HAL
open science

A review of RT-PCR technologies used in veterinary virology and disease control: sensitive and specific diagnosis of five livestock diseases notifiable to the World Organisation for Animal Health

Bernd Hoffmann, Martin Beer, Scott M. Reid, Peter Mertens, Chris A.L. Oura, Piet A. van Rijn, Marek J. Slomka, Jill Banks, Ian H. Brown, Dennis J. Alexander, et al.

To cite this version:

Bernd Hoffmann, Martin Beer, Scott M. Reid, Peter Mertens, Chris A.L. Oura, et al.. A review of RT-PCR technologies used in veterinary virology and disease control: sensitive and specific diagnosis of five livestock diseases notifiable to the World Organisation for Animal Health. Veterinary Microbiology, 2009, 139 (1-2), pp.1. 10.1016/j.vetmic.2009.04.034 . hal-00520658

HAL Id: hal-00520658

https://hal.science/hal-00520658

Submitted on 24 Sep 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: A review of RT-PCR technologies used in veterinary virology and disease control: sensitive and specific diagnosis of five livestock diseases notifiable to the World Organisation for Animal Health

Authors: Bernd Hoffmann, Martin Beer, Scott M. Reid, Peter Mertens, Chris A.L. Oura, Piet A. van Rijn, Marek J. Slomka, Jill Banks, Ian H. Brown, Dennis J. Alexander, Donald P. King

PII: S0378-1135(09)00251-X
DOI: doi:10.1016/j.vetmic.2009.04.034
Reference: VETMIC 4435

To appear in: *VETMIC*

Received date: 5-11-2008
Revised date: 15-4-2009
Accepted date: 28-4-2009

Please cite this article as: Hoffmann, B., Beer, M., Reid, S.M., Mertens, P., Oura, C.A.L., van Rijn, P.A., Slomka, M.J., Banks, J., Brown, I.H., Alexander, D.J., King, D.P., A review of RT-PCR technologies used in veterinary virology and disease control: sensitive and specific diagnosis of five livestock diseases notifiable to the World Organisation for Animal Health, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2009.04.034

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

A review of RT-PCR technologies used in veterinary virology and disease control: sensitive and specific diagnosis of five livestock diseases notifiable to the World Organisation for Animal Health

Bernd Hoffmann[1], Martin Beer[1*], Scott M. Reid[2], Peter Mertens[2], Chris A. L. Oura[2]

Piet A. van Rijn[3], Marek J. Slomka[4], Jill Banks[4], Ian H. Brown[4], Dennis J. Alexander[4], Donald P. King[2]

[1] *Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, D-17493 Greifswald-Insel Riems, Germany*

[2] *Institute for Animal Health, Ash Road, Pirbright, Surrey. GU24 0NF, United Kingdom*

[3] *Central Veterinary Institute of Wageningen University Research Center (CVI-Lelystad), P.O. Box 2004, 8203 AA Lelystad, The Netherlands*

[4] *OIE, FAO and EU Community Reference Laboratory (CRL) for Avian Influenza and Newcastle Disease, Virology Dept, Veterinary Laboratories Agency (VLA-Weybridge), Addlestone, Surrey, KT15 3NB, United Kingdom*

*corresponding author:

Dr. Martin Beer
Institute of Diagnostic Virology
Friedrich-Loeffler-Institut
Südufer 10
17493 Greifswald-Insel Riems
Tel. 0049-38351-7-200
Fax 0049-38351-7-275
e-mail: martin.beer@fli.bund.de

Accepted Manuscript

ACCEPTED MANUSCRIPT

Abstract:

Real-time, reverse transcription polymerase chain reaction (rRT-PCR) has become one of the most widely used methods in the field of molecular diagnostics and research. The potential of this format to provide sensitive, specific and swift detection and quantification of viral RNAs has made it an indispensable tool for state-of-the-art diagnostics of important human and animal viral pathogens. Integration of these assays into automated liquid handling platforms for nucleic acid extraction increases the rate and standardisation of sample throughput and decreases the potential for cross-contamination. The reliability of these assays can be further enhanced by using internal controls to validate test results. Based on these advantageous characteristics, numerous robust rRT-PCRs systems have been developed and validated for important epizootic diseases of livestock. Here, we review the rRT-PCR assays that have been developed for the detection of five RNA viruses that cause diseases that are notifiable to the World Organisation for Animal Health (OIE), namely: foot-and-mouth disease, classical swine fever, bluetongue disease, avian influenza and Newcastle disease. The performance of these tests for viral diagnostics and disease control and prospects for improved strategies in the future are discussed.

Keywords: polymerase chain reaction; real-time PCR; FMDV; AIV; NDV; CSFV; BTV

Accepted Manuscript

ACCEPTED MANUSCRIPT

1. Real-time PCR technology

Over the past decade, fluorescence-based polymerase chain reaction assays in real-time (real-time PCR) have become an established scientific tool for the detection of RNA, DNA and cDNA. This approach is a highly sensitive technique enabling simultaneous amplification and quantification of specific nucleic acid sequences. In addition to enhanced sensitivity, the benefits of real-time PCR assays over conventional endpoint detection methods include their large dynamic range, a reduced risk of cross-contamination, an ability to be scaled up for high throughput applications and the potential for accurate target quantification (Nazarenko et al., 1997; Schweiger et al., 2000; Black et al., 2002). Real-time PCR is suitable for a wide range of applications, such as gene expression analysis, determination of viral load, detection of genetically modified organisms, SNP (Single Nucleotide Polymorphisms) genotyping, and allelic discrimination. Detection of target sequences occurs by monitoring the fluorescence generated by intercalating dyes, fluorophore-labeled primers or sequence-specific probes. The use of a specific probe facilitates an increase in specificity compared to conventional agarose-gel based PCR assays (Bustin, 2000; Bustin et al., 2005). Quantification of target sequences is typically achieved by determining the number of amplification cycles required to generate PCR product (and corresponding fluorescence) at the beginning of the exponential phase of the PCR, in order to cross a threshold fluorescence line (threshold cycle (Ct) = the number of cycles required to reach the threshold). In contrast with conventional PCR, where endpoint detection occurs in the plateau phase, preventing accurate quantification of nucleic acids, Ct values in real-time PCR correlate closely with the original quantity of target sequences.

The development of multi-colour real-time PCR cyclers and “ready-to-use” commercial multiplex real-time PCR kits has also made it possible to combine up to four assays within a single tube. Multiplex-PCR is the simultaneous amplification of two or more DNA (or cDNA) targets in a single reaction vessel and can only be carried out using uniquely-labelled probes for each target sequence. Typically, up to four target sequences can be detected in a multiplex 5' nuclease assay when the appropriate instrument and the right combination of fluorophores are used. Major advantages of multiplexing include a reduced sample requirement, which is especially important when sample material is scarce (Persson et al. 2005, Belak; 2007), and the

ACCEPTED MANUSCRIPT

ability to combine assays with an internal control system (Hoffmann et al., 2006). However, it is important to optimize these assays in order to limit competitive interactions that may significantly impact upon assay sensitivity.

The combined properties of high sensitivity and specificity, low contamination risk, and speed has made real-time PCR technology a highly attractive alternative to tissue culture- or immunoassay-based methods for diagnosing many infectious diseases (Espy et al., 2006). Clinical diagnostic applications and the use of real-time PCRs are growing exponentially, and real-time PCR is rapidly replacing conventional PCR and other established diagnostic methods such as antigen-ELISA and cell culture isolation. More general information is provided in a series of review articles (Mackay et al., 2002; Mackay, 2004; Bustin, 2000, 2002, 2005; Clementi et al., 1995; Niesters, 2002, 2004; Ratcliff et al., 2007; Gunson et al., 2006; Espy et al., 2006; Kaltenboeck and Wang, 2005; Kubista et al, 2006). This review considers the current scope and capability of this powerful technique, especially when detecting important notifiable viral pathogens of livestock such as foot-and-mouth disease virus (FMDV), classical swine fever virus (CSFV), bluetongue virus (BTV), avian influenza virus (AIV) and Newcastle disease virus (NDV).

1.1 Detection formats

Detection formats without sequence confirmation of the PCR product

Fluorescent dyes such as ethidium bromide (Higuchi et al., 1993; Wittwer et al., 1997a; LePecq, 1966) that are specific for double-stranded DNA (dsDNA) were the first systems employed in real-time PCR assays. Other intercalating dyes such as YO-PRO-1 have also been used (Ishiguro et al., 1995; Tseng et al., 1997). SYBR Green I is currently the most frequently used intercalating dye in real-time PCR. It has a 100 times higher binding affinity than ethidium bromide, and the fluorescence of bound dye is more than 1000-fold higher than that of free dye. These properties make SYBR Green I highly suitable for monitoring product accumulation during PCR (Wittwer et al., 1997b; Morrison et al., 1998). An often-stated disadvantage of this format using intercalating dyes, is that non-specific dsDNA products, such as primer dimers can interfere with the assay signal and yield false positive results. Therefore, it is important that the assay is optimized to avoid non-specific amplification products.

ACCEPTED MANUSCRIPT

Furthermore, melt curve analysis can be used to differentiate specific from non-specific PCR products (Helps et al., 2002).

Another detection technology known as 'LUX (Light Upon eXtension)' utilizes a modification to one of the two primers such that it possesses a fluorophore located near the 3´end in a hairpin structure (Nazarenko, 2006; Kusser, 2006).
The Amplifluor Quantitative PCR detection system uses a similar approach with labelled and unlabelled primers (Nazarenko et al., 1997; Nuovo et al., 1999; Khripin, 2006). Both of these systems are described as highly flexible and beneficial, but have not been frequently exploited for diagnostic tests.

Detection formats with increased target specificity

The incorporation of a specific probe into real-time PCR confirms the specificity of the amplicon. Fluorophore-labelled oligonucleotide probes are most commonly used for the specific detection of target sequences (Cardullo et al., 1988; Clegg, 1995; Wu and Brand, 1994). In these assays, an increase in fluorescence signal proportional to the accumulation of PCR product arises as a consequence of fluorescence resonance energy transfer (FRET) between separate fluorogenic labels (known as the reporter and quencher) conjugated to the probe (or primers). FRET, also called Förster transfer, is a spectroscopic process by which energy is passed over a maximum distance of 70 Å between reporter and acceptor molecules possessing overlapping emission and absorption spectra (Selvin and Hearst, 1994). For real-time PCR, several formats have been used: however, most assays exploit a decrease in quench (compared to an increase in FRET). The most commonly used fluorogenic quenchers are TAMRA and DABCYL, while Black Hole Quencher (BHQ) is also widely used and disperses energy from the reporter as heat rather than fluorescence (Didenko, 2001).

The most widely used detection chemistries are briefly reviewed below.

Hybridisation probes

Hybridisation probes, also known as 'HybProbes', use a pair of adjacent, fluorogenic hybridisation oligos and are the only detection format that directly measures FRET (Cardullo et al., 1988). These probes have become the preferred chemistry of the

ACCEPTED MANUSCRIPT

manufacturer for the capillary-based LightCycler system (Roche Molecular Biochemicals; Wittwer et al., 1997a, 1997b) with special filters for the detection of the acceptor fluorophores Red 640 and Red 705.

Hydrolysis probes (5'-exonuclease assay)

Hydrolysis probes (commercially called *TaqMan*® probes) are dual-fluorophore-labelled oligonucleotides, with a 5'-terminal reporter (e.g. FAM) and a 3'-terminal quencher (e.g. TAMRA). Once the labels are separated by destroying the *TaqMan*® probe based on the 5´-exonuclease activity of the DNA polymerase (e.g. Taq polymerase), the increase in reporter fluorescence caused by the removal of the adjacent quencher is monitored by a real-time PCR instrument (Heid, et al., 1996; Livak et al., 1995; Gibson, 1996). A modification of this strategy exploits the so-called minor groove binding (MGB) probes. MGB probes form extremely stable duplexes with single-stranded DNA targets mediated via van der Waals forces (Afonina et al., 2002): as a consequence shorter-length probes are required for hybridization. In comparison with unmodified DNA, MGB probes have higher T_m and are reported to hybridize with greater sequence specificity (Afonina et al., 1996; Kutyavin et al., 2000). These short MGB probes are ideal for allele discrimination studies or for detection of single-nucleotide polymorphisms (SNPs) because they are more significantly destabilized by nucleotide changes within the hybridisation site compared with probes of longer length (de Kok et al., 2002; Belousov et al., 2004; Itabashi et al., 2004).

Molecular beacons

Molecular beacons are hairpin-shaped oligoprobes terminally labelled with a reporter and a quencher fluorophore (Tyagi and Kramer, 1996; Tyagi et al., 1998; Vet et al., 2002).

Scorpion primer

The Scorpion technology is mainly used in allelic discrimination (Whitcombe et al., 1999; Thelwel et al., 2000) and in SNP genotyping (Roberts, 2000).

Locked nucleic acid (LNA) probes

ACCEPTED MANUSCRIPT

Incorporation of LNA residues increases the T_m of the oligonucleotide sequence, allowing the use of markedly shorter probes as allele-specific tools in genotyping assays (Costa et al., 2004; Latorra et al., 2003; Braasch and Corey, 2001).

1.2 Internal controls

The use of an internal control (IC) is an important aspect of quality control. An IC is necessary for ensuring adequate efficiency of RNA extraction and confirming the absence of PCR inhibitors in each sample, thereby avoiding false negative results (Belak and Thoren, 2001). An IC is a valuable tool when testing nucleic acids extracted from potentially difficult biological matrices (such as degraded samples) where the presence of PCR-inhibitors could be problematic. In these cases, co-amplification of an IC increases the reliability of the results and is used to validate negative results. In practice, different IC systems can be used. One approach is to detect an endogenous gene that occurs naturally in the test specimen. To be a reliable indicator of assay performance, the gene selected should exhibit a constant, basal cell cycle-independent level of transcription that is not influenced by the cellular pathology associated with the disease targeted by the real-time assay in question. Genes fulfilling these criteria are commonly known as housekeeping genes. Those used most often include glyceraldehydes-3-phosphate (GADPH), β-actin, 18S ribosomal RNA, glutamate decarboxylase (GAD), and β_2-microglobulin (Oleksiewicz et al., 2001; Korimbocus et al., 2002; Gorzelniak et al., 2001; Hüssy et al., 2001; Kim and Dubovi, 2003; Huggett et al., 2005; Kompalic-Cristo et al., 2007; West et al., 2007; Müller et al., 2007; Toussaint et al., 2007). In contrast to an endogenous IC, an exogenous IC does not occur naturally within the nucleic acid preparation. This type of IC can be designed to contain an all-purpose heterologous target sequence (Hoffmann et al., 2006; Olson et al., 2004; Drosten et al., 2001) or a complete heterologous viral genome (Niesters, 2004; Stranska et al., 2004), unrelated to the sequence to be detected. To avoid apparent false-negative results, exogenous ICs can be added to each test sample before either the template purification step or prior to co-amplification in the real-time PCR. Depending upon the particular design of these ICs, amplification of the control sequences may require that an additional set of primer pairs are included in the reaction. Alternatively, 'mimic' ICs (*in-vitro* transcripts, plasmids or chimeric viruses) can be engineered containing homologous target sequences for the same primer pair as the diagnostic test. In addition, these

ACCEPTED MANUSCRIPT

mimic ICs can contain internal sequences not present in the target amplicon to yield an amplification product with a different length (Belak and Thoren, 2001), which can be differentiated from wild-type amplicon by a second, IC-specific probe (King et al., 2003; Hofmann, 2003; Hodgson et al., 2007; Fedele et al., 2006; King et al., 2007). Since these controls are amplified using the assay target-specific primers, a benefit of this type of control is that it directly monitors the performance of diagnostic primers set ensuring that the correct PCR components have been added. However, in contrast to the previously-described exogenous ICs, these target-specific control systems cannot be transferred to other assays, although a cassette approach can be designed to incorporate primer sequences of multiple diagnostic tests (King et al., 2007).

1.3 Multiplexing

In most cases the detection of co-amplified PCR products in one tube (multiplexing) were performed by hybridising probes and hydrolysis probes. Hybridising probes use the different melting points for differentiation of amplicons (Olson et al., 2004). On the other hand different emission spectra (colours) of sequence specific probes (*TaqMan*® or Hyb probes) allow also the detection of multiple sequences in a single reaction tube (Dupont et al., 2002; Wittwer et al., 2001). Some real-time PCR machines enable the detection and differentiation of up to 6 colours. This feature of real-time PCR becomes more and more important since it allows the cost-effective answer to several diagnostic questions within one sample in one reaction like agent detection together with sub-typing or the combined detection of several different pathogens (Gunson et al., 2008). Examples are established multiplex assays for the detection and sub-typing of influenza viruses (Wu et al., 2008; Payungporn et al., 2006) or the differentiation of Porcine circovirus-2 genotypes (Gagnon et al., 2008).

1.4 Experiences of real-time PCR deployment in different laboratories

A questionnaire campaign was conducted between the 19 collaborating partner institutes in Europe, Turkey and China that participate in an EU-funded research project for epizootic diseases “EPIZONE”. Many of the participants are engaged in detection of the important pathogens of veterinary significance. Partners were asked about the strategies, techniques and equipment they utilise for performing real-time PCR. We will briefly summarise the findings of this survey before discussing specific information relating to the real-time PCR assays used for individual selected

ACCEPTED MANUSCRIPT

pathogens. Within the EPIZONE project, utilization of real-time PCR was widespread with many laboratories having a range of molecular tests in routine diagnostic use. These assays included a broad assortment of the different detection chemistries (described above) and it is important to note that there are currently very few instances of assay harmonisation between the laboratories. All of the partners have at least one assay in the dual-labelled probe format (such as TaqMan® or MGB), while intercalating dyes were used by about 40% of the participants. About 10% also used alternative probe formats such as FRET probes (hybridization probes), molecular beacons or Primer-probe energy transfer (PriProET: to be described later) for some of their tests. Scorpion technology was not used. Nearly 90% of the participants undertake a one-step (RT and PCR in one reaction) rather than a two-step (separate RT and PCR step) strategy. Nearly half of the laboratories have internal controls, mostly endogenous housekeeping genes included in the assays which were incorporated as duplex targets in the tests.

2. Detection of foot-and-mouth disease virus by rRT-PCR

Foot-and-mouth disease (FMD) is a highly contagious disease affecting cloven-hoofed livestock (cattle, sheep, pigs and goats). The causative agent is an RNA virus (FMDV: genus *Aphthovirus,* family *Picornaviridae*) with a positive sense genome approximately 8300 nucleotides in length. This virus exists as seven antigenically distinct serotypes (O, A, C, Asia 1, SAT 1, SAT 2 and SAT 3), as well as numerous and constantly evolving subtypes. FMD is endemic in large areas of Africa, Asia and South America and can readily cross international boundaries to cause epidemics in previously free areas. In disease-free countries, sporadic outbreaks of FMD can result in devastating losses to the agricultural industry due to the restrictions to international trade and the implementation of control measures to eradicate the disease. The significance of FMD as a major threat to the farming industry was dramatically illustrated during the 2001 FMD epidemic in the United Kingdom (UK). This outbreak lasted for 8 months requiring the slaughter of approximately 6.5 million animals for its control, and is estimated to have cost between £2 - 8 billion to the national economy.

