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2 **A review of RT-PCR technologies used in veterinary virology and**
3 **disease control: sensitive and specific diagnosis of five livestock**
4 **diseases notifiable to the World Organisation for Animal Health**
5

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33

34 **Abstract:**

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

51

52

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

55

56

57 **1. Real-time PCR technology**

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

81
82 The development of multi-colour real-time PCR cyclers and “ready-to-use”
83 commercial multiplex real-time PCR kits has also made it possible to combine up to
84 four assays within a single tube. Multiplex-PCR is the simultaneous amplification of
85 two or more DNA (or cDNA) targets in a single reaction vessel and can only be
86 carried out using uniquely-labelled probes for each target sequence. Typically, up to
87 four target sequences can be detected in a multiplex 5' nuclease assay when the
88 appropriate instrument and the right combination of fluorophores are used. Major
89 advantages of multiplexing include a reduced sample requirement, which is especially
90 important when sample material is scarce (Persson et al. 2005, Belak; 2007), and the

91 ability to combine assays with an internal control system (Hoffmann et al., 2006).

92 However, it is important to optimize these assays in order to limit competitive

93 interactions that may significantly impact upon assay sensitivity.

94

95 The combined properties of high sensitivity and specificity, low contamination risk,
96 and speed has made real-time PCR technology a highly attractive alternative to tissue

97 culture- or immunoassay-based methods for diagnosing many infectious diseases

98 (Espy et al., 2006). Clinical diagnostic applications and the use of real-time PCRs are

99 growing exponentially, and real-time PCR is rapidly replacing conventional PCR and

100 other established diagnostic methods such as antigen-ELISA and cell culture isolation.

101 More general information is provided in a series of review articles (Mackay et al.,

102 2002; Mackay, 2004; Bustin, 2000, 2002, 2005; Clementi et al., 1995; Niesters, 2002,

103 2004; Ratcliff et al., 2007; Gunson et al., 2006; Espy et al., 2006; Kaltenboeck and

104 Wang, 2005; Kubista et al, 2006). This review considers the current scope and

105 capability of this powerful technique, especially when detecting important notifiable

106 viral pathogens of livestock such as foot-and-mouth disease virus (FMDV), classical

107 swine fever virus (CSFV), bluetongue virus (BTV), avian influenza virus (AIV) and

108 Newcastle disease virus (NDV).

109

110 **1.1 Detection formats**

111

112 *Detection formats without sequence confirmation of the PCR product*

113 Fluorescent dyes such as ethidium bromide (Higuchi et al., 1993; Wittwer et al.,

114 1997a; LePecq, 1966) that are specific for double-stranded DNA (dsDNA) were the

115 first systems employed in real-time PCR assays. Other intercalating dyes such as YO-

116 PRO-1 have also been used (Ishiguro et al., 1995; Tseng et al., 1997). SYBR Green I

117 is currently the most frequently used intercalating dye in real-time PCR. It has a 100

118 times higher binding affinity than ethidium bromide, and the fluorescence of bound

119 dye is more than 1000-fold higher than that of free dye. These properties make SYBR

120 Green I highly suitable for monitoring product accumulation during PCR (Wittwer et

121 al., 1997b; Morrison et al., 1998). An often-stated disadvantage of this format using

122 intercalating dyes, is that non-specific dsDNA products, such as primer dimers can

123 interfere with the assay signal and yield false positive results. Therefore, it is

124 important that the assay is optimized to avoid non-specific amplification products.

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

127

128 Another detection technology known as 'LUX (Light Upon eXtension)' utilizes a
129 modification to one of the two primers such that it possesses a fluorophore located
130 near the 3' end in a hairpin structure (Nazarenko, 2006; Kusser, 2006).

131 The Amplifluor Quantitative PCR detection system uses a similar approach with
132 labelled and unlabelled primers (Nazarenko et al., 1997; Nuovo et al., 1999; Khripin,
133 2006). Both of these systems are described as highly flexible and beneficial, but have
134 not been frequently exploited for diagnostic tests.

135

136 *Detection formats with increased target specificity*

137 The incorporation of a specific probe into real-time PCR confirms the specificity of
138 the amplicon. Fluorophore-labelled oligonucleotide probes are most commonly used
139 for the specific detection of target sequences (Cardullo et al., 1988; Clegg, 1995; Wu
140 and Brand, 1994). In these assays, an increase in fluorescence signal proportional to
141 the accumulation of PCR product arises as a consequence of fluorescence resonance
142 energy transfer (FRET) between separate fluorogenic labels (known as the reporter
143 and quencher) conjugated to the probe (or primers). FRET, also called Förster
144 transfer, is a spectroscopic process by which energy is passed over a maximum
145 distance of 70 Å between reporter and acceptor molecules possessing overlapping
146 emission and absorption spectra (Selvin and Hearst, 1994). For real-time PCR, several
147 formats have been used: however, most assays exploit a decrease in quench
148 (compared to an increase in FRET). The most commonly used fluorogenic quenchers
149 are TAMRA and DABCYL, while Black Hole Quencher (BHQ) is also widely used
150 and disperses energy from the reporter as heat rather than fluorescence (Didenko,
151 2001).

152

153 The most widely used detection chemistries are briefly reviewed below.

154

155 *Hybridisation probes*

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

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

162

163 *Hydrolysis probes (5'-exonuclease assay)*

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

181

182 *Molecular beacons*

183 Molecular beacons are hairpin-shaped oligoprobes terminally labelled with a reporter
184 and a quencher fluorophore (Tyagi and Kramer, 1996; Tyagi et al., 1998; Vet et al.,
185 2002).

186

187 *Scorpion primer*

188 The Scorpion technology is mainly used in allelic discrimination (Whitcombe et al.,
189 1999; Thelwel et al., 2000) and in SNP genotyping (Roberts, 2000).

190

191 *Locked nucleic acid (LNA) probes*

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

195

196 **1.2 Internal controls**

197 The use of an internal control (IC) is an important aspect of quality control. An IC is
198 necessary for ensuring adequate efficiency of RNA extraction and confirming the
199 absence of PCR inhibitors in each sample, thereby avoiding false negative results
200 (Belak and Thoren, 2001). An IC is a valuable tool when testing nucleic acids
201 extracted from potentially difficult biological matrices (such as degraded samples)
202 where the presence of PCR-inhibitors could be problematic. In these cases, co-
203 amplification of an IC increases the reliability of the results and is used to validate
204 negative results. In practice, different IC systems can be used. One approach is to
205 detect an endogenous gene that occurs naturally in the test specimen. To be a reliable
206 indicator of assay performance, the gene selected should exhibit a constant, basal cell
207 cycle-independent level of transcription that is not influenced by the cellular
208 pathology associated with the disease targeted by the real-time assay in question.
209 Genes fulfilling these criteria are commonly known as housekeeping genes. Those
210 used most often include glyceraldehydes-3-phosphate (GADPH), β -actin, 18S
211 ribosomal RNA, glutamate decarboxylase (GAD), and β_2 -microglobulin (Oleksiewicz
212 et al., 2001; Korimbocus et al., 2002; Gorzelniak et al., 2001; Hüsey et al., 2001; Kim
213 and Dubovi, 2003; Huggett et al., 2005; Kompalic-Cristo et al., 2007; West et al.,
214 2007; Müller et al., 2007; Toussaint et al., 2007). In contrast to an endogenous IC, an
215 exogenous IC does not occur naturally within the nucleic acid preparation. This type
216 of IC can be designed to contain an all-purpose heterologous target sequence
217 (Hoffmann et al., 2006; Olson et al., 2004; Drosten et al., 2001) or a complete
218 heterologous viral genome (Niesters, 2004; Stranska et al., 2004), unrelated to the
219 sequence to be detected. To avoid apparent false-negative results, exogenous ICs can
220 be added to each test sample before either the template purification step or prior to co-
221 amplification in the real-time PCR. Depending upon the particular design of these
222 ICs, amplification of the control sequences may require that an additional set of
223 primer pairs are included in the reaction. Alternatively, 'mimic' ICs (*in-vitro*
224 transcripts, plasmids or chimeric viruses) can be engineered containing homologous
225 target sequences for the same primer pair as the diagnostic test. In addition, these

