DIAGNOSIS OF RABIES BY REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

Abstract
The nucleic acid based technique Polymerase chain reaction is becoming very popular in the field of diagnosis of diseases and also in molecular characterization of the etiological agents. It allows the detection of nucleic acid in minute amount of samples and even in the autolysed samples which become unfit for other laboratory tests. This technique is commonly used in most of the rabies diagnostic laboratories. In this study, one step reverse transcriptase polymerase chain reaction (RT-PCR) was standardized on 20 rabies positive (in FAT) brain samples from different species (goat 10, sheep 3, cow 2, camel 2, fox 3). All these rabies positive cases were found positive by RT-PCR using N gene specific and G gene specific primer sets. In N gene, specific products of 200 bp and in G gene products of 406 bp were got amplified and detected on agarose gel electrophoresis. The similar brain samples were also found positive for rabies on immunofluorescence and histopathological examinations. Rabies suspected animals showed nervous signs like paralysis and inability or unwillingness to eat or drink. Animals survived variably for 2 to 42 days after showing the nervous symptoms. Besides brain, other samples like body fluids (saliva, cerebrospinal fluid) and archival samples from rabies suspected cases could also be used in RT-PCR for diagnosis of the disease.

INTRODUCTION
Rabies (OIE List-'B' disease) is one of the most dreadful disease and a major viral zoonosis. It is caused by RNA virus of family Rhabdoviridae, genus lyssa virus, which infects all warm-blooded animals and birds. The genus Lyssavirus contains 7 serotypes/genotypes includes Classical rabies virus (RABV), Lagos bat virus (LBV), Mokola virus (MOKV), Duvenhage virus (DUVV), European bat lyssavirus 1 (EBLV 1), European bat lyssavirus 2 (EBLV 2), Australian bat lyssavirus (ABLV). Different genotypes fall into two distinct phylogroups. Phylogroup I comprises, genotype I ( Classical rabies Virus), genotype IV ( Duvenhage virus), genotype V (EBLV1), genotype VI (EBLV2) and genotype VII (Australian bat lyssavirus) while, phylogroup II comprises the divergent African genotypes II (Lagos bat virus) and III (Mokola virus). The disease is almost always transmitted by an animal bite and characterized by a prolonged and variable incubation period (usually 3-8 weeks; as short as 10 days or long as 9 months or more) followed by fatal encephalomyelitis, ganglioneuritis and parotid adenitis in mammals. It is prevalent in all parts of the Sultanate. Rabies, being a major zoonosis, its precise and rapid diagnosis is important in suspected cases for early treatment and effective prevention/control measures.

Lyssaviruses have a 12kb long non-segmented negative sense RNA genome encoding 5 viral proteins (3’ to 5’): group specific nucleoprotein N (1334 bp), phsophoprotein P (978 bp), matrix protein M (840 bp), glycoprotein G (1674 bp) and polymerase L (6381 bp). The ectodomain of the G protein (carrying the main antigenic sites)
Reverse transcriptase polymerase chain reaction (RT-PCR):
The nucleic acid based PCR tests allow rabies diagnosis even in the autolysed samples which become unfit for immunofluorescence or virus isolation (Biswal et al 2007). In this technique viral RNA is first converted into c-DNA by the enzyme reverse transcriptase and then in vitro amplification by the enzyme polymerase. The RNA work should be carried out at separate place with separate set of pipettes, chemicals, reagents bottles, etc. A very small amount of specimen is required. Fresh specimen is preferred to decomposed ones. This technique consists of 3 major processes; denaturation, annealing, extension. The amplified product is then run under gel-electrophoresis and is compared with a known positive control and DNA ladder. It should be noted that viral RNA secretion in saliva, urine and cerebrospinal fluid is intermittent.

All the procedures of handling rabies suspected material should be carried out in bio-safety cabinet and should comply with National Biocontainment and Bio-Safety regulations.

MATERIALS AND METHODS

100 mg of rabies suspected brain samples from different species of animals including goat (10), sheep (3), cow (2), camel (2), fox (3), were taken for extraction of viral RNA using QIAamp Viral RNA kit (QIAGEN) following the manufacturer’s protocol. The RNA was dissolved in the RNase free buffer provided with the kit. 50 to 100 ng RNA was used as template for cDNA synthesis by reverse transcriptase polymerase chain reaction. The Qiagen OneStep RT-PCR kit was used for amplification of the RNA template. The oligo primers were designed and got synthesized commercially. The set 1 pair of primers were designed from the conserved regions of nucleoprotein N gene of rabies virus using computer software. The set 2 pair of primers from conserved regions of glycoprotein G gene were also used for diagnosis of rabies in disease suspected brain samples. The primers set 1 for N gene of rabies amplify the product of 200 bp and the primers set 2 for G gene amplify the product of 406 bp.

