Pathological and molecular diagnosis of rabies in clinically suspected food animals using different diagnostic tests

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SUMMARY

Introduction - Rabies is a fatal viral disease of the nervous system caused by a RNA virus, which belongs to the genus lyssavirus of the family Rhabdoviridae. Rabies can affect all mammals including humans. It is a serious veterinary and public health problem in Jordan and many other countries around the world. Therefore, early diagnosis with highly sensitive and specific tests will reduce unnecessary prophylaxis and treatment.

Aim - The aim of this present study was to diagnose rabies in the clinically suspected cows, donkeys, horses and goats in Jordan by using different diagnostic tests and to compare the results of these tests.

Materials and methods - During the years 2012-2013, a total of 11 brain samples were collected from different food animal species (5 cows, 3 donkeys, 1 horse and 2 goats) that were provided by the Vaccine and Sera Department / Al-Basheer central hospital in Amman /Jordan. Clinically, rabies was suspected in these animals. These brain tissues were examined by fluorescent antibody test (FAT), histopathology, immunohistochemistry (IHC) and reverse transcriptase polymerase chain reaction (RT-PCR).

Results and discussion - The results showed that 55%, 45%, 82% and 91% of 11 brain tissues were positive for rabies by FAT, histopathology, IHC and RT-PCR respectively. The results of 5 animals out of 11 (45.5%) were consistent in all diagnostic tests where 4 (80%) of them were rabies positive. Two cases were rabies negative by FAT and proven to be rabies positive by the other tests. None of the examined clinically rabies cases was only detected by FAT. No significant difference was found when comparing between any two diagnostic tests.

Conclusions - Although FAT is considered the primary standard confirming test that is used to distinguish rabies encephalitis from other viral encephalitidis, these results do emphasize the importance of conducting more than FAT to diagnose rabies in animals.

KEY WORDS

Food animals, rabies, fluorescent antibody test, histopathology, immunohistochemistry, reverse transcriptase polymerase chain reaction.

INTRODUCTION

Rabies is a neuroinvasive viral disease that causes encephalitis and death to human beings and mammals. The disease is caused by lyssavirus genus, which belongs to Rhabdoviridae family. The genus has seven genotypes: Rabies virus, Lagos bat virus, Makola virus, Duvenhage virus, European bat lyssavirus 1 and 2 and Australian bat lyssavirus genotypes¹. There are differences in the ability to infect the host, and differences in spreading through the host body. Rabies virus genotypes are divided to two phylogroups: phylogroup 1 and phylogroup 2. Phylogroup 1 contains genotype 1, 4, 5, 6 and 7 while phylogroup 2 contains Lagos bat virus and Mokola virus. Each phylogroup differs in its biological properties, pathogenicity, induction of apoptosis and recognition of receptors. The genotypes in phylogroup 1 are more pathogenic than the genotypes in phylogroup 2.

Rabies is a major problem to public health since three billion people in Asia and Africa are threatened by this disease². Its importance came from the fact that Rabies is fatal to both human and animals and for this reason a World Rabies Day was established by CDC, OIE and WHO at 2007. Throughout the world, 40,000 to 100,000 human deaths were related to rabies³.

In Jordan, a retrospective epidemiological study was carried out investigating the trend of rabies and animal bites from 2000 to 2007. This study showed that Rabies was increased from 1 case in 2003 to 50 cases in 2007 [dogs (56.52%), cattle (23.21%) sheep (7.6%), and goats (6.6%)]. In another epidemiological study carried by Al Qudah et al.,³ showed that stray dogs were the most common rabid animals and represented 45.12% of rabid cases. The same author stated that domestic animals were also affected: cattle 19.51%, donkeys 4.87%, sheep, and goats 3.65%.
Different diagnostic methods were developed and recognized internationally for rabies diagnosis either in animals or in human. In animals, WHO recommended many diagnostic methods for detection of rabies. The fluorescent antibody test is considered the gold standard for rabies diagnosis because it is rapid, sensitive and accurate. However, other methods could be used to diagnose rabies. These methods include: histopathology, virus antigen detection, mouse inoculation technique, and virus cultivation. The aim of this research was to diagnose rabies using brains from the clinically suspected food animals via different diagnostic tests (FAT, histopathology, IHC and RT-PCR) in Jordan.

