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Virus isolation in cell culture for confirmatory diagnostic of rabies in bovine specimens

Isolamento viral em cultivo celular para o diagnóstico confirmatório de raiva em amostras de bovinos

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– NOTE –

ABSTRACT

This study investigated the suitability of virus isolation (VI) in mouse neuroblastoma cells (N2A) and baby hamster kidney cells (BHK-21) as a confirmatory test for diagnosis of bovine rabies. Fourty-eight brain samples from cattle suspected of rabies were initially submitted to fluorescent antibody test (FAT) and mouse inoculation test (MIT) for routine diagnostic. Subsequently, these specimens were submitted to three protocols of VI in each cell line: a single 24h or 72h passage (T1, T2), or three 48h passages (T3). The FAT and MIT combined detected 32/48 positive samples, from which MIT detected 32 and FAT 31. The average time required for final MIT results was 12.3 days (8 – 21). VI in BHK-21 cells provided definitive, positive results in 100% of the samples in 72h (T2) and in 96.9% after three 48h passages (T3). VI in N2A cells yielded positive results in 100% in 72h (T2) and in 93.7% of samples after three 48h passages (T3). Sensitivity, specificity, positive and negative predictive values were 100% in T2 in N2A and BHK-21 cells, and the Kappa *value was excellent in both cells (k=1). A single 24h passage (T1) in both cell lines performed poorly, detecting less than 40% of the positive samples. Taking together, these results indicate that VI in both cell lines, especially in BHK-21 cells that grow faster and are much easier to maintain, does represent an adequate alternative for MIT as a confirmatory test for rabies diagnostic in bovine specimens, yielding reliable results in reduced time.*

Key words: *rabies, cattle, outbreaks, diagnostic, virus isolation.*

RESUMO

Este estudo investigou a sensibilidade do isolamento viral (VI) em células de neuroblastoma murino (N2A) e células de rim de hamster (BHK-21) como um teste confirmatório para o diagnóstico de raiva bovina. Quarenta e oito amostras de cérebro de bovinos com suspeita de raiva foram inicialmente submetidas aos testes diagnósticos de rotina, imunofluorescência direta (FAT) e inoculação intracerebral em camundongos (MIT). Subsequentemente, essas amostras foram submetidas a três protocolos de VI em cada linhagem celular: uma única passagem de 24h ou 72h (T1, T2), ou três passagens de 48h (T3). Os testes FAT e MIT combinados detectaram 32/48 amostras positivas, das quais o MIT detectou 32 e a FAT 31. O tempo médio requerido para o resultado final no MIT foi 12,3 dias (8-21 dias). O teste de VI em células BHK-21 obteve resultados positivos em 100% das amostras em 72h (T2), e em 96,9% após três passagens de 48h (T3). O teste de VI em células N2A forneceu resultado positivo em 100% em 72h (T2), e em 30 das 32 amostras após três passagens de 48h (T3). Sensibilidade, especificidade, valores preditivos positivos e negativos foram de 100% tanto em N2A quanto em BHK-21 após 72h de incubação (T2). Além disso, o valor Kappa *foi excelente em ambas as células (k=1). Uma única passagem de 24h (T1) em ambas as linhagens celulares não apresentou resultados satisfatórios, detectando menos de 40% das amostras positivas. Esses resultados indicam que o isolamento viral em ambas as linhagens celulares, especialmente em BHK-21 - que multiplica mais rápido e é de mais fácil manutenção - representa uma alternativa adequada para a substituição do MIT como um teste confirmatório para o diagnóstico da raiva em amostras de bovinos, fornecendo resultados confiáveis em tempo reduzido.*

Palavras-chave: *raiva, bovino, surtos, diagnóstico, isolamento viral.*

Rabies is as an acute and fatal viral disease affecting a wide range of domestic and wild mammals, including man, caused by the ***Lyssavirus*** rabies virus (RabV) (WHO, 2005). Bovine rabies transmitted mainly by the hematophagous bat ***Desmodus rotundus*** is endemic in most Brazilian states, being responsible for important losses to farmers and livestock industry (MAPA, 2013). In Rio Grande do Sul state (RS), vampire bat-associated

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bovine rabies (and equine rabies as well) historically occurred endemically in well defined, restricted regions rather than being widely distributed (MAPA, 2013). Hence, massive vaccination of cattle was not a common practice in most regions. From 2012 to the present, however, an unprecedent rabies outbreak is underway in RS, crossing boundaries and expanding dramatically over otherwise free areas. To date, this outbreak has produced an estimate of 40-50.000 bovine deaths (SEAPA/RS, 2014). As a consequence, diagnosing bovine rabies has become an overwhelming task for some laboratories, whose diagnostic routine was historically set up for a limited number of cases year round.