226 mimic ICs can contain internal sequences not present in the target amplicon to yield
227 an amplification product with a different length (Belak and Thoren, 2001), which can
228 be differentiated from wild-type amplicon by a second, IC-specific probe (King et al.,
229 2003; Hofmann, 2003; Hodgson et al., 2007; Fedele et al., 2006; King et al., 2007).
230 Since these controls are amplified using the assay target-specific primers, a benefit of
231 this type of control is that it directly monitors the performance of diagnostic primers
232 set ensuring that the correct PCR components have been added. However, in contrast
233 to the previously-described exogenous ICs, these target-specific control systems
234 cannot be transferred to other assays, although a cassette approach can be designed to
235 incorporate primer sequences of multiple diagnostic tests (King et al., 2007).

236

237 **1.3 Multiplexing**

238 In most cases the detection of co-amplified PCR products in one tube (multiplexing)
239 were performed by hybridising probes and hydrolysis probes. Hybridising probes use
240 the different melting points for differentiation of amplicons (Olson et al., 2004). On
241 the other hand different emission spectra (colours) of sequence specific probes
242 (*TaqMan*[®] or Hyb probes) allow also the detection of multiple sequences in a single
243 reaction tube (Dupont et al., 2002; Wittwer et al., 2001). Some real-time PCR
244 machines enable the detection and differentiation of up to 6 colours. This feature of
245 real-time PCR becomes more and more important since it allows the cost-effective
246 answer to several diagnostic questions within one sample in one reaction like agent
247 detection together with sub-typing or the combined detection of several different
248 pathogens (Gunson et al., 2008). Examples are established multiplex assays for the
249 detection and sub-typing of influenza viruses (Wu et al., 2008; Payungporn et al.,
250 2006) or the differentiation of Porcine circovirus-2 genotypes (Gagnon et al., 2008).

251

252 **1.4 Experiences of real-time PCR deployment in different laboratories**

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

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

274

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

276

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

292

293 Control of outbreaks is dependent upon early recognition of infected animals, which
294 requires familiarity with clinical signs of FMD and the ability to accurately and
295 rapidly detect FMDV in clinical samples using laboratory tests. Of the established
296 diagnostic approaches, virus isolation (VI) in cell culture is considered to be the “gold
297 standard”. This method can be highly sensitive (depending upon the cell culture
298 system used), although it can be slow taking between 1-4 days to generate a result.
299 Other assays such as antigen-detection ELISA are more rapid, but have lower
300 analytical sensitivity and are inappropriate for use with certain sample types. It is now
301 recognised that RT-PCR assays can play an important role for the rapid and sensitive
302 detection of FMDV in a wide range of clinical sample types. Over the past decade,
303 improvements have been made to RT-PCR protocols for the detection of FMDV that
304 have paralleled the evolution of molecular diagnostics. Initially, assays utilising
305 agarose-gel electrophoresis for the detection of amplified products and targeting
306 conserved regions of the genome (3D: Meyer et al., 1991; Rodríguez et al., 1994 and
307 5' untranslated region [5' UTR], Reid et al., 2000) were developed. However, these
308 labour intensive procedures have a high risk of generating false positives due to carry-
309 over of PCR amplicons and are therefore not ideal for routine testing of large numbers
310 of samples. Recent development of rRT-PCR methodology employing a
311 fluorescently-labelled probe to detect PCR amplicons has allowed the diagnostic
312 potential of molecular assays to be realised. These assays (outlined in Table 1) are
313 highly sensitive and obviate tube opening after amplification thereby reducing the
314 potential for cross-contamination of test samples by post-PCR products. In addition to
315 the widely exploited 5'-nuclease (TaqMan[®]) system using dual-labelled probes (Reid
316 et al., 2002; Callahan et al., 2002; Oem et al., 2005) and modified MGB probes
317 (Mowika et al., 2007), assays have also been developed using other rRT-PCR formats
318 such as those using hybridisation probes (Moonen et al., 2003) and PriProET
319 (Rasmussen et al., 2003). In order to increase assay throughput and minimise operator
320 errors, rRT-PCR assays for FMDV can be automated using robots for nucleic acid
321 extraction and liquid handling equipment to set-up the reaction mixes (Reid et al.,
322 2003; Moonen et al., 2003). Together with the implementation of quality control
323 systems, these improvements have increased the acceptance of the rRT-PCR assays
324 for statutory diagnostic purposes. Although on-going studies continue this work, there
325 is already a wealth of data focusing on different aspects of validation to support the
326 use of rRT-PCR for routine FMD diagnosis:

327

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

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

337

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

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

354

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

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

361 et al., 2002) and 3D region (Callahan et al., 2002). An initial study (Boyle et al., 2004)
362 using a small selection of inactivated viruses has recently been expanded to include a
363 more diverse selection of clinical samples from field cases of FMD (King et al.,
364 2006). There was concordance between the results generated by the two assays for the
365 majority of RNA samples extracted from epithelial tissue obtained from suspect FMD
366 cases. However, the comparison between the two tests highlighted a small number of
367 samples which failed to produce a signal in one assay while giving a positive signal in
368 the other. The sequence of the genomic targets for selected isolates highlighted
369 nucleotide substitutions in the primer and/or probe regions, thereby providing an
370 explanation for these negative results. Individually each rRT-PCR had higher
371 sensitivity than VI. However, the combined use of multiple and independent
372 diagnostic targets could further enhance the sensitivity of molecular methods for the
373 detection of FMDV.

374

375 *(iv) inter-laboratory equivalence testing*

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

385

386 *Future opportunities for RT-PCR diagnosis*

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

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

411

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

413

414 Classical swine fever (CSF) is a highly contagious and often fatal viral disease of pigs
415 and wild boar. The causative agent of CSF, *Classical Swine Fever Virus* (CSFV), is a
416 member of the genus *Pestivirus* within the family *Flaviviridae* (Fauquet et al., 2005).
417 Further members within the genus *Pestivirus* are *Bovine Viral Diarrhoea Virus*
418 (BVDV) and *Border Disease Virus* (BDV). CSFV is an enveloped virus with a 12.5
419 kb single-stranded RNA genome of positive sense (Horzinek, 1991). The genome
420 consists of a single large open reading frame (ORF) which is flanked by highly
421 conserved 5' and 3' non-translated regions (NTR) (Wirz et al., 1993; Boye et al.,
422 1991). The ORF encodes a 4,000-amino acid polyprotein that is co- and post-
423 translationally processed by viral and cellular proteases into 12 polypeptides (Meyers
424 et al., 1996). CSF causes major economic losses, especially in countries with an
425 industrialized pig production system (Horst et al., 1999), and is therefore a disease
426 notifiable to the Office International des Epizooties (OIE, 2006) and in the European
427 Union (Anonymous, 1982).

