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Single reaction, real time RT-PCR detection of all known avian and human metapneumoviruses

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ABSTRACT

Current molecular methods for the detection of avian and human metapneumovirus (AMPV, HMPV) are specifically targeted towards each virus species or individual subgroups of these. Here a broad range SYBR Green I real time RT-PCR was developed which amplified a highly conserved fragment of sequence in the N open reading frame. This method was sufficiently efficient and specific in detecting all MPVs. Its validation according to the NF U47-600 norm for the four AMPV subgroups estimated low limits of detection between 1000 and 10 copies/µL, similar with detection levels described previously for real time RT-PCRs targeting specific subgroups. RNA viruses present a challenge for the design of durable molecular diagnostic test due to the rate of change in their genome sequences which can vary substantially in different areas and over time. The fact that the regions of sequence for primer hybridization in the described method have remained sufficiently conserved since the AMPV and HMPV diverged, should give the best chance of continued detection of current subgroups and of potential unknown or future emerging MPV strains.

1. Introduction

Metapneumoviruses have recently been classified in the new family *Pneumoviridae*, genus *Metapneumovirus* (MPV) (Afonso et al., 2016) where they are subdivided into avian and human strains. In both species these viruses cause important respiratory diseases (Cook, 2000; Gough et al., 1988; Picault et al., 1987; Toquin et al., 1999; van den Hoogen et al., 2001; Williams et al., 2004). Respiratory disease retrospectively reported to be caused by avian metapneumovirus (AMPV) was first described in turkeys in South Africa in the late 1970s, then shortly after in Europe (France and UK) in the early 1980s. Today AMPV has been detected in most regions of the world with the exception of Australasia (Jones and Rautenschlein, 2013). Human metapneumoviruses (HMPV) were first detected in the Netherlands in 2001 but were shown to have been circulating since the 1950's (van den Hoogen et al., 2001). They also have been detected worldwide as a major cause of bronchiolitis and respiratory infections in infants, the elderly, or immunocompromised adults (Falsey et al., 2003; van den Hoogen et al., 2001).

To date, four AMPV subgroups (A to D) have been described based on genetic and antigenic variation (Alvarez et al., 2003; Bayon-Auboyer et al., 2000; Juhasz and Easton, 1994). Subgroup A and B viruses are present in most countries around the world with the exception of North America and Australia (Bayon-Auboyer et al., 1999; Naylor et al., 1997). Subgroup C viruses have only been reported in North America (Senne et al., 1997), France (Toquin et al., 1999) China and Korea (Lee et al., 2007; Wei et al., 2013). Subgroup D has so far only been detected in France (Bayon-Auboyer et al., 2000). For HMPV, four genetic lineages have been described (A1, A2, B1, and B2), also based on antigenic and genetic variability (van den Hoogen et al., 2004). HMPV subgroups appear to have an even distribution worldwide and can co-circulate in any given location (Schildgen et al., 2011).

MPV isolation is difficult to carry out as virus excretion is short, the infection is often recognized when bacterial superinfection occurs – often after the virus has been cleared – and laboratory methods for virus isolation are time consuming and labour-intensive. As a result, MPV infection is best detected routinely using either serological, molecular PCR tests or a combination of the two, and which method should be used is often decided according to the sample timing. For example, tests such as the enzyme linked immunosorbent assay (ELISA) or virus neutralization assay (Eterradossi et al., 1992; van den Hoogen et al., 2004; Wyeth et al., 1987) that detect antibodies elicited by MPV infection are used in cases where infection was suspected and sampling was performed long after the virus had been cleared. However,

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molecular PCR tests are favoured when samples such as respiratory or oro-pharyngeal swabs have been collected during the infectious period, as molecular assays are generally more sensitive and allow sequence-based sub-grouping of the viral RNA.

Several conventional MPV RT-PCRs (Bayon-Auboyer et al., 1999; Cavanagh et al., 1999; Huck et al., 2006; Mackay et al., 2003), real time RT-PCRs using fluorescent oligonucleotide probes (Taqman® probe or molecular beacon probe) (Cecchinato et al., 2013; Guionie et al., 2007; Jokela et al., 2010; Kwon et al., 2010; Maertzdorf et al., 2004; Velayudhan et al., 2005) or intercalating dyes such as SYBR Green I (Franzo et al., 2014) have been developed for AMPV or HMPV with high sensitivity and specificity. However, no real time RT-PCR test currently exists to detect all known AMPVs and no one test has been described as capable of detecting all members of the genus *Metapneumovirus*. Such an assay would prove useful, as it cannot be excluded that unknown AMPV or HMPV subgroups currently exist or will emerge in the future. To develop such a broad-spectrum test, a highly conserved region in the genome of AMPV and HMPV, such as that within the HMPV N gene used for detecting the four HMPV sub-lineages (Maertzdorf et al., 2004), or the Nd/Nx primers described for pan AMPV detection of AMPV (Bayon-Auboyer et al., 1999), needed to be identified.

This paper describes the development of a SYBR Green I single reaction, real time RT-PCR that detects all members of the genus *Metapneumovirus*. The target region of the genome for this PCR was selected in the N open reading frame (ORF) of MPVs.

