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Research article

Abattoir based study of rabies virus in brain tissues of slaughtered animals using conventional diagnostic techniques

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Abstract

Rabies is considered a deadly zoonotic disease that still has been causing huge fatalities in all over the world. The objective of the present study was to investigate the histopathological, immunohistochemical and molecular diagnosis of rabies in randomly selected ruminant's brain tissue samples, slaughtered at eight abattoirs of Faisalabad, Pakistan. A total number of 192 brain samples of sheep (n^1=48), goats (n^2=48), cows (n^3=48) and buffaloes (n^4=48) were procured and, immersed in 10% neutral buffer formalin for fixation purpose. Most of the samples were quite healthy and their percentage distribution of variable pathological lesions (neuronal necrosis, perivascular cuffing and vascular congestion) in all brain samples was noted, but found negative for rabies virus infection. However, one sample of goat and buffalo were diagnosed positive for rabies virus by exhibiting characteristic pathological lesions such as babe's nodule, cavernous lesions, perivascular cuffing, satellitosis and degenerative changes in various sections of the brain. Similarly, immunohistochemistry showed intense characteristic antigen-antibody reaction by producing viral masses in cerebrum and cerebellum of the brain. Reverse transcriptase PCR (RT-PCR) further confirmed the findings by generating nucleic acid band against a standard DNA ladder. This study was the second insight into rabies cases scattered among domestic animals of Faisalabad. Proper awareness, reliable prophylactic measures and diagnostic methods must be established to stop the concurrent spread of the rabies virus in humans and animals.

Keywords: brain sample; rabies virus; histopathology; immunohistochemistry

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Introduction

Rabies is a deadly infectious disease that causes severe encephalitis in all warm blooded animals and, represents the greatest economic problems related to livestock and public health in many countries (Camila et al., 2014, Feng et al., 2014). It is caused by single stranded, non-segmented negative sense RNA genome

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of genus *Lyssavirus* and of family *Rhabdoviridae*. A large number of people (>30,000 in Asia and 2,500 in Pakistan) died of rabies, mainly due to dog bites which is the main reservoir of rabies in Asia (Yousaf et al., 2012; Zaidi et al., 2013). The incubation period of naturally infected animal varies from 20 to 90 days depending upon the site of the bite (Jackson, 2011; Johnson et al., 2011). The virus travels in a retrograde fashion from peripheral nerves to central nervous system where, it causes severe encephalomyelitis resulting in inevitable death (Jackson, 2011).

Natural and experimental RV infection in ruminants exhibit salient clinical signs and symptoms such as fever, anorexia, sexual excitement, off feed, bellowing, aggressiveness, salivation, uncoordinated movements and paralysis of limbs (Brookes et al., 2007; Beigh et al., 2014). However, these features are not always prominent in ruminants, as they are apparent in dogs (Woldehiwet, 2005). Prophylactic vaccination is the mainstay in controlling rabies among animals and humans, administrated either with or without immunoglobulin (McElhinney et al., 2008; Yousaf et al., 2012).

At present, a range of diagnostic techniques has been well established by WHO and OIE to diagnose rabies at ante-mortem and post-mortem, including Fluorescent Antibody Test (FAT) as gold standard test (McElhinney et al., 2008). Nevertheless, immune-histochemistry also provides actual and precise results with an equal sensitivity and specificity to that of FAT (Stein et al., 2010; Faizee et al., 2011). Clinical diagnosis can be achieved in biological samples such as skin biopsy, saliva and cerebrospinal fluid using RT-PCR that helps us in virus characterization by detecting specific nucleic acid on the gel (Hsu et al., 2005). This technique is reliable, quick and sensitive tool to diagnose rabies in all animal species, even in denatured and decomposed samples (Biswal et al., 2007; Faizee et al., 2011). At necropsy, formalin fixed brain tissue samples are processed by routinely used diagnostic techniques such as histopathology and immunohistochemistry which are being used worldwide in various animals and humans to reveal characteristic lesions and distribution of viral aggregates respectively (Jamadagni et al., 2007; Manickam et al., 2009; Stein et al., 2010; Beigh et al., 2014). It is because in developing states, the preservation and processing of fresh sample seem difficult and burdensome due to mismanagement during sample collection and secondly, due to possible risk of environmental infection with rabies virus (Beigh et al., 2013). Moreover, formalin fixed treated tissue samples are not only cost effective and save time, but also preserve the actual form of a sample without rendering further autolysis until processing (Abreu et al., 2012).

