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Short communication

Disinfection protocols for necropsy equipment in rabies laboratories: Safety of personnel and diagnostic outcome

Roberta Aiello*, Barbara Zecchin, Silvia Tiozzo Caenazzo, Giovanni Cattoli, Paola De Benedictis

FAO and National Reference Centre for Rabies, OIE Collaborating Centre and National Reference Centre for Diseases at the Animal-Human Interface, Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università 10, 35020 Italy

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A B S T R A C T

In the last decades, molecular techniques have gradually been adopted for the rapid confirmation of results obtained through gold standard methods. However, international organisations discourage their use in routine laboratory investigations for rabies post-mortem diagnosis, as they may lead to false positive results due to cross-contamination. Cleaning and disinfection are essential to prevent cross-contamination of samples in the laboratory environment. The present study evaluated the efficacy of selected disinfectants on rabies-contaminated necropsy equipment under organic challenge using a carrier-based test. The occurrence of detectable Rabies virus (RABV) antigen, viable virus and RNA was assessed through the gold standard Fluorescent Antibody Test, the Rabies Tissue Culture Infection Test and molecular techniques, respectively.

None of the tested disinfectants proved to be effective under label conditions. Off label disinfection protocols were found effective for oxidizing agents and phenolic, only. Biguanide and quaternary ammonium compound were both ineffective under all tested conditions. Overall, discordant results were obtained when different diagnostic tests were compared, which means that in the presence of organic contamination common disinfectants may not be effective enough on viable RABV or RNA.

Our results indicate that an effective disinfection protocol should be carefully validated to guarantee staff safety and reliability of results.

Rabies is an acute, progressive encephalitis caused by non-segmented single stranded RNA viruses belonging to the order *Mononegavirales*, family *Rhabdoviridae*, genus Lyssavirus. Rabies diagnosis in animals is based on the laboratory evidence of the Rabies virus (RABV) infecting the central nervous system. The Fluorescent Antibody Test (FAT) on acetone-fixed smears of hippocampus, cerebellum or medulla oblongata, is currently the reference test for laboratory diagnosis (WHO, 2013; OIE, 2013). In the last decades, molecular methods have been largely introduced in the diagnostic pipelines of reference laboratories and they have been particularly exploited to trace the origin of an infection (Fooks et al., 2009; De Benedictis et al., 2013; Fusaro et al., 2013). Molecular methods have already proved their high sensitivity and are undoubtedly useful in case of decomposed or improperly stored samples, in forensic investigations and whenever standard techniques are likely to fail (McElhinney et al., 2014; Beltran et al., 2014; Markotter et al., 2015). However, their use in routine post-mortem rabies diagnosis has been strongly discouraged when brain tissue is available and the gold standard technique applicable. International organisations limit the application of molecular tests to epidemiological surveys which should be conducted in well-experienced laboratories, with the adoption of stringent quality controls (WHO, 2013; OIE, 2013; Cliquet et al., 2010). As a matter of fact, the occurrence of false positive results due to nucleic acid cross-contamination has long been recognised for molecular methods (OIE, 2013) and especially observed in international inter-laboratory trials on rabies diagnosis, with a higher false positive rate obtained from molecular rather than standard techniques (Robardet et al., 2011; Robardet, 2015).

As for post-mortem rabies diagnosis in animals, many constraints make the application of molecular techniques still difficult to implement on a routine basis. Contamination may occur at any step of the procedure, from RNA extraction through the DNA amplification and gel-electrophoresis. Molecular techniques for rabies

* Corresponding author.
E-mail addresses: raiello@izsvenezie.it (R. Aiello), bazecchin@izsvenezie.it (B. Zecchin), dsbio.izsve@izsvenezie.it (S. Tiozzo Caenazzo), gcattoli@izsvenezie.it (G. Cattoli), pdebenedictis@izsvenezie.it (P. De Benedictis).

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diagnosis are still widely based on nested and hemi-nested protocols (Robardet et al., 2011; McElhinney et al., 2014), thus increasing the probability of cross-contaminating a negative sample with amplified nucleic acids. For this reason, a One Step PCRs for rabies diagnosis has been validated by our group (De Benedictis et al., 2011) and has been applied also in other reference laboratories (Lojkić et al., 2012; Robardet et al., 2011) with the aim of reducing sample cross-contamination (by reducing manipulation steps) and maintaining excellent diagnostic performances. Thus, although cross-contamination events have a high probability to be traced-back in case of inconsistent laboratory results (i.e. FAT and RTCIT negative vs PCR positive), those due to an improper sampling procedure may be assumed but seldom demonstrated with certitude. Moreover, when contamination of samples occurs from positive controls (usually obtained from standard rabies strains) during PCR procedures, sequencing may clarify inconsistent findings.

Apart from cross-contamination intrinsically linked to the procedure itself, the application of stringent quality controls at the sampling stage, including proper decontamination of necropsy equipment, is a paramount pre-requisite for a reliable laboratory diagnosis.

Evidences of nucleic acids decontamination failures have been extensively reported in relation to other viruses belonging to Category A infectious agents (De Benedictis et al., 2007; Bowman et al., 2015), as Rhabdoviridae are (Noll and Youngner, 1959). Moreover, common disinfection protocols, proving effective on previously cleaned laboratory surfaces, are expected to fail under heavy organic challenge and cannot be applied without any assessment of their efficacy.