Laboratory diagnosis of animal rabies is routinely performed by fluorescent antibody test (FAT) in brain tissue, usually followed by a confirmatory biological assay, e.g. the mouse inoculation test (MIT) (RUDD et al., 1980; OIE, 2013). Although MIT is highly sensitive, reliable and easy to perform, the relative long time required for the definitive results (up to 21 – 30 days) and the inherent restrictions and issues concerning animal use have led for its gradual replacement. Hence, virus isolation (VI) in cell culture has been proposed as an alternative method to replace MIT as a confirmatory test for rabies. Isolation systems based on mouse neuroblastoma (N2A) and baby hamster kidney (BHK-21) cells have been evaluated regarding to sensitivity to RabV from different animal species (RUDD et al., 1980; 1989; RUDD & TRIMARCHI, 1987; ANTÚNEZ et al., 2013). Considering the fastidious growth and the special culture medium requirements for N2A cells, the use of BHK-21 cells for VI as confirmatory rabies diagnosis would compare favorably since BHK-21 cells display a fast growth *in vitro* and are largely used in many laboratories.

The dramatic increase in submissions for diagnosis of rabies in bovine samples in the last years and the consequent overwhelming demand for diagnosis, in addition to the crescent restrictions for animal use prompted us to evaluate VI in cell culture as a confirmatory test for diagnosis of rabies in bovine specimens.

Bovine specimens (n=48) submitted to the Virology Section of the Universidade Federal de Santa Maria (SV/UFSM) between May, 2013 and January, 2015 were routinely processed for rabies diagnosis, e.g. FAT in brain smears followed by MIT (according to OIE, 2013). Subsequently, the samples were submitted to three protocols of virus isolation in N2A cells (passage 12-15, Pasteur Institute, SP) and BHK-21 cells (passage 30-35, ATCC, 334). VI was performed following three protocols: a single 24h passage (T1) or 72h (T2), or three 48h passages (T3), all followed by FAT for RabV antigens at the end of the last passage. At least one RabV positive and one negative brain were included in all steps of VI. For VI, brain fragments of samples (hippocampus, cortex, olfactory bulb and cerebellum) were macerated, homogenized and resuspended to 10% (weight/volume) in culture medium. After low speed centrifugation, the supernatants were inoculated onto monolayers of cells and monitored according to the described protocols at 37°C with 5% of CO_2.

The results of the respective tests, validation values and protocols are summarized in table 1. RabV and/or viral antigens were detected in 32 out of 48 examined specimens. From these, 31 were positive in FAT and 32 were positive in MIT (confirmatory/gold-standard test). Both tests agreed in 31/32 samples and disagreed in a single sample. Subsequently, this FAT false negative was found positive by VI in both N2A and BHK-21 cells. The average time required to achieve a positive result in MIT was 12.3 days (8 –

Table 1 - Results of the tests used for diagnosis of rabies in bovine specimens and validation values.

	---------- VI[1] in N2A cells/protocol ----------			---------- VI in BHK-21 cells/protocol ----------		
	T1[3]	T2[4]	T3[5]	T1	T2	T3
Positive/MIT[2] positive samples	03/13	32/32	30/32	02/13	32/32	31/32
Sensitivity %	23.0	100	93.8	15.4	100	96.9
Specificity %	100	100	100	100	100	100
Positive predictive value %	100	100	100	100	100	100
Negative predictive value %	61.5	100	88.9	59.3	100	94.1
Concordance %	65.5	100	95.8	62.0	100	97.9
Kappa value	0.2	1	0.9	0.16	1	0.95

[1]Virus isolation; [2]Mouse inoculation test; [3]T1: protocol 1: a single passage of virus in cells for 24h; [4]T2: protocol 2: a single passage of virus in cells for 72h; [5]T3: protocol 3: three virus passages in cells of 48h each; All validation values of VI were calculated in relation to MIT (gold-standard test to rabies diagnosis).