ACCEPTED MANUSCRIPT

Control of outbreaks is dependent upon early recognition of infected animals, which requires familiarity with clinical signs of FMD and the ability to accurately and rapidly detect FMDV in clinical samples using laboratory tests. Of the established diagnostic approaches, virus isolation (VI) in cell culture is considered to be the "gold standard". This method can be highly sensitive (depending upon the cell culture system used), although it can be slow taking between 1-4 days to generate a result. Other assays such as antigen-detection ELISA are more rapid, but have lower analytical sensitivity and are inappropriate for use with certain sample types. It is now recognised that RT-PCR assays can play an important role for the rapid and sensitive detection of FMDV in a wide range of clinical sample types. Over the past decade, improvements have been made to RT-PCR protocols for the detection of FMDV that have paralleled the evolution of molecular diagnostics. Initially, assays utilising agarose-gel electrophoresis for the detection of amplified products and targeting conserved regions of the genome (3D: Meyer et al., 1991; Rodríguez et al., 1994 and 5' untranslated region [5' UTR], Reid et al., 2000) were developed. However, these labour intensive procedures have a high risk of generating false positives due to carry-over of PCR amplicons and are therefore not ideal for routine testing of large numbers of samples. Recent development of rRT-PCR methodology employing a fluorescently-labelled probe to detect PCR amplicons has allowed the diagnostic potential of molecular assays to be realised. These assays (outlined in Table 1) are highly sensitive and obviate tube opening after amplification thereby reducing the potential for cross-contamination of test samples by post-PCR products. In addition to the widely exploited 5'-nuclease (TaqMan®) system using dual-labelled probes (Reid et al., 2002; Callahan et al., 2002; Oem et al., 2005) and modified MGB probes (Mowika et al., 2007), assays have also been developed using other rRT-PCR formats such as those using hybridisation probes (Moonen et al., 2003) and PriProET (Rasmussen et al., 2003). In order to increase assay throughput and minimise operator errors, rRT-PCR assays for FMDV can be automated using robots for nucleic acid extraction and liquid handling equipment to set-up the reaction mixes (Reid et al., 2003; Moonen et al., 2003). Together with the implementation of quality control systems, these improvements have increased the acceptance of the rRT-PCR assays for statutory diagnostic purposes. Although on-going studies continue this work, there is already a wealth of data focusing on different aspects of validation to support the use of rRT-PCR for routine FMD diagnosis:

ACCEPTED MANUSCRIPT

(i) evaluation of real-time assays with RNA prepared from different biological matrices:

Vesicular epithelium is the sample of choice to collect from affected animals since it is rich in virus during the acute phase of infection. In the absence of epithelium, blood (collected during the acute phase), milk, oesophageal-pharyngeal scrapings ('probangs') and mucosal swabs are suitable sample types for laboratory examination. In addition to extensive testing of vesicular epithelium samples, the performance of rRT-PCR has recently been assessed on milk (Reid et al., 2006) and "probang" samples (Sammin et al., 2006).

(ii) comparison of rRT-PCR to other established diagnostic approaches

The performance of automated rRT-PCR has been compared with VI and antigen-detection ELISA using samples submitted to the FAO/OIE Reference Laboratory (Pirbright, UK). The results (Shaw et al., 2004) showed that all VI-positive samples and those samples positive by VI and ELISA combined were also positive by rRT-PCR. Depending on the rRT-PCR cut-off value used, FMDV genome was detected in an additional 18% of samples tested. These findings indicate that the rRT-PCR has higher sensitivity than VI for the detection of FMDV in epithelial samples. However, in a subsequent study (Ferris et al., 2006), a number of VI-positive samples were missed by a rRT-PCR detecting the 5' UTR. These assay failures were attributed to nucleotide mismatches in the probe recognition region of a subset of related viruses from the 2001 epidemic in the UK. This is a consequence of the high variability and rapid mutation rate of the RNA genome of FMDV and highlights the importance of constantly monitoring representative field FMDV strains by nucleotide sequencing to ensure that the primers/probe set selected for the diagnostic rRT-PCR is fit for purpose.

(iii) comparison of performance between different rRT-PCR assays

An important aspect of these pan-serotype assays is that they target regions of the FMDV genome which are conserved across the seven FMDV serotypes. The nucleotide variability of six of these assays is shown in Figure 1. Nucleotide substitutions in primer or probe recognition sites were also thought to underline differences between the performance of rRT-PCR assays targeting the 5' UTR (Reid

ACCEPTED MANUSCRIPT

et al., 2002) and 3D region (Callahan et al., 2002). An initial study (Boyle et al., 2004) using a small selection of inactivated viruses has recently been expanded to include a more diverse selection of clinical samples from field cases of FMD (King et al., 2006). There was concordance between the results generated by the two assays for the majority of RNA samples extracted from epithelial tissue obtained from suspect FMD cases. However, the comparison between the two tests highlighted a small number of samples which failed to produce a signal in one assay while giving a positive signal in the other. The sequence of the genomic targets for selected isolates highlighted nucleotide substitutions in the primer and/or probe regions, thereby providing an explanation for these negative results. Individually each rRT-PCR had higher sensitivity than VI. However, the combined use of multiple and independent diagnostic targets could further enhance the sensitivity of molecular methods for the detection of FMDV.

(iv) inter-laboratory equivalence testing

Such is the diverse spectrum of approaches (Table 1) currently employed by different National Reference Laboratories, it is unlikely that harmonisation of RT-PCR protocols will be achieved in the short-term. To investigate inter-assay/laboratory equivalence, five European reference laboratories participated in an exercise to compare the sensitivity and specificity of their routinely-employed RT-PCR (and VI) tests for detection of FMDV (Ferris et al., 2006b). The best of the RT-PCR assays used in each laboratory gave comparable results (unlike the VI results which were highly variable). A larger inter-laboratory comparison of RT-PCR methods involving more than thirty laboratories is currently underway.

Future opportunities for RT-PCR diagnosis

During the 2001 epidemic, the UK government adopted a control strategy that required slaughtering of animals on infected premises within 24 hours. In this scenario, the time taken to transport material to a centralised laboratory was too long to allow laboratory confirmation of suspect cases based on clinical signs. In order to dramatically speed up the time taken for diagnosis, mobile or portable rRT-PCR machines could potentially be used close to the suspect cases in the field, or in regional laboratories. The desired characteristics of these assays are speed, sensitivity and simplicity of use. With this in mind, the portable SmartCycler (Cepheid; Hearps

ACCEPTED MANUSCRIPT

et al., 2002; Callahan et al., 2002; Moniwa et al., 2007) and BioSeeq™ (Smiths Detection; King et al., 2008) platforms have been evaluated for diagnosis of FMD. The sensitivity and specificity of these assays were comparable to previously published real-time assays. However, further assay development and validation work is required in order that these assays will be suitable for detection of FMDV in field conditions. In particular, the development of simple-to-use and robust nucleic acid purification strategies is a priority, since the presence of tissue-derived factors may inhibit the RT-PCR and current extraction protocols are too complicated for field use. Isothermal amplification strategies (such as RT-loop-mediated isothermal amplification (LAMP) and nucleic acid sequence based amplification (NASBA)) offer an alternative to rRT-PCR which may be particularly appropriate for field-based assays since they are not reliant upon sophisticated instrumentation and have been reported to be less sensitive to tissue inhibitors. An RT-LAMP assay which has equivalent analytical sensitivity to rRT-PCR has been recently developed for FMDV (Dukes et al., 2006). This assay may form the basis of a highly sensitive, extremely rapid, specific, and cost-effective device for field diagnosis of FMD.

3. Detection of Classical swine fever virus by rRT-PCR

Classical swine fever (CSF) is a highly contagious and often fatal viral disease of pigs and wild boar. The causative agent of CSF, *Classical Swine Fever Virus* (CSFV), is a member of the genus *Pestivirus* within the family *Flaviviridae* (Fauquet et al., 2005). Further members within the genus *Pestivirus* are *Bovine Viral Diarrhoea Virus* (BVDV) and *Border Disease Virus* (BDV). CSFV is an enveloped virus with a 12.5 kb single-stranded RNA genome of positive sense (Horzinek, 1991). The genome consists of a single large open reading frame (ORF) which is flanked by highly conserved 5' and 3' non-translated regions (NTR) (Wirz et al., 1993; Boye et al., 1991). The ORF encodes a 4,000-amino acid polyprotein that is co- and post-translationally processed by viral and cellular proteases into 12 polypeptides (Meyers et al., 1996). CSF causes major economic losses, especially in countries with an industrialized pig production system (Horst et al., 1999), and is therefore a disease notifiable to the Office International des Epizooties (OIE, 2006) and in the European Union (Anonymous, 1982).

ACCEPTED MANUSCRIPT

Diagnosis of CSF comprises observation of clinical signs in the field, pathological findings and laboratory detection of the virus or virus-specific antibodies. Clinical and pathological signs of CSF are rather variable and unspecific, and the severity of the clinical signs depends on many factors, such as age of the animals, virulence, presence of other pathogens or by host factors (Kaden et al., 2004, Floegel-Niesmann et al., 2003; Mittelholzer et al., 2000; Depner et al., 1997; Kamolsiriprichaiporn et al., 1992a, 1992b). Therefore, laboratory diagnostics of CSF plays a central role in the confirmation of an outbreak, and rapid and precise detection of CSFV is crucial for disease containment. Besides the established virological methods, PCR techniques have become increasingly important for CSF diagnostics during the last decade (Paton et al., 2000), and PCR has been accepted by the European Union as an official method for confirming this disease (Anonymous, 2002). Several rRT-PCR assays for CSF diagnostics have been described (LePotier et al., 2006; Risatti et al., 2003, 2005; Hoffmann et al., 2005; Uttenthal et al., 2003). The benefits of rRT-PCR are described elsewhere in this review (Gibson et al., 1996; Wittwer et al., 2001; Mackay et al., 2002; Mackay, 2004). Depner at al. (2006, 2007) and Schnyder et al. (2002) have highlighted the enhanced sensitivity and specificity of rRT-PCR for CSFV confirmation over the conventional diagnostic methods, such as VI or antigen-detection ELISA. Liu et al. (2007) compared conventional and rRT-PCR assays for the sensitive and specific detection of CSFV. The authors demonstrated identical sensitivities with these strategies but favoured the conventional, gel-based detection method for laboratories without facilities for rRT-PCR assays.

All published rRT-PCR assays for the detection of pestiviral sequences utilize the 5' NTR (see Table 2) as target, and all those reported for specific detection of CSFV use TaqMan® probes with a specificity of 100%. However, only four assays included an IC to monitor RNA isolation, reverse transcription and amplification efficacy (extraction and inhibition controls). Hoffmann et al. (2005) integrated an exogenous IC serving both as an extraction and PCR inhibition control. Two commercially-available assays amplify the cellular housekeeping gene GAPDH as an endogenous IC (Le Dimna et al., 2006, 2007; Le Potier et al., 2006) while the housekeeping gene β-actin was amplified in the assays described by Deng et al. (2005).

ACCEPTED MANUSCRIPT

Published quantitative rRT-PCRs have also been used to analyse the virus load in blood and tissue samples collected from CSFV-infected pigs (Koenig et al., 2007; Depner et al., 2006, 2007; Ophuis et al., 2006; Risatti et al., 2005, 2003; Uttenthal et al., 2003). Uttenthal et al. (2003) used a SYBR Green assay for quantification but the others favoured a TaqMan® format. Jamnikar Ciglenečki et al. (2008) compared a SYBR Green assay with a TaqMan® MGB rRT-PCR for the detection of CSFV genomes. While both assays were useful for CSFV detection, the TaqMan MGB version was thought to be more appropriate based on a reduced risk of false positives and the increased speed provided by the one-step format of the assay. Koenig et al. (2007) also used a TaqMan® assay to investigate the distribution of a conventional C-strain vaccine and a modified live marker vaccine candidate in pigs. The authors demonstrated the rRT-PCR to be more sensitive than VI even though the viral genomes of both vaccine strains were successfully detected in blood very rarely. Long-term detection of CSFV genomes in tonsils was, however, shown in the same study.

Ophuis et al. (2006) used specific primers and probes to detect the selected CSFV strains NSW, Baker and Weybridge, for detailed pathogenesis studies. In this study, CSFV was first detected in the tonsil (day 1), and subsequently in the submandibular lymph node, spleen, ileum and mesenteric lymph node (by day 3). Thereafter, virus spread to the visceral organs and finally to the pancreas and brain. These authors concluded that tonsil samples, nasal and rectal swabs and whole blood samples were the most favourable materials for rapid detection of CSFV using TaqMan®-based rRT-PCR assays.

For quick and cost-saving validation of techniques for molecular diagnostics, quantification of genomes using real-time PCR assays has some key advantages: Firstly, the sensitivity of different real-time PCR assays can be compared in one run using a small number of identical templates; secondly, a real-time PCR assay can quickly compare different genome extraction procedures. Deng et al. (2005) used conventional and rRT-PCR assays to validate six RNA extraction kits for effective detection of CSFV in blood and tissue samples, and concluded that all six methods were more or less useful for the detection of CSFV by both assays in these samples types. However, some of the methods offered certain advantages not common to all

ACCEPTED MANUSCRIPT

extraction procedures. For example, RNA extracted by the TRIzol LS reagent constantly had the highest yield, while the RNAqueous kit showed the highest A260/A280 ratio for almost all samples. Samples extracted using the NucleoSpin RNA II and the GenElute mammalian total RNA kits were demonstrated to be free of contamination with genomic DNA.

Modern real-time PCR cyclers can now measure up to 5 separate reporter fluorophores in one tube. This is a prerequisite for the application of multiplex real-time PCR assays. Beside the co-amplification of one target gene and an IC (Le Dimna et al., 2006, 2007; Le Potier et al., 2006, Hoffmann et al. 2005), different target genes can be detected in a single well (Wu et al., 2007; Legoff et al., 2008). Zho et al. (2008) as well as Leifer et al. (2009) developed a multiplex rRT-PCR assay for the simultaneous detection and differentiation of CSFV field strains and the C-strain vaccine virus.

The options provided by the current real-time PCR cylers, in combination with high quality commercial kits, should support the development of novel specific and highly sensitive multiplex rRT-PCR assays for the concurrent detection of several swine pathogens in the near future.

4. Detection of Bluetongue virus by rRT-PCR

Bluetongue disease (BT) is a non-contagious viral disease of domestic and wild ruminants that is transmitted by arthropod vectors of the genus *Culicoides* (Biting midges - reviewed by Mellor et al., 2000). Severe BT is primarily seen in sheep and in some species of deer, however significant levels of clinical disease were also seen in cattle during the recent bluetongue virus serotype 8 (BTV-8) outbreak in northern Europe. Bluetongue is endemic in many parts of the world including Africa and regions in the USA, Australia and Asia (Walton, 2004). Since 1998, multiple strains of BTV belonging to five different serotypes (1, 2, 4, 9 and 16) have been circulating in more than 12 countries within the Mediterranean basin (reviewed by Purse et al., 2005), and in August 2006 a strain of BTV-8 was reported in The Netherlands, then soon afterwards in Belgium, Germany, France and Luxembourg. By October 2008 BTV-8 had spread into many more countries in Europe including the UK,

ACCEPTED MANUSCRIPT

Switzerland, Spain, Czeck Republic, Hungary, Denmark and Sweden. Also since 2006 bluetongue virus serotype 1 (BTV-1) spread rapidly from North Africa through Spain and into the south of France. These recent outbreaks of BT in northern Europe have highlighted a need for rapid, high-throughput, sensitive and specific diagnostic assays to detect and identify BTV.

The BTV genome consists of 10 segments of linear double-stranded RNA (dsRNA) which encode 7 structural proteins (VP1-VP7) and 3 non-structural proteins (NS1, NS2 and NS3/NS3A) (Mertens et al., 1984; Roy, 1989, Roy et al., 1990). There are 24 serotypes of BTV, determined by the specificity of interactions between neutralising antibodies and the outer capsid of the virus, primarily with protein VP2 (encoded by genome segment 2- the most variable region of the virus genome). The remainder of the BTV genome plays no significant part in determining virus serotype (with the possible exception of genome segment 6 – encoding the other outer coat protein VP5). The smallest genome segment (Seg-10) codes for a small non-structural membrane protein NS3, and its truncated form NS3a, which also show a relatively high degree of variability, although this is independent of virus serotype (Nikolakaki et al., 2005; Balasuriya et al 2008). Genome segment 7 encodes the BTV core surface protein VP7, which is also the major (immunodominant) bluetongue-serogroup specific antigen. Perhaps surprisingly, Seg-7 also shows a relatively high degree of variability across the bluetongue virus species as a whole (Bonneau et al 2000). In a recent study of European BTV serotyopes it was detected as the third most variable region of the virus genome (after Seg-2 and Seg-6) (Maan et al 2008). The majority of the other BTV genome segments (which code for the virus core proteins or non-structural proteins NS1 and NS2) are more highly conserved between BTV serotypes and strains. However, significant variations are still detected in most of the BTV genome segments (including segments 2 and 6), which reflect the geographical origins of the virus, allowing isolates to be divided into different groups (e.g. ‘east’ and ‘west’) or topotypes (Gould and Pritchard, 1990 Maan et al., 2007, 2008; Mertens et al., 2007; Shaw et al., 2007; Anthony et al., 2007).

When designing primers for molecular assays it is important to select areas of the BTV genome that are sufficiently conserved to enable the detection of all 24 serotypes

ACCEPTED MANUSCRIPT

and related topotypes, but also sufficiently divergent from sequences of the members of other closely related *Orbivirus* species (other 'serogroups') to avoid cross-reactions. The degree of variation detected within individual BTV genome segments (e.g. between geographic groups) complicates the selection of appropriate targets. However, several of the more conserved BTV genome segments have previously been used as targets for molecular diagnostic methods, including: genome segment 5, encoding the highly conserved non-structural 'tubule' protein NS1 (currently recommended as an RT-PCR target by the OIE (OIE, 2008); genome segment 7 (despite its variability), encoding the BTV core surface protein and major BTV virus-species / serogroup-specific antigen, VP7 (Gumm and Newman, 1982; Huismans, 1981; Mertens et al., 1987); genome segment 10 (encoding NS3/3a), in which nucleotide variation between gene sequences of up to 20% have been detected (Pierce et al., 1998; van Niekerk et al., 2003) and genome segment 1 encoding VP1, the viral RNA-dependent RNA polymerase, one of the most highly conserved proteins of the reoviruses (members of the family *Reoviridae* - Huang et al., 1995, Mertens, 2004). Many of the conventional RT-PCR assays that have been developed are able to detect BTV RNA in clinical samples (blood and spleen), including those targeting genome segments 2, 3, 6, 7 and 10 (reviewed by Zientara et al., 2004), however, these methods require agar gel electrophoresis to show the amplification of the target sequence, which severely limits the speed of testing.