One-Step RT-PCR for rabies diagnosis using N gene primers (Qiagen kit):
PCR was carried out in a 25 µl reaction mix using 5µl (100 ng) of purified RNA template, 5 µl of 5X QIAGEN One-step RT-PCR Buffer, 3 µl of 5X QIAGEN Q solution, 1.5 µl of dNTP Mix (10 mM each), 3 µl (30 pmol) each of N gene specific forward and reverse primers, 1 µl of QIAGEN One-step RT-PCR Enzyme Mix, 0.5µl of RNase inhibitor (20 U/ µl), and 3 µl of H2O.

The reaction mix in thin walled 0.2 ml PCR tubes was incubated at 60 °C for 1 min, 42 °C for 10 min, and 50 °C for 30 min for cDNA synthesis (step 1) followed by denaturation at 95°C for 15min (step 2). After denaturation of the template at 95°C for 15 min, amplification was carried out for 30 cycles of three steps, at 95°C for 30 sec, 55°C for 30 sec, 70°C for 45 sec (step 3), with final extension at 72°C for 5 min.

One-step RT-PCR for rabies diagnosis using G gene primers (Qiagen kit):
The RNA extracted from rabies suspected brains of different animal species was used for diagnosis of the disease using G gene specific primers. The protocol used was almost similar to N gene except little modifications in annealing temperature and cycling time. Similar to N gene, the PCR for G gene was also carried out in a 25 µl
reaction mix using 5 µl (100 ng) of purified RNA template, 5 µl of 5X QIAGEN One-step RT-PCR Buffer, 3 µl of 5X QIAGEN Q solution, 1.5 µl of dNTP Mix (10 mM each), 3 µl (30 pmol) each of G gene specific forward and reverse primers, 1 µl of QIAGEN One-step RT-PCR Enzyme Mix, 0.5 µl of RNase inhibitor (20 U/µl), and 3 µl of H2O.

The reaction mix in thin walled 0.2 ml PCR tubes was incubated at 60 °C for 1 min, 42 °C for 10 min, and 50 °C for 30 min for cDNA synthesis (step 1) followed by denaturation at 95°C for 15min (step 2). After denaturation of the template at 95°C for 15min, amplification was carried out for 35 cycles of three steps, at 94°C, 55°C, and 72°C, each for 1 min (step 3), with final extension at 72°C for 5 min. After completion of the PCR reactions, 5 µl of the each amplicon was run on 1.5% agarose gel at 80 volt for 60 min and the electrophoreses gel was visualized in gel documentation system under UV light, picture of the gel was captured.

Set 1 primers sequence: (200 bp product)
Rab/N/688F    5’ GGATTGAGCATCTATATTCAGC 3’
Rab/N/888R    5’ GAGGAACGGCGGTCTCCTG 3’

Set 2 primers sequence: (406 bp product) (Gupta et al 2001)
Rab/G1/F    5’ TAATCCCAGAGATGCAATCA 3’
Rab/G1/R   5’ CCTCACAGTCTGGTCTCCTG 3’

RESULTS AND DISCUSSION

Clinical diagnosis is difficult in the early stages of the disease and it can easily be confused with other diseases or with normal aggressive behavior of the animals. The rapid identification and confirmation of rabies suspected infection is essential in animals to allow specific control strategies. The routine diagnostic methods used for rabies diagnosis are fluorescent antibody test (FAT) on brain impression smear after the death of the animal and also histopathological examination of the brain for Negri bodies (Mahadevan et al 2004). FAT is the OIE recommended test for rabies diagnosis as it is sensitive, specific and easy to perform (OIE 2004). It became a standard diagnostic procedure and is the preferred test for rabies diagnosis (Dean et al 1996). The PCR technique is much more sensitive in comparison with FAT particularly in decomposed state of brain tissue.