21). Sixteen samples were negative in FAT and in the biological assays (MIT and VI). Comparing to MIT, the FAT presented a sensitivity of 96.9%, missing one positive sample (probably contained low viral amounts). Thus, MIT fulfilled its major objective, i.e. to enhance the sensitivity of rabies diagnosis by detecting weakly positive samples that may go undetected by FAT (RUDD & TRIMARCHI, 1989).

Viral isolation in N2A and BHK-21 cells presented sensitivity, specificity, positive and negative predictive values of 100% upon a single 72h passage (T2), inclusively detecting a positive sample missed by FAT. In these protocol (T2) of both cells, the *Kappa* values were excellent (k=1) (Table 1). In the T3 protocol, however, BHK-21 cells presented a higher sensitivity, detecting 31/32 (96.9%) samples against 30/32 (93.8%) detected in N2A cells. A single 24h passage (T1) followed by FAT performed poorly in both cell lines in 13 specimens and was not tested further. Considering the time required for the final results, both T2 (72h) and T3 (3 x 48h) compared positively against MIT (mean time for final diagnosis: 12.3 days). Thus, virus isolation in BHK-21 and N2A cells in a single 72h (T2) passage provided reliable results, which were obtained much faster than in MIT. No cytopathic effect was observed in any protocol.

Our results indicate that an adequate incubation time for reaching maximal sensitivity (comparable to that of MIT) would be 72h, faster than the OIE protocol (96h). The T3 protocol (three passages of 48h each) failed in detecting one positive sample in BHK-21 cells and two in N2A. Previous studies have suggested a minimal incubation period of three (ANTÚNEZ et al., 2013) or 4-5 days (RUDD & TRIMARCHI, 1989), while others suggested that a 24h period would be adequate (BOURHY et al., 1989; CHITRA et al., 1988). These discrepancies may reflect different VI protocols or cells (cell lines, passage number, etc.), species origin, sample conservation, variable virus titers in specimens, among others (RUDD & TRIMARCHI, 1989). Since the purpose of a confirmatory test (VI in this case) is mainly to detect FAT false negative results – those containing low amounts of virus – a minimum of 72h incubation would be indicated for detecting RabV in bovine specimens.

The MIT has long been used as a confirmatory, backup test for FAT, and is especially indicated to confirm FAT negative result in cases of human exposure, in which post-exposure treatment may be urgently required (RUDD et al., 1980; RUDD & TRIMARCHI, 1989). A major drawback of this test is the long incubation period of many field RabV isolates (7 to 18 days), which would unacceptably delay the final diagnosis, the medical decision and, thus, compromise the success of the treatment (RUDD et al., 1980). Hence, a confirmatory backup test for FAT yielding a final result in less time would greatly improve rabies diagnosis in field specimens (RUDD et al., 1980). Our results demonstrate that VI in cell cultures may be an alternative for MIT as a confirmatory test for FAT in the diagnosis of rabies in bovine specimens. Using this system, the definitive results were obtained in 72h in 100% of the cases, much faster than in MIT (8-21 days). In addition to provide reliable results in less time, the 72h VI protocol dispenses the use of animals and its implications. Molecular diagnosis (e.g. PCR) may be another alternative for fast rabies diagnosis, but VI is more prone to be compared with MIT since both tests detect viable virus.

Previous studies have indicated a higher sensitivity of N2A cells over BHK-21 cells for VI of street RabV (RUDD & TRIMARCHI, 1987). Nonetheless, our VI results demonstrate that BHK-21 cells do present an adequate sensitivity – even slightly superior to N2A cells in our conditions – for bovine RabV samples. This study was conducted only with bovine samples because herbivore rabies is highly prevalent in southern Brazil and the diagnosis is routinely performed in our laboratory. In contrast, carnivore rabies has been virtually absent from our state in the last two decades. Thus, in addition to N2A cells, already incorporated into the rabies diagnosis in many laboratories, VI in BHK-21 cell provide an adequate alternative, yielding reliable and faster results, at least for samples of bovine origin.

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