Prior to the BTV-8 outbreak in northern Europe in the summer of 2006, few real-time PCR assays had been published for the detection of BTV. The first rRT-PCR assay published for the detection of BTV, used primers designed from the NS1 gene (Seg-5) (Wilson et al., 2004). However this assay detected only 11 out of the 19 serotypes tested (serotypes 20-24 were not tested). The same year another rRT-PCR was published using FRET-probe technology targeting genome segment 2 (VP2) (Orrù et al., 2004, De Santis et al., 2004). This assay was used in Italy to differentiate wild-type BTV-2 from the vaccine strain and was able to distinguish between vaccinated and infected animals. In 2006 an rRT-PCR assay was developed using a conserved region in RNA segment 5 of BTV-2 and BTV-4 (Jimenez-Clavero., 2006). This assay detected all of the recent Mediterranean isolates that were tested (including isolates of serotypes 2, 4 and 16), BTV vaccine strains for serotypes 2 and 4 and also 15 out of the 24 BTV reference strains. The primers and probe were positioned in a part or

ACCEPTED MANUSCRIPT

segment 5 that had various mismatches which reduced the sensitivity of the assay. Moreover, the assay was only able to detect field isolates, but not the reference strain, of BTV-4 and BTV-16 and in the recent northern European outbreak of BTV-8, this assay showed a reduced sensitivity for the detection of the field strain of BTV-8 compared to other assays (Batten et al., 2008a). Subsequent studies have indicated that the field strain of BTV-16 from Italy 2002 that was tested was a reassortant virus, containing genome segment 5 derived from a BTV-2 vaccine strain (Batten et al 2008b), potentially explaining differences in the sensitivity of the assay for field and reference strains of this serotype. In the same year, a quantitative rRT-PCR was developed using a Molecular Beacon (MB) fluorescent probe designed within the NS3 conserved region of segment 10 (Orrù et al., 2006). The MB is a circular oligonucleotide characterised by a high specificity for the chosen target and by its ability to develop a colour-forming reaction when used in a conventional RT-PCR test. This assay successfully detected 10 serotypes (BTV-1, 2, 3, 4, 5, 6, 7, 8, 9, 16) and detected 10^3 molecules/PCR reaction.

Since the start of the northern European outbreak in August 2006 many rRT-PCR assays have been developed, which are currently being used in many countries across Europe. All the rRT-PCR assays that have been published up to 2008 are described in Table 3. Some assays remain unpublished and, at the time of writing, 3 rRT-PCR assays are published that are able to detect representative strains from all 24 serotypes of BTV. Two of these assays were published in the same manuscript by Toussaint et al., (2007). These two assays detect strains of the different BTV serotypes from the Mediterranean region, as well as prototype strains of all 24 serotypes. Both assays have similar detection limits of below 0.01 ECE_{50} and detect 100 RNA copies. The primers were designed to target different genomic segments; a one-step procedure, combining the reverse transcription (RT) and the PCR steps, to amplify BTV genome segment 1 and a two-step procedure, with separate RT and PCR reactions, to amplify genome segment 5. These assays have the advantage of a quantitative output which gives an estimate of viral load and both assays include an internal beta-actin control (Toussaint et al., 2007).

A further duplex rRT-PCR assay has been recently published by Shaw et al., (2007) in which two sets of primers and probes were designed to target segment 1 of eastern

ACCEPTED MANUSCRIPT

and western group viruses respectively. This combined 'duplex' assay was evaluated using a wide variety of test samples, including tissue culture derived viruses, infected tissue and blood samples from cattle and sheep and infected *Culicoides* midges. This assay showed no cross-reactions with closely related orbiviruses and gave positive Ct results and amplification plots with all of the viruses tested, including a wide range of 129 different BTV isolates derived from different geographical locations (different topotypes), reference strains of all 24 BTV serotypes, and multiple field strains of BTV serotypes 1, 2, 4, 8, 9 and 16 from European outbreaks that have occurred since 1998, (although the BTV-9 vaccine and a Sicilian isolate of BTV-9 gave weaker but still positive responses). This assay was able to detect less than 10 template copies per reaction (equivalent to 0.5 $TCID_{50}$/ml in BHK21 cells). Differences in the sequences of Seg-1 also made it possible to use the two primer sets from the duplex assay, separately, to distinguish viruses of eastern and western origins.

A one step rRT-PCR method has also been described (Polci et al., 2007) that detects strains of BTV serotypes 2, 4, 9 and 16 isolated in Italy as well as their respective vaccine strains. The limit of detection of the assay fluctuated between 0.005 and 0.05 $TCID_{50}$/ml depending on the virus strain. Preliminary tests also showed that this assay was able to identify serotype 1, which was introduced into Italy towards the end of 2006, and also serotypes 8, 13, 14 and 19.

Given the high variability of BTV and the fact that real-time probes are very sensitive to probe-target mismatches (Jiminez-Clavero et al., 2006), it is important to emphasise that these assays may not be able to recognise all BTV strains and that the sensitivity of the assays may not be the same for every strain. It would thus be wise, as emphasised by Toussaint et al., (2007), to run two methods that amplify different genomic regions in parallel, as this would avoid the risk of missing a mutant, a recombinant or a reassortant strain. Results of PCR-based BTV diagnosis need to be interpreted with caution as BTV RNA has been detected in blood from both infected cattle and sheep for at least 30 days and sometimes up to 90 days after virus isolation has ceased to be positive (Bonneau et al., 2002, Katz et al., 1993, MacLachlin et al., 1994). Thus detection of virus-specific nucleic acid by these methods indicates a recent virus infection, but does not necessarily indicate the presence of infectious virus in the animal.

Accepted Manuscript

ACCEPTED MANUSCRIPT

5. Detection of avian influenza virus by rRT-PCR

Avian influenza viruses (AIV) belong to the genus *Influenzavirus A*, family *Orthomyxoviridae*, possessing a negative stranded RNA genome of eight segments. AIVs are classified into subtypes according to antigenic variation of the glycoproteins, haemagglutinin (H) and neuraminidase (N), there are sixteen H types and nine N types currently known. Wild birds, in which overt disease due to AI has been rarely recorded, are the natural hosts for AIV and their migration is believed to be important in its spread (Brown et al, 2006). Most AIV subtypes are termed low pathogenicity avian influenzas (LPAIs) because little morbidity and mortality is normally observed in infected chickens. LPAI virus infections are typically localised to the avian respiratory and/or intestinal tract. However, H5 and H7 LPAI infections in poultry may result in mutations in the viral haemagglutinin genes that bring about changes at the HA0 "cleavage site" (CS, Figure 2) causing phenotypic change to highly pathogenic avian influenza (HPAI) virus which spreads systemically. HPAI is highly infectious to poultry and is characterised by rapid and aggressive spread of the disease within a flock with very high mortality, typically within 48 hrs (Swayne & Suarez, 2000; OIE, 2005a). Both LPAI and HPAI poultry outbreaks caused by H5 and H7 AIVs are notifiable diseases (CEC, 2006; OIE, 2005b), and the biology of such outbreaks and AIV wild bird isolates are under continuous scrutiny (Alexander, 2007; Senne, 2007).

HPAI is a constant threat to poultry in both developed and developing world countries, especially in recent years as H5N1 HPAI virus originating in SE Asia (Webster et al, 2006) spread westwards into Europe in late 2005 (FAO, 2006), and into Africa in 2006 (Ducatez et al 2006). The risk of zoonotic AI infections in humans also remains a concern for public health authorities (Alexander et al., 2005; WHO Global Influenza Program Surveillance Network 2005; Chen et al 2006). In terms of AI diagnosis, VI in embryonated fowls' eggs (EFEs) is the gold standard method used followed by serological typing to identify the H and N subtypes by haemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests. Although VI followed by HI and NI tests can take up to several days, these methods remain obligatory to identify the index case of any new AI outbreak (OIE, 2005a; EU 2006).

ACCEPTED MANUSCRIPT

Clinical specimens appropriate for AI isolation include cloacal and oropharyngeal swabs, *ie* from enteric and oral-respiratory shedding sites. Tissue specimens may also be a source of AI, particularly from systemic HPAI infections, but for LPAI the virus tends to be restricted to the respiratory and / or enteric tract.

It has been established that rRT-PCR provides rapidity, sensitivity and specificity for diagnosis directly from clinical specimens. These are ideal qualities for AI outbreak management, where prompt and unequivocal diagnosis may be crucial to decision making by the relevant veterinary authority. Many real-time PCR platforms operate in a 96-well format, and can be combined with high-throughput robotic RNA extraction from specimens (Agüero et al., 2007). For AIV, this review will focus primarily on TaqMan® (hydrolysis probe) approaches to rRT-PCR, and will emphasise the importance of AI rRT-PCR validation. This is coupled to the appropriate application of AI rRT-PCRs for poultry outbreaks and wild bird surveys (WBSs). It is intended to provide the reader with guidelines for assessing any future publications on AI rRT-PCR methodology. In this respect it is important to distinguish genuine and practically relevant developments in AI diagnosis from methods that may be scientifically interesting, but represent little or no practical progress in AI diagnosis.

Conventional AI RT-PCRs

Laboratories have used conventional RT-PCR for AI characterisation since the 1990s, but this involves initial biological amplification of AI specimens in EFEs in which high AI titres (*eg* 1 x 10^6 EID_{50}/ml or greater) are obtained in the chorioallantoic fluid (CAF) and used for RNA extraction. Sequencing of amplicons that span the region coding for the HA0 CS region (Figure 2) may determine pathogenicity for H5 or H7 AIVs (OIE, 2005a; EU 2006; Senne et al 2006). This has served as a very useful adjunct to *in vivo* pathogenicity testing in poultry (Senne et al, 2006). Conventional RT-PCR continues to be used to generate AIV sequence data, including haemagglutinin gene sequences of H5/H7 isolates from outbreaks (Bao et al, 2008). This provides phylogenetic data to track the molecular epidemiology of AI outbreaks, particularly if the variable HA1 region of the haemagglutinin gene (Figure 2) is analysed (Banks et al, 2001). American H5 isolates are clearly phylogenetically

ACCEPTED MANUSCRIPT

distinct from Eurasian H5 isolates, and the same applies to American and Eurasian H7 isolates (Rohm et al, 1995).

However, there has been little regular use of conventional AI PCR in a diagnostic setting where RNA has been directly extracted from clinical specimens. Swabs are known to yield far lower AI titres than those in CAF harvests, particularly in the case of LPAI infections (Wood et al, 1995). Ring trials conducted recently in the EU FP5 AVIFLU project identified H5 and H7 conventional RT-PCRs that can directly amplify from extracted poultry specimens, at least from swabs containing high virus titres obtained from HPAI infections (Figure 2, Slomka et al 2007a).

M gene, H5 & H7 testing by rRT-PCR

M gene (generic) rRT-PCR:

The matrix (M) gene is highly conserved for all sixteen H subtypes from all geographical regions, and is an ideal target for global generic AIV detection (Figure 2). M gene rRT-PCR has been described and validated in testing clinical specimens obtained from live bird markets (LBMs) in the USA and from experimental infection studies (Spackman et al 2002; Lee & Suarez 2004).

H5 and H7 specific rRT-PCRs:

The HA2 region has served as the target region for H5 and H7 TaqMan® rRT-PCRs as it is relatively conserved within these respective haemagglutinin genes (Figure 2; Spackman et al, 2002). These rRT-PCRs have also been shown to function efficiently as H5 and H7 quantitative (Q)-rRT-PCRs in studies of virus shedding (Lee & Suarez 2004). Spackman et al (2002) cautioned that their H5 and H7 primer / probe sequences had been designed for the detection of North American H5 and H7 isolates and were unlikely to be applicable to Eurasian viruses. The extent of sequence divergence between viruses from the different hemispheres is so great that, even within the HA2 region, it appears difficult to design a consistently sensitive rRT-PCR for global detection of H5 or H7 AIVs. The primer and probe sequences of Spackman et al (2002) were modified for detection of "Asian lineage" H5N1 AIV and also other Eurasian H5 AIVs that have been isolated within the past decade (Figure 2; Slomka et al, 2007b). This Eurasian H5 rRT-PCR has demonstrated its value in the investigation

ACCEPTED MANUSCRIPT

of many H5N1 HPAI clinical specimens submitted from Europe, Africa and Asia since autumn 2005 (Slomka et al 2007b). Monne et al (2008) have also described H5 and H7 rRT-PCRs that amplify within the HA2 region of Eurasian H5 and H7 AIVs (Figure 2).

Eurasian H7 AIVs can also be detected by two validated H7 rRT-PCRs that target the HA2 and the CS region (Slomka & Voermans, 2007). Sequencing of the latter amplicon provides molecular pathotyping data (Figure 2). Hoffmann et al. (2007) described a H5 rRT-PCR in which the probe is included for the specific detection of H5N1 HPAI. In this rRT-PCR the probe was carefully designed to bind to the characteristic CS sequence of this particular H5 virus (Figure 2).

Validation of AI rRT-PCRs

Guidelines for validation of new molecular methods, in particular rRT-PCRs, have been outlined by the OIE (OIE, 2004). Rigorous validation of AI rRT-PCRs requires the following aspects to be addressed:

Sensitivity: the new method must successfully detect a range of different AI viruses. Generic AI PCRs should detect representative isolates from among all sixteen H subtypes. H5 and H7 rRT-PCRs should similarly detect a range of isolates among the corresponding H subtype. While historic AI isolates may be available in many laboratories, it is important to include contemporary AIVs from various geographic locations. As mentioned above, it is also important to take into account genetic diversity between Eastern and Western Hemisphere H5 and H7 subtype AIVs (Rohm et al 1995). Furthermore, ongoing evaluation of 'fitness for purpose' has been done using a large number of contemporary viruses and this will continue, especially important given the highly mutable nature of the influenza virus genome.

Specificity: it must be shown that the new AI rRT-PCR does not produce false positive signals, e.g. testing of all non-H5 / H7 AIVs with H5 / H7 rRT-PCRs must demonstrate clear specificity. Specificity testing should also include major non-AI avian pathogens (especially Newcastle disease virus (NDV)) and a panel of clinical specimens shown to be AI negative by VI.

ACCEPTED MANUSCRIPT

Testing of clinical specimens:

It is important to demonstrate that the new method is applicable to clinical materials. Specimens should be tested by both the new AI rRT-PCR and VI in EFEs (ie the 'gold standard'). This will determine whether or not the new AI rRT-PCR is more sensitive than VI. Clinical specimens may be obtained from AI positive birds infected experimentally, during outbreaks or from wild birds.

The following studies describe AI rRT-PCRs which have included the above sample populations for validation: Spackman et al (2002) included extensive M gene rRT-PCR testing on clinical specimens collected from LBMs in 2001. Comparative testing alongside VI revealed some discrepant results, which were discussed in detail (Spackman et al, 2002). Samples from poultry infected experimentally served to further validate the American H5/H7 type-specific assays (Lee & Suarez, 2004). All three AI rRT-PCRs were shown to be generally more sensitive than VI (Spackman et al, 2002; Lee & Suarez, 2004). Validation of the Eurasian H5 rRT-PCR included a wide range of Eurasian H5 AIVs (both LP and HP) and non-H5 AIVs (Slomka et al., 2007b). Clinical specimens were obtained from a number of suspect H5N1 HPAI submissions from Europe, Africa and Asia from autumn 2005 to spring 2006. It was concluded that the Eurasian H5 rRT-PCR is more sensitive than VI (Slomka et al. 2007b). Monne et al (2008) used a broad range of known AI isolates, plus large numbers of clinical specimens from poultry and wild birds to validate their sensitive and specific H5 and H7 rRT-PCRs in comparison with VI.

Few veterinary institutions possess substantial collections of AIVs or have access to clinical specimens that are key materials for such thorough validations. Lack of robust validation data will enevitably provide areas of uncertainty and result in such AI rRT-PCRs being classified as proposed or proof of principle methods.

Use of AI rRT-PCRs in poultry outbreaks

Validated AI rRT-PCRs can bring great advantages to outbreak management and to decision making by the relevant veterinary authorities. AI rRT-PCR was used prospectively during the H7N2 LPAI poultry outbreak in Virginia, USA in 2002, in which 3,628 tracheal swabs were obtained (Akey, 2003). H7N2 LPAI was also detected by rRT-PCR in LBMs in 2002 and in commercial layers in 2003 (Swayne &

ACCEPTED MANUSCRIPT

Akey, 2005). AI rRT-PCR demonstrated its value in these LPAI outbreaks where clinical signs are not as dramatically clear as those observed in HPAI poultry outbreaks.

During the HPAI poultry outbreak in British Columbia, Canada, in 2004, caused by a virus of H7N3 subtype, swabs were tested initially by VI and M gene rRT-PCR, and positives were tested by the American H7 rRT-PCR. M gene rRT-PCR was also used for screening in the surveillance region that was established during the outbreak (Lees et al, 2004; Pasick et al, 2007). Identification of likely progenitor H7N3 LPAI isolates in poultry indicated the importance of accurate, sensitive and rapid laboratory diagnosis.

Westward spread of H5N1 HPAI during the summer of 2005 (Chen et al 2005; Liu et al 2005) accelerated EU emergency preparedness to tackle any subsequent European outbreaks. This included recommendation of the M gene rRT-PCR (Spackman et al 2002) that had fared very effectively and reproducibly in blind testing of AI panels organised within six EU laboratories (Slomka et al 2007a). Ongoing validation of the Eurasian H5 rRT-PCR (Slomka et al 2007b) resulted in the inclusion of this method among the EU recommended AI protocols (EU 2006). This contingency planning was timely, with the first incursions of H5N1 HPAI in wild birds and/or poultry occurring during autumn 2005 in Turkey, Romania, Croatia and Romania (FAO 2006; Alexander 2007). These and subsequent European H5N1 reports in 2006 included a high proportion of wild waterfowl cases, notably swans, and the Eurasian H5 rRT-PCR demonstrated its value in the direct testing of clinical specimens (Slomka et al 2007b). European H5N1 HPAI outbreaks continue to be diagnosed by this method, e.g. the three turkey outbreaks in the UK and Poland in 2007 (EU 2007).

The EU recommended H7 HA2 rRT-PCR (Slomka & Voermans, 2007) has also served in the diagnosis of LPAI poultry outbreaks caused by H7N3 (UK 2006; Manvell et al., 2008), H7N2 (UK 2007; EU, 2007) and H7N1 (Denmark 2008; EU, 2008). An outbreak of H7N7 HPAI in chickens in the UK was diagnosed and investigated in June 2008, where the H7 HA2 rRT-PCR again demonstrated its value (Defra, 2008).

ACCEPTED MANUSCRIPT

Wild bird surveys (WBSs) and AI rRT-PCR

Recent years have seen renewed interest in AI WBSs, particularly to identify H5 and H7 AIVs (Munster et al 2005). M gene rRT-PCR has been used as the primary screening tool, with the inoculation of positive samples into EFEs for VI (Munster et al, 2005 & 2006; Terregino et al, 2007). It has been observed consistently that VI is not 100% successful from all M gene rRT-PCR positive swabs (Terregino et al, 2007; Wallensten et al, 2007), inferring that a proportion of specimens contain detectable AI viral RNA that may not be infectious and recoverable in EFEs. This appears to be supported by high M gene rRT-PCR Ct values observed for these specimens, relating to very low AI titres (Slomka et al, unpublished). It is also possible that such WBS swabs may have their viability compromised during storage or transport. Such interpretation difficulties occur when M gene rRT-PCR results cannot be confirmed by VI nor conventional PCR, although in low titre specimens H5 and H7 subtypes may be confirmed by H5/H7 rRT-PCRs.

Controls in AI rRT-PCR

Van Borm et al. (2007) described the parallel use of M gene and β-actin rRT-PCRs, in which the latter had been extensively validated for successful detection of avian β-actin RNA present in swabs and tissue specimens from forty-seven different bird species. Use of this endogenous internal control allows detection of false negatives in clinical specimens, or may resolve interpretation problems associated with high Ct value M gene rRT-PCR positives described above for WBSs by revealing sub-optimal levels of sampled specimen.