Pakistan is still lacking the general awareness among the public regarding the implementing of integrated control strategies, data management and establishment of proper diagnostic & prophylactic centers to minimize rabies incidence (Zaidi et al., 2013). The present study, therefore, was designed to screen the brain samples of slaughtered animals (sheep, goats, cows and buffaloes) for the rabies virus and pathological changes associated with these infected cases.

Materials and Methods

Study area & sample collection

Faisalabad is the third largest metropolitan city, located in Punjab province of Pakistan. Relatively longer period of hot weather occupies throughout all the cities of Punjab. The area of the city has been divided into eight towns/sites from where samples were collected. The maximum temperature values in summer and winter reach up to 50°C and −2°C, respectively. A total of 192 brain samples of 4 animal species were procured from eight abattoirs, located in each town of Faisalabad. In other words, six brain samples of each animal species were obtained from each abattoir as shown below:

$\Sigma n^1 = 6 \times 8 = 48$ cows, $\Sigma n^2 = 6 \times 8 = 48$ buffaloes, $\Sigma n^3 = 6 \times 8 = 48$ sheep and $\Sigma n^4 = 6 \times 8 = 48$ goats, $\Sigma^{n1+n2+n3+n4} = 192$ brain samples

Procurement of dog bite cases

To assess the overall burden of dog bites, a total number of dog bite cases in domestic animals were procured from the main Civil Veterinary Hospital, Faisalabad, Pakistan from the year 2006 to 2010.

Paraffin sectioning

The typical histopathological technique was followed for fixation, dehydration, embedding, sectioning and mounting of paraffin section as previously described (Bancraft and Gamble, 2008).

Immunohistochemistry

Commonly used methodology was adopted for immunohistochemical staining using hematoxylin as counter stain as described earlier (Camila et al., 2014). Polyclonal rabbit anti-rabies antibody (1:500 dilutions) was used as primary antibodies for 1 hr at room temperature while anti-rabbit antibodies (Vector Lab.) were used as linker secondary antibodies to incubate the sections for 20 minutes at room temperature. The use of polyclonal antibodies facilitates in detecting a wide range of antigenic types present among various animal species as compared to the monoclonal antibodies.

Reverse transcriptase polymerase chain reaction (RT-PCR)

The polymerase reaction was performed on the suspected samples using Trizol LSreagent® for RNA extraction and cDNA kit (Fermentas) to prepare complementary DNA according to the manufacturer's recommendations as described earlier (Numan et al., 2010). While for PCR reaction, 5'-3' TTT GAG ACT GCT CCT TTT G and 5'-3' CCC ATA TAG CAT CCTAC were used as forward and reverse primers in the reaction tube. The PCR products were loaded in 1.5% agarose gel and analyzed after electrophoresis in gel documentation system.

Results

Assessment of dog bite cases

A huge number of dog bite cases among domestic animals were obtained from the year 2006 to 2010. Unfortunately, health workers do not give due consideration to save the detail record of each animal bite history at hospitals because, rabies is still considered as neglected disease. However, recorded bite cases including livestock species (sheep, goat, cow, buffalo, horse and donkey) were 3519, 223, 1720, 2760 and 4806 during the years 2006, 2007, 2008, 2009 and 2010, respectively.

Gross pathology

Grossly, all the brain samples were devoid of pathological changes and appeared healthy in consistency, color, shape and size. However, fewer brain samples of small (sheep and goats) and large ruminants (cows and buffaloes) demonstrated certain areas of discoloration and edema in the cerebrum. Rabies suspected brain samples of goats showed congestion and edema and that of buffaloes viewed petechial hemorrhages at the dorsal surface of cerebrum respectively.