428

429 Diagnosis of CSF comprises observation of clinical signs in the field, pathological
430 findings and laboratory detection of the virus or virus-specific antibodies. Clinical and
431 pathological signs of CSF are rather variable and unspecific, and the severity of the
432 clinical signs depends on many factors, such as age of the animals, virulence,
433 presence of other pathogens or by host factors (Kaden et al., 2004, Floegel-Niesmann
434 et al., 2003; Mittelholzer et al., 2000; Depner et al., 1997; Kamolsiriprichaiporn et al.,
435 1992a, 1992b). Therefore, laboratory diagnostics of CSF plays a central role in the
436 confirmation of an outbreak, and rapid and precise detection of CSFV is crucial for
437 disease containment. Besides the established virological methods, PCR techniques
438 have become increasingly important for CSF diagnostics during the last decade (Paton
439 et al., 2000), and PCR has been accepted by the European Union as an official method
440 for confirming this disease (Anonymous, 2002). Several rRT-PCR assays for CSF
441 diagnostics have been described (LePotier et al., 2006; Risatti et al., 2003, 2005;
442 Hoffmann et al., 2005; Uttenthal et al., 2003). The benefits of rRT-PCR are described
443 elsewhere in this review (Gibson et al., 1996; Wittwer et al., 2001; Mackay et al.,
444 2002; Mackay, 2004). Depner et al. (2006, 2007) and Schnyder et al. (2002) have
445 highlighted the enhanced sensitivity and specificity of rRT-PCR for CSFV
446 confirmation over the conventional diagnostic methods, such as VI or antigen-
447 detection ELISA. Liu et al. (2007) compared conventional and rRT-PCR assays for
448 the sensitive and specific detection of CSFV. The authors demonstrated identical
449 sensitivities with these strategies but favoured the conventional, gel-based detection
450 method for laboratories without facilities for rRT-PCR assays.

451

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

461

462 Published quantitative rRT-PCRs have also been used to analyse the virus load in
463 blood and tissue samples collected from CSFV-infected pigs (Koenig et al., 2007;
464 Depner et al., 2006, 2007; Ophuis et al., 2006; Risatti et al., 2005, 2003; Uttenthal et
465 al., 2003). Uttenthal et al. (2003) used a SYBR Green assay for quantification but the
466 others favoured a TaqMan[®] format. Jamnikar Ciglencčki et al. (2008) compared a
467 SYBR Green assay with a TaqMan[®] MGB rRT-PCR for the detection of CSFV
468 genomes. While both assays were useful for CSFV detection, the TaqMan MGB
469 version was thought to be more appropriate based on a reduced risk of false positives
470 and the increased speed provided by the one-step format of the assay. Koenig et al.
471 (2007) also used a TaqMan[®] assay to investigate the distribution of a conventional C-
472 strain vaccine and a modified live marker vaccine candidate in pigs. The authors
473 demonstrated the rRT-PCR to be more sensitive than VI even though the viral
474 genomes of both vaccine strains were successfully detected in blood very rarely.
475 Long-term detection of CSFV genomes in tonsils was, however, shown in the same
476 study.

477

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

486

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

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

501

502 Modern real-time PCR cyclers can now measure up to 5 separate reporter
503 fluorophores in one tube. This is a prerequisite for the application of multiplex real-
504 time PCR assays. Beside the co-amplification of one target gene and an IC (Le Dimna
505 et al., 2006, 2007; Le Potier et al., 2006, Hoffmann et al. 2005), different target genes
506 can be detected in a single well (Wu et al., 2007; Legoff et al., 2008). Zho et al.
507 (2008) as well as Leifer et al. (2009) developed a multiplex rRT-PCR assay for the
508 simultaneous detection and differentiation of CSFV field strains and the C-strain
509 vaccine virus.

510

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

515

516 **4. Detection of Bluetongue virus by rRT-PCR**

517

518 Bluetongue disease (BT) is a non-contagious viral disease of domestic and wild
519 ruminants that is transmitted by arthropod vectors of the genus *Culicoides* (biting
520 midges - reviewed by Mellor et al., 2000). Severe BT is primarily seen in sheep and in
521 some species of deer, however significant levels of clinical disease were also seen in
522 cattle during the recent bluetongue virus serotype 8 (BTV-8) outbreak in northern
523 Europe. Bluetongue is endemic in many parts of the world including Africa and
524 regions in the USA, Australia and Asia (Walton, 2004). Since 1998, multiple strains
525 of BTV belonging to five different serotypes (1, 2, 4, 9 and 16) have been circulating
526 in more than 12 countries within the Mediterranean basin (reviewed by Purse et al.,
527 2005), and in August 2006 a strain of BTV-8 was reported in The Netherlands, then
528 soon afterwards in Belgium, Germany, France and Luxembourg. By October 2008
529 BTV-8 had spread into many more countries in Europe including the UK,

530 Switzerland, Spain, Czech Republic, Hungary, Denmark and Sweden. Also since
531 2006 bluetongue virus serotype 1 (BTV-1) spread rapidly from North Africa through
532 Spain and into the south of France. These recent outbreaks of BT in northern Europe
533 have highlighted a need for rapid, high-throughput, sensitive and specific diagnostic
534 assays to detect and identify BTV.

535

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

560

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

563 and related topotypes, but also sufficiently divergent from sequences of the members
564 of other closely related *Orbivirus* species (other ‘serogroups’) to avoid cross-
565 reactions. The degree of variation detected within individual BTV genome segments
566 (e.g. between geographic groups) complicates the selection of appropriate targets.
567 However, several of the more conserved BTV genome segments have previously been
568 used as targets for molecular diagnostic methods, including: genome segment 5,
569 encoding the highly conserved non-structural ‘tubule’ protein NS1 (currently
570 recommended as an RT-PCR target by the OIE (OIE, 2008); genome segment 7
571 (despite its variability), encoding the BTV core surface protein and major BTV virus-
572 species / serogroup-specific antigen, VP7 (Gumm and Newman, 1982; Huisman,
573 1981; Mertens et al., 1987); genome segment 10 (encoding NS3/3a), in which
574 nucleotide variation between gene sequences of up to 20% have been detected (Pierce
575 et al., 1998; van Niekerk et al., 2003) and genome segment 1 encoding VP1, the viral
576 RNA-dependent RNA polymerase, one of the most highly conserved proteins of the
577 reoviruses (members of the family *Reoviridae* - Huang et al., 1995, Mertens, 2004).
578 Many of the conventional RT-PCR assays that have been developed are able to detect
579 BTV RNA in clinical samples (blood and spleen), including those targeting genome
580 segments 2, 3, 6, 7 and 10 (reviewed by Zientara et al., 2004), however, these
581 methods require agar gel electrophoresis to show the amplification of the target
582 sequence, which severely limits the speed of testing.