2. Materials and methods

Two real time RT-PCRs using SYBR Green I were developed, one specific for MPV and one specific for a house keeping gene (glyceraldehyde-3-phosphate deshydrogenase (GAPDH)) which was used as an endogenous non target control. Both PCRs were validated according to the Association Française de Normalisation (AFNOR) NF U47-600 norm. RNA transcripts were produced *in vitro* for the validation of the RT-PCR. Reference AMPV strains were used for the validation of the complete method.

2.1. Primer design

All primers were evaluated *in silico* using OligoAnalyser Tool (https://www.idtdna.com) for secondary structures and self and hetero-dimers, the specificity was investigated by Primer-BLAST.

2.1.1. MPV primers

All available MPV N gene sequences in the GenBank database were downloaded (accession numbers List S1) then aligned using MEGA7.0 together with several in- house sequences. Based on a highly conserved region, primers (PanMPV/N1fwd, PanMPV/N1AMPVDfwd and MPV/N1Rev) were defined (Table 1). Two forward primers were used to maximize hybridization.

2.1.2. GAPDH primers

A variety of GAPDH sequences were downloaded from the GenBank database (accession numbers List S1) and aligned using MEGA7.0. Based on the most conserved regions Primer pair PanGAPDH Forward and Reverse were defined (Table 1).

2.2. Samples

2.2.1. Viruses

To assess the inclusivity of the assay, 28 AMPV field strains (Table 2) composed of eight subgroup A, eleven subgroup B, seven subgroup C and two subgroup D viruses (Bayon-Auboyer et al., 1999; Toquin et al., 2003; Toquin et al., 2006), plus three attenuated vaccine strains (two subgroup A and one B), were used. All viruses had been propagated on Vero cells. One reference strain of each subgroup was selected to evaluate the sensitivity of the method (A = 85051, B = 86004, C = 99178 and D = 85035 at titres $10^{5,2}$, $10^{4,97}$, $10^{5,42}$ and $10^{5,6}$ $TCID_{50}$/mL respectively). Six HMPV clinical samples collected in France from human patients (nasopharyngeal swabs in universal transport medium, COPAN) composed of three subgroup A2 and one of subgroup A1, B1 and B2 were also used.

To assess the exclusivity of the assay, three human respiratory syncytial viruses (HRSV) classified within family *Pneumoviridae* (two from serotype A and one from serotype B) and 25 avian, non-AMPV viruses were used. The latter were composed of: avian paramyxovirus 1, 2, 6–9 and 11; orthomyxovirus H1N1, H5N2, H5N3, H7N1 and H7N3; Fowl adenovirus, reovirus, infectious bronchitis virus, turkey coronavirus, turkey haemorrhagic enteritis virus, infectious bursal disease virus serotypes I and II, avian encephalomyelitis virus (List S2). These viruses had been previously propagated on specific pathogen free (SPF) chicken or turkey eggs, or in cell culture.

2.2.2. Experimental materials

Fourteen tracheal swabs (Table 4) taken from SPF turkeys and ducks or from previous experimental infections, of these two species with AMPV A, B, C and D (Table 4) were taken according to the protocol of Whitworth et al., 2007, however swabs were collected into 2 mL of cell culture medium as opposed to viral transport media (MEMH Themo Fischer Scientific France, Villebon supplemented with 0.2% of Penicillin/Streptomycin and 0.4% of Amphoterin B). Experimental infections had been performed according to previously reported protocols approved by the authors' institute ethical committee (Ling et al., 2008).

2.3. RNA extraction

RNA was extracted using 140 µL for avian samples by QIAamp® Viral RNA Mini Kit (Qiagen, France, Les Ulis) and using 200 µL for human samples by High Pure RNA Isolation Kit (Roche, Germany, Mannheim) according to the manufacturers' instructions, respectively.

2.4. In vitro synthesis of AMPV RNA transcripts

RNA transcripts for each AMPV subgroup were produced to determine the limit of detection (LoD) of the RT-PCR. In vitro transcription reactions were used to synthesize RNA transcripts from DNA templates using a T7 promoter system.

Viral RNA extract was reverse transcribed with superscript II (Invitrogen, France, Illkirch) for 42 °C, 50 min; 70 °C, 15 min according to the manufacturers' recommendations, using primer AMPVA/B71Fwd for subgroups A and B, FrC63Fwd for subgroup C and AMPVD131Fwd for subgroup D (Table 1) (Fig. 1, step 1).

cDNA was amplified with subgroup-specific forward primers AMPVA, B, C or DtFwd (Table 1), each incorporating a T7 polymerase recognition sequence at the immediate 5′ end and reverse primers AMPVA, B, C or DtRev, respectively (Table 1) (Fig. 1 step 2). PCR reactions were carried out using the Expand high fidelity PCR System (Roche Diagnostics, France, Meylan) according to the manufacturer's instructions. Amplicons were separated in 2% agarose gels for 40 mins at 110 V. DNA was extracted from the agarose gels using a MinElute Gel Extraction Kit (Qiagen, France, Les Ulis), then was quantified by Qubit® Fluorometer (Life Technology, France, Saint Aubin).