MATERIALS AND METHODS

Sample Collection
Eleven different brain tissue samples were provided by the Vaccine and Sera Department / Al-Basheer central hospital in Amman/Jordan. These brain samples were collected from different food animal species: cows (5 cases), donkeys (3 cases), goats (2 cases) and horse (1 case). These animals were clinically suspected for rabies. Half or part of the submitted brain tissues samples were fixed in 10% formalin after being examined by FAT.

Diagnostic tests
Fluorescent Antibody Test - The FAT was carried out in the Vaccine and Sera Department as it considered the reference diagnostic laboratory for rabies in Jordan. The procedure was conducted according to Bingham and van der Merwe, 2002. In summary, touch impression were made from three parts (cerebrum, cerebellum and brain stem) and fixed at -20 °C in acetone for 30 minute. After that conjugate polyclonal antibody (fluorescein isothiocyanate) was applied and incubated for 30 minute at 37 °C the slides were washed and glycerol drops were applied. Finally, the slides were covered and results were recorded under florescent microscope.

Histopathology - The formalin fixed brain tissue samples were transported to the Veterinary Pathology Laboratory at the Department of Veterinary Pathology and Public Health / Faculty of Veterinary Medicine at Jordan University of Science and Technology (JUST). The brain tissues were trimmed and routinely processed in an automatic histoki -to. The brain tissues were trimmed and routinely processed in an automatic histokinetic machine. The tissues routinely embedded and 4-5 μm tissue sections were made and stained for histopathological examination with the ordinary Hematoxylin and Eosin stain (H&E).

Immunohistochemistry - The brain tissue samples, from paraffin-wax embedded blocks, 3-4 μm tissue sections were cut and mounted on coated slides. The slides were deparaffinized by heating at 70 °C for 10 min, then by xylene for 40 min and air dried. The slides were quenched by endogenous peroxidase 3% H2O2 for 10 min and then were rinsed under running water for 10 min. The slides were exposed to citrate buffer 1 X solution and heat-induced epitope retrieval was performed in a microwave for 4, 3, 3 min periods followed by washing with phosphate buffered saline with tween 20 (PBST) for 5 minutes. Protein block (Biogenex, USA) was added to the slides for 5 min followed by washing with PBST for 2 min. Primary rabies polyclonal antibody (Millipore, USA) was diluted in TBST according to manufacturer's instruction and applied to the slides and incubated for 2 h at 37 °C. The antibody solution was washed twice by PBST for 2 min. Biotinylated secondary antibody (Abcam, UK), diluted in TBST according to manufacturer's instruction, was added to each slide and incubated for 3 h at 37 °C. After incubation, secondary antibody solution was washed twice by PBST for 5 min each. ABC reagents were prepared according to manufacturer's instruction 30 min prior to use. ABC reagents were added to the slides and incubated in humid chamber for 1h at 37 °C then washed twice by PBST for 5 min each. DAB solution was added for each slide until color was developed. The slides then immersed in dH2O followed by staining with Meyer’s hematoxylin. Finally the slides were dehydrated routinely and prepared for evaluation. The immunohistochemically stained tissue sections were examined by light microscope and the IHC reactivity were recorded.

RNA extraction - RNA extraction was carried out from formalin fixed brain tissues according to the manufacturer’s instructions (iQeasy, Plus Viral DNA/RNA Extraction Kit - Intron Biotechnology, Korea). The extraction technique was previously described by Faizee et al. (2012). At the end, 2-5 μl of eluted solution was used for RT-PCR. However, at the beginning, formalin was removed from the brain tissue samples by treating them with xylene then with absolute ethanol alcohol for 5 min each for two times. After that phosphate buffer saline was added to the treated brain tissues and were homogenized by a homogenizer. After homogenization, the samples were centrifuged to remove un-lysed tissue particles.

