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Original Research

A Comparative Study of The Immunofluorescence in Brain, Salivary Gland and Cornea in Rabid Carcasses

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Abstract

The present study has compared the immunofluorescence observed in the brain, cornea, and salivary gland of rabid carcasses. It has enabled a relative assessment of the reliability, sensitivity, and effectiveness of using corneal and salivary gland impression smears for the diagnosis of rabies in live animals. The study was conducted on 352 carcasses belonging to various species of domestic and wild animals. Direct Fluorescent Antibody Test (dFAT) was performed on the brain of carcasses to detect rabies. The smear from the cornea and salivary gland of rabies-positive animals were also subjected to dFAT and a comparison was made between each with regard to the intensity of staining and the area of distribution as indicated by fluorescence. The study revealed that the sensitivity of corneal impression smear for rabies diagnosis in animals was found to be lesser when compared with impression smear from the salivary gland.

Keywords: Animals, Brain, Cornea, dFAT, Rabies, Immunofluorescence, Salivary Gland

Introduction

Rabies has been recognized as one of the most dangerous and feared zoonotic disease ever since the dawn of human civilization. It affects almost all species of warm-blooded animals including humans. There exist no effective curative measures once the clinical symptoms are manifested. The disease is most often transmitted through close contact with the saliva of a rabid animal via bites or scratches. Rabies is a highly fatal disease with varying incubation periods ranging from a few days to years. The affected animal exhibits either furious signs characterized by aggressiveness, unprovoked biting, uninterrupted and frequent howling, irritability, and hyper-excitability; or dumb signs characterized by paralysis of masticatory muscles, inability to swallow, profuse salivation, dropping of the lower jaw, and paralysis of rear limbs. This viral disease causes acute encephalitis in warm-blooded animals, ultimately resulting in death due to respiratory failure (Warrell *et al*., 1976).

Though clinical symptoms can be considered as a factor suggestive of rabies, the disease has to be confirmed by reliable laboratory tests. The confirmatory diagnosis of rabies is usually done by the examination of the brain on necropsy. The fluorescent Antibody Technique (FAT) on impression smears from the brain is considered to be the gold standard test for the diagnosis of rabies as approved by WHO and OIE on account of its sensitivity and reliability (Bourhy *et al.*, 1989; Meslin, 1996). Though the procedure is expensive and demands appropriate infrastructure and well-trained technicians, the speed and reliability of the dFA test have made it the stalwart of rabies diagnosis. Another challenging aspect in this regard is the procurement of the brain which is cumbersome and equally risky and attainable only in deceased animals.

In live animals, the diagnosis is usually carried out by examination of the corneal smear and saliva (Crepin *et al*., 1998; Zaidman and Billingsley., 1998), but the results are never conclusive. Recent researches reveal that the sensitivity of FAT on corneal impression smear is very less in comparison to FAT on brain tissue smears (Awahan *et al*., 2011). A systematic study to verify and streamline the reliability, sensitivity, and effectiveness of using the corneal and salivary gland impression smears for the diagnosis of rabies in live animals is a felt need in the diagnosis of rabies. But a study of this nature is cumbersome and difficult due to the problems associated with the handling of the suspected cases and collection of the right test material. Hence a study to compare the sensitivity and reliability of corneal impression and the salivary gland impression in dead animals with that of the immunofluorescence in the hippocampus was designed in confirmed positive cases of rabies.

Materials and Methods

The study was performed at the Rabies diagnosis and Research cell, Department of Veterinary Pathology, College of Veterinary and Animal Sciences, Mannuthy, under the Kerala Veterinary and Animal Sciences University.

Sample Collection

The study was conducted on carcasses of various species of domestic and wild animals brought for rabies diagnosis to the Department of Veterinary Pathology, College of Veterinary and Animal Sciences, Mannuthy, over a period of 16 months. A total of 352 carcasses comprising of 246 dogs, 61cats, 22 cattle, 12 goats, four rabbits, one pig, three squirrels, one leopard, one bat, and one mongoose formed the material for study.

An impression smear from the brain was collected as per the method described by Tierkel (1973). The head was decapitated from the body at the occipito-axial articulation. From the disarticulated head, the skin was reflected craniocaudal and temporal muscles on the dorsolateral surface of the skull were removed. The skull was then opened, meninges dissected and the brain examined for gross lesions if any. Impression smears from the hippocampus, mandibular salivary gland, and cornea of all the carcasses was collected in pre-marked areas of one end frosted clean microscopic glass slides.