In vitro transcription using 300–500 ng of DNA template was done at 37 °C for 4 h using RiboMAX™ Large Scale RNA Production Systems (Promega, France, Charbonnières-les-Bains). Products were then treated twice with 7.5 U of DNaseI (RNase-free DNase Set, Qiagen/France/Les Ulis) to remove template DNA. Purification of RNA transcripts was then performed using RNeasy® Mini Kit (Qiagen, France, Les Ulis) followed by quantification using Qubit®. RNA concentrations were converted to numbers of molecular copies using the following formula:

Table 1
RT-PCR primers.

Name	5′-3′ nt sequence	SYBR RT-PCR
PanMPV/N1fwd[A]	**CTGTTTGTGAACATTTTYATGCA**	**Target MPV**
PanMPV/N1AMPVDfwd[A]	**CTGGTTGTGAACATATTCATGCA**	**Target MPV**
PanMPV/N1Rev[B]	**ACAGAGACATGGCCTAACATDAT**	**Target MPV**
PanGAPDHFwd	**TGAGTATGTTGTGGAGTCCACT**	**Target GAPDH**
PanGAPDHRev	**GCCAGGCAGTTGGTGGTGCA**	**Target GAPDH**
		RNA transcripts
AMPVA/B71Fwd	**CAATAAGGAGAGATGTTGGTGC**	**RT-AMPV A/B**
AMPVAtFwd	**TTAATAATACGACTCACTATAGGGGTACATCCACCGGAAGC**	**PCR-AMPV A**
AMPVAtRev	**GGTCATAGACCTCAGATACTTGCCTCAACTCAG**	**PCR-AMPV A**
AMPVBtFwd	**TTAATAATACGACTCACTATAGGGGTACGTCTTCCGGGAGT**	**PCR-AMPV B**
AMPVBtRev	**GATCATACACTTCTGAGACCTGCCTTAACTCTG**	**PCR-AMPV B**
FrC63Fwd	**CAGGGGATTCAGCTTAGCG**	**RT-AMPV C**
AMPVCtFwd	**TTAATAATACGACTCACTATAGGGCTTCTTCCACAGGAAGC**	**PCR-AMPV C**
AMPVCtRev	**GATCATATACCTCCGTGACCTGTTTGAGTTCCG**	**PCR-AMPV C**
AMPVD131Fwd	**TATCCACATTGTGTGGCATGGTG**	**RT-AMPV D**
AMPVDtFwd	**TTAATAATACGACTCACTATAGGGCATCTACTGGGAGCAG**	**PCR-AMPV D**
AMPVDtRev	**CACTAGATCATAAACTTCTGACACTTGTTTAAGTTCAG**	**PCR-AMPV D**

(These positions were determined using all AMPV and HMPV N gene sequences available in the database).Underlined = T7 polymerase recognition sequence.
[A] Hybridization in N gene at position 718–740 for AMPV A/B/D and at 727–749 for AMPV C/HMPV.
[B] Hybridization in N gene at position 824–802 for AMPV A/B/D and at 833–811 for AMPV C/HMPV.

Table 2
RT-PCR for 37 MPV samples.

