*Indian Journal of Animal Sciences* **81** (3): 205–08, March 2011

# Ante-mortem diagnosis of rabies from body secretions and mucosal impression smears in experimentally infected buffalo calves

H SINGH[1] and C K SINGH[2]

*Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab 141 004 India*

Received: 15 January 2010; Accepted: 1 December 2010

ABSTRACT

An experimental study was carried out on 9 buffalo calves that were divided into 3 groups of 3 buffalo calves each. In group 1, buffalo calves (2) were inoculated with 656865 $MICLD_{50}$ of Street Rabies Virus (SRV) via oral route. 5 ml of normal mice brain (NMB) suspension was inoculated via similar route to third buffalo calf which served as control of this group. In group 2, buffalo calves (2) were inoculated with 394119 $MICLD_{50}$ of SRV via intra-thecal route. 3 ml of NMB suspension was inoculated via similar route to third buffalo calf which served as control of this group. In group 3, buffalo calves (2) were inoculated with SRV by instilling 131373 $MICLD_{50}$ SRV in either eye. SRV could be detected earliest in salivary secretion, nasal secretion and rectal secretion at 15 days post-inoculation (DPI) in group 1, 20 DPI in group 1 and 3 and 35 DPI in group 1 and 2 respectively. In corneal and prepucial impression smears, virus was found as early as 25 DPI in groups 3 and 35 DPI in groups 1 and 3, respectively. It is suggested that simple detection of rabies virus by immune-fluorescence from body secretions and impression smears from mucosal surfaces could be of ante-mortem diagnostic value in rabies.

**Key words**: Ante-mortem, Diagnosis, Nasal secretion, Rabies, Rectal secretion, Saliva

Ante-mortem diagnosis of rabies has been globally limited to molecular biologically equipped laboratories that employ polymerase chain reaction (PCR) for detection of rabies virus in secretions biopsy of rabid animals. Although latter is a sensitive technique but its use has been limited to a few laboratories. Thus, the application of molecular approaches for routine ante-mortem detection of rabies is still quite uncommon for want of highly equipped laboratories as well as highly skilleld laboratory personnel. The present study was, therefore, envisaged to develop a routinely used technique, viz. fluorescent antibody technique (FAT) whose sensitivity has been established by various workers (Devis *et al*. 1997, Tepsumethanon *et al*. 1997, Jamadagni *et al*. 2009, Sandhu et al. 2009) for ante-mortem detection of rabies virus from body secretions and mucosal impression smears of experimentally infected buffalo.

## MATERIALS AND METHODS

*Isolation of street rabies virus (SRV):* SRV was isolated from spontaneous case of a dog with history of aggressive behaviour and hyper salivation. The brain tissue impression smears was stained with Seller's stain (Meslin *et al*. 1996) and duplicate brain tissue smear was fixed in chilled acetone after drying for FAT by using anti-rabies fluorescent isothiocyanate conjugate. After pooling and weighing of hippocampus, cerebellum, cerebrum, pons and medulla of infected brain of dog, a 10% suspension with sterile distilled water containing 2% inactivated normal horse serum, was prepared and inoculated intre-cerebrally (Sehgal *et al*. 1987) into mice.

*Propagation of rabies virus:* The mice infected with the virus isolate were observed for 21 days. The symptoms observed were depression, rough hair coat, paralysis and finally death. Following death of mice, the brains were pooled, weighed and homogenized in sterile pestle and mortar to make a fine paste. A 10% suspension was prepared with sterile distilled water containing 2% inactivated normal horse serum. The suspension was centrifuged at 1000 rpm for 5 min. Penicillin and streptomycin @ 100 IU/ml and 50 µgm/ml were added to supernatant respectively and kept at 4°C for 30 min. 0.03 ml of this supernatant was inoculated intra-cerebrally in 3–4 weeks and albino mice. The SRV isolate was subjected to 3 passages into mice brain to accomplish multiplication of SRV.