Fluorescent Antibody Technique (FAT)

Direct Fluorescent Antibody Test was performed (as per the procedure described by Dean *et al.,* 1996) on impression smears prepared from the cornea and salivary gland of 98 animals comprising of 79 dogs, 5 cats, 9 cattle, and 5 goats which were confirmed to be rabid. Impression smears collected from different areas of the brain, cornea, and salivary gland were fixed in cold acetone (immersed in a Coplin jar containing cold acetone and refrigerated) for a

minimum of 30 minutes. The fixed smears were taken out of the refrigerator and kept outside to dry in air. To the air-dried smear, 100µl of lyophilized adsorbed antirabies nucleocapsid fluorescein isothiocyanate conjugate from prediluted aliquots was added using a micropipette. The slides were then placed in a moist chamber, covered, and incubated at 37ºC for 30 minutes. After incubation, the slides were taken out and thoroughly washed in two changes of phosphate-buffered saline (PBS) pH 7.2, each for a time period of 5 minutes. The slides were then taken out and mounted with a 22 sq mm coverslip using glycerine buffer as the mounting medium. The slides were then viewed under the 40X objective of a fluorescent microscope *(Carl Zeiss AXIO)* with excitation filter and barrier filter systems selected for excitation in the ultraviolet spectrum (380nm). A known positive sample was kept as the positive control and a smear incubated with quartz distilled water alone was taken as the negative control.

Table 1: Composition of PBS used for dFAT

Chemical ingredient	**Quantity**
Stock solution A	
Sodium dihydrogen orthophosphate	3.12g
Distilled water	up to 100ml
Stock solution B	
Disodium hydrogen orthophosphate	2.83g
Distilled water	up to 100 ml
Working buffer solution	
Stock solution A	28 ml
Stock solution B	72 ml

Table 2: Composition of glycerin buffer used as mounting media in dFAT

Chemical ingredient	**Quantity**
Glycerin	5 ml
PBS at pH 7.2	5 ml

Microscopic Examination

The slides were scanned over approximately 50 fields at a magnification of 400, for the presence of characteristic apple green fluorescence. Based on the intensity of staining and the distribution of nucleocapsid antigen as indicated by fluorescence, the different slides were graded on a scale ranging from 0 to 4.

Table 3: Criteria followed for grading the fluorescence (Rupprecht *et al*., 2018)

DESCRIPTION	**GRADE**
Massive infiltration of large and small-sized inclusions in almost every area of the impression (at least in 90% area) with glaring apple green brilliance. Numerous inclusions in all microscopic fields.	4
Inclusions of varying size and shape in 75 – 80% of the area of impression with slightly diminished staining intensity. Numerous inclusions in most fields.	3
Inclusions of varying size and shape are present in 10 – 50% of the area of impression smear with a slight loss of glare. Few to a moderate number of inclusions in most microscopic fields.	2
Inclusions of varying size and shape are present in <10% of the microscopic fields with noticeably dull fluorescence. Few inclusions per field.	1
Clear blackout areas in all microscopic fields.	0

Statistical Analysis

The grades assigned for fluorescence on impression smears from the cornea and salivary gland were correlated with that of the hippocampus independently by means of the Karl Pearson correlation coefficient (Mukaka, 2012). Sensitivity, specificity, positive predictive value, and negative predictive value of corneal smear and smear from

salivary gland in the diagnosis of rabies were calculated using formulae (Parikh *et al.,* 2008).

Table 4: Formulae for calculating sensitivity, specificity, positive predictive value, and negative predictive value

Condition (as determined by the gold standard – dFAT on hippocampus)				
Test outcome		Condition positive	Condition negative	
	Test outcome positive	True positive (TP)	False positive (FP)	Positive predictive value (PPV) = TP / (TP + FP)
	Test outcome negative	False negative (FN)	True negative (TN)	Negative predictive value (NPV) = TN / (FN + TN)
		Sensitivity = TP / (TP + FN)	Specificity = TN / (FP + TN)	

Results

Out of the 352 carcasses subjected to the diagnosis of rabies by dFAT, 155 (44.03%) animals (belonging to canine, feline, bovine, and caprine species) were found to be positive for rabies. The age of the animals infected with the disease ranged from 45 days to 10 years. Out of 155 animals found positive for rabies, 15 animals (9.68 %) belonged to the vaccinated group. A more detailed analysis of their vaccination history revealed that four of them including three dogs and a calf had received only a single dose of post-exposure vaccine. The remaining 11 had been subjected to pre-exposure prophylactic vaccination, but without adhering to the proper schedules for prophylactic and booster vaccinations.

