Review Article

Real Time PCR and Its Application in Diagnosis of Current Veterinary Diseases: A Brief Review

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A B S T R A C T

Diagnosis of disease is the backbone of control and treatment in veterinary field. In addition to the antemortem and post mortem methods, currently several laboratory-based tools and technique are also being used for early diagnosis. Since few decades, polymerase chain reaction (PCR) has emerged as the most preferred molecular diagnostic technique for disease diagnosis due to its high specificity. But it only detects the presence of the target nucleic acid in the sample without quantifying the same. Additionally, the detection of amplified DNA requires one extra step of gel electrophoresis followed by visualization under ultraviolet rays which involves radiation hazards. Hence, a more sophisticated technique called real time polymerase chain reaction (PCR) has been discovered for developing rapid assay for the diagnosis of many diseases. Along with the detection of particular nucleotide sequence, quantification of the latter can also be performed using this assay. Real time PCR was either of two specific chemistry: Nonspecific DNA binding dye or specific hybridization probe. The fluorescence generated from either of the above during the assay is directly proportional to the quantity of target being amplified at the real time. Although field application of real time PCR is infrequent, nevertheless its rapidity, high sensitivity & specificity and less contamination risk may lead to its enhanced application in screening and epidemiological study in the veterinary field in recent future. In this review we attempted to brief about the chemistries of real time PCR and its application in diagnosis of different veterinary diseases worldwide.

Introduction

Real time polymerase chain reaction (PCR)

Real time polymerase chain reaction is a molecular biology technique used to monitor the progress of a PCR reaction in real time. In real-time PCR, reactions are characterized by the point in time during cycling when amplification of a target is first detected rather than the amount of target accumulated after a fixed number of cycles, hence, this is also

called quantitative PCR (qPCR) (Navarro *et al.*, 2015). There are two different chemistries behind the PCR product quantification in real-time PCR, however, both involve quantification based on the fluorescence produced: (a) firstly the intercalation of non-specific dye to double-stranded DNA emitting fluorescence, thus the reporter signal indicates the quantity of amplified DNA and (b) secondly the hybidization of sequence-specific fluorescent labelled probes (containing fluorophore at 5'-end and quencher at 3'-end) to the complementary DNA strand, which gets cleaved by the 5'→3' exonuclease activity of Taq polymerase from the PCR reaction during amplification, hence separating the fluorophor away from quencher and allowing the fluorescence emission from the former (this is based on the principle of fluorescence resonance energy transfer (FRET)) (Mackay *et al.*, 2002).

Unlike conventional PCR, agarose gel electrophoresis is not performed for the amplified qPCR product, rather melting curve analysis is done *in silico* for real time quantification of products. In addition, visualization of DNA under ultraviolet illumination is not required in qPCR thus eliminating the risk of radiation hazards. Furthermore, qPCR can be used for both absolute and relative quantification of the nucleic acids (Schena *et al.*, 2004)

Fluorescent chemistries in real-time PCR

Two different chemistries of real-time PCR are explained in figure 1.

DNA binding dyes

SYBR green-I is a commonly used fluorescent dye that intercalates between two strands of all kinds of dsDNA including nonspecific PCR products and primer-dimers. The dye fluoresces when bound to the dsDNA. An increase in DNA product during amplification leads to an increase in fluorescence intensity and this can be measured at each cycle by the detector present in the instrument. In real-time PCR with dsDNA binding dyes the reaction is prepared as usual, with the addition of fluorescent dsDNA dye (Morrison *et al.*, 1998).

The biggest disadvantage of SYBR is that it binds to any dsDNA. To avoid this problem one needs to carefully optimize the PCR reaction to reduce formation of primer-dimers. Secondly, hot start techniques like Taq Start antibody can be helpful in reducing primer-dimers also. Another disadvantage is multiplexing cannot be done using SYBR green dye.