Anses ref	Source	virus	Species	*Results GAPDH*		*Results MPV*	
				Ct	Tm	Ct	Tm
TRT Nobilis	UK	**AMPV A**	Vaccine	24.3	81.5	17.0	79.0
Turkadin	UK	**AMPV A**	Vaccine	19.3	83.0	17.6	78.5
85051	France	**AMPV A**	Turkeys	23.5	83.0	18.3	78.5
93084	Israël	**AMPV A**	Unknown	21.5	82.5	19.5	78.5
STG761/88	Germany	**AMPV A**	Unknown	26.3	82.5	24.2	78.5
STG854/88	Germany	**AMPV A**	Unknown	26.7	82.5	24.8	78.5
STGIII/88	Germany	**AMPV A**	Unknown	19.5	83.0	18.4	78.5
3BOC18	UK	**AMPV A**	Turkeys	20.8	83.0	16.1	78.5
14/86/2	UK	**AMPV A**	Turkeys	20.1	83.0	17.9	78.5
TRT 1125/91	Germany	**AMPV A**	Unknown	25.0	83.0	19.0	78.5
			Mean Tm AMPV A ± SD				**78.6 ± 0,2**
Aviffa RTI	France	**AMPV B**	Vaccine	20.1	83.0	18,3	76,5
86004	France	**AMPV B**	Turkeys	24.4	83.0	19,2	78,5
98103	France	**AMPV B**	Turkeys	19.6	83.0	19,2	76,0
85231	France	**AMPV B**	Turkeys	19.1	83.0	18,5	76,5
85234	France	**AMPV B**	Turkeys	20.5	83.0	18,0	76,5
86016	France	**AMPV B**	Chickens	25.9	83.0	23,4	76,5
86019	France	**AMPV B**	Turkeys	17.0	83.0	20,1	76,5
95021	France	**AMPV B**	Turkeys	18.9	83.0	21,8	75,5
96252	France	**AMPV B**	Turkeys	19.9	83.0	17,1	76,5
97104	France	**AMPV B**	Turkeys	19.3	82.5	17,5	75,5
98238	France	**AMPV B**	Chickens	24.6	83.0	20,9	76,0
00185	France	**AMPV B**	Turkeys	23.4	83.0	22,4	75,5
			Mean Tm AMPV B ± SD				**76.3 ± 0.8**
99178	France	**AMPV C**	Ducks	24.2	83.0	14,9	77,5
99214	France	**AMPV C**	Ducks	25.8	83.0	13,4	77,0
99350	France	**AMPV C**	Ducks	26.4	83.0	15,3	77,0
00094	France	**AMPV C**	Ducks	25.4	83.0	14,0	77,0
04268	France	**AMPV C**	Ducks	20.3	83.0	12,5	77,0
193ADV9802	USA	**AMPV C**	Turkeys	24.6	83.0	17,2	75,5
AV247/97	USA	**AMPV C**	Turkeys	18.2	81.0	15,5	76,0
			Mean Tm AMPV C ± SD				**76.7 ± 0.7**
85035	France	**AMPV D**	Turkeys	21.0	83.0	16,4	78,5
85053	France	**AMPV D**	Turkeys	21.0	82.5	16,6	78,0
			Mean Tm AMPV D ± SD				**78.3 ± 0.4**
150054a	France	**HMPV A1**	Human	22,3	83,5	23,2	78,0
172622-5	France	**HMPV A2**	Human	19,2	81,5	27,1	78,0
172622-7	France	**HMPV A2**	Human	18,4	82,0	22,9	78,0
172622-12	France	**HMPV A2**	Human	24,1	81,5	26,1	77,5
172622-9	France	**HMPV B1**	Human	26,3	82,0	32,4	77,5
172622-23	France	**HMPV B2**	Human	20,5	82,0	33,3	77,0

$$\left(\frac{X\,(g/\mu\,lRNA)}{(transcript\ \ length\ in\ nucleotides \times 340}\right) \times 6.023 \times 10^{23} = Y\,(molecules/\mu\,l)$$

2.5. SYBR green I RT-PCR assays

One step real time RT-PCR for MPV or GAPDH was carried out using *Power* SYBR® Green RNA-to-CT™ 1-Step Kit (Applied Biosystems, Saint Aubin, France) in a 20 µL final volume. The reaction mixture was made up of 10 µL of master mix (2x), 0.16 µL of enzyme mix (reverse transcriptase and Taq DNA polymerase), 5 µL of RNA template and either a combination of PanMPV/N1Fwd, PanMPV/N1Rev and PanMPV/N1AMPVD (0.4 µM, 0.6 µM and 0.2 µM final concentrations, respectively) for MPV targets or PanGAPDHFwd and Rev (both at 0.4 µM final concentration) for the GAPDH target. The RT-PCR cycle used for both targets was as follows: 48 °C for 30 min (reverse transcription), 95 °C for 10 min, then 40 cycles each of 95 °C for 15 s, 54 °C for 30 s and 60 °C for 30 s, followed by a melting curve analysis step as defined by the ABI7500 software (Applied Biosystems).

Analyses of cycle threshold (Ct) and melting temperature (Tm) were performed using the Applied Biosystems 7500 Software.

2.6. Method validation

2.6.1. Specificity (AMPV/HMPV)

The 31 AMPV, six HMPV, three HRSV and 25 non-MPV viruses listed in section 2.2.1 were tested. The results of the sensitivity (or true positive rate) and the specificity (or true negative rate) were expressed using the following formulae: Sensitivity = [number of true positives/(number of true positives + number of false negatives)] x 100 and specificity = [number of true negatives/(number of true negatives + number of false positives)] x 100.

2.6.2. LoD and repeatability (AMPV) according to the NF U47-600 norm (requirements and recommendations for the implementation, development and validation of PCR in animal health)

The LoD was determined experimentally by preparing a serial dilution of each reference material, either RNA transcripts diluted in Rnase free water for assessing the LoD of the RT-PCR uniquely, or viruses diluted in supernatant of SPF turkey tracheal swabs for assessing the complete method.

For the LoD of the RT-PCR, eight replicates of ten-fold serial dilutions in three separate assays were performed. For the LoD of the complete method, four repeats at each ten-fold dilution point in two separate assays were performed on AMPV subgroup A, B, C and D reference viruses.

The LoD was considered as the last positive dilution which gave a specific amplification at a confidence level of 95%. The cycle threshold (Ct) and the melting temperature (Tm) were analysed. A cut off value set at Ct > 35 was applied.

Repeatability of the assay was evaluated by calculating the Ct mean values, standard deviation (SD) and coefficient of variation (CV) for each replicate within the same assay (intra-assay variability) and between separate assays (inter-assay variability).

