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Review Paper

REAL-TIME REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION TECHNIQUE: USEFULNESS AS AN ANIMAL VIRAL DISEASE DIAGNOSTIC

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Summary: Quick and early diagnosis of the causative agents is critical for countries which are enzootic to particular disease. In these diagnostics speed is paramount; Real-time Reverse Transcription PCR (rRT-PCR) is being utilized increasingly in novel clinical diagnostic assays in molecular biology. The combination of excellent sensitivity and specificity made this technique an alternative to cell culture and other laboratory testing methods for disease diagnosis. In this review, the usefulness or applications of rRT-PCR assays in the diagnosis of some of the important animal viral infections are summarized.

Key words: animal diseases; virus diseases - diagnosis; reverse transcriptase polymerase chain reaction - methods; RNA, viral - genetics

Introduction

Real-time Reverse Transcription Polymerase Chain Reaction (rRT-PCR) is a powerful tool for quantitative analysis of nucleic acids (1, 2). rRT-PCR techniques are increasingly used to quantify RNA viruses for diagnosis (3), the standard for the detection and quantification of RNA targets (4) and is firmly established as a mainstream research technology (5). This technique is a refinement of the original PCR developed by Kary Mullis and co-workers in the mid 1980ís (2, 6). The amplification is detected by using either probe (specific) or non-probe (non-specific) and is discussed elaborately (7). In rRT-PCR the amount of product formed is monitored during the course of the reaction by monitoring the fluorescence of dyes or probes introduced into this reaction which is proportional to the amount of product formed, and the number of amplification cycles required to obtain a particular amount of DNA molecules (2). The advantages of using rRT-PCR are as follows, 1] traditional PCR is measured at end-point, while rRT-PCR collects data in the exponential growth phase, 2] an increase in reporter fluorescent signal is directly proportional to the number of amplicons generated, 3] a permanent record amplification of an amplicon, 4] increased dynamic range of detection, 5] requirement of 1000-fold less RNA than conventional assays, 6] no-post PCR processing 7] detection ranges down to a 2-fold change 8] small amplicon size results in increased amplification efficiency and 9] less time-consuming. rRT-PCR can be applied to traditional PCR applications as well as new applications that would have been less effective with traditional PCR. With the ability to collect data in the exponential growth phase, the power of rRT-PCR has been expanded into applications such as, a] quantization of gene expression (8) including NK cell KIR gene expression (9), b] drug therapy efficacy/drug monitoring (10), c] Viral quantization (11), and d] pathogen detection (12). rRT-PCR when compared to ELISA, RT-PCR and virus Isolation in cell culture, have greater versatility, sensitivity and specificity (13, 14). The technique was and is being used in the diagnosis of a wide variety of diseases caused by RNA viruses in animals and birds and some of them are being summarized in this article.

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rRT-PCR In diagnosis of Animal Viral Infections

A. Positive-strand RNA viruses

rRT-PCR assays are also useful in the diagnosis of animal viral diseases which are economically and as well as zoonotically important. These include some of the viruses listed by the *Office Internationl des Epizootics* (OIE). Foot and mouth disease (FMD) is the number 1 animal viral disease of OIE list and presently the rRT-PCR technique is being widely used in the diagnosis of FMD which causes huge economic crisis world wide. The technique has proven to be more efficient and sensitive than the conventional RT-PCR and virus isolation in cell culture. rRT-PCR assay was found to be very efficient for quantitation of Foot and mouth disease virus (FMDV) in porcine tissues (15); in detecting FMDV from experimentally affected animals (16, 17) and shows 100% specific diagnosis of all the 7 serotypes of FMDV in less than 2 hrs (18, 19). The assay is also used to diagnose swine vesicular disease virus and its differential diagnosis from other vesicular disease viruses like FMD, vesicular stomatitis and vesivirus (20). Another 1000-fold more sensitive TaqMan rRT-PCR was used for the specific detection of FMDV in both cell culture and clinical samples and also for the differential diagnosis of other vesicular diseases and bovine viral diarrhea (21). The two independent rRT-PCR techniques were studied and then compared to detect FMDV in clinical samples; the results suggested that the techniques could be used to enhance sensitivity of molecular methods for further FMDV detection (22). Surveillance and for field deployment another RT-loop mediated isothermal amplification technique was found to be very effective for rapid detection of FMDV (23).