583

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

597 segment 5 that had various mismatches which reduced the sensitivity of the assay.
598 Moreover, the assay was only able to detect field isolates, but not the reference strain,
599 of BTV-4 and BTV-16 and in the recent northern European outbreak of BTV-8, this
600 assay showed a reduced sensitivity for the detection of the field strain of BTV-8
601 compared to other assays (Batten et al., 2008a). Subsequent studies have indicated
602 that the field strain of BTV-16 from Italy 2002 that was tested was a reassortant virus,
603 containing genome segment 5 derived from a BTV-2 vaccine strain (Batten et al
604 2008b), potentially explaining differences in the sensitivity of the assay for field and
605 reference strains of this serotype. In the same year, a quantitative rRT-PCR was
606 developed using a Molecular Beacon (MB) fluorescent probe designed within the NS3
607 conserved region of segment 10 (Orrù et al., 2006). The MB is a circular
608 oligonucleotide characterised by a high specificity for the chosen target and by its
609 ability to develop a colour-forming reaction when used in a conventional RT-PCR
610 test. This assay successfully detected 10 serotypes (BTV-1, 2, 3, 4, 5, 6, 7, 8, 9, 16)
611 and detected 10^3 molecules/PCR reaction.

612

613 Since the start of the northern European outbreak in August 2006 many rRT-PCR
614 assays have been developed, which are currently being used in many countries across
615 Europe. All the rRT-PCR assays that have been published up to 2008 are described in
616 Table 3. Some assays remain unpublished and, at the time of writing, 3 rRT-PCR
617 assays are published that are able to detect representative strains from all 24 serotypes
618 of BTV. Two of these assays were published in the same manuscript by Toussaint et
619 al., (2007). These two assays detect strains of the different BTV serotypes from the
620 Mediterranean region, as well as prototype strains of all 24 serotypes. Both assays
621 have similar detection limits of below 0.01 ECE₅₀ and detect 100 RNA copies. The
622 primers were designed to target different genomic segments; a one-step procedure,
623 combining the reverse transcription (RT) and the PCR steps, to amplify BTV genome
624 segment 1 and a two-step procedure, with separate RT and PCR reactions, to amplify
625 genome segment 5. These assays have the advantage of a quantitative output which
626 gives an estimate of viral load and both assays include an internal beta-actin control
627 (Toussaint et al., 2007).

628

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

631 and western group viruses respectively. This combined ‘duplex’ assay was evaluated
632 using a wide variety of test samples, including tissue culture derived viruses, infected
633 tissue and blood samples from cattle and sheep and infected *Culicoides* midges. This
634 assay showed no cross-reactions with closely related orbiviruses and gave positive Ct
635 results and amplification plots with all of the viruses tested, including a wide range of
636 129 different BTV isolates derived from different geographical locations (different
637 topotypes), reference strains of all 24 BTV serotypes, and multiple field strains of
638 BTV serotypes 1, 2, 4, 8, 9 and 16 from European outbreaks that have occurred since
639 1998, (although the BTV-9 vaccine and a Sicilian isolate of BTV-9 gave weaker but
640 still positive responses). This assay was able to detect less than 10 template copies per
641 reaction (equivalent to 0.5 TCID₅₀/ml in BHK21 cells). Differences in the sequences
642 of Seg-1 also made it possible to use the two primer sets from the duplex assay,
643 separately, to distinguish viruses of eastern and western origins.

644

645 A one step rRT-PCR method has also been described (Polci et al., 2007) that detects
646 strains of BTV serotypes 2, 4, 9 and 16 isolated in Italy as well as their respective
647 vaccine strains. The limit of detection of the assay fluctuated between 0.005 and 0.05
648 TCID₅₀/ml depending on the virus strain. Preliminary tests also showed that this assay
649 was able to identify serotype 1, which was introduced into Italy towards the end of
650 2006, and also serotypes 8, 13, 14 and 19.

651

652 Given the high variability of BTV and the fact that real-time probes are very sensitive
653 to probe-target mismatches (Jiménez-Clavero et al., 2006), it is important to
654 emphasise that these assays may not be able to recognise all BTV strains and that the
655 sensitivity of the assays may not be the same for every strain. It would thus be wise,
656 as emphasised by Toussaint et al., (2007), to run two methods that amplify different
657 genomic regions in parallel, as this would avoid the risk of missing a mutant, a
658 recombinant or a reassortant strain. Results of PCR-based BTV diagnosis need to be
659 interpreted with caution as BTV RNA has been detected in blood from both infected
660 cattle and sheep for at least 30 days and sometimes up to 90 days after virus isolation
661 has ceased to be positive (Bonneau et al., 2002, Katz et al., 1993, MacLachlin et al.,
662 1994). Thus detection of virus-specific nucleic acid by these methods indicates a
663 recent virus infection, but does not necessarily indicate the presence of infectious
664 virus in the animal.

665

666 **5. Detection of avian influenza virus by rRT-PCR**

667

668 Avian influenza viruses (AIV) belong to the genus *Influenzavirus A*, family
669 *Orthomyxoviridae*, possessing a negative stranded RNA genome of eight segments.
670 AIVs are classified into subtypes according to antigenic variation of the
671 glycoproteins, haemagglutinin (H) and neuraminidase (N), there are sixteen H types
672 and nine N types currently known. Wild birds, in which overt disease due to AI has
673 been rarely recorded, are the natural hosts for AIV and their migration is believed to
674 be important in its spread (Brown et al, 2006). Most AIV subtypes are termed low
675 pathogenicity avian influenzas (LPAIs) because little morbidity and mortality is
676 normally observed in infected chickens. LPAI virus infections are typically localised
677 to the avian respiratory and/or intestinal tract. However, H5 and H7 LPAI infections
678 in poultry may result in mutations in the viral haemagglutinin genes that bring about
679 changes at the HA0 “cleavage site” (CS, Figure 2) causing phenotypic change to
680 highly pathogenic avian influenza (HPAI) virus which spreads systemically. HPAI is
681 highly infectious to poultry and is characterised by rapid and aggressive spread of the
682 disease within a flock with very high mortality, typically within 48 hrs (Swayne &
683 Suarez, 2000; OIE, 2005a). Both LPAI and HPAI poultry outbreaks caused by H5
684 and H7 AIVs are notifiable diseases (CEC, 2006; OIE, 2005b), and the biology of
685 such outbreaks and AIV wild bird isolates are under continuous scrutiny (Alexander,
686 2007; Senne, 2007).

687

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

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

703

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

718

719 Conventional AI RT-PCRs

720 Laboratories have used conventional RT-PCR for AI characterisation since the 1990s,
721 but this involves initial biological amplification of AI specimens in EFEs in which
722 high AI titres (*eg* 1×10^6 EID₅₀/ml or greater) are obtained in the chorioallantoic fluid
723 (CAF) and used for RNA extraction. Sequencing of amplicons that span the region
724 coding for the HA0 CS region (Figure 2) may determine pathogenicity for H5 or H7
725 AIVs (OIE, 2005a; EU 2006; Senne et al 2006). This has served as a very useful
726 adjunct to *in vivo* pathogenicity testing in poultry (Senne et al, 2006). Conventional
727 RT-PCR continues to be used to generate AIV sequence data, including
728 haemagglutinin gene sequences of H5/H7 isolates from outbreaks (Bao et al, 2008).
729 This provides phylogenetic data to track the molecular epidemiology of AI outbreaks,
730 particularly if the variable HA1 region of the haemagglutinin gene (Figure 2) is
731 analysed (Banks et al, 2001). American H5 isolates are clearly phylogenetically