Now a days molecular technique polymerase chain reaction (PCR) is becoming common in most of the diagnostic laboratories (Gupta et al 2001). In this study, one step RT-PCR was standardized on 20 rabies suspected brain samples from different species (goat 10, sheep 3, cow 2, camel 2, fox 3). All these rabies suspected cases were found positive by RT-PCR using N gene specific and G gene specific primer sets. In N gene, a specific product of 200 bp was detected on agarose gel electrophoresis (Fig. 1). G gene specific primers amplified the product of 406 bp seen on agarose gel electrophoresis (Fig 2). Further the N gene specific 200 bp amplified product was also obtained in brain samples of different species (Fig 3). Rabies suspected animals showed nervous signs like paralysis and inability or unwillingness to eat or drink. Animals survived variably for 2 to 42 days after showing the nervous symptoms. The variable incubation period in rabies lasting 20 to 90 days and some times even 1 year has also been reported by earlier workers (Kaplan and Koprowski, 1973). Charlton et al. (1997) experimentally infected the skulls with Canadian isolates of street rabies virus and sacrificed them at 62 to 64 days post inoculation. They used reverse transcriptase polymerase chain reaction amplification and reported the presence of viral nucleic acid in the inoculation muscle, but not in either spinal ganglia or the spinal cord.
Many workers reported that RT-PCR was an important test for diagnosis of rabies (Jayakumar et al. 2004, Nagarajan et al. 2006). The PCR coupled with sequencing of amplified product can further provide accurate genotyping of rabies virus. Heaton et al. (1999) detected the rabies virus from decomposed brain samples using nested and hemi nested RT-PCR after 72 hours and 360 hours respectively. Many workers (Nagarajan, et al. 2006, Biswal et al. 2007) recommended that RT-PCR should be method for antemortem (in saliva) and postmortem (in archival tissues) diagnosis of rabies. The rabies virus (RV) and rabies related viruses (RRVs) can be rapidly detected using either RT-PCR (Heaton, 2000) or heminested RT-PCR and a microwell capture hybridization assay using PCR ELISA kit within 10 hours. This can detect all six genotypes of rabies and RRVs. Warner et al. (1997) used paraffin embedded formalin fixed tissue for viral RNA extraction and employed this RNA template for RT-PCR amplification. They amplified a specific region of the N gene yielding approximately 120 nucleotides product and characterized the virus as vampire bat virus. Biswal et al. (2007) used RT-PCR for diagnosis of rabies in archival tissue sections. One tube reverse transcription polymerase chain reaction can be used as practical application as confirmatory test to fluorescence antibody or mouse inoculation in busy rabies diagnostic laboratories (Gupta et al., 2001).

The same brain samples were processed for routine haematoxylin and eosin (H & E) staining and showed the lesions of non-suppurative encephalitis, haemorrhages, perivascular cuffing, satellitosis and presence of eosinophilic intracytoplasmic inclusion bodies indistinguishable from Negri bodies. Similar brain samples were also tested by immunofluorescence staining techniques and found positive for rabies antigen. Based on this study, it was found that the RT-PCR is very sensitive and specific technique for rabies diagnosis which can even work in the decomposed brain samples and also in body fluids. This technique can also be used in formalin fixes tissues and archival samples.

**Fig. 1** Agarose gel electrophoresis (1.5%) of N gene 200 bp PCR products generated from rabies suspected brain samples. Lane M, 50 bp DNA ladder (Qingen), Lanes 1, 2, 3 of goats died at 42, 21 & 20 days respectively, Lane 4 sheep died at 2 days and Lane 5 cow died at 3 days, after corneal impression test positive for rabies by FAT.

**Fig. 2** Agarose gel electrophoresis (1.5%) of G gene 406 bp PCR products generated from rabies suspected brain samples. Lane M, 50 bp DNA ladder (Qingen), Lanes 1, 2, 3 of goats died at 42, 21 & 20 days respectively, Lane 4 sheep died at 2 days and Lane 5 cow died at 3 days, after corneal impression test positive for rabies by FAT.
REFERENCES


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REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR) FOR DETECTION OF H5N1 AVIAN INFLUENZA VIRUS