Reverse Transcriptase-Polymerase Chain Reaction - RT-PCR was carried out as previously described by Faizee et al.7. In summary, 4 μl of template RNA and 16 μl of DNase/RNase-free water was added into the RT-PCR premix tube according to manufacturer’s instruction (VeTeK™ RV Detection Kit, Intron Biotechnology, Korea). Two μl of positive control and 18 μl of RNase-free water was added into a RT-PCR premix tube for monitoring of amplification and easy interpretation (VeTeK™ RV Detection Kit, Intron Biotechnology, Korea). The following thermocycling program was used: 45 °C for 30 min (Reverse transcription step) and 94 °C for 5 min (Inactivation of reverse transcriptase enzymes) 1 cycle, 94 °C for 30 second (Denaturation), 50 °C for 30 seconds (Annealing), and 72 °C for 40 seconds (Extension), and 72 °C for 5 min (Final extension) 1 cycle.

Detection of Amplified Products - Detection of the amplified products was carried out using agarose gel electrophoresis. A 1.5% of agarose gel was prepared and then loaded with 7 μl of PCR product and 7 μl of positive control. Electrophoresis was run for 30 minute /100 volt. Then results were recorded under Ultra violet trans-illuminator.
RESULTS

Fluorescent Antibody Test
Rabies virus replicate inside infected cells cytoplasm as oval inclusions bodies, contains mainly Nucleoprotein or N protein, it appears as green bright apple particles by FAT. Eleven brain tissue samples were included in this study; all were tested for FAT in the vaccine and sera department / Al Basheer Central Hospital, Amman / Jordan. Six (55%) out of eleven submitted animal brain samples were positive in FAT. Table 1 summarizes the FAT results in different animal species.

Histopathology
Presence of Negri bodies within the affected neural cells was considered positive regardless of the other lesions. However, absence of Negri bodies with presence of nonsuppurative inflammation was considered a suspected case. Out of eleven examined brain samples, 5 (45%) of them were considered positive. Table 2 summarizes histopathological results in different animal species. In general, throughout the examined sections in various animals the histopathological findings consisted mainly of variable degrees of non-suppurative meningencephalitis. The blood vessels within the parenchyma and meninges were cuffed with one layer or more of mononuclear cells, primarily lymphocytes (Figure 1). Purkinje cells and neuronal necrosis with or without Negri bodies were seen (Figure 2). The Negri bodies appeared as single or multiple, eosinophilic intracytoplasmic Negri bodies were seen (Arrow). H&E. 40X.

Immunohistochemistry
Eleven brain tissue samples were examined by immunohistochemistry test. The positive signals appeared as variably sized rounded brownish inclusions within the cytoplasm of the neurons and Purkinje cells. The signal was stronger in the Purkinje cells as well as their dendrites (Figure 3). Nine cases (82%) out of 11 showed positive signals in the examined tissues. Table 3 summarizes the results of IHC.

Reverse Transcriptase-Polymerase Chain Reaction
In RT-PCR examinations, the examined samples which had a band of approximately 263 bp was considered positive and the samples which did not show a band at the same location of the positive control band was considered negative.

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Table 1 - Shows the results of FAT in different food animal species.

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Table 2 - Shows the results of histopathology in different food animal species.

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Figure 1 - Cerebellum; Cow. The blood vessels within the parenchyma and meninges were cuffed with one layer or more of mononuclear cells, primarily lymphocytes (arrows). H&E. 4X.

Figure 2 - Cerebellum; Cow. Purkinje cell necrosis (*) and one contains more than one eosinophilic intracytoplasmic Negri bodies were seen (Arrow). H&E. 40X.
consequential damage of any type of disease introduction or novel emergence are to maintain diagnostic capabilities at specialty labs and to assess threats posed by outbreaks in the other countries. Moreover, early diagnosis with highly sensitive and specific tests will reduce unnecessary prophylaxis and treatment. In the present study, four different diagnostic tests were used to diagnose rabies in the clinically suspected cows, donkeys, horses and goats in Jordan, during the years 2012-2013. The brain tissue samples were examined by using FAT, histopathology, IHC, RT-PCR.

Florescent Antibody Test is considered a golden test, recommended by WHO and OIE, because it has high specificity and sensitivity for rabies diagnosis. It is a primary standard confirming test that is used to distinguish rabies encephalitis from other viral encephalitis diseases. It can be used on frozen, fresh or formalin fixed brain tissue samples. Whitfield et al. validated the use of FAT for routine diagnosis of rabies.