Pathological lesions in sheep & goats

The summary of pathological lesions has been presented in Table 1. Formalin fixed paraffin embedded tissue sectioning revealed clear structure of neuronal cell layers residing in the cerebrum, cerebellum and hippocampus. A variable amount of minute pathological lesions were also found in cerebrum and cerebellum of various samples.

However, the degree of severity was not the same in all areas of the brain samples. Certain areas of cerebrum showed severe perivascular cuffs and cavernous lesions. These specific pathological lesions were not observed in all the brain samples. However, mild reactive inflammatory changes such as necrosis and severe congestion appeared in few brain samples that could hardly be attributed to ascertain the presence of rabies virus or to further diagnose them via immunohistochemistry.

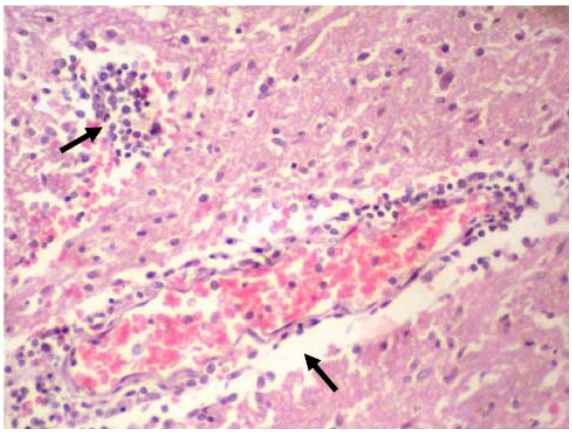

Fig. 1: Arrows showing typical babe's nodule (upper) and perivascular cuff (lower) surrounded by inflammatory cells in rabies positive cerebrum of goat brain sample (H&E, X-400).

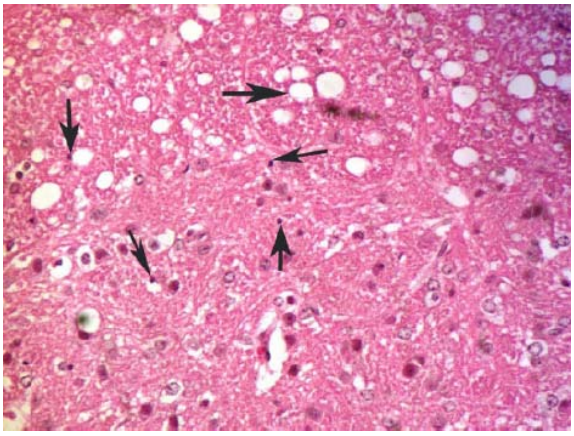

Fig. 2: Arrows showing typical honey comb cavernous lesions in rabies infected cerebrum of goat brain sample along with necrotic and degenerative changes (H&E, X-200).

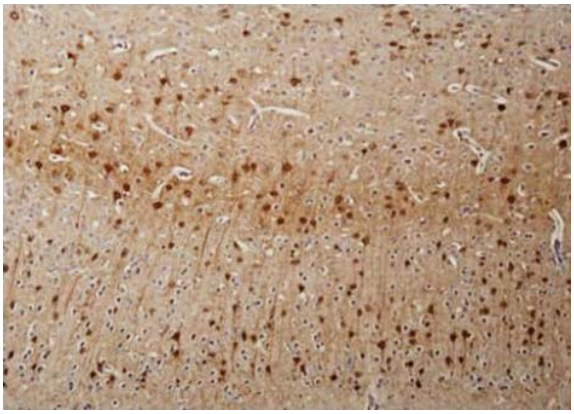

Fig. 3: Goat brain's cerebrum exhibiting several brownish colored immuno-stained neuronal soma and dendrites (DAB substrate, X-200)

Table 1: Percentage distribution of pathological lesions in brain samples of sheep, goat, cow and buffaloes