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

734

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

742

743 *M gene, H5 & H7 testing by rRT-PCR*

744

745 *M gene (generic) rRT-PCR:*

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

751

752 *H5 and H7 specific rRT-PCRs:*

753 The HA2 region has served as the target region for H5 and H7 TaqMan[®] rRT-PCRs as
754 it is relatively conserved within these respective haemagglutinin genes (Figure 2;
755 Spackman et al, 2002). These rRT-PCRs have also been shown to function efficiently
756 as H5 and H7 quantitative (Q)-rRT-PCRs in studies of virus shedding (Lee & Suarez
757 2004). Spackman et al (2002) cautioned that their H5 and H7 primer / probe
758 sequences had been designed for the detection of North American H5 and H7 isolates
759 and were unlikely to be applicable to Eurasian viruses. The extent of sequence
760 divergence between viruses from the different hemispheres is so great that, even
761 within the HA2 region, it appears difficult to design a consistently sensitive rRT-PCR
762 for global detection of H5 or H7 AIVs. The primer and probe sequences of Spackman
763 et al (2002) were modified for detection of “Asian lineage” H5N1 AIV and also other
764 Eurasian H5 AIVs that have been isolated within the past decade (Figure 2; Slomka et
765 al, 2007b). This Eurasian H5 rRT-PCR has demonstrated its value in the investigation

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

770

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

777

778 *Validation of AI rRT-PCRs*

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

782

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

793

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

799

800 Testing of clinical specimens:

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

806

807 The following studies describe AI rRT-PCRs which have included the above sample
808 populations for validation: Spackman et al (2002) included extensive M gene rRT-
809 PCR testing on clinical specimens collected from LBMs in 2001. Comparative testing
810 alongside VI revealed some discrepant results, which were discussed in detail
811 (Spackman et al, 2002). Samples from poultry infected experimentally served to
812 further validate the American H5/H7 type-specific assays (Lee & Suarez, 2004). All
813 three AI rRT-PCRs were shown to be generally more sensitive than VI (Spackman et
814 al, 2002; Lee & Suarez, 2004). Validation of the Eurasian H5 rRT-PCR included a
815 wide range of Eurasian H5 AIVs (both LP and HP) and non-H5 AIVs (Slomka et al.,
816 2007b). Clinical specimens were obtained from a number of suspect H5N1 HPAI
817 submissions from Europe, Africa and Asia from autumn 2005 to spring 2006. It was
818 concluded that the Eurasian H5 rRT-PCR is more sensitive than VI (Slomka et al.
819 2007b). Monne et al (2008) used a broad range of known AI isolates, plus large
820 numbers of clinical specimens from poultry and wild birds to validate their sensitive
821 and specific H5 and H7 rRT-PCRs in comparison with VI.

822

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

827

828 *Use of AI rRT-PCRs in poultry outbreaks*

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

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

837

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

845

846 Westward spread of H5N1 HPAI during the summer of 2005 (Chen et al 2005; Liu et
847 al 2005) accelerated EU emergency preparedness to tackle any subsequent European
848 outbreaks. This included recommendation of the M gene rRT-PCR (Spackman et al
849 2002) that had fared very effectively and reproducibly in blind testing of AI panels
850 organised within six EU laboratories (Slomka et al 2007a). Ongoing validation of the
851 Eurasian H5 rRT-PCR (Slomka et al 2007b) resulted in the inclusion of this method
852 among the EU recommended AI protocols (EU 2006). This contingency planning
853 was timely, with the first incursions of H5N1 HPAI in wild birds and/or poultry
854 occurring during autumn 2005 in Turkey, Romania, Croatia and Romania (FAO 2006;
855 Alexander 2007). These and subsequent European H5N1 reports in 2006 included a
856 high proportion of wild waterfowl cases, notably swans, and the Eurasian H5 rRT-
857 PCR demonstrated its value in the direct testing of clinical specimens (Slomka et al
858 2007b). European H5N1 HPAI outbreaks continue to be diagnosed by this method,
859 e.g. the three turkey outbreaks in the UK and Poland in 2007 (EU 2007).

860

861 The EU recommended H7 HA2 rRT-PCR (Slomka & Voermans, 2007) has also
862 served in the diagnosis of LPAI poultry outbreaks caused by H7N3 (UK 2006;
863 Manvell et al., 2008), H7N2 (UK 2007; EU, 2007) and H7N1 (Denmark 2008; EU,
864 2008). An outbreak of H7N7 HPAI in chickens in the UK was diagnosed and
865 investigated in June 2008, where the H7 HA2 rRT-PCR again demonstrated its value
866 (Defra, 2008).

867

868 Wild bird surveys (WBSs) and AI rRT-PCR

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

882

883 Controls in AI rRT-PCR

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

891

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

900

901 AI rRT-PCR Proficiency Testing

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

916

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

926

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

933

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935

936 **6. Detection of Newcastle disease virus by rRT-PCR**

937

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

950

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

959

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

969

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

977

978 In addition to RT-PCR methods, other molecular approaches, such as RT-LAMP,
979 have been recently developed for detection of NDV (Pham et al. 2005a). The big
980 advantage of all these molecular techniques is that isolation of infectious NDV, and
981 subsequent egg passage and/or cell line passages are not needed for diagnostic
982 purposes. Therefore, detection of genetic material specific for NDV directly from a
983 wide range of specimens, i.e. blood, faeces, tissues, and from different avian species
984 speeds up the diagnostic process significantly. Another advantage of molecular
985 techniques is the possibility of differential diagnosis by multiplex-RT-PCRs between
986 pathogens causing similar clinical signs, i.e. Avian influenza and ND (Farkas et al.,
987 2007). RT-PCR can be achieved by universal primers that amplify targeted regions of
988 NDV, and thus detect its presence (Barbezange & Jestin, 2002; Kant et al., 1997;
989 Gohm et al., 1999). However, even highly conserved genes like the M-gene shows
990 variability resulting in failures to detect NDV-isolates. A complementary assay based
991 on the L-gene has been developed to cover all NDVs, irrespective of virulence (Kim
992 et al., 2007). In contrast, many approaches have narrowed the specificity to virulent
993 NDV, and thus distinguish between virulent and vaccine strains. However, these
994 assays generally only detect a subset of all virulent NDVs, lacking the necessary
995 performance criteria for adoption into routine use.

996

997 Depending on the particular molecular method used, it is possible to detect, serotype,
998 and define the virulence of NDV in parallel. These integrated assay characteristics
999 speed up the diagnosis and characterisation of virulent NDV strains significantly.

1000 Detection of NDV by amplification of the region encoding the cleavage site of the
1001 fusion protein opens the possibility to identify the virulence by studying the presence
1002 of additional basic amino acid codons. Using open amplification systems, this has
1003 been achieved by several methods, i.e. subsequent sequencing of the amplicon (Gohm

1004 et al. 1999), a specific nested PCR (Barbezange and Jestin, 2002; Kant et al., 1997;
1005 Kou et al., 1999; Kho et al., 2000), agarose-gel electrophoresis, or restriction enzyme
1006 analysis (Creelan and McCullough, 2006). A major risk of these approaches is
1007 contamination of samples by amplicon from previously amplified material during
1008 post-PCR manipulations.