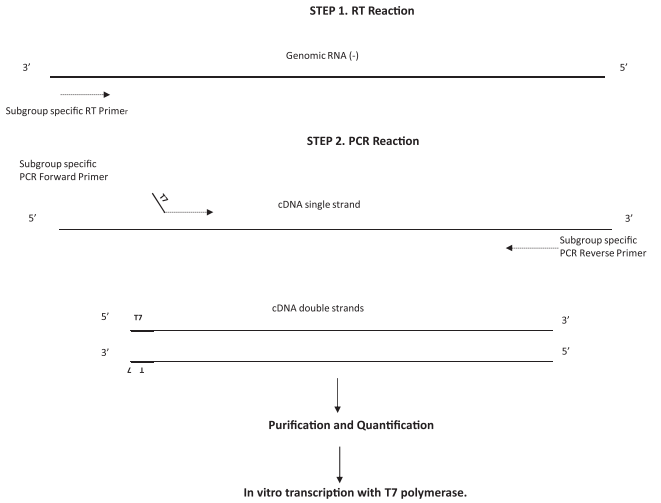

Fig. 1. Production of the dsDNA for use as a template in-vitro transcription. (Step 1) Reverse Transcription reaction with superscript II using a primer specific of each subgroup. (Step 2) PCR reaction with Expand High Fidelity enzyme using a forward primer specific of each subgroup that incorporated a T7 promoter recognition sequence at its immediate 5′end, and a reverse primer specific of each subgroup.

2.6.3. Amplification efficiency (AMPV)

Either the log of the number of RNA copies per µL or of infectious virus titres expressed as tissue culture infectious doses $(TCID)_{50}$/mL were plotted against the Ct following the equation Y = ax + b. Using the slope of the regression line, the PCR efficiency was calculated with formula E = 100 x $(10^{-1/slope} - 1)$.

An amplification efficiency of 75%–125% corresponding to a slope between −2.8 and −4.1 was considered acceptable, in agreement with the NF U47-600 norm.

2.6.4. Linearity (AMPV)

The linearity was evaluated by the expression of the coefficient of determination (R^2) of the linear regression curve. The R^2 should be ≥0.99 (Broeders et al., 2014).

3. Results

3.1. RNA transcripts

The concentration of RNA transcripts AMPV A, B, C and D were 1.3×10^{13}, 1.5×10^{13}, 1.3×10^{13} and 9.6×10^{12} copies/µL respectively. All concentrations were adjusted to1×10^9 copies/µL before the LoD studies.

3.2. Specificity of the RT-PCR assays

3.2.1. Target MPV

All AMPVs belonging to subgroups A, B, C, and D were positive. Ct values between 16.1 and 24.8 were obtained for subgroup A, between 17.1 and 23.4 for subgroup B, between 12.5 and 17.2 for subgroup C and between 16.4 and 16.6 for subgroup D (Table 2).

Tm analysis showed a mean value of 78.6 (SD 0.2, N = 10) for subgroup A, 76.3 (SD 0.8, N = 12) for subgroup B, 76.7 (SD 0.7, N = 7) for subgroup C and of 78.3 (SD 0.4, N = 2) for subgroup D (Table 2).

All HMPVs belonging to subgroups HMPV-A1, A2, B1 and B2 were positive. Ct values varied between 22.9 and 27.1 for subgroup A and between 32.4 and 33.3 for subgroup B (Table 2).

RNA templates isolated from non-MPV strains, from SPF turkey or duck tracheal swabs and non-infected Vero cells were all negative, except for two out of three HRSV samples which gave positive Ct values of 34.3 and 34.6 close to the detection threshold.

For MPVs the sensitivity of the method was shown to be equal to 100% and the specificity was 93%.

3.2.2. Target GAPDH

Housekeeping gene GAPDH was detected in all cases. For all samples Cts between 17.0 and 26.7 were observed with a mean Tm value of 82.1 (SD 0.7 N = 37) (Table 2.)