In this study, four different diagnostic techniques were used. The summary of the different technique results was presented in Table 5. The table showed that the results of five animals out of 11 (45.5%) were consistent in all diagnostic tests where four of them (80%) were rabies positive. Histopathology was the least sensitive diagnostic test to diagnose rabies while RT-PCR was the most sensitive. No significant difference was found when comparing any two diagnostic tests. It is worth mentioning that one case [donkey (7)] was negative only by FAT and only two cases (cow 1 and cow 5) where only negative by histopathology.

**DISCUSSION**

Hanlon and Child wrote, “It is of paramount importance to keep in mind that the first essentials in reducing the risk and confirming test that is used to distinguish rabies encephalitis from other viral encephalitis diseases. It can be used on frozen, fresh or formalin fixed brain tissue samples. Whitfield et al. validated the use of FAT for routine diagnosis of rabies.

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Rabies virus antigen appears as oval green bright particles, vary in their sizes. The results of this test are obtained within two hours by well-trained laboratory staff however; the test needs florescent microscope$^{12}$. Bingham, J. and Van Der Merwe, M.$^{13}$ conducted a study to determine the most reliable regions of the brain where rabies antigen was found and to make recommendations for sampling of brains from different animal species for FAT. The authors found that the cerebellum, hippocampus and different parts of the cerebrum were negative in, respectively, 4.5, 4.9 and 3.9-11.1% of positive brains while thalamus was positive in examined brains.

In this current study, FAT revealed 6 (55%) positive out of 11 examined brain tissue samples from different animal species. In a previous study conducted by Faizee et al. 2012, out of 27 brain tissue samples examined by FAT from different animal species, 21 (77.77%) were rabies positive. FAT sensitivity depends on the expertise diagnostic staff and the quality of the submitted brain samples in addition to the conjugate used in the procedure, Bansal, K. et al.14. In another study carried out by Zimmer et al.$^{15}$, FAT detected 98% positivity of 187 brain tissue sample from clinically suspected animals of different species. It has been reported that false positive results can occur due the presence of cross-reaction of rabies with other viral encephalitides when using FAT to detect rabies$^{16}$ which indicate that the sensitivity of the FAT does not reach 100%. Histopathological techniques were used as a routine technique for rabies diagnosis. Some histopathology techniques were very rapid like Mann's stain and H and E stain on paraffin-embedded tissues. The pathognomonic lesions of rabies are encephalitis with Negri bodies. The pathognomonic lesions of rabies are encephalitis with Negri bodies$^{12}$. Mann's stain and Geimsa and Seller can differentiate inclusion bodies of rabies from other related lyssaviruses by using specific primer for each genotype$^{12}$. Moreover, it detects the rabies antigen earlier than the other diagnostic tests$^{20}$. However false positive results were reported to occur in RT-PCR$^{22}$. It was not recommended for routine postmortem diagnosis of rabies due to high false positive and false negative results$^{21}$.

This method could overtake FAT as the primary test for rabies diagnosis because RT-PCR is successful for sequencing of rabies virus genome by using one or more specific primers. It is also used to distinguish between rabies virus and different related lyssaviruses by using specific primer for each genotype$^{12}$. This method could overtake FAT as the primary test for rabies diagnosis because RT-PCR is successful for sequencing of rabies virus genome by using one or more specific primers. It is also used to distinguish between rabies virus and different related lyssaviruses by using specific primer for each genotype$^{12}$. Moreover, it detects the rabies antigen earlier than the other diagnostic tests$^{20}$. However false positive results were reported to occur in RT-PCR$^{22}$. It was not recommended for routine postmortem diagnosis of rabies due to high false positive and false negative results$^{21}$.

When a comparison where done between the results of FAT and the result of the histology, no significant difference was found (P value > 0.05). Similar results were seen when comparison between FAT and IHC was conducted. Furthermore, no statistical significant difference was found when comparing between any two different tests. These insignificant dif-

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Table 5 - Summarizes the test results of FAT, Histopathology, IHC, and RT-PCR in different food animal species.
ferences might be explained because of the small number of samples tested although variation in the results were present.

CONCLUSION

In Jordan, Rabies is more frequently occurred in cows more than any other food animals. In the present study, although there was no significant difference between the different tests to diagnose rabies, FAT alone should not be rely upon to diagnose rabies. At least one more confirmatory test should be carried out to confirm FAT results. RT-PCR is a good candidate but false positive results might be of concern.

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References