For use with the M gene rRT-PCR (Spackman et al 2002), Das et al. (2007) described an internal positive control (IPC) RNA, transcribed in vitro, which was carefully titrated and included without compromising the sensitivity of the M gene rRT-PCR. The IPC possessed the same primer binding sequences as the M gene rRT-PCR, but a different probe binding sequence. This exogenous IPC detected PCR inhibitors in RNA extracted from blood, organs and cloacal swabs, but not in CAF, serum or tracheal swabs (Das et al 2007). Di Trani et al (2006) used a commercially available IPC for inclusion with a different M gene rRT-PCR (Figure 2).

AI rRT-PCR Proficiency Testing

ACCEPTED MANUSCRIPT

Proficiency testing assesses competence for the practise of molecular diagnostic methods (OIE, 2002). In the EU, six AI National Reference Laboratories (NRLs) were originally engaged in the AVIFLU project, where a programme of blind ring trials was organised to identify sensitive and specific methods, which included M gene (generic AI) rRT-PCR and H5/H7 conventional PCRs for molecular pathotyping by amplicon sequencing (Slomka et al 2007a). As a result, EU AI PCR protocols for inclusion in the Diagnostic Manual (EU 2006) were recommended and this trial served as a template for the organisation of a larger annual pan-European AI PCR proficiency testing panel for EU NRLs and other laboratories. This has been organised since 2006 (Slomka 2006, 2007 & 2008) with the aim of harmonising diagnostic AI PCR approaches among veterinary laboratories across the EU, and included AI rRT-PCRs for M gene and H5 and H7 subtype detection. These are now in use in twenty seven NRLs in EU member states (Slomka 2008). Similar proficiency programmes have also been introduced in the US (Suarez et al 2007).

In conclusion, this review has summarised the current published literature concerning AIV rRT-PCR which are based on TaqMan® (hydrolysis probe) technology. The importance of validation has been emphasised. The problem of evolving sequence variability has been noted elsewhere in this review, and for AI high sequence variability is observed in the haemagglutinin gene. Changes in primer or probe binding sequences may affect the diagnostic sensitivity of a currently validated AI rRT-PCR. Vigilance for future sequence changes in the Asian lineage H5N1 HPAI (Smith et al., 2006) and other H5/H7 genes is crucial, as modifications to protocols may be necessary to restore optimal assay performance.

While validated AI rRT-PCRs have recently attained a very high standard of diagnostic performance in terms of sensitivity and specificity, other molecular methods will emerge within the coming years which may challenge the present standing of AI rRT-PCRs. Regardless of the nature of any new molecular diagnostic techniques, an appropriate and robust validation strategy will remain key for their acceptance.

Accepted Manuscript

ACCEPTED MANUSCRIPT

6. Detection of Newcastle disease virus by rRT-PCR

Outbreaks of Newcastle disease (ND), caused by virulent Newcastle disease virus (NDV), result in serious losses in poultry with an enormous economic impact. NDV is an avian paramyxovirus with a single stranded negative sense RNA genome of approximately 15.2 kilobases. NDV can differ in pathotype, defined as lentogenic, mesogenic, and velogenic related to low, moderate, and high virulence, respectively. The group of NDV represents one serotype (APMV-1) out of nine serotypes within avian paramyxoviruses (APMV´s). The NDV-group consists of at least six genetic groups or genogroups. Recent analysis has revealed two distinct classes. (Czegledi et al., 2006). Lentogenic strains are routinely used as vaccines. The diagnostic challenge for ND is on the one hand the development of diagnostic tools differentiating between vaccine strains and virulent NDVs, and on the other hand detecting all genetic groups of NDV.

Suspicions of Newcastle disease cannot be conclusively diagnosed by clinical signs. Many diseases, i.e. Fowl cholera, Avian influenza (HPAI), Laryngotracheitis, Fowl pox (diphtheritic form), Ornithosis (psittacosis or chlamydiosis) (psittacine birds and pigeons), Infectious bronchitis, Pacheco's parrot disease (psittacine birds), Gumboro disease (very virulent strains), Salmonellosis (pigeons) show similar clinical signs or one or more clinical signs that are also observed with ND. In addition, non-optimal housing, i.e. withdrawal of water, air, or feed, or acute poisoning could result in misdiagnosis of ND.

Conventional techniques, i.e. immunohistology (Hilbink et al., 1982), immunoperoxidase-based methods (Lockaby et al., 1993), haemagglutination test, inhibition test of haemagglutination using monospecific sera, and an *in vitro* test detecting plaque formation in the absence of trypsin, are available. In addition, *in vivo* tests are available to determine pathogenicity of NDV; intracerebral pathogenicity index (ICPI) test, and mean death time in eggs (MDT). All these methods have one or more disadvantages; they are laborious, expensive, use unacceptable numbers of animals in experiments, and above all, are time-consuming delaying adequate control measures after NDV-introduction.

ACCEPTED MANUSCRIPT

Plaque formation, as associated with virulence of NDV, appeared to be dependent on additional basic amino acids at the processing site of the fusion protein. The detection of codons for these basic amino acids is now accepted in place of *in vivo* and *in vitro* tests for identification of virulent NDV (OIE, 2008). This, together with the fast development of molecular diagnostic techniques, has stimulated many researchers to develop new and fast methods to detect NDV and to identify virulent NDV in various specimens.

In addition to RT-PCR methods, other molecular approaches, such as RT-LAMP, have been recently developed for detection of NDV (Pham et al. 2005a). The big advantage of all these molecular techniques is that isolation of infectious NDV, and subsequent egg passage and/or cell line passages are not needed for diagnostic purposes. Therefore, detection of genetic material specific for NDV directly from a wide range of specimens, i.e. blood, faeces, tissues, and from different avian species speeds up the diagnostic process significantly. Another advantage of molecular techniques is the possibility of differential diagnosis by multiplex-RT-PCRs between pathogens causing similar clinical signs, i.e. Avian influenza and ND (Farkas et al., 2007). RT-PCR can be achieved by universal primers that amplify targeted regions of NDV, and thus detect its presence (Barbezange & Jestin, 2002; Kant et al., 1997; Gohm et al., 1999). However, even highly conserved genes like the M-gene shows variability resulting in failures to detect NDV-isolates. A complementary assay based on the L-gene has been developed to cover all NDVs, irrespective of virulence (Kim et al., 2007). In contrast, many approaches have narrowed the specificity to virulent NDV, and thus distinguish between virulent and vaccine strains. However, these assays generally only detect a subset of all virulent NDVs, lacking the necessary performance criteria for adoption into routine use.

Depending on the particular molecular method used, it is possible to detect, serotype, and define the virulence of NDV in parallel. These integrated assay characteristics speed up the diagnosis and characterisation of virulent NDV strains significantly. Detection of NDV by amplification of the region encoding the cleavage site of the fusion protein opens the possibility to identify the virulence by studying the presence of additional basic amino acid codons. Using open amplification systems, this has been achieved by several methods, i.e. subsequent sequencing of the amplicon (Gohm

ACCEPTED MANUSCRIPT

et al. 1999), a specific nested PCR (Barbezange and Jestin, 2002; Kant et al., 1997; Kou et al., 1999; Kho et al., 2000), agarose-gel electrophoresis, or restriction enzyme analysis (Creelan and McCullough, 2006). A major risk of these approaches is contamination of samples by amplicon from previously amplified material during post-PCR manipulations.

NDV-specific rRT-PCR have utilised intercalating dyes such as SYBR-green (Tan et al. 2004). A drawback of this method is that amplification must be very specific as non-specific amplification contributes to the fluorescent signal. However, specificity of the amplicon can be checked afterwards by melt curve analysis and this approach has even been used for pathotyping (Pham et al. 2005b). Use of hybridization probes improved assay reliability: not only by offering a higher specificity, but also by raising the analytical sensitivity of detection of NDV-RNA, as approximately 10 EID_{50} can be routinely detected by this method (Tan et al. 2004, Wise et al. 2004).

All these combinations of detection of virulent NDV by rRT-PCR have one major disadvantage; the targeted gene (F-gene) encoding the fusion protein is considerably variable. Since the primers used in the assay must be reliably amplify all virulent NDVs, it is diffcult (or perhaps impossible) to develop a single rRT-PCR to detect all virulent NDVs.. Alternatively, several RT-PCRs based on the F-gene can be performed in parallel or in the same tube to achieve amplification of the cleavage site of all NDVs. However, since this region is less conserved there will be a higher chance of failure for the amplification of new virus isolates. These concerns are also evident when considering the use of probes to detect products from these rRT-PCR reactions. The ability of a probe to hybridise to a specific product is very sensitive to mutations (Kim et al. 2006), however, this can be overcome by the use of many probes in one reaction (Aldous et al., 2001). Preferably a second RT-PCR based on a more conserved target, that detects all NDVs can be used to exclude false negative results by an F-gene based RT-PCR. However, such assays may still not cover all NDVs and so a second assay, based on L-gene may offer advantages since it will be applicable to most if not all strains (Kim et al., 2007). However, such assays targeting relatively conserved regions cannot be applied in vaccinated populations to diagnose ND, without recourse to tests leading to virulence determination. Published rRT-PCR assays used for NDV genome detection are listed in Table 4.

ACCEPTED MANUSCRIPT

Reliability of laboratory diagnosis for ND is very important, since misdiagnosis has enormous consequences. In case of false negative results, a new outbreak remains undetected, and control measures are unnecessarily delayed. Therefore, frequent testing of rRT-PCR assays with new isolates to confirm sensitivity must be part of the sustainability of the laboratory diagnosis for ND. Concerning false positive results for ND, the consequences could be even worse. In particular, the impact of a false positive index case in a ND-free country would be enormous. In addition to these diagnostic tests, it is important to sequence all (or part of) the F-gene of the isolate causing the outbreak for analysis of the cleavage site and epidemiological studies. It is always strongly recommended that virus is isolated from index cases to facilitate further analyses.

Nevertheless, after introduction of ND, high-throughput diagnostics is needed to control the outbreak. Focusing on molecular diagnostics for ND, in particular high throughput rRT-PCR diagnostics, only one report has been published using a validated 96-well format isolation- and rRT-PCR procedure (Crossley et al., 2005). Compared to VI, this assay had a diagnostic sensitivity of 100%, and a diagnostic specificity of 95% with excellent reproducibility. Many rRT-PCR assays have been published but are validated to a less rigorous extent. Most of these methods use manually extracted RNA or other isolation methods that are not amenable to automation. The particular benefits and disadvantages of these procedures must be taken into account and varies from use for vaccinated poultry to wild birds and from allantoic fluids to faeces. Application as a diagnostic tool in vaccinated poultry is limited to the assays based on a target on the F-gene, including the region encoding the cleavage site in order to be able to distinguish between vaccine and virulent strains (Kim et al., 2006). Application as a diagnostic tool for samples from sources such as different non-vaccinated avian species, including wild birds, is focused on more conserved regions such as the M-gene, although a second assay may be required to cover all NDVs (Kim et al., 2007).

In summary, molecular diagnostics for ND can essentially be divided into two categories depending on the purpose Detection of ND in avian species, and determination of virulent NDV. For detection of NDV, even if highly conserved targets like the M- and L-gene are chosen, it is very difficult to develop an rRT-PCR

ACCEPTED MANUSCRIPT

assay capable of detecting all NDVs (six genogroups to date). For characterisation and recognition of virulent NDV, part of the F-gene, including the region encoding the cleavage site, is the target of interest. However, it is extremely difficult to develop an assay to detect all virulent NDVs especially considering geographical variation. The risk of false negatives due to variation in primer or probe locations is still a challenge to assay-developers. Nevertheless, it should be recommended that confirmatory assays be developed and utilised to avoid unnecessary delay of implementing control measures in case of a new outbreak. Only one extensive validation of a high-through PCR-based assay for ND has been published. In spite of recent progress, there is still a greater need for more reliable and improved validated rRT-PCR assays for the detection of NDV in general, as well as for identification of virulent strains of NDV.

7. Discussion and general conclusions

This review outlines the remarkable progress made over the past decade by real-time PCR techniques to improve pathogen detection for diagnostics and disease control. Beside the high sensitivity and ability to quantify viral targets, the substantial gain in specificity and the reduced risk of cross-contamination are important features of this technology. Integration of these assays onto automated high-throughput platforms provides diagnostic laboratories with the capability to test large numbers of samples that might be received during outbreaks of livestock disease. Although this review emphasises the positive features of rRT-PCR assays, it is important to recognise that there are a number of factors that can negatively impact upon the performance of these tests. In particular, the design of assays capable of detecting RNA viruses such as FMDV, CSFV, BTV, AIV and NDV with highly variably genomes can pose specific challenges. In addition to identifying conserved regions in the genome for primer-binding sites, the requirement to find further regions for the location of probe sites can reduce the diagnostic sensitivity of real-time PCR assay. In order to avoid this phenomenon, more than one real-time RT-PCR targeting different regions of the genome can be employed in parallel. As a consequence, the likelihood that mutations will cause failure of detection in both independent assays is very low (King et al., 2006). This “double check strategy” can be particularly important in the context of first line or exclusive diagnostics of pathogens using rRT-PCR. rRT-PCR assays can generate objective results regarding the presence of a particular pathogen in a sample.

ACCEPTED MANUSCRIPT

However, border-line (or doubtful) results can arise when high Ct values below the cut-off of the test are generated. Unfortunately, non-PCR antigen detection methods are often not sensitive enough for a reliable independent confirmation of these signals. Therefore, a robust approach for dealing with these results should be adopted and standardised for each individual assay. It is recommended that an inconclusive interval (e.g. based on Ct-values or genome copies) is defined. Specimens with a doubtful result should be further analysed, e.g. using a second real-time PCR, a new extraction or a new sample from the same animal. In addition, for livestock investigations these effects can be mitigated or negated by the use of a robust statistically valid sampling frame at the level of epidemiological group. A further point to consider is putting in place systems to minimise the potential for cross-contamination. Although real-time PCR assays are closed tube, sub-optimal seals, the use and propagation of positive controls (e.g. plasmids, virus stocks), as well as further molecular procedures for the characterisation of detected genomes (e.g. sequencing) can cause false positive results in the test. In order to minimise cross-contamination, these different activities should be clearly separated away from the laboratory areas used to prepare nucleic acid template for the rRT-PCR.

Modern real-time PCR cyclers can measure up to five separate reporter fluorophores in one tube. This technical feature is a prerequisite for the application of multiplex real-time PCR assays. Many of the rRT-PCR assays reviewed here co-amplified internal controls. It is recommended that these internal controls are included for each real-time PCR analysis. The minimum requirement for a state-of-the-art real-time PCR should be the use of an internal inhibition control in a duplex assay format. The currently published rRT-PCR assays for the detection of FMDV, CSFV, AIV, NDV and BTV summarized in this review are an excellent basis for the establishment of qualified and reliable molecular diagnostics. In Europe, these tests have recently provided critical support to the efficient control of notifiable diseases like FMD, AI, BT and CSF. Future work will continue to assess the performance of these assays, providing validation data for different sample types. Furthermore, continued inter-laboratory comparative exercises are also import to gauge the relative performance of tests used at different sites, and may ultimately lead to a more rational harmonisation of the rRT-PCR assays.

ACCEPTED MANUSCRIPT

In conclusion, over the past decade rRT-PCR has become a central laboratory tool for the diagnosis of important livestock diseases. In light of the performance of these tests, it is evident that molecular diagnostics will be the main tool for eradication and control of epizootic diseases in the future.

Acknowledgements:

This work was supported by the EU Network of Excellence, EPIZONE (Contract No FOOD-CT-2006-016236); UK Defra projects SE1121, SE1124, SE2613, SV3029, SV3033 and SV3400; BBSRC project BBS/Q/Q2004/05484, and EU supported Med Reo Net Project No.: 044285..

Accepted Manuscript

ACCEPTED MANUSCRIPT

References

Afonina, I., Kutyavin, I., Lukhtanov, E., Meyer, R.B., Gamper, H. (1996) Sequence-specific arrest of primer extension on single-stranded DNA by an oligonucleotide-minor groove binder conjugate. Proc Natl Acad Sci 93(8):3199-204.

Afonina, I.A., Reed, M.W., Lusby, E., Shishkina, I.G., Belousov, Y.S. (2002) Minor groove binder-conjugated DNA probes for quantitative DNA detection by hybridization-triggered fluorescence. Biotechniques 32(4):940-4, 946-9.

Anonymous. Council Directive 82/894/EEC of 21 December 1982 on the notification of animal diseases within the Community.Official Journal 1982;L 378:0058–62.

Anonymous, 2002. Commission decision 2002/106/EC of 1 February 2002 approving a diagnostic manual establishing diagnostic procedures, sampling methods and criteria for evaluation of the laboratory tests for the confirmation of classical swine fever

Anthony, S., Jones, H., Darpel, K. E., Elliott, H., Maan, S., Samuel, A. R., Mellor, P. S. & Mertens, P. P. C. 2007. A duplex RT-PCR assay for detection of genome segment 7 (VP7 gene) from 24 BTV serotypes. J. Virol. Meth. 141, 188-197

Agüero, M., San Miguel, E., Sánchez, A., Gómez-Tejedor, C., Jiménez-Clavero, M.A., 2007. A fully automated procedure for the high-throughput detection of avian influenza virus by real-time reverse transcription–polymerase chain reaction. Avian Dis. 51, 235-241.

Akey, B. L., 2003. Low-pathogenicity H7N2 avian influenza outbreak in Virginia during 2002. Avian Dis. 47, 1099–1103.

Aldous EW, Fuller CM, Mynn JK, Alexander DJ. A molecular epidemiological investigation of isolates of the variant avian paramyxovirus type 1 virus (PPMV-1) responsible for the 1978 to present panzootic in pigeons. Avian Pathol. 2004 Apr;33(2):258-69.

Alexander, D.J., 2007. Summary of Avian Influenza Activity in Europe, Asia, Africa, and Australasia, 2002–2006. Avian Dis. 51, 161-166.

Alexander, D.J., Capua, I., Brown, I.H., 2005. Avian influenza viruses and influenza in humans. In: “Avian Influenza: Prevention and Control”, eds: Shrijver, R.S, Koch, G., pp1-8, Springer.

Balasuriya UB, Nadler SA, Wilson WC, Pritchard LI, Smythe AB, Savini G, Monaco F, De Santis P, Zhang N, Tabachnick WJ, Maclachlan NJ. (2008) The NS3 proteins of global strains of bluetongue virus evolve into regional topotypes through negative (purifying) selection. Vet Microbiol. 126, 91-100.

Banks, J., Speidel, E. S., Moore, E., Plowright, L., Piccirillo, A., Capua, I., Cordioli, P., Fioretti, A., Alexander, D. J., 2001. Changes in the haemagglutinin and the neuraminidase genes prior to the emergence of highly pathogenic H7N1 avian influenza viruses in Italy. Arch. Virol. 146, 963–973.

Barbezange C, and Jestin V. Development of a RT-nested PCR test detecting pigeon Paramyxovirus-1 directly from organs of infected animals. J Virol Methods. 2002 Dec;106(2):197-207.

Batten CA, Bachanek-Bankowska K, Bin-Tarif A, Kgosana L, Swain AJ, Corteyn M, Darpel K, Mellor PS, Elliott HG, Oura CAL, 2008a . Bluetongue virus: European

ACCEPTED MANUSCRIPT

Community inter-laboratory comparison tests to evaluate ELISA and RT-PCR detection methods. Vet Microbiol. 129 (1-2): 80-8..