Abstract

Avian influenza (AI) is a highly contagious disease caused by type A influenza virus of the family Orthomyxoviridae. The AI viruses are divided into subtypes on the basis of two surface glycoproteins- haemagglutinin (HA) and neuraminidase (NA). Sixteen HA and 9 NA subtypes have been identified so far. All the subtypes of the AI virus are found in aquatic birds, which serve as the reservoir of all influenza A viruses. The subtypes H5 and H7 are found highly virulent in poultry. The surveillance for type A and highly pathogenic H5 subtypes is underway in the Sultanate. About 5000 birds of different species including wild birds were tested for type A avian influenza viral and avian influenza subtype H5 antigen using rapid dipstick strip test. Pooled organs (lungs, trachea) and cloacal swabs from the birds under study were also found negative for highly pathogenic H5N1 avian influenza virus in one-step RT-PCR test. Based on the study, it can be concluded that the local poultry and other imported birds in the Sultanate are free from the highly pathogenic H5 subtype of the avian influenza virus till date.

INTRODUCTION

Avian influenza (AI) is one of the important public health concerns. There has been a sharp increase in the number of outbreaks of AI in poultry in recent past. The influenza viruses are enveloped particles, have segmented, negative sense single stranded RNA genome belong to family Orthomyxoviridae (Lamb and Krug, 1996). The influenza viruses are classified into types A, B; C based on antigenic differences of their nucleocapsid and matrix proteins. Avian influenza virus belong to type A. The antigenicity of influenza viruses changes gradually by point mutation (antigenic drift) or drastically by genetic reassortment (antigenic shift). The major surface glycoproteins- the haemagglutinin (HA) and neuraminidase (NA) are always under immunological pressure and thought to responsible for antigenic drift and thereby subtypes of the virus (Easterday et al 1997). There are 16 H and 9 N recognized subtypes of influenza virus, and some are nonpathogenic. All the HA subtypes of influenza virus are found in aquatic birds, which serve as the potential reservoir of all influenza A viruses (Webster et al, 1997). Highly pathogenic avian influenza viruses are associated with the H5 and H7 subtypes (Alexander 1995).
Reverse transcriptase polymerase chain reaction (RT-PCR)

The nucleic acid based PCR test provides a fast and accurate early diagnosis of avian influenza virus, specially the H5N1 strain. RT-PCR is sensitive, accurate and rapid test for the detection of the infection. Such test is important for the early diagnosis which facilitate early antiviral therapy. RT-PCR has been used for the detection of AIV and each of the 15 HA subtypes by earlier workers (Lee et al 2001, Munch et al 2001, Starick et al 2000). The World Health Organization (WHO) has also recommended a pair of primers for H5 subtype detection as a laboratory test (WHO 2005). In this technique viral RNA is first converted into c-DNA by the enzyme reverse transcriptase and then in vitro amplification by the enzyme polymerase. The RNA work should be carried out at separate place with separate set of pipettes, chemicals, reagents bottles, etc. A very small amount of specimen is required. This technique consists of 3 major processes; denaturation, annealing, extension. The amplified product is run under gel-electrophoresis and is compared with a known positive control and DNA ladder.

All the procedures of handling highly pathogenic avian influenza virus H5N1 suspected material should be carried out in bio-safety cabinet and should comply with National Biocontainment and Bio-Safety regulations.

MATERIALS AND METHODS

Samples: Pooled organs (lungs, trachea) from 25 healthy chickens and cloacal swabs in phosphate buffer saline from 50 chickens were collected for extraction of viral RNA using QIAamp Viral RNA kit. Other samples like tracheal swab and infected allantoic fluid can also be used for the diagnosis of the disease. About 5000 birds of different species including wild birds were tested for type A avian influenza and avian influenza subtype H5 antigens using rapid dipstick strip test (Anigen, Animals Genetics Inc. Korea).

Procedure: Small amount of sample (50-100 mg homogenized tissue or 150 µl cloacal swab sample) was taken for extraction of viral RNA using QIAamp Viral RNA kit (QIAGEN) according to the manufacturer’s instructions. The RNA was dissolved in the RNase free buffer provided with the kit. 50 to 100 ng RNA was used as template for reverse transcriptase polymerase chain reaction (RT-PCR). The One-Step RT-PCR (Qiagen, USA) and AIV- H5N1 (Veredus Laboratories, Singapore) kits were used for amplification of the RNA template along with positive and negative controls provided in the kit.