Fluorescent reporter probes

Fluorescent reporter probes hybridize with specific complementary DNA and is based on the principle of FRET (Didenko, 2001). Using different-coloured labels, fluorescent probes can be used in multiplex assays where many target sequences can be detected in the same tube. Use of the reporter probe significantly increases specificity and enables performing the technique even in the presence of any other non-specific dsDNA. The specificity of fluorescent reporter probes also prevents interference of measurements caused by primer-dimers. The method relies on a DNA-based probe with a fluorescent reporter at one end and a quencher of fluorescence at the opposite end of the probe. Various fluorophores used are 6-carboxyfluorescein (FAM) or tetrachlorofluorescein (TET) and quenchers like tetramethylrhodamine (TAMRA) are available (Kutyavin *et al.,* 2000). The close proximity of the reporter to the quencher prevents detection of its fluorescence, however the breakdown of probe by the 5'→3' exonuclease activity of the Taq polymerase breaks the reporter-quencher

proximity and thus allows unquenched emission of fluorescence, which can be detected after excitation with a laser (Ponchel *et al.,* 2003).

Various examples fluorescent probes are: TaqMan, molecular beacon, scorpion probe, FRET (Förster Resonance Energy Transfer) probes etc. The primary disadvantage of the fluorescent probes is that the synthesis of different probes is required for different template sequences which may cost higher to the researcher (Tyagi *et al*. 1996, Thelwell *et al*. 2000, Didenko *et al*., 2001).

Absolute quantification vs relative quantification

By absolute quantification a standard curve is plotted using the fluorescent signals obtained from the serially diluted samples. Further, quantification of unknown samples is done by comparison with the standard curve. While in case of relative quantification, expression of a gene of interest in treated samples is compared to expression of the same gene in untreated sample (also called control) and the results are expressed as fold change.

Different terms related to real time PCR are explained in brief in table 1.

Veterinary disease diagnosis by real-time PCR

Conventional disease diagnosis is performed by specific clinical signs or post-mortem examination but laboratory techniques aids in better diagnosis of the disease and eliminates the doubts of non-specific pathological disorders. However, there are some diseases for which no available cost-effective serological assays have been developed like Jaagsiekte sheep retrovirus (JSRV), since the virus does not induce a specific antibody response in infected animals (Ortin *et al*.,

1998). In some cases, isolation of virus or detection of specific antibody is time consuming and may kill the patient before diagnosis, like in case of Zika virus infection (Faye *et al*., 2013). But in other cases, alternative laboratory methods like Indirect antibody fluorescent test (IFAT) can lack sensitivity and specificity compared to molecular detection methods and in addition multiplexing cannot be performed with help of former (Thonur *et al*., 2012). To combat these issues, many researchers are building interest in developing real-time PCR for detection of diseases with high specificity and sensitivity which can be performed within less time to obtain the result. In the case of conventional PCR, the analysis of the results requires an additional step of agarose gel electrophoresis using factors like ethidium bromide and UV light and the latter are hazardous for human health, nevertheless detection and analysis of real-time PCR product is performed simultaneously during the amplification process by the software provided with the instrument (Schena *et al*., 2004; Hoffmann *et al*., 2009). Hence real-time PCR possess advantages such as speed, high specificity, sensitivity, cost-effectiveness, and reduced contamination risk (Espy *et al*., 2006). Here we have briefed some world-wide reported recent animal diseases for which real-time PCR has been developed as a detection method.

Ovine pulmonary adenomatosis

The LTR region in JSRV genome was detected in biological materials from experimentally and naturally infected sheep by real-time PCR and the results were compared to that of heminested PCR (hnPCR) and subsequently found that the earlier results are rapid, more sensitive and less error-prone than latter (Kycko and Reichert, 2010). For the first time Kycko and Reichert reported that rRT-PCR may be used either to confirm the

infection in clinically suspected animals or employed as a screening method in disease eradication programmes (Kycko and Reichert, 2010). Further, a TaqMan real-time PCR technique was developed to investigate Jaagsiekte sheep retrovirus (JSRV) proviral DNA in whole blood samples of sheep for diagnosis of ovine pulmonary adenomatosis. The results were compared with the histopathological lesions of lung tissue which revealed the rate of viral infection detected by real-time PCR is much higher as compared to histopathological examination (Bahari *et al*., 2016).