Batten, C.A., Maan, S., Shaw, A.E., Maan, N.S., Mertens, P.P.C. 2008b A European field strain of bluetongue virus derived from two parental vaccine strains by genome segment reassortment. Virus Res. 137, 56-63.

Bao, Y., Bolotov, P., Dernovoy, D., Kiryutin, B., Zaslavsky, L. Tatusova, T., Ostell, J., Lipman, D., 2008. The Influenza Virus Resource at the National Center for Biotechnology Information. J. Virol. 82, 596-601. http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/select.cgi

Belák S. Molecular diagnosis of viral diseases, present trends and future aspects A view from the OIE Collaborating Centre for the Application of Polymerase Chain Reaction Methods for Diagnosis of Viral Diseases in Veterinary Medicine.Vaccine. 2007 Jul 26;25(30):5444-52.

Belak, S., Thoren, P. (2001) Molecular diagnosis of animal diseases: some experiences over the past decade. Expert Rev Mol Diagn 1(4):434-43.

Belousov, Y.S., Welch, R.A., Sanders, S., Mills, A., Kulchenko, A., Dempcy, R., Afonina, I.A., Walburger, D.K., Glaser, C.L., Yadavalli, S., Vermeulen, N.M., Mahoney, W. (2004) Single nucleotide polymorphism genotyping by two colour melting curve analysis using the MGB Eclipse Probe System in challenging sequence environment. Hum Genomics 1(3):209-17.

Black, E.M., Lowings, J.P., Smith, J., Heaton, P.R., McElhinney, L.M. (2002) A rapid RT-PCR method to differentiate six established genotypes of rabies and rabies-related viruses using TaqMan technology. J Virol Methods. 105(1):25-35.

Bonneau, K.R., DeMaula, C.D., Mullens, B.A., MacLaughlan, N.J., (2002). Duration of viraemia infectious to *Culicoides sonorensis* in Bluetongue virus-infected cattle and sheep. Vet. Microbiol. 88, 115-125.

Bonneau, K.R., Zhang, N.Z., Wilson, W.C., Zhu, J.B., Zhang, F.Q., Li, Z.H., Zhang, K.L., Xiao, L., Xiang, W.B., MacLchlan, N.J. (2000) Phylogenetic analysis of the S7 gene does not segregate Chinese strains of bluetongue virus into a single topotype. Arch Virol.;145(6):1163-71.

Boye, M., S. Kamstrup, and K. Dalsgaard. (1991). Specific sequence amplification of bovine virus diarrhea virus (BVDV) and hog cholera virus and sequencing of BVDV nucleic acid. Vet. Microbiol. 29:1–13.

Boyle DB, Taylor T, Cardoso M. (2004) Implementation in Australia of molecular diagnostic techniques for the rapid detection of foot and mouth disease virus. Aust. Vet. J. 82, 421-425.

Brown, I.H., Banks, J., Manvell, R.J., Essen, S.C., Shell, W., Slomka, M., Löndt, B., Alexander, D.J., (2006). Recent epidemiology and ecology of influenza A viruses in avian species in Europe and the Middle East. Dev. Biol., 124, 45-50.

Bustin, S.A. (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrinol. 25(2):169-93.

Bustin, S.A.(2002). Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J. Mol. Endocrinol. 29: 23-39

ACCEPTED MANUSCRIPT

Bustin, S.A., Benes, V., Nolan, T., Pfaffl, M.W. (2005) Quantitative real-time RT-PCR--a perspective. J Mol Endocrinol. 34(3):597-601.Anonymous. Council Directive 82/894/EEC of 21 December 1982 on the notification of animal diseases within the Community.Official Journal 1982;L 378:0058–62.

Callahan JD, Brown F, Osorio FA, Sur JH, Kramer E, Long GW, Lubroth J, Ellis SJ, Shoulars KS, Gaffney KL, Rock DL, Nelson WM. (2002) Use of a portable real-time reverse transcriptase-polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus J Am Vet Med Assoc 220(11):1636-42

Callens M, De Clercq K. (1997) Differentiation of the seven serotypes of foot-and-mouth disease virus by reverse transcriptase polymerase chain reaction. J. Virol. Methods 67, 35-44.

Cardullo, R.A., Agrawal, S., Flores, C., Zamecnik, P.C., Wolf, D.E. (1988) Detection of nucleic acid hybridization by nonradiative fluorescence resonance energy transfer. Proc Natl Acad Sci 85(23):8790-4.

CEC, 2006. Council Directive 2005/94/EC of 20 December 2005 on Community measures for the control of avian influenza and repealing 92/40/EEC. Official Journal of European Commission, L10, 16-65.

Chen, H., Smith, G.J.D., Zhang, S.Y., Qin, K., Wang, J., Li, K.S., et al, 2005. Avian flu: H5N1 virus outbreak in migratory waterfowl. Nature 436, 191–192.

Chen, H., Smith, G.J.D., Li, K.S., Wang, J., Fan, X.H., Rayner, J.M., et al., 2006. Establishment of multiple sublineages of H5N1 influenza virus in Asia: implications for pandemic control. Proc. Natl. Acad. Sci. U S A 103, 2845–2850.

Clegg, R.M. (1995) Fluorescence resonance energy transfer. Curr Opin Biotechnol 6(1):103-10.

Clementi M, Menzo S, Manzin A, Bagnarelli P. Quantitative molecular methods in virology. Arch Virol. 1995;140(9):1523-39. Review.

Costa JM, Ernault P, Olivi M, Gaillon T, Arar K. (2004) Chimeric LNA/DNA probes as a detection system for real-time PCR. Clin Biochem. Oct;37(10):930-2.

Crossley BM, Hietala SK, Shih LM, Lee L, Skowronski EW, Ardans AA. High-throughput real-time RT-PCR assay to detect the exotic Newcastle Disease Virus during the California 2002--2003 outbreak. J Vet Diagn Invest. 2005 Mar;17:124-32.

Creelan JL, McCullough SJ. Detection and differentiation of pathogenicity of avian paramyxovirus serotype 1 (APMV-1) from field cases using one-step real-time RT-PCR. Dev Biol (Basel). 2006;126:149-57; discussion 325-6.

Czeglédi A, Ujvári D, Somogyi E, Wehmann E, Werner O, Lomniczi B. Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. Virus Res. 2006 Sep;120(1-2):36-48.

Das, A., Spackman, E., Senne, D., Pedersen, J., Suarez, D.L., 2006. Development of an internal positive control for rapid diagnosis of avian influenza virus infections by real-time reverse transcription-PCR with lyophilized reagents. J. Clin. Microbiol. 44, 3065-3073.

Defra, National Emergency Epidemiology Group (NEEG), 2008. Highly Pathogenic Avian Influenza H7N7, Oxfordshire, June 2008.

ACCEPTED MANUSCRIPT

http://www.defra.gov.uk/animalh/diseases/notifiable/disease/ai/latest-situation/index.htm

de Kok, J.B., Wiegerinck, ET., Giesendorf, B.A., Swinkels, D.W. (2002) Rapid genotyping of single nucleotide polymorphisms using novel minor groove binding DNA oligonucleotides (MGB probes). Hum Mutat 19(5):554-9.

Deng MY, Wang H, Ward GB, Beckham TR, McKenna TS. Comparison of six RNA extraction methods for the detection of classical swine fever virus by real-time and conventional reverse transcription-PCR. J Vet Diagn Invest. 2005 Nov;17(6):574-8.

Depner, K.R., Greiser-Wilke, I., Moennig, V., Liess, B. (1997) Breed dependent variations influence the outcome of classical swine fever virus infection in pigs. In: Edwards S, Paton DJ, Wenswoort G, editors. Proceedings of the third ESVV symposium on pestivirus infections. p. 59–61.

Depner, K., Bunzenthal, C., Heun-Mu¨nch, B., Strebelow, G., Hoffmann, B., Beer, M. (2006) Diagnostic evaluation of a real-time RT-PCR assay for routine diagnosis of classical swine fever in wild boar. J. Vet. Med. B 53, 317–320.

Depner, K., Hoffmann, B., Beer, M. (2007) Evaluation of real-time RT-PCR assay for the routine intra vitam diagnosis of classical swine fever Veterinary Microbiology 121: 338–343.

De Santis, P., Orru, G., Solinas, F., Piras, V., Savini, G., Caporale, V., 2004. Molecular differentiation of field and vaccine strains of bluetongue virus serotype 2 using the real-time polymerase chain reaction and fluorescene resonance energy transfer hybridisation probe, Vet Ital. 40, 572-576.

Didenko, V.V. (2001) DNA probes using fluorescence resonance energy transfer (FRET): designs and applications. Biotechniques 31(5):1106-16, 1118, 1120-1.

Di Trani, L., Bedini, B., Donatelli, I., Campitelli, L., Chiappini, B., De Marco, M.A., Delogu, M., Buonavoglia, C., Vaccari, G., 2006. A sensitive one-step real-time PCR for detection of avian influenza viruses using a MGB probe and an internal positive control. BMC Infect. Dis. 6, 87.

Drosten, C., Seifried, E., Roth, W.K. (2001) TaqMan 5'-nuclease human immunodeficiency virus type 1 PCR assay with phage-packaged competitive internal control for high-throughput blood donor screening. J Clin Microbiol 39(12):4302-8

Ducatez, M.F., Olinger, C.M., Owoade, A.A., de Landtsheer, S., Ammerlaan, W., Niesters, H.G., et al 2006. Avian flu: multiple introductions of H5N1 in Nigeria. Nature 442, 37.

Dukes JP, King DP, Alexandersen S. (2006) Novel reverse transcription loop-mediated isothermal amplification for rapid detection of foot-and-mouth disease virus. Arch. Virol. 151(6), 1093-1106.Braasch, D.A., Corey, D.R. (2001) Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA. Chem Biol 8(1):1-7.

Dupont, M., Goldsborough, A., Levayer, T., Savare, J., Rey, J.M., Rossi, J.F., Demaille, J., Lavabre-Bertrand, T. (2002) Multiplex fluorescent RT-PCR to quantify leukemic fusion transcripts. Biotechniques 33(1):158-60, 162, 164.

Espy MJ, Uhl JR, Sloan LM, Buckwalter SP, Jones MF, Vetter EA, Yao JD, Wengenack NL, Rosenblatt JE, Cockerill FR 3rd, Smith TF. (2006) Real-time PCR in

ACCEPTED MANUSCRIPT

clinical microbiology: applications for routine laboratory testing. Clin Microbiol Rev. Jan;19(1):165-256

EU, 2006. Diagnostic manual for avian influenza. http://eur-lex.europa.eu/LexUriServ/site/en/oj/2006/l_237/l_23720060831en00010027.pdf

EU, 2007. Avian influenza (AI): Extensive chronology of main events and list of decisions adopted by the European Commission in 2007. http://ec.europa.eu/food/animal/diseases/controlmeasures/avian/31-12-2007_ai_chronology.pdf

EU, 2008. Low pathogenic avian influenza H7N1 in Denmark. http://ec.europa.eu/food/committees/regulatory/scfcah/animal_health/presentations/ai_78052008_dk.pdf

FAO, 2006. Evolution of highly pathogenic avian influenza type H5N1 in Europe: review of disease ecology, trends and prospects of spread in autumn-winter 2006. In: "Empres Watch. Emergency prevention systems", October 2006. http://www.fao.org/docs/eims/upload/214878/EW_europe_oct06_hpai.pdf

Farkas T, Antal M, Sámi L, Germán P, Kecskeméti S, Kardos G, Belák S, Kiss I. Rapid and simultaneous detection of avian influenza and newcastle disease viruses by duplex polymerase chain reaction assay. Zoonoses Public Health. 2007;54(1):38-43.

Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds), 2005. Virus Taxonomy, VIIIth Report of the ICTV. London: Elsevier/Academic Press.

Fedele, C.G., Negredo, A., Molero, F., Sanchez-Seco, M.P., Tenori, A. (2006) Use of internally controlled real-time genome amplification for detection of variola virus and other orthopoxviruses infecting humans. J Clin Microbiol. 44(12):4464-70.

Ferris NP, Dawson M. (1988) Routine application of enzyme-linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot-and-mouth and swine vesicular diseases. Vet. Microbiol. 16, 201-209.

Ferris NP, King DP, Reid SM, Shaw AE, Hutchings GH. (2006a) Comparisons of original laboratory results and retrospective analysis by real-time reverse transcriptase-PCR of virological samples collected from confirmed cases of foot-and-mouth disease in the UK in 2001. Vet. Rec. 16;159(12):373-8.

Ferris NP, King DP, Reid SM, Hutchings GH, Shaw AE, Paton DJ, Goris N, Haas B, Hoffmann B, Brocchi E, Bugnetti M, Dekker A, De Clercq K. (2006b) Foot-and-mouth disease virus: a first inter-laboratory comparison trial to evaluate virus isolation and RT-PCR detection methods. Vet. Microbiol. 117(2-4), 130-140.

Floegel-Niesmann G, Bunzenthal C, Fischer S, Moennig V. (2003). Virulence of recent and former classical swine fever virus isolates evaluated by their clinical and pathological signs. J. Vet. Med B, 50:214–20.

Gagnon, C.A., del Castillo, J.R., Music, N., Fontaine, G., Harel, J., Tremblay, D. (2008). Development and use of a multiplex real-time quantitative polymerase chain reaction assay for detection and differentiation of Porcine circovirus-2 genotypes 2a and 2b in an epidemiological survey. J. Vet. Diagn. Invest., 20:545-58.

Gibson, U.E., Heid, C.A., Williams, P.M. (1996) A novel method for real time quantitative RT-PCR. Genome Res 6(10):995-1001.

ACCEPTED MANUSCRIPT

Gohm D, Schelling E, Audigé L, Thür B. (1999). Newcastle disease--seroepidemiologic study of a highly contagious epizootic in poultry and in wild birds in Switzerland Schweiz Arch Tierheilkd.141:549-58. German. J Vet Med Sci. 1999 Nov;61(11):1191-5.

Gorzelniak, K., Janke, J., Engeli, S., Sharma, A.M. (2001). Validation of endogenous controls for gene expression studies in human adipocytes and preadipocytes. Horm Metab Res 33(10):625-7

Gould, A.R., Pritchard, L.I., 1990. Relationships amongst bluetongue viruses revealed by comparisons of capsid and outer coat protein nucleotide sequences. Virus Res. 17, 31-52.

Gumm, I.D., Newman, J.F., 1982. The preparation of purified bluetongue virus group antigen for use a as a diagnostic reagent. Arch. Virol. 72, 83-93.

Gunson RN, Collins TC, Carman WF. Practical experience of high throughput real time PCR in the routine diagnostic virology setting. J Clin Virol. 2006 Apr;35(4):355-67.

Gunson, R., Bennett, S., Maclean, A., Carman, W. (2008) Using multiplex real time PCR in order to streamline a routine diagnostic service. *Journal of Clinical Virology*, 43: 372-375

Heid, C.A., Stevens, J., Livak, K.J., Williams, P.M. (1996) Real time quantitative PCR. Genome Res 6(10):986-94.

Helps, C., Lait, P., Tasker, S., Harbour, D. (2002) Melting curve analysis of feline calicivirus isolates detected by real-time reverse transcription PCR. J Virol Methods. 106(2):241-4.

Hearps A, Zhang Z, Alexandersen S. (2002) Evaluation of the portable Cepheid SmartCycler real-time PCR machine for the rapid diagnosis of foot-and-mouth disease. Vet Record 150, 625-628.

Higuchi, R., Fockler, C., Dollinger, G., Watson, R. (1993) Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology 11(9):1026-30.

Hilbink F, Vertommen M, Tibben J, van't Veer W. The fluorescent antibody technique in the diagnosis of a number of poultry diseases: manufacture of conjugates and use (author's transl) Tijdschr Diergeneeskd. 1982 Mar 1;107(5):167-73.

Hodgson, J., Zuckerman, M., Smith, M. (2007) Development of a novel internal control for a real-time PCR for HSV DNA types 1 and 2. J Clin Virol. 38(3):217-20.

Hoffmann, B., Depner, K., Schirrmeier, H., Beer, M. (2006) A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses. J Virol Methods 136(1-2):200-9.

Hoffmann, B., Beer, M., Schelp, C., Schirrmeier, H., Depner, K. (2005) Validation of a real-time RT-PCR assay for sensitive and specific detection of classical swine fever. J. Virol. Methods 130, 36–44.

Hoffmann, B., Harder, T., Starick, E., Depner, K., Werner, O., Beer, M., 2007. Rapid and highly sensitive pathotyping of avian iInfluenza A H5N1 virus by using real-time reverse transcription-PCR. J. Clin. Microbiol. 45, 600-603.

Hofmann, M.A. (2003) Construction of an infectious chimeric classical swine fever virus containing the 5'UTR of bovine viral diarrhea virus, and its application as a

ACCEPTED MANUSCRIPT

universal internal positive control in real-time RT-PCR. J Virol Methods 114(1):77-90.

Horst, H.S., Meuwissen, M.P.M., Smak, J.A., Van de Meijs, C.C.J.M., 1999. The involvement of the agriculture industry and government in animal disease emergencies and the funding of compensation in Western Europe. Rev. Sci. Tech. Off. Int. Epiz. 18, 30–37.

Horzinek, M. C. 1991. Pestiviruses—taxonomic perspectives. Arch. Virol. Suppl. 3:1–5.

Huang, I.J., Hwang, G.Y., Yang, Y.Y., Hayama, E. and Li, J.K., 1995. Sequence analyses and antigenic epitope mapping of the putative RNA-directed RNA polymerase of five U.S. bluetongue viruses. Virology 214, 280-8.

Huismans, H.a.E., B.J., 1981. Identification of the seroype-specific and group specific antigens of Bluetongue virus. Ond. J. Vet. Res. 48, 51-58.

Hüssy, D., Stauber, N., Leutenegger, C.M., Rieder, S., Ackermann, M. (2001) Quantitative fluorogenic PCR assay for measuring ovine herpesvirus 2 replication in sheep. Clin. Diagn. Lab. Immunol. 8, 123– 128.

Huggett, J., Dheda, K., Bustin, S., Zumla, A. (2005) Real-time RT-PCR normalisation; strategies and considerations. Genes Immun 6(4):279-84

Ishiguro, T., Saitoh, J., Yawata, H., Yamagishi, H., Iwasaki, S., Mitoma, Y. (1995) Homogeneous quantitative assay of hepatitis C virus RNA by polymerase chain reaction in the presence of a fluorescent intercalater. Anal Biochem 229(2):207-13.

Itabashi, T., Maesawa, C., Uchiyama, M., Higuchi, T., Masuda, T. (2004) Quantitative detection of mutant alleles of the K-ras gene with minor groove binder-conjugated fluorogenic DNA probes. Int J Oncol 24(3):687-96.

Jamnikar Ciglenečki U, Grom J, Toplak I, Jemeršić L, Barlič-Maganja D. Real-time RT-PCR assay for rapid and specific detection of classical swine fever virus: Comparison of SYBR Green and TaqMan MGB detection methods using novel MGB probes. J Virol Methods. 2008 Feb;147(2):257-64

Jimenez-Clavero, M.A., Aguero, M., San Miguel, E., Mayoral, T., Lopez, M.C., Ruano, M.J., Romero, E., Monaco, F., Polci, A., Savini, G. and Gomez-Tejedor, C., 2006. High throughput detection of bluetongue virus by a new real-time fluorogenic reverse transcription-polymerase chain reaction: application on clinical samples from current Mediterranean outbreaks. J Vet Diagn Invest 18, 7-17.