*Titration of $MICLD_{50}$:* $MICLD_{50}$ of SRV isolates was

Present address: [1]Formerly M.V.Sc. Student.

[2]Senior Veterinary Pathologist, Incharge (e mail: rabiesck@gmail.com), Rabies Research cum Diagnostic Laboratory, Department of Veterinary Pathology, College of Veterinary Science.

Table 1. Detection of rabies virus in body secretions of experimentally infected buffalo calves

| DPI | Group 1 | | | Group 2 | | | Group 3 | | |
|---|---|---|---|---|---|---|---|---|---|
| | N | S | R | N | S | R | N | S | R |
| 0 | - | - | - | - | - | - | - | - | - |
| 3 | - | - | - | - | - | - | - | - | - |
| 5 | - | - | - | - | - | - | - | - | - |
| 10 | - | - | - | - | - | - | - | - | - |
| 15 | - | + | - | - | - | - | - | - | - |
| 20 | + | + | - | - | - | - | + | - | - |
| 25 | - | - | - | - | + | - | + | - | - |
| 30 | - | - | - | + | - | - | - | - | - |
| 35 | - | - | + | + | - | + | + | + | - |
| 40 | + | + | - | + | - | - | - | + | - |
| 45 | - | + | - | - | + | - | - | + | - |
| 50 | - | - | - | - | + | + | + | - | - |
| 55 | - | - | - | - | + | - | + | + | - |
| 60 | + | - | - | - | + | - | - | + | + |

DPI: Days post inoculation; N, nasal secretion; S, salivary secretion; R, rectal secretion.

Table 2. Detection of rabies virus in impression smears of experimentally infected buffalo calves

| DPI | Group 1 | | Group 2 | | Group 3 | |
|---|---|---|---|---|---|---|
| | C | P | C | P | C | P |
| 0 | - | - | - | - | - | - |
| 3 | - | - | - | - | - | - |
| 5 | - | - | - | - | - | - |
| 10 | - | - | - | - | - | - |
| 15 | - | - | - | - | - | - |
| 20 | - | - | - | - | - | - |
| 25 | - | - | + | - | - | - |
| 30 | - | - | + | - | - | - |
| 35 | - | - | - | + | + | + |
| 40 | - | - | - | + | + | - |
| 45 | + | - | - | - | - | - |
| 50 | + | - | + | - | + | + |
| 55 | + | - | - | - | + | + |
| 60 | + | - | - | + | + | + |

DPI, days post inoculation; C, corneal impression; P, prepucial impression.

titrated by intra-cerebral inoculation of serial dilutions ranging from $10^{-1}$ to $10^{-5}$ into 3 weeks-old albino mice. The calculation was carried out as per Reed and Muench (1938).

*Preparation of inoculum for experimental study:* After the third passage, all infected mice brains were pooled, weighed and homogenized in a sterile pestle and mortar to make a fine paste. Chilled sterile distilled water containing 2% inactivated normal horse serum was added to make a 10% suspension and was centrifuged at 1000 rpm for 5 min. 100 IU/ml of penicillin and 50 µgm/ml of streptomycin were added to the supernatant and were kept at 4°C for 30 min. This supernatant was inoculated via different routes to experimental buffalo calves divided into 3 groups.

*Preparation of normal brain tissue suspension:* Brains of normal mice were collected, pooled, weighed and homogenized. Chilled sterile distilled water containing 2% inactivated normal horse serum was added to make 10% normal mice brain (NMB) suspension. This suspension was centrifuged at 1000 rpm for 5 min. Penicillin @100 IU/ml and streptomycin @50 µgm/ml were added to supernatant and kept at 4°C for 30 min. This supernatant was inoculated to experimental buffalo calves, kept as control.

*Experimental study:* Male buffalo calves (9) aged 2–5 months were subjected to elementary examination for a week before starting the experimental study. During these days, their general behaviour, hematological parameters, body secretions/excretions and body temperature (°F), were analyzed. Modified counter immuno-electrophoresis was employed to rule out the anti-rabies antibiotics in sera samples of these calves before the start of the experiment.