The worldwide occurrence and re-occurrence of trans-boundary diseases like FMD or classical swine fever (CSF) indicates that there is a high need for the development of powerful, robust and high-capacity new diagnostic methods that are able to detect the causative agents before they could spread to large populations and cause tremendous losses (24). The rapid, powerful and internationally standardized molecular diagnosis contributes to the reduction of losses caused by the trans-boundary viral diseases to a larger extent (24). Thus, rRT-PCR forms an important assay in the detection and species-specific differentiation of pestiviruses like CSF virus (25). CSF in an experimentally infected swine will produce the same extent of disease that of natural outbreaks and its detection made easier by TaqMan rRT-PCR assays in less than 2 hrs thus providing a rapid method for the diagnosis of CSF virus on herd basis (26, 27) and also for the quantitative pathogenesis study of this virus (28).

Japanese encephalitis virus (JEV) is one of the most important zoonotic diseases of swine, in areas where it is endemic, such as East Asia (29). TaqMan rRT-PCR assay using fluorogenic probes was developed to distinguish JEV with West Nile virus (WNV) and the method was tested on experimentally infected animal tissues which showed clear discrimination between WNV and JEV (29). Quantitative detection of JEV by rRT-PCR using virus specific primers showed no cross-reactions with other swine viruses and bovine viral diarrhea virus (30). TaqMan rRT-PCR assay was also used for the detection of equine arteritis virus in seminal plasma and nasal secretions of infected horses (31) and then for differentiating the avian infectious bronchitis virus isolates in clinical samples thus by identifying the serotypes involved in disease outbreak (32).

B. Negative-strand RNA viruses

Typical influenza is an acute respiratory herd disease and commonly observed during autumn, winter and early summer (33). Influenza viruses affect all animal and avian species. Immuno-fluorescent techniques were used initially which showed evidence of viral antigen in bronchial epithelial cells within 2 hours after infection (34). Recently, a rRT-PCR assay was used to detect swine influenza A viruses and the test was found to be 100% specific and 88-100% sensitive in screening numerous nasal swab specimens and also very efficient and specific for the respective viral genes thus able to distinguish between their viral subtypes (35) and are largely used to detect and differentiate the North American swine influenza viruses (33). rRT-PCR based on LC technology was used to detect equine influenza virus over two influenza seasons, analyzing 171 samples and they could get a positive correlation between the quantitative rRT-PCR in both cases (36) indicating the high specificity of the assay. Rapid diagnosis of H5N1 influenza A virus was performed by using multiplex rRT-PCR from 75 clinical specimens isolated from both poultry and mammals. The results highlights that the assay could be feasible and very effective for large-scale screening during times of H5N1 outbreaks (37) and also as a good tool for the rapid screening of

flocks and live bird markets (38, 39). Simultaneous detection of influenza viruses A and B was carried out using TaqMan based rRT-PCR assay which was found to be more sensitive than the combination of viral culturing and shell vial culturing of influenza viruses (40).

Bovine respiratory syncytial virus (BRSV) causes respiratory disease in infected animals. Detection of BRSV was done by quantitative rRT-PCR assays based on fluorogenic probe using BioRad's iCycler iQ (41) was found to be 100 fold more sensitive than conventional RT-PCR used previously for BRSV diagnosis (42). Rabies is an enzootic fatal disease and is still a major problem in developing countries. Several RT-PCR methods have been reported for the detection of rabies and rabies-related viruses. Distinguishing the classical rabies virus and its genotypes was described in a single tube, non-nested rRT-PCR with TaqMan technology in real-time and found to be very useful in the detection and differentiation of members of the genus Lyssavirus (43).