Kaden, V., Lange, E., Polster, U., Klopfleisch, R., Teifke, J.P. (2004) Studies on the virulence of two field isolates of the classical Swine Fever virus genotype 2.3 rostock in wild boars of different age groups. J Vet Med B Infect Dis Vet Pub Health 51:202–8.

Kaltenboeck B, Wang C. Advances in real-time PCR: application to clinical laboratory diagnostics. Adv Clin Chem. 2005;40:219-59. Review.

Kamolsiriprichaiporn, S., Hooper, P.T., Morrissy, C.J., Westbury, H.A. (1992a) A comparison of the pathogenicity of two strains of hog cholera virus. 1. Clinical and pathological studies. Aust Vet J 69:240–4.

ACCEPTED MANUSCRIPT

Kamolsiriprichaiporn, S., Morrissy, C.J., Westbury, H.A. (1992b) A comparison of the pathogenicity of two strains of hog cholera virus. 2. Virological studies. Aust Vet J. 69:245–8.

Kant A, Koch G, Van Roozelaar DJ, Balk F, Huurne AT. Differentiation of virulent and non-virulent strains of Newcastle disease virus within 24 hours by polymerase chain reaction. Avian Pathol. 1997 Dec;26(4):837-49.

Katz, J.B., Alstad, A.D., Gustafson, G.A., Moser, K.M., 1993. Sensitive identification of bluetongue virus serogroup by a colorimetric dual oligonucleotide sorbent assay of amplified viral nucleic acid. J. Clin. Microbiol. 31, 3028-3030.

Kho CL, Mohd-Azmi ML, Arshad SS, Yusoff K. Performance of an RT-nested PCR ELISA for detection of Newcastle disease virus. J Virol Methods. 2000 Apr;86(1):71-83.

Khripin, Y. (2006) High-throughput genotyping with energy transfer-labeled primers. Methods Mol Biol 335: 215-40.

Kim LM, Afonso CL, Suarez DL. Effect of probe-site mismatches on detection of virulent Newcastle disease viruses using a fusion-gene real-time reverse transcription polymerase chain reaction test. J Vet Diagn Invest. 2006 Nov;18(6):519-28.

Kim LM, King DJ, Curry PE, Suarez DL, Swayne DE, Stallknecht DE, Slemons RD, Pedersen JC, Senne DA, Winker K, Afonso CL. Phylogenetic diversity among low-virulence newcastle disease viruses from waterfowl and shorebirds and comparison of genotype distributions to those of poultry-origin isolates. J Virol. 2007 81:12641-53

Kim, S.G., Dubovi, E.J. (2003) A novel simple one-step single-tube Rtduplex PCR method with an internal control for detection of bovine viral diarrhoea virus in bulk milk, blood, and follicular fluid samples. Biologicals 31, 103–106.

King, D.P., Reid, S.M., Hutchings, G.H., Grierson, S.S., Wilkinson, P.J., Dixon, L.K., Bastos, A.D., Drew, T.W. (2003) Development of a TaqMan PCR assay with internal amplification control for the detection of African swine fever virus. J Virol Methods. 107(1):53-61

King D. P., Dukes J. P., Reid S. M., Ebert K., Shaw A. E., Mills C. E., Boswell L. and Ferris N. P. (2008) Prospects for rapid diagnosis of foot-and-mouth disease in the field using RT-PCR. *Veterinary Record* 162 (10): 315-6.

King DP, Ferris NP, Shaw AE, Reid SM, Hutchings GH, Giuffre AC, Robida JM, Callahan JD, Nelson WM, Beckham TR. (2006) Detection of foot-and-mouth disease virus: comparative diagnostic sensitivity of two independent real-time reverse transcription-polymerase chain reaction assays. J Vet Diagn Invest. 18(1):93-7.

King D. P., Montague N., Ebert K., Reid S. M., Dukes J. P., Schädlich L., Belsham G. J., and Lomonossoff G. P. (2007) Development of a novel recombinant encapsidated RNA particle: evaluation as an internal control for diagnostic RT-PCR. *Journal of Virological Methods* 146: 218-225

Koenig P, Hoffmann B, Depner KR, Reimann I, Teifke JP, Beer M. Detection of classical swine fever vaccine virus in blood and tissue samples of pigs vaccinated either with a conventional C-strain vaccine or a modified live marker vaccine. Vet Microbiol. 2007 Mar 10;120 (3-4):343-51.

ACCEPTED MANUSCRIPT

Kompalic-Cristo, A., Frotta, C., Suarez-Mutis, M., Fernandes, O., Britto, C. (2007) Evaluation of a real-time PCR assay based on the repetitive B1 gene for the detection of Toxoplasma gondii in human peripheral blood. Parasitol Res Mar 25 (Epub)

Korimbocus, J., Coates, D., Baker, I., Boonham, N., 2002. Improved detection of sugarcane yellow leaf virus using a real-time fluorescent (TaqMan) RTPCR assay. J. Virol. Meth. 103, 109–120.

Kou YT, Chueh LL, Wang CH. Restriction fragment length polymorphism analysis of the F gene of Newcastle disease viruses isolated from chickens and an owl in Taiwan.

Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonák J, Lind K, Sindelka R, Sjöback R, Sjögreen B, Strömbom L, Ståhlberg A, Zoric N. The real-time polymerase chain reaction.Mol Aspects Med. 2006 Apr-Jun;27(2-3):95-125. Epub 2006 Feb 3. Review.

Kusser, W. (2006) Use of self-quenched, fluorogenic LUX primers for gene expression profiling. Methods Mol Biol 335:115-33.

Kutyavin, I.V., Afonina, I.A., Mills, A., Gorn, V.V., Lukhtanov, E.A., Belousov, E.S., Singer, M.J., Walburger, D.K., Lokhov, S.G., Gall, A.A., Dempcy, R., Reed, M.W., Meyer, R.B., Hedgpeth, J. (2000) 3'-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. Nucleic Acids Res 28(2):655-61.

Latorra, D., Arar, K., Hurley, J.M. (2003) Design considerations and effects of LNA in PCR primers. Mol Cell Probes 17(5):253-9.

Le Dimna, M., Kuntz-Simon, G., Louguet, Y., Bougeard, S., Le-Potier, M.F. (2006) Diagnosis validation of new real time RT-PCR kits for detection of classical swine fever virus. J Recherche Porcine 38:365–70.

Le Dimna M, Vrancken R, Koenen F, Bougeard S, Mesplede A, Hutet E, Kuntz-Simon G, Le Potier MF. Validation of two commercial real-time RT-PCR kits for rapid and specific diagnosis of classical swine fever virus. J Virol Methods. 2007 Oct 1.

Le Pecq, J., Paoletti, C. (1966) A new fluorometric method for RNA and DNA determination. Anal Biochem 17, 100-107

Lee, C.-W., Suarez, D. L., 2004. Application of real-time RT-PCR for the quantitation and competitive replication study of H5 and H7 subtype avian influenza virus. J. Virol. Methods 119, 151–158.

Lees, W., Chown, L., Inch, C., Moll, K., Brassel, P., Wu, T., Kahn, S., Pasick, J., 2004. A short summary of the 2004 outbreak of high pathogenicity avian influenza (H7N3) in British Columbia, Canada. http://www.inspection.gc.ca/english/anima/heasan/disemala/avflu/2004sum/summaryhpaie.shtml

Legoff J, Kara R, Moulin F, Si-Mohamed A, Krivine A, Bélec L, Lebon P. Evaluation of the one-step multiplex real-time reverse transcription-PCR ProFlu-1 assay for detection of influenza A and influenza B viruses and respiratory syncytial viruses in children. J Clin Microbiol. 2008 Feb;46(2):789-91.

Leifer, I., Depner, K., Blome, S., Le Potier,M-F., Le Dimna, M., Beer, M. , Hoffmann, B. 2009. Differentiation of C-strain “Riems” or CP7_E2alf vaccinated

ACCEPTED MANUSCRIPT

animals from animals infected by classical swine fever virus field strains using real-time RT-PCR. J. Virol. Meth., *in press.*

Le Potier, M.F., Le Dimna, M., Kuntz-Simon, G., Bougeard, S., Mesplede, A. (2006) Validation of a real-time RT-PCR assay for rapid and specific diagnosis of Classical Swine Fever virus. Dev Biol.; 126:179-86; discusssion 326-7.

Liu L, Widen F, Baule C, Belak S. A one-step, gel-based RT-PCR assay with comparable performance to real-time RT-PCR for detection of classical swine fever virus.J Virol Methods. 2007 Feb;139(2):203-7.

Liu, J., Xiao, H., Lei, F., Zhu, Q., Qin, K., Zhang, X.-W., Zhang, X.-l., Zhao, D., Wang, G., Feng, Y., Ma, J., Liu, W., Wang, J., Gao, G. F., 2005. Highly pathogenic H5N1 influenza virus infection in migratory birds. Science 309, 1206.

Livak, K.J., Flood, S.J., Marmaro, J., Giusti, W., Deetz, K. (1995) Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl 4(6):357-62.

Lockaby SB, Hoerr FJ, Ellis AC, Yu MS. Immunohistochemical detection of Newcastle disease virus in chickens. Avian Dis. 1993 Apr-Jun;37(2):433-7.

Maan S, Maan NS, Ross-Smith N, Batten CA, Shaw AE, Anthony SJ, Samuel AR, Darpel KE, Veronesi E, Oura CA, Singh KP, Nomikou K, Potgieter AC, Attoui H, van Rooij E, van Rijn P, De Clercq K, Vandenbussche F, Zientara S, Bréard E, Sailleau C, Beer M, Hoffman B, Mellor PS, Mertens PP. (2008) Sequence analysis of bluetongue virus serotype 8 from the Netherlands 2006 and comparison to other European strains. Virology 377, 308–318.

Maan, S., Maan, N. S., Samuel, A. R., Rao, S., Attoui, H. & Mertens, P. P. C. 2007. Analysis and phylogenetic comparisons of full-length VP2 genes of the 24 bluetongue virus serotypes. J. Gen.Virol. 88, 621-630

Mackay IM, Arden KE, Nitsche A.2002. Real-time PCR in virology. Nucleic Acids Res. 30(6):1292-305. Review.

Mackay, I.M. (2004) Real-time PCR in the microbiology laboratory. Clin. Microbiol. Infect. 10, 190–212

MacLachlan, N.J., 1994. The pathogenesis and immunology of bluetongue virus infection of ruminants. Comp. Immunol, Microbiol. Infect. Dis. 17, 197-206.

McGoldrick, A., Bensaude, E., Ibata, G., Sharp, G., Paton, D.J. (1999) Closed onetube reverse transcription nested polymerase chain reaction for the detection of pestiviral RNA with fluorescent probes. J Virol Meth 79:85–95.

Manvell, R.J, Londt, B.Z., Ceeraz, V., Cox, W.J., Essen, S., Banks, J., Slomka, M.J., Pavlidis, T., Irvine, R.M., Wilesmith, J.W., Sharpe, C.E., Hurst, A., Alexander, D.J., Brown, I.H., 2008. Low pathogenic avian influenza in domestic fowl in Norfolk, England, March-April 2006. Vet. Rec. 162, 278-280.

Mellor, P.S., Boorman, J., Baylis, M., 2000. Culicoides biting midges: their role as arbovirus vectors. Annu. Rev. Entemol. 45, 307-340.

ACCEPTED MANUSCRIPT

Mertens, P.P., Brown, F., Sangar, D.V., 1984. Assignment of the genome segments of bluetongue virus type 1 to the proteins which they encode. Virology. 135 (1), 207-17.

Mertens, P.P., Pedley, S., Cowley, J. and Burroughs, J.N., 1987. A comparison of six different bluetongue virus isolates by cross-hybridization of the dsRNA genome segments. Virology 161, 438-47.

Mertens, P.C., Diprose., 2004. The bluetongue virus core: a nano-acale transcription machine. Virus Res. 101, 29-43.

Mertens, P. P. C., Maan, N. S., Prasad, G., Samuel, A. R., Shaw, A. E., A.C., P., Anthony, S. & Maan, S. 2007. The design of primers and use of RT-PCR assays for typing European BTV isolates: Differentiation of field and vaccine strains. J. Gen. Virol. (in press).

Meyer RF, Brown CC, House C, House JA, Molitor TW (1991) Rapid and sensitive detection of foot-and-mouth disease virus in tissues by enzymatic RNA amplification of the polymerase gene. J. Virol. Methods 34, 161-172.

Meyers G., H. J. Thiel, and T. Rumenapf. 1996. Classical swine fever virus: recovery of infectious viruses from cDNA constructs and generation of recombinant cytopathogenic defective interfering particles. J. Virol. 70:1588–1595.

Mittelholzer, C., Moser, C., Tratschin, J.D., Hofmann, M.A. (2000) Analysis of classical swine fever virus replication kinetics allows differentiation of highly virulent from avirulent strains. Vet Microbiol 74:293–308.

Monne, I., Ormelli, S., Salviato, A., De Battisti, C., Bettini, F., Salomoni, A., Drago, A., Zecchin, B., Capua, I., Cattoli, G., 2008. Development and Validation of a one-step RealTime PCR Assay for simultaneous detection of subtype H5, H7, and H9 avian influenza viruses. J Clin Microbiol 46, 1769-1773.

Moniwa M, Clavijo A, Li M, Collignon B, Kitching PR. (2007) Performance of a foot-and-mouth disease virus reverse transcription–polymerase chain reaction with amplification controls between three real-time instruments J Vet Diagn Invest 19, 9-20

Moonen P, Boonstra, J, Hakze-van der Honing R, Boonstra-Leendertse C, Jacobs L, Dekker A. (2003) Validation of a LightCycler-based reverse transcription polymerase chain reaction for the detection of foot-and-mouth disease virus. J. Virol. Methods 113, 35-41.

Morrison, T.B., Weis, J.J., Wittwer, C.T. (1998) Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification.. Biotechniques 24(6):954-8, 960, 962.

Müller, J., Eis-Hübinger, A.M., Däumer, M., Kaiser, R., Rox, J.M., Gürtler, L., Hanfland, P., Pötzsch, B. (2007) A novel internally controlled real-time reverse transcription-PCR assay for HIV-1 RNA targeting the pol integrase genomic region. J Virol Methods. Feb 23; (Epub)

Munster, V.J., Wallensten, A., Baas, C., Rimmelzwaan, G.F., Schutten, M., Olsen, B., Osterhaus, A.D., Fouchier, R.A., 2005. Mallards and highly pathogenic avian influenza ancestral viruses, northern Europe. Emerg. Infect. Dis. 11, 1545-1551.

ACCEPTED MANUSCRIPT

Munster, V.J., Veen J., Olsen, B.,Vogel, R., Osterhaus, A.D., Fouchier, R.A., 2006. Towards improved influenza A virus surveillance in migrating birds. Vaccine 24, 6729-6733.

Nazarenko, I.A., Bhatnagar, S.K., Hohman, R.J. (1997) A closed tube format for amplification and detection of DNA based on energy transfer. *Nucleic Acids Res.* **25**, 2516–2521

Nazarenko, I. (2006) Homogeneous detection of nucleic acids using self-quenched polymerase chain reaction primers labeled with a single fluorophore (LUX primers). Methods Mol Biol 335:95-114

Nikolakaki, S.V., Nomikou, K., Koumbati, M., Mangana, O., Papanastassopoulou, M., Mertens, P.P., Papadopoulos, O., 2005. Molecular analysis of the NS3/NS3A gene of Bluetongue virus isolates from the 1979 and 1998-2001 epizootics in Greece and their segregation into two distinct groups. Virus Res. 114(1-2), 6-14.

Niesters HG. Clinical virology in real time. J Clin Virol. 2002 Dec;25 Suppl 3:S3-12. Review.

Niesters, H.G. (2004) Molecular and diagnostic clinical virology in real time. Clin Microbiol Infect 10(1):5-11.

Nuovo, G.J., Hohman, R.J., Nardone, G.A., Nazarenko, I.A. (1999) In situ amplification using universal energy transfer-labeled primers. J. Histochem. Cytochem. 47**,** 273–280.

Oem JK, Kye SJ, Lee KN, Kim YJ, Park JY, Park JH, Joo YS, Song HJ. (2005) Development of a Lightcycler-based reverse transcription polymerase chain reaction for the detection of foot-and-mouth disease virus. J Vet Sci. 6(3):207-12.

OIE (World Organisation for Animal Health) 2002. OIE Guide 3: Laboratory Proficiency Testing. In: OIE Quality Standard and Guidelines for Veterinary Laboratories: Infectious Diseases. OIE, Paris, France, 53-63.

OIE Manual for Diagnostic Tests and Vaccines for Terrestrial Animals 2004. OIE World Organisation for Animal Health Chapter 2.1.15.

OIE (World Organisation for Animal Health), 2008. Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases. In: Manual of diagnostic tests and vaccines for terrestrial animals. Chapter 1.1.4., 6th ed. Paris, France. http://www.oie.int/eng/normes/mmanual/A_00014.htm

OIE (World Organisation for Animal Health), 2008. Bluetongue. In: Manual of Diagnostic Tests and Vaccines for Terrestrial animals, Chapter 2.1.3.

OIE (World Organisation for Animal Health), 2005a. Avian influenza. In: Manual of diagnostic tests and vaccines for terrestrial animals. Chapter 2.7.12. Paris, France. http://www.oie.int/eng/normes/mmanual/A_00037.htm

OIE (World Organisation for Animal Health), 2005b. World Health Organisation for Animal Health, Terrestrial Animal Health Code, 14th chapter 2.7.12.1 on avian influenza. www.oie.int/eng/normes/mcode/en_chapter_2.7.12.htm

OIE. Office International des Epizooties. Diseases notifiable to the OIE; 2006, http://wwwoieint/eng/maladies/en classificationhtm.

ACCEPTED MANUSCRIPT

Oleksiewicz, M.B., Donaldson, A.I., Alexandersen, S., 2001. Development of a novel real-time RT-PCR assay for quantitation of foot-and-mouth disease virus in diverse porcine tissues. J. Virol. Meth. 92, 23–35.

Olson, V.A., Laue, T., Laker, M.T., Babkin, I.V., Drosten, C., Shchelkunov, S.N., Niedrig, M., Damon, I.K., Meyer, H. (2004) Real-time PCR system for detection of orthopoxviruses and simultaneous identification of smallpox virus. J Clin Microbiol. 42(5):1940-6

Ophuis RJ, Morrissy CJ, Boyle DB. Detection and quantitative pathogenesis study of classical swine fever virus using a real time RT-PCR assay. J Virol Methods. 2006 Jan;131(1):78-85.

Orru, G., Santis, P.D., Solinas, F., Savini, G., Piras, V. and Caporale, V., 2004. Differentiation of Italian field and South African vaccine strains of bluetongue virus serotype 2 using real-time PCR. J. Virol. Methods 122, 37-43.

Orru, G., Ferrando, M.L., Meloni, M., Liciardi, M., Savini, G., De Santis, Poala., 2006. Rapid detection and quantification of bluetongue virus (BTV) using a Molecular Beacon fluorescent probe assay. J. Virol. Meth. 137, 34-42.

Pasick, J., Robinson, J., Hooper-McGrevy, K., Wright, P., Kitching, P., Handel, K., Copps, J., Ridd, D., Kehler H., Hills, K., Cottam-Birt, C., 2007. The roles of national and provincial diagnostic laboratories in the eradication of highly pathogenic H7N3 avian influenza virus from the Fraser Valley of British Columbia, Canada. Avian Dis. 51, 309-312.