One-Step RT-PCR for H5N1 (Veredus Laboratories, Singapore, Qiagen USA Kits):

PCR was carried out in a 25 µl reaction mix using 5µl (100 ng) of purified RNA template, 5 µl of 5X QIAGEN One-step RT-PCR Buffer, 3 µl of 5X QIAGEN Q solution, 1.5 µl of dNTP Mix (10 mM each), 4 µl (40 pmol) of AIV-H5N1 Primer mix, 1 µl of QIAGEN One-step RT-PCR Enzyme Mix, 0.5µl of RNase inhibitor (20 U/ µl), and 5 µl of H2O.

The reaction mix in thin walled 0.2 ml PCR tubes was incubated at 60 °C for 1 min, 42 °C for 10 min, and 50 °C for 30 min for cDNA synthesis (step 1) followed by denaturation at 95°C for 15min (step 2). After denaturation of the template at 95°C for 15 min, amplification was carried out for 40 cycles of three steps, at 94°C for 30 sec, 50°C for 30 sec, 72°C for 60 sec (step 3), with final extension at 72°C for 10 min.
RESULTS AND DISCUSSION

Surveillance of avian influenza including highly pathogenic subtype H5 is going on in the Sultanate for last 3 years. About 5000 birds of different species including wild birds were tested for type A avian influenza and avian influenza subtype H5 antigen. The cloacal swabs were collected from the birds and tested by the rapid dipstick strip test. The samples which gave doubtful results on avian influenza type A dipstick test, further tested by Anigen rapid H5 avian influenza virus antigen test. Few of the samples found positive for low pathogenic H9N2 on virus isolation in 9 to 11 days old chicken embryos. But none of the sample found positive for subtype H5N1 viral antigen. In one-step RT-PCR test, H5N1 positive samples were expected to generate two specific bands of 456 bp (HA gene) and 306 bp (NA gene) in same reaction tube. The amplicons of both the genes (HA & NA) were visualized on 1.5% agarose gel electrophoresis under gel documentation UV light. Pooled organs (lungs, trachea) and cloacal swabs from all the samples were found negative for highly pathogenic H5N1 avian influenza virus (Fig 1).

The highly pathogenic avian influenza virus H5N1 was first isolated from humans during the 1997 outbreak in Hong Kong. The recent recurrence of influenza A H5N1 was first reported in Southeast Asia in mid-December 2003. This highly pathogenic strain not only posed a global human public health risk but also endangered the poultry industry (Viseshakul et al 2004).

Rapid, sensitive and accurate tests like PCR and Real Time PCR are important for the early reliable diagnosis which is very much needd for the timely control of the disease. The timely diagnosis is also important to start with early antiviral therapy. Other diagnostic techniques like cell culture, haemagglutinin (HA) and neuraminidase subtyping by serological testing require time and thus they are less useful in making therapeutic and infection control strategies. The commercially available rapid monoclonal based antigen detection dipstick test for type A and subtype H5 is rapid, simple but subtyping (H1 and H5) of viruses shows cross-reactivity (WHO, 2005). Molecular diagnosis of influenza A H5 by conventional and real time RT-PCR is rapid and also subtyping of the

![Fig. 1](image-url)

**Fig. 1** Agarose gel electrophoresis (1.5%) of HA and NA gene of the H5N1 strain of the avian influenza virus. The RT-PCR used for amplification of HA (456 bp) and NA (306 bp) genes from avian influenza suspected faecal and pooled tissue samples originated from different species of birds. Lane M, 50 bp DNA ladder (Qiagen), Lane 1 chicken faecal sample, Lane 2 chicken tissue sample, Lane 3 pigeon faecal sample, Lane 4 pigeon tissue sample, Lane 5 H5N1 positive control and Lane 6 H5N1 negative control (Veredus AIV-H5N1 RT-PCR kit).
viruses is also possible in minimum possible time interval. Real time PCR further offers the advantages of speed, viral load analysis, sensitivity and specificity compared to conventional RT-PCR (Weijun et al. 2007). Horimoto and Kawaoka, (1995) reported that the RT-PCR amplification of HA gene followed by sequence analysis of the HA cleavage site was useful for rapid determination of the virulence potential of H5 and H7 viruses in birds.

Based on the study, it can be concluded that the local poultry and other imported birds in the Sultanate are free from the highly pathogenic H5N1 subtype of the avian influenza virus till date. The RT-PCR was found specific, sensitive and quick test for the diagnosis of avian influenza virus as compared to rapid dipstick test. Further, Real time RT-PCR can be used for sub typing and virulence determination of the virus.

REFERENCES