1009

1010 NDV-specific rRT-PCR have utilised intercalating dyes such as SYBR-green (Tan et
1011 al. 2004). A drawback of this method is that amplification must be very specific as
1012 non-specific amplification contributes to the fluorescent signal. However, specificity
1013 of the amplicon can be checked afterwards by melt curve analysis and this approach
1014 has even been used for pathotyping (Pham et al. 2005b). Use of hybridization probes
1015 improved assay reliability: not only by offering a higher specificity, but also by
1016 raising the analytical sensitivity of detection of NDV-RNA, as approximately 10
1017 EID₅₀ can be routinely detected by this method (Tan et al. 2004, Wise et al. 2004).

1018

1019 All these combinations of detection of virulent NDV by rRT-PCR have one major
1020 disadvantage; the targeted gene (F-gene) encoding the fusion protein is considerably
1021 variable. Since the primers used in the assay must be reliably amplify all virulent
1022 NDVs, it is difficult (or perhaps impossible) to develop a single rRT-PCR to detect all
1023 virulent NDVs.. Alternatively, several RT-PCRs based on the F-gene can be
1024 performed in parallel or in the same tube to achieve amplification of the cleavage site
1025 of all NDVs. However, since this region is less conserved there will be a higher
1026 chance of failure for the amplification of new virus isolates. These concerns are also
1027 evident when considering the use of probes to detect products from these rRT-PCR
1028 reactions. The ability of a probe to hybridise to a specific product is very sensitive to
1029 mutations (Kim et al. 2006), however, this can be overcome by the use of many
1030 probes in one reaction (Aldous et al., 2001). Preferably a second RT-PCR based on a
1031 more conserved target, that detects all NDVs can be used to exclude false negative
1032 results by an F-gene based RT-PCR. However, such assays may still not cover all
1033 NDVs and so a second assay, based on L-gene may offer advantages since it will be
1034 applicable to most if not all strains (Kim et al., 2007). However, such assays targeting
1035 relatively conserved regions cannot be applied in vaccinated populations to diagnose
1036 ND, without recourse to tests leading to virulence determination. Published rRT-PCR
1037 assays used for NDV genome detection are listed in Table 4.

1038 Reliability of laboratory diagnosis for ND is very important, since misdiagnosis has
1039 enormous consequences. In case of false negative results, a new outbreak remains
1040 undetected, and control measures are unnecessarily delayed. Therefore, frequent
1041 testing of rRT-PCR assays with new isolates to confirm sensitivity must be part of the
1042 sustainability of the laboratory diagnosis for ND. Concerning false positive results for
1043 ND, the consequences could be even worse. In particular, the impact of a false
1044 positive index case in a ND-free country would be enormous. In addition to these
1045 diagnostic tests, it is important to sequence all (or part of) the F-gene of the isolate
1046 causing the outbreak for analysis of the cleavage site and epidemiological studies. It is
1047 always strongly recommended that virus is isolated from index cases to facilitate
1048 further analyses.

1049

1050 Nevertheless, after introduction of ND, high-throughput diagnostics is needed to
1051 control the outbreak. Focusing on molecular diagnostics for ND, in particular high
1052 throughput rRT-PCR diagnostics, only one report has been published using a
1053 validated 96-well format isolation- and rRT-PCR procedure (Crossley et al., 2005).
1054 Compared to VI, this assay had a diagnostic sensitivity of 100%, and a diagnostic
1055 specificity of 95% with excellent reproducibility. Many rRT-PCR assays have been
1056 published but are validated to a less rigorous extent. Most of these methods use
1057 manually extracted RNA or other isolation methods that are not amenable to
1058 automation. The particular benefits and disadvantages of these procedures must be
1059 taken into account and varies from use for vaccinated poultry to wild birds and from
1060 allantoic fluids to faeces. Application as a diagnostic tool in vaccinated poultry is
1061 limited to the assays based on a target on the F-gene, including the region encoding
1062 the cleavage site in order to be able to distinguish between vaccine and virulent strains
1063 (Kim et al., 2006). Application as a diagnostic tool for samples from sources such as
1064 different non-vaccinated avian species, including wild birds, is focused on more
1065 conserved regions such as the M-gene, although a second assay may be required to
1066 cover all NDVs (Kim et al., 2007).

1067

1068 In summary, molecular diagnostics for ND can essentially be divided into two
1069 categories depending on the purpose Detection of ND in avian species, and
1070 determination of virulent NDV. For detection of NDV, even if highly conserved
1071 targets like the M- and L-gene are chosen, it is very difficult to develop an rRT-PCR

1072 assay capable of detecting all NDVs (six genogroups to date). For characterisation
1073 and recognition of virulent NDV, part of the F-gene, including the region encoding
1074 the cleavage site, is the target of interest. However, it is extremely difficult to develop
1075 an assay to detect all virulent NDVs especially considering geographical variation.
1076 The risk of false negatives due to variation in primer or probe locations is still a
1077 challenge to assay-developers. Nevertheless, it should be recommended that
1078 confirmatory assays be developed and utilised to avoid unnecessary delay of
1079 implementing control measures in case of a new outbreak. Only one extensive
1080 validation of a high-through PCR-based assay for ND has been published. In spite of
1081 recent progress, there is still a greater need for more reliable and improved validated
1082 rRT-PCR assays for the detection of NDV in general, as well as for identification of
1083 virulent strains of NDV.

1084

1085 **7. Discussion and general conclusions**

1086 This review outlines the remarkable progress made over the past decade by real-time
1087 PCR techniques to improve pathogen detection for diagnostics and disease control.
1088 Beside the high sensitivity and ability to quantify viral targets, the substantial gain in
1089 specificity and the reduced risk of cross-contamination are important features of this
1090 technology. Integration of these assays onto automated high-throughput platforms
1091 provides diagnostic laboratories with the capability to test large numbers of samples
1092 that might be received during outbreaks of livestock disease. Although this review
1093 emphasises the positive features of rRT-PCR assays, it is important to recognise that
1094 there are a number of factors that can negatively impact upon the performance of
1095 these tests. In particular, the design of assays capable of detecting RNA viruses such
1096 as FMDV, CSFV, BTV, AIV and NDV with highly variably genomes can pose
1097 specific challenges. In addition to identifying conserved regions in the genome for
1098 primer-binding sites, the requirement to find further regions for the location of probe
1099 sites can reduce the diagnostic sensitivity of real-time PCR assay. In order to avoid
1100 this phenomenon, more than one real-time RT-PCR targeting different regions of the
1101 genome can be employed in parallel. As a consequence, the likelihood that mutations
1102 will cause failure of detection in both independent assays is very low (King et al.,
1103 2006). This “double check strategy” can be particularly important in the context of
1104 first line or exclusive diagnostics of pathogens using rRT-PCR. rRT-PCR assays can
1105 generate objective results regarding the presence of a particular pathogen in a sample.