Paton, D.J., McGoldrick, A., Belak, S., Mittelholzer, C., Koenen, F., Vanderhallen, H., Biagetti, M., De Mia, G., Stadejek, T., Hofmann, M.A., Thuer, B. (2000) Classical swine fever virus: a ring test to evaluate RT-PCR detection methods. Vet. Microbiol. 73, 159–174.

Payungporn, S., Chutinimitkul, S., Chaisingh, A., Damrongwantanapokin, S., Buranathai, C., Amonsin, A., Theamboonlers, A., Poovorawan, Y. (2006). Single step multiplex real-time RT-PCR for H5N1 influenza A virus detection. J. Virol. Methods. 131:143-7

Persson K, Hamby K, Ugozzoli LA. Four-color multiplex reverse transcription polymerase chain reaction--overcoming its limitations. Anal Biochem. 2005 Sep 1;344(1):33-42.

Pham HM, Konnai S, Usui T, Chang KS, Murata S, Mase M, Ohashi K, Onuma M. Rapid detection and differentiation of Newcastle disease virus by real-time PCR with melting-curve analysis. Arch Virol. 2005 Dec;150(12):2429-38.

Pham HM, Nakajima C, Ohashi K, Onuma M. Loop-mediated isothermal amplification for rapid detection of Newcastle disease virus. J Clin Microbiol. 2005 Apr;43(4):1646-50.

Pierce, C.M., Balasuriya, U.B., Maclachlan, N.J., 1998. Phylogenetic analysis of the S10 gene of field and laboratory strains of bluetongue virus from the United States. Virus Res. 55, 15-27.

Polci, A., Cammà, C, Serini, S., Di Gialleonardo, L., Monaco, F., Savini, G., 2007. Real-time polymerase chain reaction to detect bluetongue virus in blood samples. Vet. Italia. 43 (1), 77-87.

ACCEPTED MANUSCRIPT

Purse, B.V., Mellor, P.S., Rogers, D.J., Samuel, A.R., Mertens, P.P. and Baylis, M., 2005. Climate change and the recent emergence of bluetongue in Europe. Nat Rev Microbiol 3, 171-81.

Rasmussen, T.B., Uttenthal, A., Reimann, I., Nielsen, J., Depner, K., Beer, M. (2007) Virulence, immunogenicity and vaccine properties of a novel chimeric pestivirus. J Gen Virol 88(Pt 2):481-6.

Rasmussen, T.B., Uttenhal, Å., de Stricker, K., Belák, S. and Storgaard, T. (2003). Development of a novel quantitative real-time RT-PCR assay for the simultaneous detection of all serotypes of foot-and-mouth disease virus. Archives of Virology, 148, 2005-2021.

Rasmussen TB, Uttenthal A, Aguero M. (2006) Detection of three porcine vesicular viruses using multiplex real-time primer-probe energy transfer. J Virol Methods 134(1-2):176-82.

Ratcliff RM, Chang G, Kok T, Sloots TP. (2007) Molecular diagnosis of medical viruses. Curr Issues Mol Biol. Jul;9(2):87-102

Reid SM, Hutchings GH, Ferris NP, De Clercq K. (1999) Diagnosis of foot-and-mouth disease by RT-PCR: evaluation of primers for serotypic characterisation of viral RNA in clinical samples. J. Virol. Methods 83, 113-123.

Reid SM, Ferris NP, Hutchings GH, Samuel AR, Knowles NJ. (2000) Primary diagnosis of foot-and-mouth disease by reverse transcriptionpolymerase chain reaction. J. Virol. Methods 89, 167-176.

Reid SM, Ferris NP, Hutchings GH, Zhang Z, Belsham GJ, Alexandersen S. (2002) Detection of all seven serotypes of foot-and-mouth disease virus by real-time, fluorogenic reverse transcription polymerase chain reaction assay J Virol Methods 105(1):67-80

Reid SM, Grierson SS, Ferris NP, Hutchings GH, Alexandersen S. (2003) Evaluation of automated RT-PCR to accelerate the laboratory diagnosis of foot-and-mouth disease virus J Virol Methods 107(2):129-39

Reid SM, Ferris NP, Hutchings GH, King DP, Alexandersen S. (2004) Evaluation of real-time reverse transcription polymerase chain reaction assays for the detection of swine vesicular disease virus. J Virol Methods. 116(2):169-76.

Reid SM, Parida S, King DP, Hutchings GH, Shaw AE, Ferris NP, Zhang Z, Hillerton JE, Paton DJ. (2006) Utility of automated real-time RT-PCR for the detection of foot-and-mouth disease virus excreted in milk. Vet Res. 37(1):121-32.

Risatti, G.R., Callahan, J.D., Nelson,W.M., Borca, M.V. (2003) Rapid detection of classical swine fever virus by a portable real-time reverse transcriptase PCR assay. J. Clin. Microbiol. 41, 500–505.

Risatti, G., Holinka, L., Lu, Z., Kutish, G., Callahan, J.D., Nelson, W.M., Brea Tio, E., Borca, M.V. (2005) Diagnostic evaluation of a real-time reverse transcriptase PCR assay for detection of classical swine fever virus. J Clin Microbiol 43(1):468-

Roberts, L. (2000) Human genome research - SNP mappers confront reality and find it daunting. Science 287 (5460): 1898-1899.

Rodríguez A, Núñez JI, Nolasco G, Ponz F, Sobrino F, De Blas C. (1994) Direct PCR detection of foot-and-mouth disease virus. J. Virol. Methods 47, 345-349.

ACCEPTED MANUSCRIPT

Rohm, C., Horimoto, T., Kawaoka, Y., Suss, J., Webster, R.G., 1995. Do hemagglutinin genes of highly pathogenic avian influenza viruses constitute unique phylogenetic lineages? Virology 209, 664–670.

Roy, P., 1989. Bluetongue virus genetics and genome structure. Virus Res. 13, 179-206.

Roy, P., Marshall, J.J.A., French, T.J., 1990. Structure of Bluetongue virus genome and its encoded proteins. In: Roy, P., Gorman, B.M. (Eds), Current Topics in Microbiology and Immunology. Springer, Berlin.

Sammin DJ, Paton DJ, Parida S, Ferris NP, Hutchings GH, Reid SM, Shaw AE. Holmes C, Gibson D, Corteyn M, Knowles NJ, Valarcher J. –F, Hamblin PA, Fleming L, Gwaze G, Sumption KJ. (2006) Evaluation of laboratory tests for SAT serotypes of foot-and-mouth disease virus with specimens collected from convalescent cattle in Zimbabwe. Vet. Record in press.

Schnyder, M., Stärk, K.D.C., Vanzetti, T., Salman, M.D., Thu¨r, B., Schleiss, W., Griot, C. (2002) Epidemiology and control of an outbreak of classical swine fever in wild boar in Switzerland. Vet. Rec. 150, 102–109

Schweiger, B., Zadow, I., Heckler, R., Timm, H., Pauli, G. (2000) Application of a Fluorogenic PCR Assay for Typing and Subtyping of Influenza Viruses in Respiratory Samples J Clin Microbiol 38 (4), 1552–1558

Selvin, PR., Hearst, J.E. (1994) Luminescence energy transfer using a terbium chelate: improvements on fluorescence energy transfer. Proc Natl Acad Sci 91(21):10024-8.

Senne, D.A., 2007. Avian influenza in North and South America, 2002–2005. Avian Dis. 51, 167-173.

Senne, D.A., Pedersen, J.C., Suarez, D.L., Panigrahy, B., 2006. Rapid diagnosis of avian influenza(AI) and assessment of pathogenicity of avian H5 and H7 subtypes by molecular methods. Dev. Biol. 126, 171-177.

Shaw AE, Reid SM, King DP, Hutchings GH, Ferris NP. (2004) Enhanced laboratory diagnosis of foot and mouth disease by real-time polymerase chain reaction. Rev Sci Tech. 23(3):1003-9.

Shaw, A., Monaghan, P., Alpar, H. O., Anthony, S., Darpel, K. E., Batten, C. A., Carpenter, S., Jones, H., Oura, C. A. L., King, D. P., Elliott, H., Mellor, P. S. & Mertens, P. P. C. (2007). Development and validation of a real-time RT-PCR assay to detect genome bluetongue virus segment 1. J. Virol. Meth. (in press).

Slomka, M., 2006. Ring trial of molecular detection/characterisation methods for avian influenza. In: "Joint Twelfth Annual Meeting of the National Reference Laboratories for Newcastle Disease and Avian Influenza of European Union Member States 2006." http://ec.europa.eu/food/animal/diseases/controlmeasures/avian/docs/jam_nwd_ailc_2006_en.pdf

Slomka, M., 2007. Interlaboratory ring trial of molecular detection / characterisation methods for AI. In: "Joint Thirteenth Annual Meeting of the National Reference Laboratories for Newcastle Disease and Avian Influenza of European Union Member States 2007."

ACCEPTED MANUSCRIPT

http://ec.europa.eu/food/animal/diseases/controlmeasures/avian/docs/programme13th_2007.pdf

Slomka, M., 2008. The third pan-European AI PCR Proficiency Panel. In: "Joint Fourteenth Annual Meetings of the National Laboratories for Avian Influenza and Newcastle Disease of European Union Member States 2008." http://ec.europa.eu/food/animal/diseases/controlmeasures/avian/docs/programme14th_2008.pdf

Slomka, M.J., Voermans, J., 2007. Development and validation of H7 RealTime RT-PCR._In: "Joint Thirteenth Annual Meeting of the National Reference Laboratories for Newcastle Disease and Avian Influenza of European Union Member States 2007." http://ec.europa.eu/food/animal/diseases/controlmeasures/avian/docs/programme13th_2007.pdf

Slomka, M.J, Coward, V.J., Banks, J., Löndt, B.Z., Brown, I.H, Voermans, J., Koch, G., Handberg, K.J., Jørgensen, P.H., Cherbonnel-Pansart, M., Jestin, V., Cattoli, G., Capua, I., Ejdersund, A., Thorén, P., Czifra, G., 2007a. Identification of sensitive and specific avian influenza PCR methods through blind ring trials in the European Union. Avian Dis. 51, 227-234.

Slomka, M. J., Pavlidis, T., Banks, J., Shell, W., McNally, A., Essen, S., Brown, I. H., 2007b. Validated H5 Eurasian real-time reverse transcriptase–polymerase chain reaction and its application in H5N1 outbreaks in 2005–2006. Avian Dis. 51, 373-377.

Smith, G.J.D, Fan, X.H, Wang, J., Li, K.S., Qin, K., Zhang, J.X., Vijaykrishna, D., Cheung, C.L., Huang, K., Rayner, J.M., Peiris, J.S.M., Chen, H., Webster, R.G., Guan, Y., 2006. Emergence and predominance of an H5N1 influenza variant in China. Proc. Natl. Acad. Sci. U S A 103, 16936-16941.

Spackman, E., Senne, D. A., Myers, T. J., Bulaga, L. L., Garber, L. P., Perdue, M. L., Lohman, K., Daum, L. T., Suarez, D. L., 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 haemagglutinin subtypes. J. Clin. Microbiol. 40, 3256–3260.

Stranska, R., Schuurman, R., de Vos, M., van Loon, A.M. (2004) Routine use of a highly automated and internally controlled real-time PCR assay for the diagnosis of herpes simplex and varicella-zoster virus infections. J. Clin. Virol. 30, 39–44.

Suarez, D.L., Das, A., Ellis, E., 2007. Review of rapid molecular diagnostic tools for avian influenza virus. Avian Dis. 51, 201-208.

Swayne, D.E, Akey, B.L, 2005. Avian influenza control strategies in the United States of America. In: "Avian Influenza: Prevention and Control", eds: Shrijver, R.S, Koch, G., pp113-130, Springer.

Swayne, D. E., Suarez, D. L., 2000. Highly pathogenic avian influenza. Rev. Sci. Tech. 19, 463–482.

Tan SW, Omar AR, Aini I, Yusoff K, Tan WS. Detection of Newcastle disease virus using a SYBR Green I real time polymerase chain reaction. Acta Virol. 2004;48(1):23-8.

Terregino, C., De Nardi, R., Guberti, V., Scremin, M., Raffini, E., Martin, A.M., Cattoli, G., Bonfanti, L., Capua, I., 2007. Active surveillance for avian influenza

ACCEPTED MANUSCRIPT

viruses in wild birds and backyard flocks in Northern Italy during 2004 to 2006. Avian Path. 36, 337-344.

Thelwell, N., Millington, S., Solinas, A., Booth, J., Brown, T. (2000) Mode of action and application of Scorpion primers to mutation detection. Nucleic Acids Res 28(19):3752-61.

Toussaint, J.F., Sailleau, C., Breard, E., Zientara, S., De Clercq, K. (2007) Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segments. J Virol Methods. 140(1-2):115-23.

Tsai HJ, Chang KH, Tseng CH, Frost KM, Manvell RJ, Alexander DJ. Antigenic and genotypical characterization of Newcastle disease viruses isolated in Taiwan between 1969 and 1996. Vet Microbiol. 2004 Nov 30;104(1-2):19-30.

Tseng, S.Y., Macool, D., Elliott, V., Tice, G., Jackson, R., Barbour, M., Amorese, D. (1997) An homogeneous fluorescence polymerase chain reaction assay to identify Salmonella. Anal Biochem 245(2):207-12.

Tyagi, S., Kramer, F.R. (1996) Molecular beacons: probes that fluoresce upon hybridization. Nat Biotechnol 14(3):303-8.

Tyagi, S., Bratu, D.P., Kramer, F.R. (1998) Multicolor molecular beacons for allele discrimination. Nat Biotechnol 16(1):49-53.

Uttenthal, A., Storgaard, T., Oleksiewicz, M.B., de Stricker, K., 2003. Experimental infection with the Paderborn isolate of classical swine fever virus in 10-week-old pigs: determination of viral replication kinetics by quantitative RT-PCR, virus isolation and antigen ELISA. Vet. Microbiol. 92, 197–212.

Van Borm, S., Steensels, M., Ferreira, H.L, Boschmans, M., De Vriese, J., Lambrecht, B., van den Berg, T., 2007. A universal avian endogenous Real-Time reverse transcriptase-polymerase chain reaction control and its application to avian influenza diagnosis and quantification. Avian Dis. 51, 213-220.

Vangrysperre W, De Clercq K (1996) Rapid and sensitive polymerase chain reaction based detection and typing of foot-and-mouth disease virus in clinical samples and cell culture isolates, combined with a simultaneous differentiation with other genomically and/or symptomatically related viruses. Arch. Virol. 141, 331-344.

Van Niekerk, M., Freeman, M., Paweska, J.T., Howell, P.G., Guthrie, A.J., Potgieter, A.C., van Staden, V., Huismans, H., 2003. Variation in the NS3 gene and protein in South African isolates of bluetongue and equine encephalosis viruses. J. Gen. Virol. 84 (Pt 3), 581-90.

Vet, J.A., Van der Rijt, B.J., Blom, H.J. (2002) Molecular beacons: colorful analysis of nucleic acids. Expert Rev Mol Diagn 2(1):77-86.

Vilcek, S., Herring, A.J., Nettleton, P.F., Lowings, J.P., Paton, D.J. (1994) Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. Arch Virol 136:309–23.

Walton, T.E., 2004. The history of bluetongue and a current global overview. Vet. Ital. 40, 31-38.

Wengler, G., D. W. Bradley, M. S. Colett, F. X. Heinz, R. W. Schlesinger, and J. H. Strauss. 1995. Flaviviridae, p. 415–427. *In* F. A. Murphy, C. M. Fauquet, D. H. L.

ACCEPTED MANUSCRIPT

Bishop, S. A. Ghabrial, A. W. Jarbis, G. P. Martelli, M. A. Mayo, and M. D. Summers (ed.), Virus taxonomy. Sixth Report of the International Committee on Taxonomy of Viruses. Springer-Verlag, New York, N.Y.

Wallensten, A., Munster, V.J., Latorre-Margalef, N., Brytting, M., Elmberg, J., Fouchier, R.A., Fransson, T., Haemig, P.D., Karlsson, M., Lundkvist, A., Osterhaus, A.D., Stervander, M., Waldenström, J., Björn, O., 2007. Surveillance of influenza A virus in migratory waterfowl in northern Europe. Emerg. Infect. Dis. 13, 404-411.

Webster, R.G., Peiris, M., Chen, H., Guan, Y., 2006. H5N1 outbreaks and enzootic influenza. Emerging Inf. Dis. 12, 3-8.

West, D.M., Sprigings, K.A-, Cassar, C., Wakeley, P.R., Sawyer, J., Davies, R.H. (2007) Rapid detection of Escherichia coli virulence factor genes using multiplex real-time TaqMan((R)) PCR assays. Vet Microbiol. Feb 4; (Epub)

Wilson, W.C., Stallknecht, D.E., Mecham, J.O., 2004. Field–deployable real-time polymerase chain reaction detection of bluetongue and epizootic haemorrhagic disease viral ribonucleic acid. Vet. Ital. 40 (4), 587-593.

Wirz, B., J.-D. Tratchin, H. K. Muller, and D. B. Mitchell. 1993. Detection of hog cholera virus and differentiation from other pestiviruses by polymerase chain reaction. J. Clin. Microbiol. 31:1148–1154.

Wittwer, C.T., Herrmann, M.G., Gundry, C.N., Elenitoba-Johnson, K.S. (2001) Real-time multiplex PCR assays. Methods 25, 430– 442

Wittwer, C.T., Herrmann, M.G, Moss, A.A., Rasmussen, R.P. (1997a) Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques. 22(1):130-1, 134-8.

Wittwer, C.T., Ririe, K.M., Andrew, R.V., David, D.A., Gundry, R.A., Balis, U.J. (1997b) The LightCycler: a microvolume multisample fluorimeter with rapid temperature control. Biotechniques 22(1):176-81.

WHO (World Health Organization) Global Influenza Program Surveillance Network, 2005. Evolution of H5N1 avian influenza viruses in Asia. Emerg Infect Dis. 11,1515–21.

Whitcombe, D., Theaker, J., Guy, S.P., Brown, T., Little, S. (1999) Detection of PCR products using self-probing amplicons and fluorescence. Nat Biotechnol 17(8):804-7.

Wise MG, Suarez DL, Seal BS, Pedersen JC, Senne DA, King DJ, Kapczynski DR, Spackman E. Development of a real-time reverse-transcription PCR for detection of newcastle disease virus RNA in clinical samples. J Clin Microbiol. 2004 Jan;42(1):329-38.

Wood, G. W., Parsons, G., Alexander, D. J., 1995. Replication of influenza A viruses of high and low pathogenicity for chickens at different sites in chickens and ducks following intranasal inoculation. Avian Pathol. 24, 545–551.

Wu, C., Cheng, X., He, J., Lv, X., Wang, J., Deng, R., Long, Q., Wang, X. (2008). A multiplex real-time RT-PCR for detection and identification of influenza virus types A and B and subtypes H5 and N1. J. Virol. Methods. 148:81-8

Wu, P., Brand, L. (1994) Resonance energy transfer: methods and applications. Anal Biochem 218(1):1-13

ACCEPTED MANUSCRIPT

Zhao JJ, Cheng D, Li N, Sun Y, Shi Z, Zhu QH, Tu C, Tong GZ, Qiu HJ.Evaluation of a multiplex real-time RT-PCR for quantitative and differential detection of wild-type viruses and C-strain vaccine of Classical swine fever virus. Vet Microbiol. 2008 Jan 1; 126(1-3):1-10.