Lesions	Sheep	Goat	Cow	Buffalo
Necrosis	13(27.08%)	22(45.83%)	17(35.41%)	8(16.66%)
Pervascular cuffs	9(18.75%)	29(60.41%)	6(12.5%)	19(39.58%)
Cell degeneration	3(6.25%)	8(16.66%)	1(2.08%)	20(41.66%)
Babe's nodules	0(0%)	1(2.08%)	0(0%)	1(2.08%)
Cytoplasmic vacuolation	10(20.83%)	5(31.25%)	7(14.58%)	6(12.5%)
Congestion	5(10.41%)	11(22.91%)	2(4.16%)	12(25%)
Satellitosis	3(6.25%)	1(2.08%)	0(0%)	4(8.33%)
Rabies virus positive	0(0%)	1(2.08%)	0(0%)	1(2.08%)

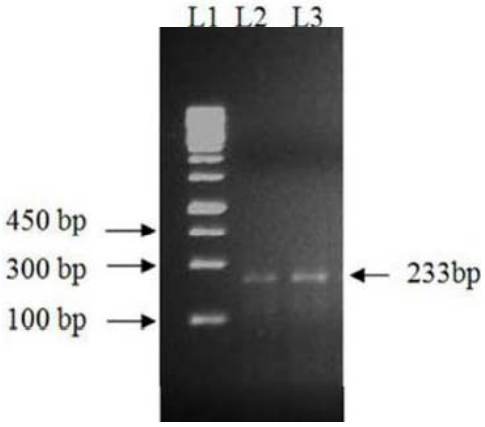

Fig. 4: RT-PCR of goat and buffalo brain samples, 233-bp RNA fragment generated by gelelectrophoresis. Lanes: L1, standard DNA Ladder; L2, positive brain sample of goat; L3, positive brain sample of buffalo

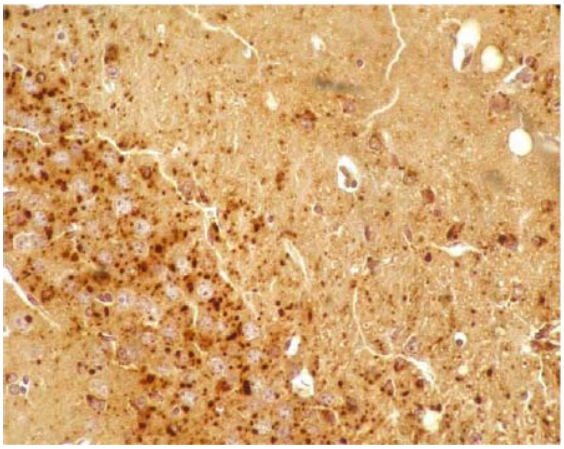

Fig. 5: A section of the cerebrum, belonging to rabies positive buffalo, showing discrete circular antigen granularities extensively dispersed in perikaryons (DAB substrate, X-200).

Rabies positive goat brain sample

However, one of brain samples from *Jinnah town*, showed severe reactive inflammatory changes such as neuronal necrosis, congestion, perivascular cuffing and babe's nodules (Fig. 1) in cerebrum and a few in cerebellums. Satellitosis was observed as an accumulation of glial cells around damaged neuronal cells occupying at few sections. In some areas, the nuclei of the cell bodies were pyknotic and at a certain place they were peripherally displaced and their dendrites were tapered at the end like pointed projections. Cavernous lesions carrying cytoplasmic vacuolation and interstitial vacuoles in the cerebrum along with necrotic and degenerative changes were likewise seen consistently, which manifested spongy appearance (Fig. 2). Nevertheless, pathognomonic Negri bodies were neither seen in the cerebrum nor in cerebellum through paraffin sectioning (Table 1).

Upon subsequent processing of this particular sample through immunohistochemistry confirmed our suspicion for rabies virus infection. The results revealed wide-spread labeling of rabies virus nucleoprotein by producing scattered viral masses and dispersed granular antigens within the perikaryons and neuronal dendrites of cerebrum (Fig. 3) and a small number of tiny scattered antigens in Purkinje cells of the cerebellum. The stained antigens were of variable size present either as single or multiple in perikaryons. Peroxidase staining confirmed that the respective sample was positive for the rabies virus infection. Furthermore, the result was also verified by running the sample RNA on 1.5% agarose gel using RT-PCR, which generated a nucleic acid band of 233 bp (Fig. 4).

