtained within 2–4 days. The FAT is then used to confirm the presence of rabies antigen in the cell monolayers. However, the sensitivities of both the FAT and RTCIT are highly dependent on the quality of the specimen (Wacharapluesadee & Hemachudha, 2010; Fooks et al., 2012).

In developing countries, resources such as the provision of a 'cold chain' may not always be available to ensure optimal carcase submission. Even in well resourced areas, logistical problems may

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result in delays finding and transporting infected carcases during surveillance programmes. The carcasses may thus be received at rabies diagnostic laboratories in various states of decomposition. Invariably, positive diagnostic results from such sources are reliable. Negative results, however, may be invalid due to deterioration in the quality of the virus or viral antigen. Previous studies have reported the effect of degradation on the validity of rabies diagnostic tests (FAT and RTCIT) (Lewis and Thacker, 1974; Valentini et al., 1991; Heaton et al., 1997; Albas et al., 1999; David et al., 2002; Araújo et al., 2008; Lopes et al., 2010).

Burkel et al. (1970) detected rabies viral antigens by FAT beyond 42 h in brain samples of striped skunk carcases exposed to outside temperatures ranging between 10 °C and 29 °C. They detected viable virus after 69 h at 10 °C but not after 20 h at 29 °C. Albas et al. (1999) similarly reported the detrimental effect of degradation on the sensitivity of conventional rabies diagnostic tests after 48 h at temperatures above 25 °C. An earlier study reported the detection of viable rabies virus, Challenge Virus Standard (CVS), in the brains of dead mice for at least 8 days at 25 °C and 20 days at 10 °C (Soave, 1966). In another study, brains from infected mice were FAT positive after storage for seven days at 25 °C but negative by viral isolation and the Negri body test (Lewis and Thacker, 1974). Debbie (1974) reported that titres of rabies virus (ERA strain) in vaccine remained fairly stable in eggs for 15 days at 6 °C, 22 °C and 37 °C, whilst Winkler et al. (1975) showed that rabies virus vaccine in baits did not retain a minimum effective dosage for foxes when exposed to 4 °C or 25 °C for 96 h.

However, previous reports employing tissue culture isolation and molecular testing have largely analysed the effect of degradation using excised brain material or cultured virus. Such anomalies in the previously published test limits reflect the variability expected when excised brain tissue or cultured viruses are prepared in different laboratories, and are then allowed to decompose under different conditions. This study aimed to assess the effect of decomposition on intact infected mouse cadavers rather than processed material, thereby better reflecting field conditions and more accurately assessing the test limitations and public health impacts. As Lewis and Thacker (1974) had previously confirmed the variation observed when different rabies street strains were used to infect the mice, a fixed and titrated laboratory strain (CVS-11) was employed to reduce variability. The limits of rabies virus viability (infectivity), antigenicity and RNA quality were assessed using RTCIT, FAT and reverse transcription polymerase chain reaction (RT-PCR), respectively.

Outbred albino female mice were inoculated intracerebrally with 40 μl 100 ID_{50} rabies virus strain CVS-11 and humanely euthanased on day 8 post-infection at a clinical score of 3 (Healy et al., 2013). All in vivo work was undertaken in BSL3/SAPO4 containment in AHVLA, following independent ethical review and complied with the Animal Scientific Procedures Act 1986. All experimental mice had access to food and water ad libitum throughout the experiment and were housed according to defined UK Home Office regulations. A P3 mask was worn during the latter stages of the study to counter the effect of the odour.

The mouse carcases were stored for different time points (2, 3, 6, 12, 18, 24, 36, 48, 60 or 70 days) at either 4 °C (refrigerator), 25 °C (laboratory room temperature) or 35 °C (incubator). The brain of an infected (I0) and uninfected mouse (C0) were removed and tested on day 0 for control purposes. At each time point the brain of the appropriate mouse carcase was removed from the head, homogenised in phosphate buffered saline and the homogenate halved. This was to minimise variability due to virus localisation in the brain. The RNA was extracted from one half brain homogenate for molecular testing and the other half was used for the OIE prescribed rabies diagnostic tests, FAT and RTCIT. Results were thus obtained for FAT, RTCIT, rabies hemi-nested RT-PCR (HnRT-PCR) and a housekeeping control 18S rRNA RT-PCR for each time point at three temperatures.

FAT was performed following the method of Dean and Abelseth (1973) using anti-nucleoprotein (N) fluorescein isothiocyanate (FITC) labelled monoclonal antibody (Fujirebio, Malvern, United States). The RTCIT was performed following the method of Webster (1987).