Zou J, Shan S, Yao N, Gong Z. Complete genome sequence and biological characterizations of a novel goose paramyxovirus-SF02 isolated in China. Virus Genes. 2005 Jan;30(1):13-21.

Zientara, S., Breard, E., Sailleau, C., 2004. Bluetongue diagnosis by reverse transcriptase-polymerase chain reaction. Vet. Ital, 40 (4), 531-537.

Accepted Manuscript

ACCEPTED MANUSCRIPT

Figure legends

Figure 1: Location of pan-serotype rRT-PCR targets within the 5' UTR (untranslated region) and 3D (RNA polymerase) genes of the FMDV genome. Boxes represent sites recognised by the primers and probes for the respective assays with * denoting the location of redundant bases which have been incorporated into the design of primers to accommodate mismatches. The bar-plots show corresponding variability at each nucleotide position assessed from analysis of 122 full genomic sequences of FMDV.

Figure 2: Diagram showing relative positions of AI PCR amplicons within different AIV ORFs. rRT PCR amplicons are shown as unbroken horizontal lines for: (1) M gene: a (99 bp size, Spackman et al 2002) and b (147bp, Di Trani et al 2006). (2) H5: c (*ca* 120-150bp, depending on precise CS size, Hoffmann et al 2007), d (152bp, Spackman et al 2002 and Slomka et al 2007b) and e (61bp, Monne et al 2008). (3) H7: f (*ca* 270-290bp) and g (132bp), both Slomka & Voermans (2007); h (98bp, Spackman et al 2002) and i (62bp, Monne et al 2008). Conventional PCR amplicons assessed by Slomka et al (2007a) are shown as broken horizontal lines and indicated by italic letters for: (2) H5: j (*ca* 300-330bp, KHA primers) and k (*ca* 300-330bp, J3/B2a primers). (3) H7: l (*ca* 200-230bp, GK primers).

Accepted Manuscript

ACCEPTED MANUSCRIPT

Table 1: Published real-time RT-PCR assays for the pan-serotype detection of FMDV

Forward primer (5' – 3')	Reverse primer (5' – 3')	Probe (5' – 3')	Genomic target	Reference
Pan-serotypic TaqMan® rRT-PCR assays				
CACYTYAAGRTGACAYTGRTACTGGTAC	CAGATYCCRAGTGWCICITGTTA	CCTCGGGGTACCTGAAGGGCATCC	IRES	Reid et al., 2002
ACTGGGTTTTACAAACCTGTGA	GCGAGTCCTGCCACGGA	TCCTTTGCACGCCGTGGGAC	3D	Callahan et al., 2002
TGTGTGCAACCCCAGCAC	CGAGTGTCGCRTGTTACC	ACAGGCTAAGGATGCCCTTCAGGTACC	IRES	Oem et al., 2005
AACACGCYGTSTTYGCSTG	GCGTCCAKGGGTARAAGTC	ACCTCCAACGGGTGGTACGCGAT	Leader	Oem et al., 2005
AGATGCAGGARGACATGTCAA	TTGTACCAGGGYTTGGCYT	AAACACGGACCCGACTTTAACCG	2B	Oem et al., 2005
ACTGGGTTTTAYAAACCTGTGATG	TCAACTTCTCCTGKATGGTCCCA	ATCCTCTCCTTTGCACGC [a]	3D	Moniwa et al., 2007
Alternative pan-reactive fluorogenic rRT-PCR formats				
TGGGTTTTACAAACCTGTGATG	CGCAGGTAAAGTGATCTGTAGC	TCTCCTGTATGGTCCCACGGCGTG [b]	3D	Moonen et al., 2003
		GCGAGTCCTGCCACGGAGATCAAC [b]		
GACAAAGGTTTTGTTCTTGGTCA	TGCGAGTCCTGCCACGGA	ATCCTCTCCTTTGCACGCCGTGGGACCAT	3D	Rasmussen et al., 2003 [c]

[a] Minor groove binder probe (MGB)
[b] Dual hybridisation probes
[c] Primer probe-energy transfer (PriProET) assay: reverse primer is labelled at the 5' end with FAM and the probe is labelled at the 3' end with CY5

ACCEPTED MANUSCRIPT

2002 **Table 2: Published real-time RT-PCR assays used for detection of CSFV genome**

Literature	Primer/ probe name	Sequence 5' → 3'	Genome position [a]	fragment size	internal control
Hoffmann et al., 2005 Depner et al., 2006	CSF 100-F CSF 192-R CSF-Probe 1	ATG CCC AYA GTA GGA CTA GCA CTA CTG ACG ACT GTC CTG TAC *FAM*-TGG CGA GCT CCC TGG GTG GTC TAA GT-*TAMRA*	100–120 192–172 141–166	93 bp	*in vitro*-transcribed EGFP-RNA
Risatti et al., 2003 Deng et al., 2005 Ophuis et al., 2006	forward reverse probe	CCCTGGGTGGTCTAAG CATGCCCTCGTCCAC *FAM*-CCTGAGTACAGGACAGTCGTCAGTAGTT-*TAMRA*	150-165 242-228 167-194	93 bp	no internal control 18s rRNA
McGoldrick et al., 1998 Vilcek et al., 1994	V324 forward V326 reverse A11-forward A14-reverse probe	1. Panpesti RT-PCR: ATG CCC ATA GTA GGA CTA GCA TCA ACT CCA TGT GCC ATG TAC 2. CSFV specific fluorogenic nested PCR: AGT ACA GGG TAG TCG TCA GTG GTT CG CTG CTG TAC ATG GCA CAT GGA GTT G *FAM*-TGA TGG GGG TAC GAC CTG ATA GGG T-*TAMRA*	100-120 383-363 171-196 382-358 296-320	284bp 212 bp	no internal control
Rassmussen et al., 2007 Uttenthal et al., 2003	CSFV6 forward CSFV7 reverse probe	CTA GCC ATG CCC IYA GTA GGA CTC CAT GTG CCA TGT ACA GCA *FAM*-CCC TGG GTG GTC TAA GTC CTG AGT ACA G-*TAMRA*	94-114 379-359 150-177	286 bp	no internal control
Le Potier et al., 2006; Le Dimna et al., 2006	not stated	not stated, commercially available kits: TaqVet CSF (LSI), Adiavet CSF (ADIAGENE)	not stated	not stated	housekeeping gene: GAPDH
Jamnikar Ciglenečki et al., 2008	CSFV primer B-L CSFV primer C-U CSFV-Probe	GGA CAG TCG TCA GTA GTT CG CTG CAG CAC CCT ATC AGG TC *FAM*-CAG GTC GTA CYC CCA TCA-*MGB*	177-196 327-308 313-296	151 bp	no internal control
Liu et al. 2007	CSF145F CSF242R TaqMan probe	GAG CTC CCT GGG TGG TCT AAG T CAT GCC CTC GTC CAC RTA GC *FAM*-ACG TCG AAC TAC TGA CGA CTG TCC TGT ACT CA-*BHQ1*	145-166 242-223 200-169	98 bp	no internal control
Zhao et al. 2008	CSFV-F CSFV-R FAM-CSFV-v111 TET-CSFV-a110	GAA CTG GGC TAG CCA TG ACT GTC CTG TAC TCA GGA C *FAM*-AGG ACT AGC AAA CGG AGG GAC TAG CCG-*TAMRA* *TET*-TAG GAC TAG CAA AAC GGA GGG ACT AGC CA-*TAMRA*	86-102 183-164 111-137 110-138[b]	98 bp (99 bp for 'Chinese' C strain)	no internal control

2003 [a] Genome position according to CSFV Alfort/ 187 (Accession No.: X87939).
2004 [b] Genome position according to CSFV 'Chinese' C strain (Accession No.: Z46258).

ACCEPTED MANUSCRIPT

2005

2006 **Table 3: Published real-time RT-PCR assays for the detection of bluetongue virus**

PCR Target	Detection method	Reference	Strain Identification
BTV Segment 1 (VP1)	duplex RT-PCR	Shaw et al., 2007	Representatives from all 24 serotypes, and multiple field strains/topotypes. Able to differentiate between 'eastern' and 'western' strains of BTV.
BTV-Segment 1 (VP1)	One step RT-PCR	Toussaint et al., 2007	Representatives from all 24 serotypes and field strains from Mediterranean basin.
BTV Segment 5 (NS1)	Two step RT-PCR	Toussaint et al., 2007	Representatives from all 24 serotypes and field strains from Mediterranean basin.
BTV Segment 5 (NS1)	One step RT-PCR	Polci et al., 2007	Representative from serotypes 2, 4, 9 and 16 isolated in Italy and their respective vaccine strains.
BTV Segment 5 (NS1)	One step RT- PCR	Jiminez-Clavero et al., 2006	Representatives from 17 serotypes (BTV – 1, 2, 3, 4, 5, 6, 8, 9, 11, 12, 14, 15, 16, 17, 18, 22, 23)
BTV Segment 5 (NS1)	One step RT-PCR	Wilson et al., 2004	Representatives from 11 serotypes (BTV-1, 5 ,6, 7, 8, 10, 11, 13, 17, 18, 19)
BTV Segment 10 (NS3)	MB fluorescent probe	Orrù et al., 2006	Representatives from 10 serotypes (BTV-1, 2, 3, 4, 5, 6, 7, 8, 9, 16)
BTV Segment 2 (VP2)	FRET RT- PCR	De Santis et al., 2004 Orrù et al., 2004.	Differentiates field and vaccine strain of BTV-2

2007 MB – Molecular Beacon
2008 FRET - Fluorescence Resonance Energy Transfer

Accepted Manuscript

ACCEPTED MANUSCRIPT

2009

2010 **Table 4: Published real-time RT-PCR assays used for detection of NDV genome**

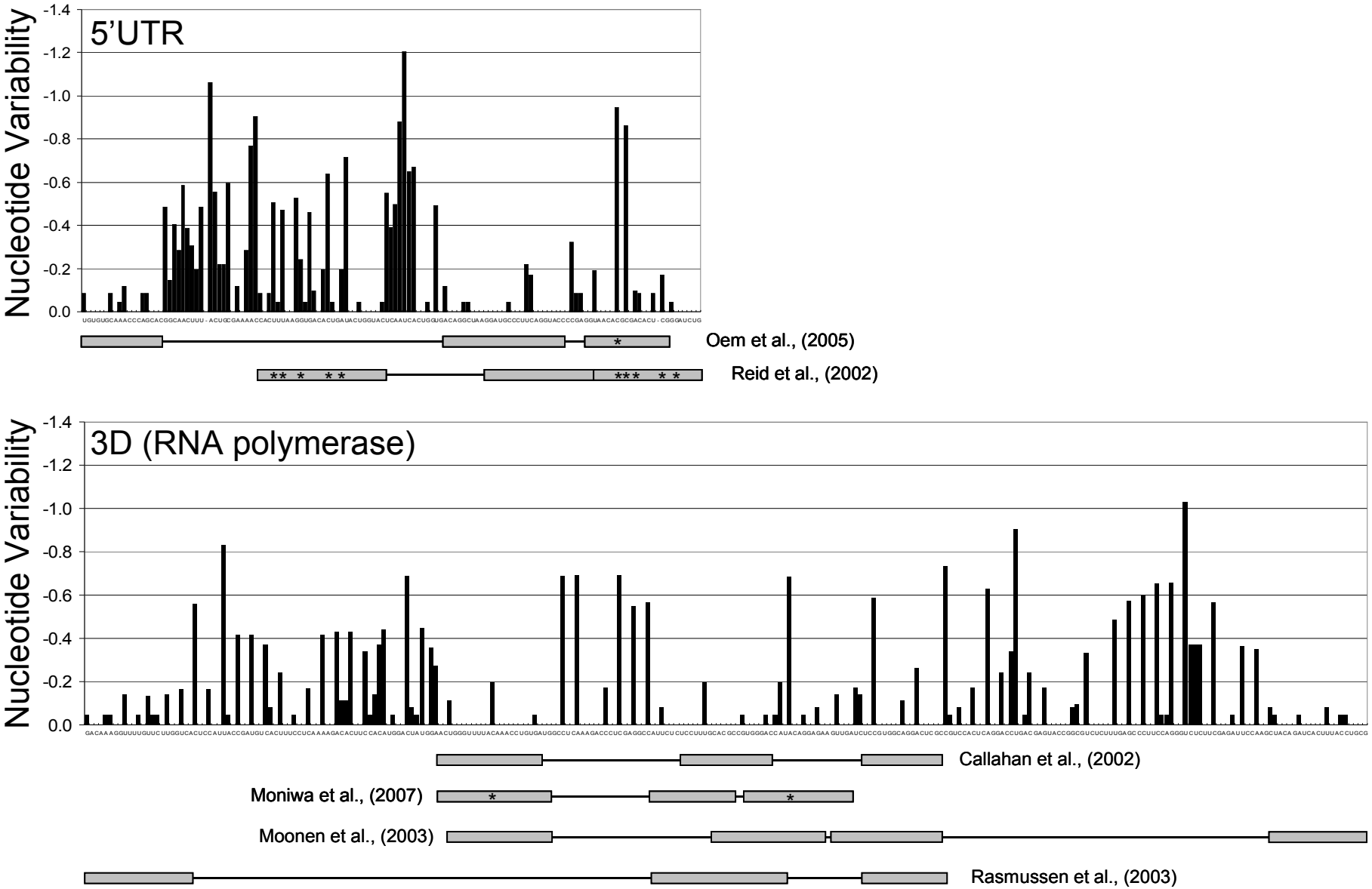
Literature	Primer/ probe name	Sequence 5' → 3'	fragment size	target
Aldous et al., 2001	taqmsf1 not stated D 1V 3A 9V 10V 11V 12A	GACCGCTGACCACGAGGTTA CGCAGCATTCTGGTTGGCTTGTATCA GGCAGCATTTTGTTTGGCTTGTATC *FAM*-AAGCGTTTCTGTCTCCTTCCTCCG-*TAMRA* *FAM*-AGACGTCCCTGTTTCCCTCCTCC-*TAMRA* *FAM*-AAACGTTTCTGTCTCCTTCCTCCGG-*TAMRA* *FAM*-AAACGTCTCTGTCTCCTTCCTCCGG-*TAMRA* *FAM*-AAGCGTTTCTGCCTCCCTCCTCC-*TAMRA* *FAM*-CCTATAAGGCGCCCCTGTCTCCC-*TAMRA*	not stated	Fusion gene
Wise et al., 2004	M+4100 M+6169 M-4220	AGTGATGTGCTCGGACCTTC *FAM*-TTCTCTAGCAGTGGGACAGCCTGC-*TAMRA* CCTGAGGAGAGGCATTTGCTA	121 bp	Matrix gene
Wise et al., 2004	F+4839 F+4894 F-4939	TCCGGAGGATACAAGGGTCT *FAM*-AAGCGTTTCTGTCTCCTTCCTCCA-*TAMRA* AGCTGTTGCAACCCAAG	101 bp	Fusion gene (velo & meso)
Wise et al., 2004	M+4213 M-4268 M-4350	TCCTCAGGTGGCCAAGATAC *FAM*-TTTTAACGCTCCGCAGGCAC-*TAMRA* TGCCCCTTCTCCAGCTTAGT	138 bp	Matrix gene (pre-1960)
Kim et al., 2005/2007	L-8784 L-8868 L+8817	CGTTCTGAGGAATTTGACAGYMT GRAGCCATGCGAAYTTGG *FAM*-CCGGCATTCTGTTTCACTCAA-3BHQ_1	85 bp	L-gene

2011

2012

ACCEPTED MANUSCRIPT

Figure 1

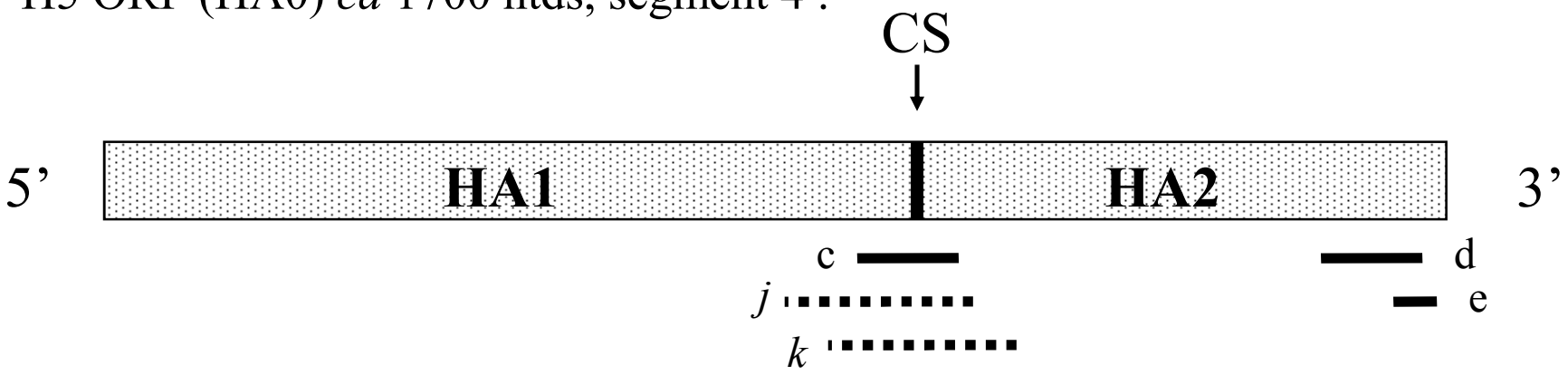
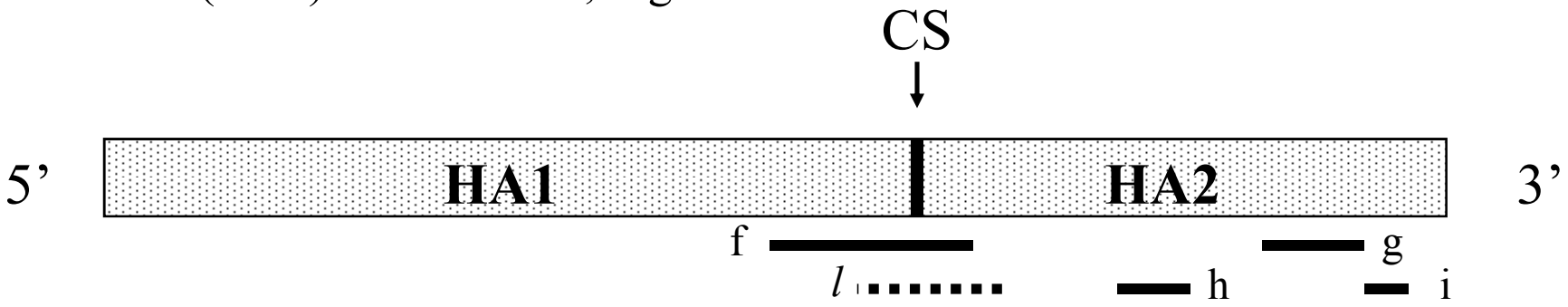


ACCEPTED MANUSCRIPT

Figure 2

1) M ORF *ca* 750 ntds, segment 7:

2) H5 ORF (HA0) *ca* 1700 ntds, segment 4 :



3) H7 ORF (HA0) *ca* 1700 ntds, segment 4 :