Pathological lesions in cows &buffaloes

Likewise, previously discussed investigations found in sheep, there were negligible rabies specific histopathological findings present among the brain samples of cow and buffaloes. However, cytoplasmic vacuolation with hyper-chromatic nuclei and perivascular cuffing surrounded by mononuclear and lymphocytic infiltrate were moderately present in the cerebrum with mild necrotic changes. There was a certain kind of alteration in the demonstration of perivascular cuffing among few brain samples, but despite of these relevant findings, the immuno-staining was negative in these probable samples (Table 1).

Rabies positive buffalo brain sample

One brain sample of buffalo from *Jaranwala town* was found rabies positive. The neurons of the cerebrum were relatively shrunk and therefore, appeared

eosinophilic. Meningoencephalitis consisted of perivascular cuffs infiltrated with mononuclear cells and lymphocytes, necrotic and degenerative changes (mesh cytoplasm or undefined cell shape) were visible in few sections of cerebrum and cerebellum. Vascular changes like congestion contained dilated blood vessels and, the perivascular space was surrounded by fewer neutrophils and macrophages characterized by enhanced space of "Virchow Robin" containing pinkish material. Like the rabies virus positive sample of goat, babe's nodule was seen in the granular cell layer of cerebrum. Even so again, Negri bodies were not confirmed in this particular sample (Table 1).

The paraffin fixed slides showed considerable immunoreactivity in the cerebrum and cerebellum using DAB substrate. Antigen deposits of variable sizes within Purkinje cells of cerebellum and in granular layers of cerebrum were seen with distinct granular appearance, suggesting strong positivity for rabies virus antigens (Fig. 5). These oval homogenous structures were almost absent or showed very weak staining in hippocampus of the brain. Later on, RT-PCR validated the results by successfully amplifying the corresponding nucleic acid band on agarose gel (Fig. 4).

Discussion

Rabies is a disease of the developing country like Pakistan, where prophylactic measures are not strictly followed and health workers, including veterinarian present insignificant attention to such kind of abandoned infectious disease. The present study evaluates different abattoirs of Faisalabad for the presence of ambiguous pathological lesions and diagnosis confirmed 2 rabies positive samples. The positive cases are extremely low which is perhaps due to small sample size and secondly, the clinical cases of rabies in animals are either not reported or merely random in Pakistan, especially in Karachi, yet huge number of stray dogs likely present lethal threat to public and thus, possibly cause occasional cases (Parviz et al., 2004; Zaidi et al., 2013). Despite that, sporadic cases of rabies have been reported previously in animals and humans and successfully diagnosed by the diagnostic techniques (Parviz et al., 2004; Numan et al., 2010) and, a similar kind of abattoir based study has been reported earlier in neighboring India (Manickam et al., 2009).

Among extensively used diagnostic test for rabies virus detection, FAT is considered as the gold standard test but, high equipment cost, transportation of live virus, high ambient temperature, and rapid use of fresh sample as well as a potential bio-hazards to public health are certain factors that limit its implementation in a country like Pakistan (Sharma et al., 2014; Stein et al., 2010). On the other hand, immunohistochemistry does not carry similar disadvantages, rather this technique is more specific and highly sensitive that enhances the detection and natural distribution of even minute rabies virus antigens in brain sections as well as tissues outside brain of various animal species (Faizee et al., 2011; Camila et al., 2014). Moreover, this technique also enables us to study the relative affinity of particular brain part for rabies virus antigens in each animal's species (Stein et al., 2010).