Nipah and Hendra viruses belong to the novel genus Henipavirus of the family *Paramyxoviridae*. Its zoonotic circulation in bats and recent emergence in Malaysia with fatal consequences for humans who were in close contact with infected pigs has made the reinforcement of epidemiological and clinical surveillance systems a priority. TaqMan rRT-PCR assay has been developed targeting the Nipah nucleoprotein so that Nipah virus RNA in field specimens or laboratory material can be characterized rapidly and specifically quantitated (44). The method was able to detect virus from hamsters infected with Nipah virus and allows a rapid detection and quantitation of Nipah RNA both from field and experimental materials used for the surveillance and specific diagnosis.

Diagnosis of Newcastle disease virus (NDV) was recently carried out by using rRT-PCR from clinical samples and a positive correlation was obtained between these assays and detecting NDV by RT-PCR and virus isolation from clinical samples (45). To obtain a large diagnostic and surveillance sample workload, a high throughput rRT-PCR assay was developed during 2002-03 outbreaks of NDV occurred in California (46). And also a two-step rRT-PCR using SYBR Green I was designed for the screening large number of NDV specimens (47).

C. Retro viruses

Simultaneous calculation of the nucleic acid recovery rate along with the copy number of viral RNA molecules of equine infectious anemia virus in the plasma was obtained by a single tube rRT-PCR reaction using a fluorogenic probe (48). At the same time a robust, ultra-sensitive quantitative assay was developed for maedi-visna virus (MVV) RNA and DNA genomic sequences and mRNA expressed at various stages of lentiviral replication (49). The assay was designed based on PCR with real-time fluorescence resonance energy transfer measurements. The quantitative assay was found to have greater use in studying the role of genetic elements in MVV infection, pathogenesis, lentiviral vectors and packaging systems based on MVV. Quantitative rRT-PCR assay of MVV RNA in culture supernatants helped in obtaining the complete genomic sequence of a sheep lentivirus isolate that presents a slow/low phenotype (50).

D. Double-stranded RNA viruses

The outbreaks of bluetongue disease in sheep can be combated by extensive vaccination. In order to do so, a rapid and sensitive technique should be used to differentiate vaccine strains of bluetongue and the field strains. A new method for bluetongue virus differentiation using fluorescence resonance energy transfer probes with rRT-PCR assay was performed with LC system and described earlier (51). Infectious bursal disease virus (IBDV) causes an immunosuppressive disease in chickens and leads to severe economic losses in the poultry industry. Vaccination may not be effective if there is exposure of the vaccinated flock to a different antigenic subtype as a result of mutation in the VP2 protein upon which the major neutralizing epitopes are located (52) also they reported that the rRT-PCR assay could be a useful tool to assist in the development of more effective vaccines and control strategies of infectious diseases.

Conclusion

The newly established methods must be standardized to maintain high quality laboratory performance. Future challenges in the study of animal viral diseases include the application of modern techniques, such as nucleic acid chips, protein chips, proteomics and new biomarkers to avoid cross-reactivity among different samples, strains or serotypes, as well as development of internationally standardized guidelines to improve the quality of these laboratory tests.

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METODA OBRATNEGA PREPISOVANJA IN VERIŽNE REAKCIJE S POLIMERAZO V REALNEM ČASU: UPORABNOST V DIAGNOSTIKI ŽIVALSKIH VIRUSNIH BOLEZNI

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Povzetek: Hitro in pravočasno odkrivanje povzročiteljev bolezni je v državah z enzootijo za določene bolezni bistvenega pomena. Pri taki diagnostiki je hitrost odločilna in rRT-PCR (obratno prepisovanje in verižna reakcija s polimerazo v realnem času) se čedalje več uporablja kot sodoben test klinične diagnostike v molekularni biologiji. Zaradi odlične občutljivosti in specifičnosti predstavlja alternativo celičnim kulturam in drugim laboratorijskim testom za diagnostiko bolezni. V preglednem članku povzemamo uporabnost in aplikacije testov rRT-PCR v diagnostiki nekaterih pomembnih živalskih virusnih bolezni.

Ključne besede: živali, bolezni; virusne bolezni - diagnostika; polimerazna verižna reakcija z reverzno transkriptazo; RNA, virusna - genetika