3.3. RT-PCR standard curves (AMPV)

3.3.1. Linearity

Standard curves for each subgroup were generated by plotting Ct

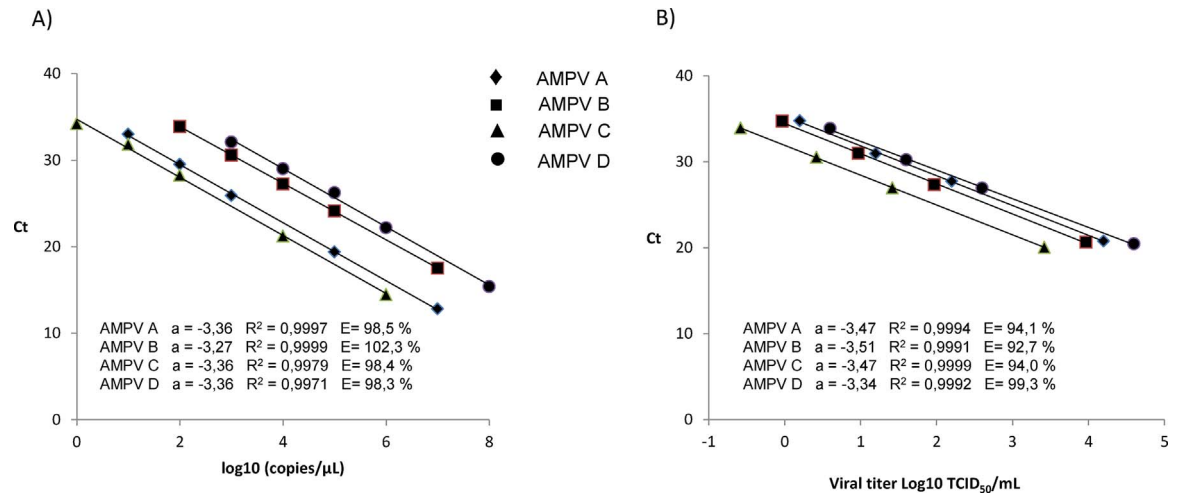

Fig. 2. Standard curves for the response of A) RNA transcripts, B) reference viruses for each subgroup AMPV A, AMPV B, AMPV C and AMPV D.
Quantities for transcripts and viruses are indicated as log10(copies number/reaction) and log10TCID50/mL, respectively. The slope (a) of the linear regression model of each subgroup, the coefficient of determination (R2) and amplification efficiency (E) are indicated.

values against the $\log_{10}$ copy number/µL of RNA transcripts or against the $\log_{10}$ $TCID_{50}$/mL of the viral titer (Fig. 2).

The standard curves from RNA transcripts exhibited a highly linear correlation for all subgroups: subgroup A from 10^7 to 10 copies/µL (R^2 = 0.9997), subgroup B from 10^7 to 100 copies/µL (R^2 = 0.9999), subgroup C from 10^6 to 10 copies/µL (R^2 = 0.9979) and subgroup D from 10^8 to 1000 copies/µL (R^2 = 0.9971) (Fig. 2A).

Standard curves with reference viruses also had highly linear correlation: subgroup A from $10^{4.2}$ to $10^{1.2}$ $TCID_{50}$/mL (R^2 = 0.9994), subgroup B from $10^{3.97}$ to $10^{-0.03}$ $TCID_{50}$/mL (R^2 = 0.9991), subgroup C from $10^{3.42}$ to $10^{-0.58}$ $TCID_{50}$/mL (R^2 = 0.9999) and subgroup D from $10^{4.6}$ to $10^{0.6}$ $TCID_{50}$/mL (R^2 = 09992) (Fig. 2B).

3.3.2. Efficiency

RT-PCR efficiency of each subgroup was calculated by using the equation of the regression line. For RNA transcripts, efficiencies of 98.5% for subgroup A, 102.3% for subgroup B, 98.4% for subgroup C and 98.3% for subgroup D were observed (Fig. 2A). For reference viruses, efficiencies of 94.1% for subgroup A, 92.7% for subgroup B, 94.0% for subgroup C and 99.3% for subgroup D were observed (Fig. 2B).

3.3.3. Limit of detection

The LoD of RNA transcripts for subgroups A, B, C and D were considered to be 10, 100, 10 and 1000 copies/µL respectively (Table 3a) as these represented the last dilution where all points gave a positive and specific amplification.

The LoD of viral RNA for subgroups A, B, C and D were considered to be $10^{1.2}$, $10^{-0.03}$, $10^{-0.58}$ and $10^{0.6}$ $TCID_{50}$/mL respectively (Table 3b) as these represented the last dilution where all points gave a positive and specific amplification at 95%.

3.3.4. Repeatability

The results for the mean Ct values, as well as the SD and the CV values estimated for intra- and inter- assay variability are summarized in Table 3a (RNA transcripts) and 3b (viral RNA).

The range of the CV was uniform regardless of the subgroup, quantity or assay tested. For RNA transcripts the intra-assay variability was 0.003–0.023 for subgroup A, 0.005–0.016 for subgroup B, 0.002–0.014 for subgroup C and 0.004–0.026 for subgroup D. The inter-assay variability was 0.008–0.022 for subgroup A, 0.011–0.015 for subgroup B, 0.006–0.010 for subgroup C and 0.018–0.024 for subgroup D.

For viral RNA the intra-assay variability was 0.003–0.019 for subgroup A, 0.005–0.026 for subgroup B, 0.004–0.018 for subgroup C and 0.006–0.026 for subgroup D. The inter-assay variability was 0.011–0.025 for subgroup A, 0.06–0.019 for subgroup B, 0.014–0.020 for subgroup C and 0.013–0.021 for subgroup D.

3.4. Experimental samples (AMPV)

RNA templates isolated from tracheal swabs taken from turkeys infected with AMPV A, B and US-C or ducks infected with Fr-AMPV C were positive for both MPV and GAPDH. RNA templates isolated from tracheal swabs of SPF turkeys and ducks were only positive for GAPDH. All detections demonstrated characteristic amplification and dissociation curves (data not shown). Ct and Tm values are reported in Table 4.