In rabies positive cases, antigen staining was moderately exhibited in cerebrum, minute in cerebellum and even absent in hippocampus of goats and buffaloes brain sample, which is in accordance with an experimental study conducted in sheep and, naturally rabies virus infected buffaloes respectively (Brookes et al., 2007; Feng et al., 2014) but yet, naturally infected buffaloes reported higher antigen staining in brainstem followed by the cerebellum and cerebrum (Sharma et al., 2014; Mundas et al., 2014; Faizee et al., 2011). Interestingly, distribution of pathological lesions in cerebrum also corresponds to the antigenic staining of the immunohistochemistry.

The gross changes observed in rabies positive samples were previously documented in natural and experimentally infected cases of different animals and humans (Hsu et al., 2005; Manickam et al., 2009). The distribution and nature of the similar characteristic pathological lesions such as satellitosis, degenerative necrosis, perivascular cuffing, cytoplasmic vacuolation and vascular congestion have been repetitively documented earlier in many domestic and wild animal species including humans (Faizee et al., 2011; Johnson et al., 2011). However, pathological alterations with variable intensity in cerebrum and cerebellum have been previously exhibited due to large sample size of rabid buffaloes (Jamadagni et al., 2007; Beigh et al., 2014).

Similar kind of study reported severe types of lesions in the cerebrum of buffaloes and sheep (Sharma et al., 2014; Nilakanth et al., 2013). The babe's nodules consisting of glial cell aggregates were present in the form of a bunch at various sections of the cerebrum in rabies positive goat and buffalo brain sample (Jamadagni et al., 2007). Although, the presence of Negri bodies is variable in rabies infected cases and typically, 20-60% of stained preparations exhibit oval shape pinkish intracytoplasmic Negri bodies (Woldehiwet, 2005), but in this study, absence of Negri bodies is in accordance with the recently published report in cattle (Castillo et al., 2015). For this reason, histopathological examination is less sensitive and specific method as compared to other diagnostic tools and therefore, the confirmatory diagnosis could not be established solely on histopathological analysis (Faizee et al., 2011; Beigh et al., 2013; Sharma et al., 2014). Hence, this study further emphasizes the requirement of

rabies specific diagnostic tools such as immunohistochemistry and RT-PCR, which are far more sensitive and specific in results as compared to histopathology (Johnson et al., 2011; Sharma et al., 2014).

Most brain samples of different animal species demonstrated a variable amount of pathological lesions such as necrosis and neuronal degeneration, but the presence of rabies virus specific lesions were not seen in these cases. These mild to moderate necrotic changes could be frequently associated with aging, salt or water toxicities (Manickam et al., 2009), pathogenic (viral/bacterial) infection (Sajid et al., 2012). Whereas, cuffs of inflammatory cells, seen in the cerebrum and cerebellum may be associated with clostridium chauvoei infection or bacterial toxins circulating in the blood (Popoff and Poulain, 2010). Condensed and dark neurons could be associated with hypoxic or ischemic injury (Manickam et al., 2009). These are consistently seen pathological alterations in necropsy examinations of small and large ruminants of slaughtered animals. Moreover, necrosis or degenerative changes related to rabies virus are perhaps due to apoptosis or degeneration of neuronal processes (Li et al., 2005, Jackson 2011). The study was merely focused on rabies virus, thus explanatory diagnosis could not be established about these lesions.

Since, the disease is endemic and incurable and yet, there is no surveillance and control policy at national level in Pakistan (WHO, 2014). Moreover, perception and knowledge regarding management of dog bite cases and generally about rabies is clearly deficient among medical practitioners of Pakistan (Siddiqui et al., 2014). This kind of underestimation and insignificant attitude of health workers is also contributing towards such kind of infections. In other developing countries like Pakistan, stray dogs contribute equally to bite both animals and humans for the transmission of rabies through deposition of saliva. Thus, Pakistan is in the line of trouble like India, where dog bites are increasing due to warmth weather of these regions.

Conclusions

Stray and unvaccinated dogs are mainly responsible in dissemination of the rabies infection among domestic animals and humans of Pakistan. Sporadic and unreported cases of rabies infection are scattered among different regions of Pakistan. Therefore, proper serosurveillance and control strategies must be designed to eradicate expected outbreaks in future.

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