Decontamination protocols currently applied for post-mortem animal brain sampling in the frame of rabies diagnosis are generally based on chemical methods in use for decades. Although they are assumed to be effective when reference techniques only are applied, they may not be as effective in disrupting nucleic acids in the presence of organic matter, possibly leading to false positive results. Necropsy equipment is commonly decontaminated by dipping with a chemical germicide of proven virucidal activity, followed in principle by steam autoclaving. Although in principle any organic matter should roughly be removed before dipping, in certain cases, such as with sharp instruments, an accurate cleaning is not feasible before disinfection because of the biological and physical hazards for the personnel. Steam autoclaving may not be applicable in practice, i.e. in case of tools for which this step is not advisable. Moreover, during an epidemic, the overall quality of the decontamination process may be challenged by the increasing diagnostic demand, i.e. by skipping the autoclaving step when the number of samples received largely exceeds the pieces available for sampling. Overall, the residual organic load in the disinfectant dip is expected to interfere with the standard disinfection process, particularly in case of an emergency and considering a high quantity of samples to be analysed daily. Apart from the impact that cross-contamination may have on the diagnostic outcome, sampling equipment often represents a major hazard for laboratory personnel (risk of cuts and injuries), therefore an improper decontamination may impact on a secondary and underestimated risk for laboratory personnel.

The aim of this study was to assess the virucidal efficacy of selected biocide products and their efficacy in nucleic acid decontamination by dipping of necropsy equipment, as well as to evaluate the most cost-effective protocol for decontamination when a molecular technique is foreseen. The worst case scenario in terms of organic matter load was intentionally set up for testing the most likely field conditions.

Five disinfectants, all belonging to biocidal categories considered effective against RABV (Bleck, 2006; Ausvetplan, 2008), were selected for this study. They included two oxidizing agents (sodium hypochlorite and Virkon® powder), a phenolic compound, a biguanide nanoemulsion and a quaternary ammonium compound (QAC). Their efficacy was assessed in the presence of a heavy organic challenge when used in compliance with the manufacturers' instructions (here referred to as "label use") and "off label" (at increasing concentrations and/or contact times), when label use tested ineffective (Table 1). As for sodium hypochlorite, the recommended concentrations of available chlorine for laboratory use (5000 ppm) have previously been reported as ineffective in the presence of organic material (Ausvetplan, 2008), so that higher concentrations were selected for this study. Similarly, the failure of one QAC in eliminating rabies virus infectivity was previously reported (Jaeger et al., 1978); nevertheless, these products are still widely used in rabies diagnostic laboratories and for this reason one QAC was included in the study as representative of the category. Since brand or trade names may vary from country to country, well-known chemical names of disinfecting substances are used throughout the manuscript rather than trade names, thus making the information universally suitable.

A slightly modified carrier-based method was applied (AOAC, 2012). Challenge Virus Standard-11 (CVS-11) at known titre ($10^{7.72}$ $TCID_{50}$/ml) was used as test organism. Test medium was composed by Dulbecco's Modified Eagle Medium (Gibco® Thermo Fisher Scientific, USA) with 1% antibiotics and 50% foetal calf serum (Sigma-Aldrich, USA). A final 20% (w/v) organic material suspension was obtained by homogenizing uninfected mouse brain and subsequently adding the CVS-11 concentrated suspension (1:1 ratio). For FAT testing (OIE, 2013), a CVS-11-positive mouse brain homogenate (20% w/v) was used. The organic matter suspensions were incubated at +37 °C for 30 min before use. Each sterile carrier (stainless steel small cylinder, 8 × 10 × 2 mm) was individually contaminated by immersion in 1.5 ml of CVS-11 infected brain homogenate for 15 min at room temperature. Ten carriers were tested for their ability to retain viral particles and then analysed without further decontamination process. The decontamination step consisted in dipping the carrier into 3.0 ml of disinfectant solution for the set concentration × contact time (C × Ct). After a rinsing step of 15 min in 1.5 ml of test medium in a new test tube, residual contamination on the carrier was assessed by Rabies Tissue Culture Infection Test (RTCIT) and molecular methods for virus infectivity and virus genome detection, respectively. Briefly, RNA extraction was performed using a NucleoSpin® RNA (Macherey Nagel GmbH & Co. KG, Germany) according to the manufacturer's instructions. RNA was eluted in a final volume of 60 μl and stored at −80 °C. Molecular tests applied were a One Step RT-PCR (amplicon length 603 bp) (De Benedictis et al., 2011) and a One Step Real-Time RT-PCR (amplicon length 87 bp) (Wakeley et al., 2005). The One Step RT-PCR was firstly applied. In case of a positive result, the disinfection protocol was automatically considered as testing positive for molecular methods without further investigations. Real-Time RT-PCR was applied only in case of negative results by One Step RT-PCR, to confirm that not only long RNA fragments but also short ones were undetectable after the disinfection protocol under evaluation, which was considered effective in disrupting detectable nucleic acid fragments when both the molecular tests were negative.

To mimic daily use conditions, disinfectants were diluted in tap water and the carrier's contamination, disinfection and rinsing steps were performed at room temperature. Three replicates were carried out for each C × Ct combination.

Stability over time of the effective working solutions of disinfectants was assessed through RTCIT and molecular testing, when the manufacturers indicated such an option. Briefly, stock ready-to-use solutions of disinfectants, at the concentration that proved effective under test conditions, were prepared and stored at room