Total RNA was extracted directly using TRIzol® (Invitrogen, Life Technologies, Paisley, UK) according to the manufacturer's instructions. Reverse transcription (RT) was performed using the pan-*Lyssavirus* messenger sense primer JW12 (ATGTAACACCYCTACAATG) as previously described (Heaton et al., 1997). The rabies hemi-nested RT-PCR assays (HnRT-PCR) were performed using JW12 and either the JW6 primer (cocktail CARTTVGCRCACATYTTRTG) for the first round (JW6/12 PCR, 46 cycles) or JW10 primer (cocktail GTCATYARWGTRTGRTGYTC) for the second round (JW10/12 PCR, 36 cycles) as described in Heaton et al. (1997). The ribosomal RNA 18S housekeeping RT-PCR (Ambion, 46 cycles) was performed as described in Smith et al. (2000).

As expected, the integrity of the skulls and brain material varied greatly between time points and temperatures. For the carcases stored at 4 °C, the skulls were intact and the brain tissue retained structural integrity, although there was a noticeable deterioration in the brain tissue by day 24. The skulls at 25 °C remained intact for each time point, however, the brain tissue deteriorated after day 3 and by day 18 the brain was liquefied and a pastette was required to transfer the material. At 35 °C the skulls showed early signs of deterioration and had no structural integrity by day 36. The brain material at 35 °C showed signs of deterioration by day 3 and had lost all integrity by day 6.

The test results obtained for each time point at each temperature are shown in Table 1. Rabies virus was detectable by virus isolation (RTCIT) on days 18, 3 and 3 at 4 °C, 25 °C and 35 °C, respectively. The FAT detected viral antigen on days 36, 12 and 3, and the rabies specific HnRT-PCR detected viral RNA on days 70, 48 and 48 at these same temperatures respectively. However, the single round RT-PCR (JW6/12) only detected rabies viral RNA up to day 36 at 35 °C, supporting the use of increased cycles or a second round of amplification (nested or hemi-nested RT-PCR) to increase the sensitivity for significantly decomposed material (Araújo et al., 2008). Alternatively, probe based assays (e.g. real-time RT-PCR), which since undertaking this study have been shown to be more sensitive than hemi-nested RT-PCR, could be employed to further extend the rabies RNA detection limits in decomposed material (Fooks et al., 2009). The abundant 18S ribosomal RNA was detected on days 70, 70 and 48 at 4 °C, 25 °C and 35 °C, respectively using a housekeeping gene 18S rRNA RT-PCR. Hence, rabies and ribosomal RNA remained detectable by the HnRT-PCR and 18S RT-PCR in the brains of mice, whose carcases had been decomposing at 35 °C for 48 days. There was not a clear cut-off for detection of 18S rRNA and for some time points, rabies virus RNA was detected in the absence of amplifiable 18S rRNA, suggesting that this may not be a wholly reliable housekeeping gene under severe decomposition conditions (Table 1). The limits of detection for each test at the three temperatures are summarised in Table 2.

These findings demonstrate the robustness of viral RNA compared to viable virus or antigen in a decomposing animal, supporting previous recommendations for the use of PCR based confirmatory testing in the diagnosis of rabies (Heaton et al., 1997; David et al., 2002; Araújo et al., 2008). Surprisingly, rabies viral RNA was detected in the decomposed mouse carcase at least 70 days (maximum tested) after death even when the integrity of the brain tissue was compromised. This contrasts to the significant detrimental effect on the detection of RABV RNA observed when tubes of brain homogenate are left to decompose for just 72 h at room temperature (Araújo et al., 2008), perhaps suggesting

Table 1
Rabies virus viability, antigenicity and RNA quality in decomposing brain material at 4 °C, 25 °C and 35 °C.