A retrospective analysis of their bite history revealed that 57 animals including 49 dogs, two cats, five cattle, and one goat were reported to have a definite history of being bit or scratched by other dogs, cats, or wild canids beyond suspicion. Another 45 rabies-positive cases had no report of bite or scratch by other carnivores. The remaining 53 animals were doubtful of whether injured by other beasts or not.

The animals confirmed as positive for rabies exhibited varying signs of nervous dysfunction characterized by changes in behavior as reported by the owners. The rabid animals were grouped under three categories based on the predominant clinical manifestation – as those exhibiting furious form, dumb/paralytic form, and obscure form. Out of the 136 rabid dogs, 72 dogs which contribute 52.94 % were found to have exhibited a “furious form of rabies” or the classical “mad–dog syndrome” characterized by aggressiveness, restlessness, and hyper-excitability. Fifty-six dogs (41.18 %) exhibited a dumb or paralytic form of rabies characterized by weakness and paralysis of the rear limbs. A total of 8 dogs (5.88 %) were reported to have succumbed to sudden death without any clinical manifestations.

Statistical Measures of Performance of dFAT on Smear from Cornea and Salivary Gland

Correlation Analysis

Comparison of dFAT for rabies virus nucleocapsid antigen between the cornea (Fig 1) and hippocampus (Fig 2) as well as between salivary gland (Fig 3) and hippocampus of 84 carnivores (dogs and cats) under study showed a significant correlation at 0.01 level with Karl Pearson correlation coefficient of 0.391 and 0.472 respectively. However, the correlation coefficient was higher between the salivary gland and the hippocampus than that observed between the cornea and hippocampus. Similarly, a comparison of dFAT between the cornea and salivary gland also revealed a positive correlation with a correlation coefficient of 0.558.

Karl Pearson’s correlation analysis revealed that the correlation of immunofluorescence between smears from salivary glands and the hippocampus was higher when compared to that of cornea and hippocampus in carnivores and ruminants (Tables 5 and 6).

Table 5: Karl Pearson's coefficient of correlation between immunofluorescence obtained from the hippocampus, cornea & salivary gland of small animals (dog & cat)

	Hippocampus	Cornea	Salivary gland
Hippocampus	1	0.391	0.472
Cornea	0.391	1	0.558
Salivary gland	0.472	0.558	1

Table 6: Karl Pearson's coefficient of correlation between immunofluorescence obtained from the cerebellum, cornea & salivary gland of ruminants (cattle & goat)

	Cerebellum	Cornea	Salivary gland
Cerebellum	1	0.510	0.625
Cornea	0.510	1	0.809
Salivary gland	0.625	0.809	1

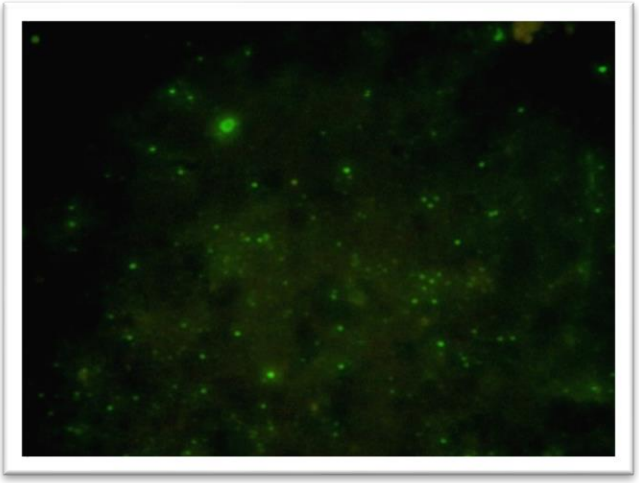

Figure 1: Rabies: Immunofluorescence in an impression smear from the cornea of a dog with grade 2 (dFAT x 400)