1106 However, border-line (or doubtful) results can arise when high Ct values below the
1107 cut-off of the test are generated. Unfortunately, non-PCR antigen detection methods
1108 are often not sensitive enough for a reliable independent confirmation of these signals.
1109 Therefore, a robust approach for dealing with these results should be adopted and
1110 standardised for each individual assay. It is recommended that an inconclusive
1111 interval (e.g. based on Ct-values or genome copies) is defined. Specimens with a
1112 doubtful result should be further analysed, e.g. using a second real-time PCR, a new
1113 extraction or a new sample from the same animal. In addition, for livestock
1114 investigations these effects can be mitigated or negated by the use of a robust
1115 statistically valid sampling frame at the level of epidemiological group. A further
1116 point to consider is putting in place systems to minimise the potential for cross-
1117 contamination. Although real-time PCR assays are closed tube, sub-optimal seals, the
1118 use and propagation of positive controls (e.g. plasmids, virus stocks), as well as
1119 further molecular procedures for the characterisation of detected genomes (e.g.
1120 sequencing) can cause false positive results in the test. In order to minimise cross-
1121 contamination, these different activities should be clearly separated away from the
1122 laboratory areas used to prepare nucleic acid template for the rRT-PCR.

1123
1124 Modern real-time PCR cyclers can measure up to five separate reporter fluorophores
1125 in one tube. This technical feature is a prerequisite for the application of multiplex
1126 real-time PCR assays. Many of the rRT-PCR assays reviewed here co-amplified
1127 internal controls. It is recommended that these internal controls are included for each
1128 real-time PCR analysis. The minimum requirement for a state-of-the-art real-time
1129 PCR should be the use of an internal inhibition control in a duplex assay format. The
1130 currently published rRT-PCR assays for the detection of FMDV, CSFV, AIV, NDV
1131 and BTV summarized in this review are an excellent basis for the establishment of
1132 qualified and reliable molecular diagnostics. In Europe, these tests have recently
1133 provided critical support to the efficient control of notifiable diseases like FMD, AI,
1134 BT and CSF. Future work will continue to assess the performance of these assays,
1135 providing validation data for different sample types. Furthermore, continued inter-
1136 laboratory comparative exercises are also important to gauge the relative performance of
1137 tests used at different sites, and may ultimately lead to a more rational harmonisation
1138 of the rRT-PCR assays.

1139

1140 In conclusion, over the past decade rRT-PCR has become a central laboratory tool for
1141 the diagnosis of important livestock diseases. In light of the performance of these
1142 tests, it is evident that molecular diagnostics will be the main tool for eradication and
1143 control of epizootic diseases in the future.

1144

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1965 **Figure legends**

1966

1967 **Figure 1:** Location of pan-serotype rRT-PCR targets within the 5' UTR (untranslated
1968 region) and 3D (RNA polymerase) genes of the FMDV genome. Boxes represent sites
1969 recognised by the primers and probes for the respective assays with * denoting the
1970 location of redundant bases which have been incorporated into the design of primers
1971 to accommodate mismatches. The bar-plots show corresponding variability at each
1972 nucleotide position assessed from analysis of 122 full genomic sequences of FMDV.

1973

1974 **Figure 2:** Diagram showing relative positions of AI PCR amplicons within different
1975 AIV ORFs. rRT PCR amplicons are shown as unbroken horizontal lines for: (1) M
1976 gene: a (99 bp size, Spackman et al 2002) and b (147bp, Di Trani et al 2006). (2) H5:
1977 c (*ca* 120-150bp, depending on precise CS size, Hoffmann et al 2007), d (152bp,
1978 Spackman et al 2002 and Slomka et al 2007b) and e (61bp, Monne et al 2008). (3)
1979 H7: f (*ca* 270-290bp) and g (132bp), both Slomka & Voermans (2007); h (98bp,
1980 Spackman et al 2002) and i (62bp, Monne et al 2008). Conventional PCR amplicons
1981 assessed by Slomka et al (2007a) are shown as broken horizontal lines and indicated
1982 by italic letters for: (2) H5: j (*ca* 300-330bp, KHA primers) and k (*ca* 300-330bp,
1983 J3/B2a primers). (3) H7: l (*ca* 200-230bp, GK primers).

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1994 **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				
CACYTYAAGRTGACAYTGRTACT GGTAC	CAGATYCCRAGTGWCICITGTTA	CCTCGGGGTACCTGAAGGGCATCC	IRES	Reid et al., 2002
ACTGGGTTTTACAAACCTGTGA	GCGAGTCCTGCCACGGA	TCCTTTGCACGCCGTGGGAC	3D	Callahan et al., 2002
TGTGTGCAACCCCAGCAC	CGAGTGTCGRTGTTACC	ACAGGCTAAGGATGCCCTTCAGGT ACC	IRES	Oem et al., 2005
AACACGCYGTSTTYGCSTG	GCGTCCAAGGGTARAAGTC	ACCTCCAACGGGTGGTACGCGAT	Leader	Oem et al., 2005
AGATGCAGGARGACATGTCAA	TTGTACCAGGGYTTGGCYT	AAACACGGACCCGACTTTAACCG	2B	Oem et al., 2005
ACTGGGTTTTAYAAACCTGTGAT G	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	ATCCTCTCCTTTGCACGCCGTGGG ACCAT	3D	Rasmussen et al., 2003 ^c

1995

1996 ^a Minor groove binder probe (MGB)1997 ^b Dual hybridisation probes1998 ^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

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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 <i>FAM</i> -TGG CGA GCT CCC TGG GTG GTC TAA GT- <i>TAMRA</i>	100–120 192–172 141–166	93 bp	<i>in vitro</i> -transcribed EGFP-RNA
Risatti et al., 2003 Deng et al., 2005 Ophuis et al., 2006	forward reverse probe	CCCTGGGTGGTCTAAG CATGCCCTCGTCCAC <i>FAM</i> -CCTGAGTACAGGACAGTCGTCAGTAGTT- <i>TAMRA</i>	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 <i>FAM</i> -TGA TGG GGG TAC GAC CTG ATA GGG T- <i>TAMRA</i>	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 <i>FAM</i> -CCC TGG GTG GTC TAA GTC CTG AGT ACA G- <i>TAMRA</i>	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 (LSD), Adiavet CSF (ADIAGENE)	not stated	not stated	housekeeping gene: GAPDH
Jamnikar Ciglencečki 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 <i>FAM</i> -CAG GTC GTA CYC CCA TCA- <i>MGB</i>	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 <i>FAM</i> -ACG TCG AAC TAC TGA CGA CTG TCC TGT ACT CA- <i>BHQ1</i>	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 <i>FAM</i> -AGG ACT AGC AAA CGG AGG GAC TAG CCG- <i>TAMRA</i> <i>TET</i> -TAG GAC TAG CAA AAC GGA GGG ACT AGC CA- <i>TAMRA</i>	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).