Buffalo calves (9) were divided into 3 groups of 3 animals each, wherein buffalo calves (2) were inoculated with infected mice brain (IMB) suspension and third buffalo calf was inoculated with normal mice brain (NMB) suspension which served as control. In group 1, buffalo calves (2) were inoculated with 5 ml of 656865 $MICLD_{50}$ of IMB suspension

Table 3. Sensitivity comparison of detection of rabies virus in body secretions and impression smears

| | | Earliest detection of rabies virus in experimental animals | | | | | |
|---|---|---|---|---|---|---|---|
| | | Group 1 DPI | | Group 2 DPI | | Group 3 DPI | |
| | | Control | Infected | Control | Infected | Control | Infected |
| 1 | Salivary | CND* | 15 (2) | CND* | 25 (1) | CND* | 35 (2) |
| 2 | Nasal | CND* | 20 (1) | CND* | 30 (1) | CND* | 20 (1) |
| 3 | Rectal secretion | CND* | CND* | CND* | 35 (1) | CND* | 45 (1) |
| 4 | Corneal impression smear | CND* | 45(2) | CND* | 25 (1) | CND* | 35 (2) |
| 5 | Prepucial impression smear | CND* | CND* | CND* | 35 (1) | CND* | 35 (1) |

*CND: Could not detect, () number of calves showing positive result.

via oral route. 5 ml of NMB suspension was inoculated via ocular route with 131373 $MICLD_{50}$ of IMB suspension wherein 0.5 ml of SRB was instilled in either eye. 0.5 ml of NMB suspension was similarly instilled in either eye in the control buffalo calf of group 3. Infected buffalo calves from 3 groups were sacrificed at 30 and 60 DPI whereas control buffalo calves of all the groups were sacrificed at 60 DPL.

*Body secretions*

The samples of salivary, nasal and rectal secretions were collected from zero DPI and subsequently on 3, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 DPI. The salivary, nasal and rectal secretions were collected as discussed here.

*Salivary secretion:* Sterile cotton swab was soaked in sterile physiological saline and wet cotton swab was swirled beneath and around the tongue surface to collect saliva. The collected swab was placed in a sterile test tube. Duplicate impression smears were made from cotton swab on the labeled glass slides and were fixed in chilled acetone for direct FAT staining.

*Nasal secretion:* The sterile cotton swab was inserted into nasal cavities and pressed against walls. Duplicate impression smears were prepared on labelled glass slides and fixed in chilleld acetone for direct FAT staining.

*Rectal secretion*: The sterile wet cotton swab was inserted into the rectum by rotatory method against walls. The swab was collected in sterile test tube. Duplicate impression smears were prepared from the swab on labelled glass slides and were fixed in chilled acetone for direct FAT staining.

*Impression smears*

The prepucial and corneal impression smears were prepared at zero DPI and subsequently on 3, 5, 10, 15. 20, 25, 30, 35, 40, 45, 50, 55 and 60 DPI as discussed here.

*Prepucial impression smears:* The samples were collected by inserting the wet sterile cotton swab into prepuce and rotating it. The swab was collected in sterile test tube. Duplicate impression smears were made from the swab on labelled glass slides and were fixed in chilled actetone for direct FAT staining.

*Corneal impression smears:* Duplicate corneal impression smears were made on labelled sterile glass slides directly and were fixed in chilled acetone for direct FAT staining.

*Fluorescent antibody technique:* Duplicate smears were prepared by swirling and circling movement of the swab on the glass slide. The smears were air-dried and fixed in chilled acetone overnight. Similarly, duplicate impression smears were made on either end of the labelled glass slides from preserved tissues kept at –20°C. The smears were air-dried and fixed in chilled acetone overnight.