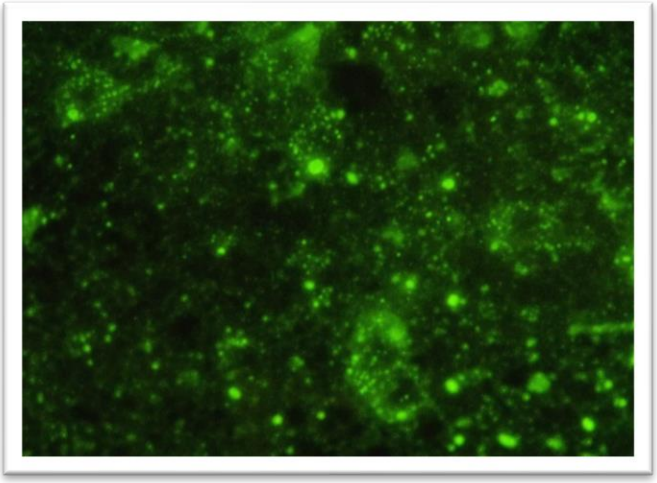

Figure 2: Rabies: Immunofluorescence in an impression smear from the hippocampus of a dog with grade 4(dFAT x 400)

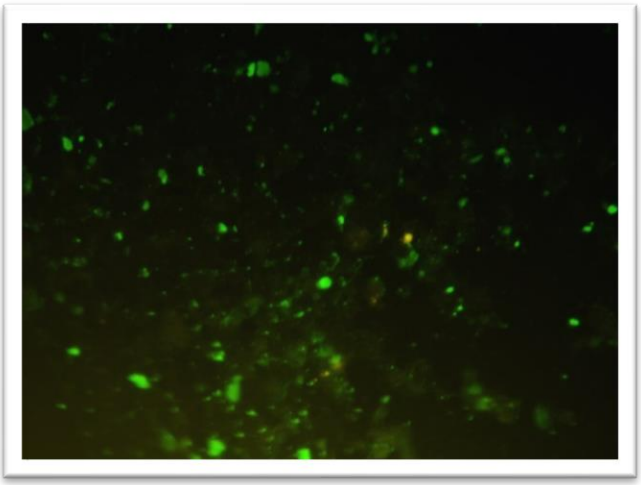

Figure 3: Rabies: Immunofluorescence in an impression smear from the salivary gland of a dog with grade 2 (dFAT x 400)

Table 7: Measure of reliability of the various methods in the diagnosis of rabies in different species

SPECIES	SENSITIVITY		SPECIFICITY		PPV		NPV	
	Cornea	Salivary gland	Cornea	Salivary gland	Cornea	Salivary gland	Cornea	Salivary gland
Dog & Cat	82.35%	94.85%	100%	100%	100%	100%	82.09%	94.08%
Cattle & Goat	50%	78.57%	100%	100%	100%	100%	74.07%	86.96%

Discussion

The study was taken up with the aim of comparing the efficiency of detecting the immunofluorescence in the brain, cornea, and salivary glands in confirmed cases of rabies. Though corneal impression and saliva smears are examined for detection of fluorescence in live suspected cases of rabies, their reliability is doubtful due to problems associated with sampling. Hence the study was directed toward a comparison of immunofluorescence in salivary gland and cornea in dead animals so that the reliability of such tests in the diagnosis of rabies in live animals can be assessed. The comparison revealed a higher correlation between the salivary gland and the brain compared to that between the cornea and brain in all species of animals.

The presence of viruses in various organs including the cornea and salivary gland has been demonstrated by various authors (Goldwasser *et al*., 1959; Dierks *et al.* 1969; Debbie and Trimarchi., 1970). The sensitivity value obtained for the diagnosis of rabies by performing dFAT on corneal impression smear and impression smear from the salivary gland is fairly higher than those obtained previously by Larghi *et al.* (1973), Baer (1991) and Awahan *et al.* (2011). The current research findings are on par with the observations made by Goldwasser *et al.* (1959) who had previously reported a sensitivity of 89.09% for dFAT on salivary glands in a study conducted on 55 rabid animals belonging to the category of carnivores and ruminants. Reports by Awahan *et al.* (2011) revealed a relatively low sensitivity of 28.57% for dFAT on salivary smears in live animals. The results of a comparative study of immunofluorescence in the cornea and salivary gland have been contradictory as described by different research workers. The conflicting reports may be due to the intermittent shedding of the virus through saliva as described by Fekadu *et al.* (1981).

Conclusion

The findings of the current research suggest that a salivary smear is a better sample for the diagnosis of rabies in live animals when compared to a smear from the cornea. A positive test result upon dFAT on salivary smear can be considered positive for rabies. However, since the test does not provide 100% sensitivity, a negative test result cannot be assumed to rule out rabies. Examination of the brain by dFAT is mandatory before declaring that the animal is not rabid.

Contribution by Authors

All the authors contributed equally to writing the manuscript. The final manuscript was read by all others and consented to publication.

Conflict of Interests

There is no conflict of interest.

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