4. Discussion

Metapneumoviruses are subdivided into avian and human strains although phylogenetic analysis have shown that the avian subgroup C viruses are more closely related to the human viruses than they are to the other avian viruses (A, B and D). For this reason it may be clearer to consider AMPV A, B and D as type I metapneumoviruses and AMPV C and HMPV as type II as has been previously suggested (Brown et al., 2014). The molecular relationships between HMPV and AMPV-C have led to the hypothesis that these viruses could be have been generated by a crossing of the species barrier (de Graaf et al., 2008). The possibility of MPVs crossing the species barrier to a different animal host species prompted the search for regions of sequence sufficiently conserved across all members of the genus *Metapneumovirus* for the development of the panMPV real time RT-PCR discussed in this paper. Regions that had been targeted previously for these types of test resided in N ORF (Bayon-Auboyer et al., 1999; Maertzdorf et al., 2004; Toquin et al., 1999) due to its high nucleotide conservation and its proximal 3'position in the viral genome. Genes closer to the 3'end of MPVs, like other viruses in the order *Mononegavirales*, are reported to be in greater abundance in infected cells due to their transcription strategy and thus, this increases the chances of detecting viral RNA molecules (Collins and Karron, 2013).

In this study a short length of sequence (107 nucleotides) in the N ORF of MPVs was deemed to be sufficiently conserved to develop a SYBR Green I real time RT-PCR. However a mixture of three primers (two in the forward sense and one reverse) was required to obtain balanced results across the four AMPV subgroups. SYBR Green I technology was chosen over a TaqMan approach because the latter technology required three zones of conserved sequence: one for each of the

Table 3a
LoD of RNA transcripts: three assays of eight replicates.

Subgroup	Copies/µLL	Assay 1 Mean Ct ± SD (CV)	Assay 2 Mean Ct ± SD (CV)	Assay 3 Mean Ct ± SD (CV)	Inter-Assay Mean Ct ± SD (CV)
A	1^E7	12.95 ± 0.14 (0.011)[A]	12.69 ± 0.13 (0.010)	12.80 ± 0.11 (0.009)	12.81 ± 0.16 (0.013)
	1^E5	19.33 ± 0.06 (0.003)	19.67 ± 0.08 (0.004)	19.40 ± 0.06 (0.003)[C]	19.47 ± 0.16 (0.008)
	1^E3	25.89 ± 0.09 (0.003)	26.49 ± 0.10 (0.004)	25.91 ± 0.55 (0.021)	26.10 ± 0.42 (0.016)
	1^E2	29.08 ± 0.14 (0.005)	30.21 ± 0.11 (0.004)	29.52 ± 0.42 (0.014)	29.60 ± 0.54 (0.018)
	1^E1	32.56 ± 0.41 (0.013)	33.75 ± 0.77 (0.023)[D]	33.01 ± 0.39 (0.012)	**33.10 ± 0.73 (0.022)**
B	1^E7	17.59 ± 0.17 (0.009)	17.73+ /−0.28 (0.016)[AD]	17.27 ± 0.09 (0.006)	17.52 ± 0.27 (0.015)
	1^E5	/	24.30 ± 0.17 (0.007)	23.96 ± 0.26 (0.011)	24.13 ± 0.27 (0.011)
	1^E4	26.81 ± 0.36 (0.014)	27.50 ± 0.16 (0.006)	27.47 ± 0.19 (0.007)	27.26 ± 0.40 (0.015)
	1^E3	30.10 ± 0.16 (0.005)[C]	30.73 ± 0.28 (0.009)	30.95 ± 0.25 (0.008)	30.59 ± 0.43 (0.014)
	1^E2	33.74 ± 0.44 (0.013)	33.66 ± 0.38 (0.011)	34.32 ± 0.46 (0.013)[A]	**33.89 ± 0.50 (0.015)**
	1^E1	0+[B]	0+[B]	/	0+/16
C	1^E6	14.55 ± 0.04 (0.003)	14.3 ± 0.09 (0.006)	14.47 ± 0.06 (0.004)	14.46 ± 0.10 (0.007)
	1^E4	21.23 ± 0.05 (0.002)[C]	21.19 ± 0.19 (0.009)	21.30 ± 0.08 (0.004)	21.24 ± 0.13 (0.006)
	1^E2	28.13 ± 0.06 (0.002)[C]	28.36 ± 0.41 (0.014)[D]	28.27 ± 0.15 (0.005)	28.25 ± 0.26 (0.009)
	1^E1	31.92 ± 0.30 (0.009)	31.91 ± 0.36 (0.011)	31.69 ± 0.31 (0.010)	**31.84 ± 0.33 (0.010)**
	1^E0	1+[B]	1+[B]	0+[B]	2+/24
D	1^E8	15.03 ± 0.15 (0.010)[A]	15.32 ± 0.12 (0.008)	15.78 ± 0.23 (0.015)	15.39 ± 0.36 (0.023)
	1^E6	21.64 ± 0.25 (0.012)	22.19 ± 0.09 (0.004)[C]	22.76 ± 0.37 (0.016)	22.20 ± 0.53 (0.024)
	1^E5	/	/	26.25 ± 0.19 (0.007)	/
	1^E4	28.72 ± 0.23 (0.008)	29.73 ± 0.22 (0.008)	28.56 ± 0.25 (0.009)	29.00 ± 0.57 (0.020)
	1^E3	31.94 ± 0.27 (0.008)	32.44 ± 0.84 (0.026)[D]	31.88 ± 0.30 (0.009)	**32.09 ± 0.58 (0.018)**