Anti rabies fluorescence isothiocynanate (FITC) conjugate was mixed with NMB suspension and infected mouse brain (IMB) suspensions to make final conjugate dilution of 1:20 each. The mixtures were kept at 4°C for 30 min. The conjugate-IMB and conjugate-NMB mixtures were added on the duplicate impression smears on every slide for each smear of secretion as well as that of tissue samples. The smears were covered with cover slips and incubated at 37°C for 30 min in a moist chamber. The slides were washed in 0.01 M phosphate buffered saline (PBS) pH 7.5 and in distilled water for 15 min each thereafter, air-dried and mounted in 90% buffered glycerol (pH 8.5). The slides were examined using an $AH_3$-RFC reflected light fluorescence microscope.

## RESULTS AND DISCUSSION

*Body secretions:* Detection of rabies virus at different time intervals in different groups of experimental animals indicated the variance in the *in-vivo* progression of rabies virus in the body with variation in the route of inoculation as reported earlier (Singh and Grewal 1999, Singh 2008). Rabies virus in salivary secretaion could be detected earliest on 15 DPI in group 1, 25 DPI in group 2 and 35 DPI in group 3 (Table 1). Isolation of rabies virus in saliva for the first time had been reported by Sikes (1962). Later, Baltazar *et al*. (1986) has also demonstrated rabies virus in the salivary secretion of sheep. Further, Fekadu (1988) in another experimental study in dogs has also reported the detection of rabies virus in salivary secretion. While there are reports of successful detection of rabies virus in salivary secretions, there are also reports of the experimental studies wherein rabies virus could not be detected in saliva of the experimentally inoculated animals. One such observation is of Hill and Beran (1992) wherein after inoculation of raccoons, the saliva samples did not reveal rabies virus even up to 92 DPI. While early detection of rabies virus in salivary secretaion lends this approach the requisite sensitivity, however, the failure in detection, thereafter, precisely due to the intermittent secretion of the virus in body secretions renders this approach unreliable because of false negative results. Nevertheless, it still is worth an attempt where the clinical investigator needs to be cautioned to ignore the negative results—which could be false negative.

Presence of viral antigen in nasal discharge was first detected on 20 DPI in groups 1 and 3 and 30DPI in group 2. SRV was earliest detected in rectal secretion of infected buffalo calves on 35 DPI in groups 1 and 2 and later on it was found on 50 DPI in group 2. While some reports are available about the detection of rabies virus in salivary secretion, however, only a few workers have attempted to detect rabies virus from the other body secretions for ante-mortem diagnosis of rabies. Steinhagen (1992) has reported that nasal and pharyngeal swabs examined by immunofluorescence gives promising results, but has recommended further research in this regard. The detection of rabies virus in the nasal discharge has likewise been found to be promising in the present sutdy.

Whereas the approach of detection of rabies virus in the

body secretions might not be quite practical in canines especially those showing furious symptoms, however, this approach can be of practical significance in case of bovines and ovines vis-a-vis ante-mortem diagnosis of rabies.

*Impression smears:* Corneal impressions revealed the viral antigen on 25 DPI as earliest in group 3, 45 DPI in group 1 and 30 DPI in group 2 (Table 2). The virus in the prepucial smear was detected earliest on 35 DPI in group 1 and 3; however, it could not be detected in group 1 throughout the experimental study.

On comparison of sensitivity of detection of rabies virus in body secretions and impression smear preparations, it was found that detection of rabies virus in secretions is more sensitive approach than detection of rabies virus in impression smears. However, the rectal secretion among all body secretions examined and the prepucial impression smear among all the impression smears tested were least sensitive. Corneal, impression smear though more sensitive than prepucial impression smear was less sensitive than salivary and nasal secretions. Comparison of salivary and nasal secretion for detection of rabies virus revealed that salivary secretion was more sensitive for detection of rabies virus in groups 1 and 2 whereas nasal secretion was more sensitive for detection of rabies virus in group 3 infected buffalo calves (Table 3).

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