Zika virus infection

In 2013, Faye *et al*. reported the detection of Zika virus by using the gene of NS5 protein of African ZIKV isolates in real-time reverse transferase PCR (rRT-PCR) where the result can be obtained within 3hrs. Here the ZIKV isolates were isolated from field-caught mosquitoes and the researchers have used TaqMan probe with locked nucleic acid that is complementary to the sequence of NS5 gene (Faye *et al*., 2013). Again in 2017, Tien *et al*. developed another SYBR green dye based rRT-PCR for surveillance of ZIKV in mosquitoes. Here the assay was faster (119bp size of amplicon) and cost-effective (due to low cost of dye) (Tien *et al*., 2017).

Nipah virus infection

Nipah virus naturally infects Pteropid fruit bats and being zoonotic and is also associated with outbreaks in humans in most parts of east Asia (Chadha *et al*., 2006; Gurley *et al*., 2007; Ching *et al*., 2015). One-step qRT-PCR assay targeting the intergenic region separating the viral F and G proteins was devised, which eliminates amplification of the viral mRNA by conventional traditional qRT-PCR (Jensen *et al*., 2018). This assay can help monitor the virus titre accurately by quantifying the

genome copy numbers independent of mRNA concentration.

Bovine viral diseases

In 2005, Boxus and team a TaqMan quantitative real-time RT-PCR assay targeting the nucleoprotein gene of bovine respiratory syncytial virus (BRSV) was developed to both detect and quantify the viral load in the respiratory tract of infected animals. In this experiment the researchers collected samples from lungs, tracheas and bronchoalveaolar fluids (BAL) from experimentally infected calves and they found that qRT-PCR is 100 times more sensitive than conventional RT-PCR for diagnosis of BRSV (Boxus *et al*., 2005).

Thonur and team has developed a one-step multiplex real-time PCR (mRT-qPCR) for diagnosis of three viral diseases of bovine such as bovine respiratory syncytial virus (BRSV), bovine herpesvirus 1 (BoHV-1) and bovine parainfluenza virus 3 (BPI3). Targets of this assay are glycoprotein B gene of BoHV-1, nucleocapsid gene of BRSV and nucleoprotein gene of BPI3. As compared to the results obtained by conventional virus isolation (VI) and IFAT. Hence this is a complete diagnostic for bovine respiratory diseases (Thonur *et al*., 2012).

***Pasteurella multocida* infection in pigs**

P. multocida as an important pathogen of respiratory disease in pigs causing progressive atrophic rhinitis and pneumonia. In Switzerland, pigs were earlier screened for progressive atrophic rhinitis (PAR) by selective culture of nasal swabs and subsequent PCR screening of bacterial colonies for the *toxA* gene of *P. multocida* (Rutter *et al*., 1984, Lichtensteiger *et al*., 1996), but this process was hectic as well as time-consuming. Hence in 2016, Scherrer *et*

al., devised a quantitative real-time PCR for detection of *Pasteurella multocida* from nasal swab of pig to diagnose PAR which eliminated the step of swab culture and hence became the faster technique. Subsequently in 2017, TaqMan qPCR targeting *sodA* gene, was