ID	°C	Day	FAT	RTCIT	18S PCR	6/12 PCR	10/12 PCR
I0	na	0	√ (4+)	√	√	√	√
C0	na	0	×	×	√	×	×
4:1	4	2	√ (3+)	√	√	√	√
4:2	4	3	√ (3+)	√	√	√	√
4:3	4	6	√ (3+)	√	√	√	√
4:4	4	12	√ (2+)	√	√	√	√
4:5	4	18	√ (2+)	√	√	√	√
4:6	4	24	√ (2+)	×	√	√	√
4:7	4	36	√ (1+)	×	√	√	√
4:8	4	48	×	×	√	√	√
4:9	4	60	×	×	√	√	√
4:10	4	70	×	NT	√	√	√
25:1	25	2	√ (2+)	√	√	√	√
25:2	25	3	√ (1+)	√	√	√	√
25:3	25	6	√ (1+)	×	√	√	√
25:4	25	12	√ (1+)	×	×	√	√
25:5	25	18	×	×	√	√	√
25:6	25	24	×	×	√	√	√
25:7	25	36	×	×	×	√	√
25:8	25	48	×	×	√	√	√
25:9	25	60	×	×	×	×	×
25:10	25	70	×	NT	√	×	×
35:1	35	2	√ (2+)	√	√	√	√
35:2	35	3	√ (1+)	√	√	√	√
35:3	35	6	×	×	√	√	√
35:4	35	12	×	×	×	×	√
35:5	35	18	×	×	×	×	√
35:6	35	24	×	×	√	√	√
35:7	35	36	×	×	√	√	√
35:8	35	48	×	×	√	×	√
35:9	35	60	×	×	×	×	×
35:10	35	70	×	NT	×	×	×

Key: √ positive test result, (1+) intensity of fluorescence in FAT, × negative test result, NT not tested.

a slower decomposition in the unexposed brain within the carcase. Molecular tools can therefore yield results in brain material harvested long after death that may have previously been believed to be of little diagnostic value. In one example, a bat carcase, submitted for rabies (lyssavirus) testing under the passive surveillance of lyssaviruses in UK, was received after being stored by a bat conservationist in a domestic freezer (approximately −20 °C) for more than a year after death (Fooks et al., 2004; Harris et al., 2006). The presence of European Bat Lyssavirus Type-2 (EBLV-2) was confirmed by FAT and hemi-nested RT-PCR in the brain of the bat, however virus was not isolated using mouse inoculation nor RTCIT. We subsequently conducted a small additional study in which CVS-11 (rabies virus) infected mouse carcases were stored at the sub-optimal temperature of −20 °C (laboratory freezer) for up to 6 months. Brain homogenate from the carcases held at −20 °C still had viable virus (RTCIT), antigen (FAT) and RNA (RT-PCR) after 6 months storage (177 days), perhaps suggesting that the multiple freeze-thaw events of the infected bat carcase (indicated in the case history and also common for domestic freezers) had resulted in a loss of viral infectivity after a year without unduly affecting the antigenicity or RNA quality.

This study represents a unique assessment of virus infectivity and detection in decomposing carcases at varying temperatures and time points, using the range of currently employed rabies assays. These data confirm the viability of rabies virus in carcases stored or found in cold conditions up to 18 days post mortem. If temperatures remain below −20 °C, carcases may be considered infectious for several months. Conversely, the lack of demonstrable virus and antigen in carcases left for more than 3 days at 35 °C may have diagnostic implications in rabies endemic areas where molecular assays are not employed for confirmatory testing.

There is serological evidence of rabies virus exposure in camels imported to Nigeria for slaughter (Al-Rawashdeh et al., 2000; Baba et al., 2005) and the likely transmission of rabies virus to two patients who became symptomatic after butchering, preparing and consuming a dog and a cat in Hanoi (Wertheim et al., 2009). The dog implicated in one of the human deaths was killed in a road traffic accident and was subsequently taken home and butchered for food. A recent study reported the presence of rabies antigen (dFAT) in 5% of apparently healthy dogs slaughtered for human consumption in Nigeria (Mshelbwala et al., 2013). Whilst rabies virus would be inactivated during the cooking process, there may be a risk of transmission during slaughter or the removal of infected organs, particularly if the unprotected individual has exposed wounds or abrasions. The limits of rabies virus viability reported in this study may thus be of interest to direct policies or working protocols for veterinarians, local authorities, workers in slaughterhouses or those preparing potentially infected animal carcases for the food industry.

Whilst molecular assays are increasingly employed for virus typing and molecular epidemiology studies, these data further support the use of a sensitive and specific RT-PCR, in particular a nested or hemi-nested assay, to accompany conventional methodologies

Table 2
Time-point limits of detection of the individual tests for carcases decomposing at various temperatures.

Temperature	FAT	RTCIT	18S rRNA	JW6/12	JW10/12
4 °C	36 days	18 days	70 days	70 days	70 days
25 °C	12 days	3 days	70 days	48 days	48 days
35 °C	3 days	3 days	48 days	36 days	48 days

70 days maximum tested.

for routine rabies diagnosis when samples are likely to be submitted in varying states of decay.

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