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	

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 GGCAGCATTTTGTGGCTTGTATC <i>FAM-AAGCGTTTCTGTCTCCTTCCTCCG-TAMRA</i> <i>FAM-AGACGTCCCTGTTTCCCTCCTCC-TAMRA</i> <i>FAM-AAACGTTTCTGTCTCCTTCCTCCGG-TAMRA</i> <i>FAM-AAACGTCTCTGTCTCCTTCCTCCGG-TAMRA</i> <i>FAM-AAGCGTTTCTGCCTCCCTCCTCC-TAMRA</i> <i>FAM-CCTATAAGGCGCCCCTGTCTCCC-TAMRA</i>	not stated	Fusion gene
Wise et al., 2004	M+4100 M+6169 M-4220	AGTGATGTGCTCGGACCTTC <i>FAM-TTCTCTAGCAGTGGGACAGCCTGC-TAMRA</i> CCTGAGGAGAGGCATTTGCTA	121 bp	Matrix gene
Wise et al., 2004	F+4839 F+4894 F-4939	TCCGGAGGATACAAGGGTCT <i>FAM-AAGCGTTTCTGTCTCCTTCCTCCA-TAMRA</i> AGCTGTTGCAACCCAAG	101 bp	Fusion gene (velo & meso)
Wise et al., 2004	M+4213 M-4268 M-4350	TCCTCAGGTGGCCAAGATAC <i>FAM-TTTTAAACGCTCCGCAGGCAC-TAMRA</i> TGCCCCTTCTCCAGCTTAGT	138 bp	Matrix gene (pre-1960)
Kim et al., 2005/2007	L-8784 L-8868 L+8817	CGTTCTGAGGAATTTGACAGYMT GRAGCCATGCGAAYTTGG <i>FAM-CCGGCATTCTGTTTCACTCAA-3BHQ_1</i>	85 bp	L-gene

2011

2012

Figure 1

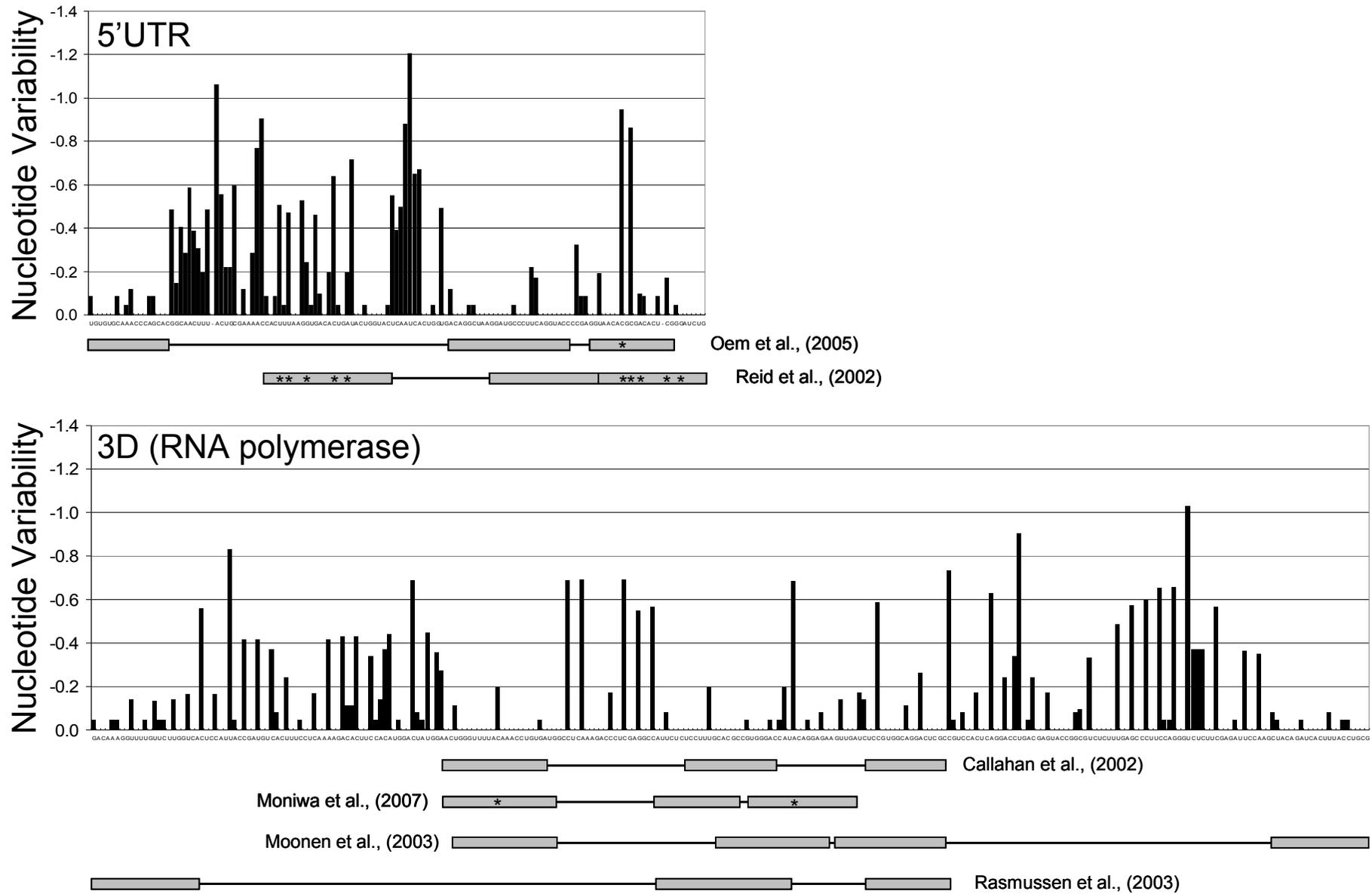
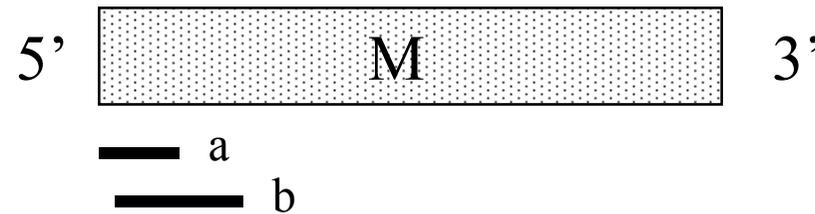
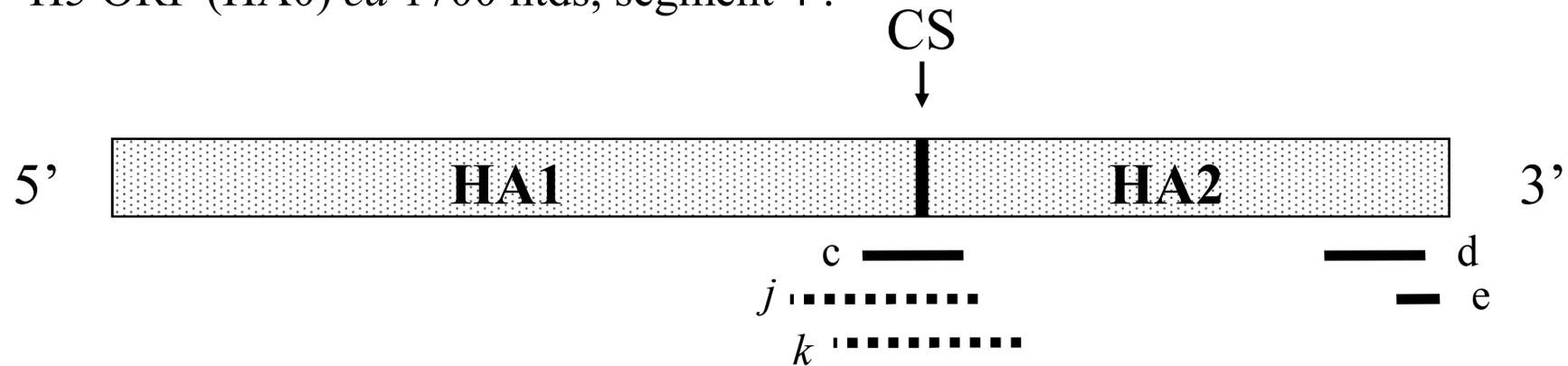


Figure 2

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 :