developed by Tocqueville *et al.*, which can be used to quantify *P. multocida* in specimens from experimentally infected live and dead pigs. Hence this can be applicable for epidemiological and transmission studies of *P. multocida* (Tocqueville *et al.*, 2017).
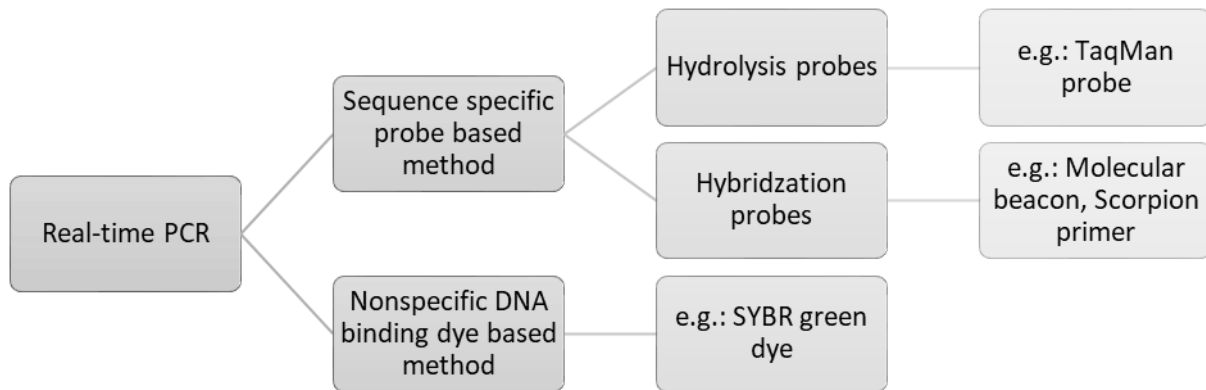
Fig.1 Different chemistries of real-time PCR

Table.1 Important terms related to real-time PCR

Ct value	Number of cycles required for the fluorescent signal to cross a predetermined (automatically or manually) threshold value
Threshold cycle	It differentiates amplification signals from the background signals
Threshold	10 times the standard deviation of the fluorescence value of the baseline which is automatically set by the PCR instrument
Baseline	Initial amplification where the fluorescent is nearly zero
Exponential phase	The phase at which the reported amplification is at its highest peak
Standard curve	A curve plotted using log of each known concentration in the dilution series in horizontal-axis against the Ct value for that concentration vertical-axis

Bluetongue and Peste des petits ruminants (PPR)

Bluetongue virus (BTV) belongs to family Reoviridae, the genus *Orbivirus* and the species Bluetongue virus, is transmitted by a few species of the genus *Culicoides* and infects most domestic and wild ruminants. This disease is included in list A of the World

Organisation for Animal Health (OIE) (Lakshmi *et al.*, 2018). Toussaint *et al.*, 2007, reported that all 24 serotypes of bluetongue viruses can be detected by targeting two different genomic segments such as segment 1 and 5 of the virus by qRT-PCR, where beta-actin gene was used as an internal control. Further in 2010, Vanbinst and team validated a duplex based real-time RT-PCR targeting

BTV for direct testing and quality control of semen for artificial insemination where glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA was used as an internal control (Vanbinst *et al*., 2010). PPR is a transboundary disease and it possess a major threat to farmers as it affects small ruminants, particularly in Asia, Middle East and Africa (Kwiatek *et al*, 2010). In 2008, Bao *et al*., developed a rapid and specific TaqMan-based, one-step real-time qRT-PCR for the detection of PPR virus (PPRV) which targeted the nucleocapsid protein gene sequence. Subsequently in 2010, another one-step real-time Taqman® RT-PCR assay was developed by Kwiatek and team for PPRV to detect all the four lineages of PPRV by targeting the nucleoprotein (N) gene of the virus. The latter assay has higher sensitivity for lineage II than the method developed by Bao *et al*., 2008 (Kwiatek *et al*., 2010).

In conclusion, although many 'gold standard' tests such as virus isolation, ELISA, combination of PCR and southern blotting etc. are available for diagnosis of various diseases, qRT-PCR remains the preferred choice for researchers now-a-days. Not only high specificity and sensitivity but its other features like rapidity, low contamination risk, reduced health hazards to handlers and faster data analysis have been explored highly in the field of clinical diagnosis. Currently this assay has been developed for many diseases of veterinary importance world-wide. But its application in field is relatively low because of high cost of instrument and requirement of highly skilled person. However, for faster screening of herd and epidemiological studies, qRT-PCR can be helpful in recent future.

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