Bold = LoD.Intra-Assay.
[A] Values obtained from 7/7 replicates.
[B] Number of positives from 8 replicates (no values calculated).
[C] Minimum CV.
[D] Maximum CV.

primers plus another inside the region amplified for the fluorescent probe. Such a region of sequence was not found in the MPV genome. In addition amplification of DNA molecules using an intercalating dye such as SYBR Green I is measured not only in terms of Ct values but also by exploiting the melting curve values assessed at the end of the amplification process. Therefore, the peak location on a melting curve depends on the sequence, GC/AT ratio and the length of the resulting amplicon (Ririe et al., 1997). In some cases the Tm values are statistically different and can be used to identify separate subgroups even in a PCR specifically designed to be “broad range”. In the current study, it was not possible to use the Tm values to differentiate between virus species or among AMPV or HMPV subgroups.

The method was shown to be specific and highly sensitive for all the MPVs tested, with only two viruses amongst the 28 non-MPV viruses giving positive signals near the limit of the cut off threshold at Ct35. Furthermore these two viruses were HRSV strains that belong to the genus Orthopneumovirus which share the same family as MPV. Further experiments are ongoing to check whether other Orthopneumoviruses could also be detected. Regarding the sensitivity, later responses were observed (Ct = 32.4 and 33.3) for the HMPV B sub-lineages when

Table 3b
LoD of viral RNA: two assays of four replicates.

Subgoup	Viral titer[A]	Assay 1 Mean Ct ± SD (CV)	Assay 2 Mean Ct ± SD (CV)	Inter-Assay Mean Ct ± SD (CV)
A	4.20	20.30 ± 0.21 (0.010	21.22 ± 0.15 (0.007)	20.76 ± 0.52 (0.025)
	2.20	27.47 ± 0.19 (0.007)	28.00 ± 0.09 (0.003)[D]	27.73 ± 0.31 (0.011)
	1.20	30.89 ± 0.21 (0.007)	31.00 ± 0.55 (0.018)	**30.94 ± 0.39 (0.013)**
	0.20	35.02 ± 0.57 (0.016)[B]	34.48 ± 0.67 (0.019)[C E]	34.75 ± 0.64 (0.018)
B	3.97	20.78 ± 0.26 (0.013)	20.52 ± 0.13 (0.006)	20.65 ± 0.24 (0.011)
	1.97	27.50 ± 0.17 (0.006)	27.13 ± 0.17 (0.006)	27.32 ± 0.25 (0.009)
	0.97	31.07 ± 0.24 (0.008)	30.94 ± 0.16 (0.005)[D]	31.00 ± 0.20 (0.006)
	−0.03	34.96 ± 0.17 (0.005)	34.48 ± 0.89 (0.026)[E]	**34.72 ± 0.65 (0.019)**
C	3.42	19.81 ± 0.15 (0.008)	20.28 ± 0.08 (0.004)[D]	20.04 ± 0.27 (0.014)
	1.42	26.64 ± 0.18 (0.007)	27.27 ± 0.26 (0.010)	26.95 ± 0.40 (0.015)
	0.42	30.15 ± 0.45 (0.015)	30.88 ± 0.57 (0.018)[E]	30.51 ± 0.61 (0.020)
	−0.58	33.79 ± 0.52 (0.015)	34.04 ± 0.47 (0.014)	**33.92 ± 0.48 (0.014)**
D	4.60	20.39 ± 0.34 (0.017)	20.54 ± 0.54 (0.026)[E]	20.45 ± 0.42 (0.021)
	2.60	27.10 ± 0.62 (0.023)	26.74 ± 0.38 (0.014)	26.92 ± 0.51 (0.019)
	1.60	30.46 ± 0.20 (0.006)[D]	29.96 ± 0.37 (0.012)	30.21 ± 0.38 (0.013)
	0.60	34.31 ± 0.54 (0.016)	33.45 ± 0.46 (0.014)	**33.88 ± 0.65 (0.019)**

Bold = LoD . Intra-Assay.
[A] Log_{10} (Viral titer) $TCID_{50}$/mL.
[B] Values obtained for 2+/4 replicates.
[C] values obtained for 3+/4 replicates.
[D] minimum CV.
[E] maximum CV.

Table 4
Ct and Tm values from experimental samples (AMPV).

Virus	Species		Results GAPDH		Results MPV	
			Ct	Tm	Ct	Tm
AMPV A	Turkey	swab 1	29.3	80.5	28.6	78.5
		swab 2	29.2	80.5	27.0	78.5
AMPV B	Turkey	swab 1	25.6	80.5	30.1	76.0
		swab 2	27.8	80.5	30.4	76.5
US-AMPV C	Turkey	swab 1	26.6	80.5	24.7	76.0
		swab 2	27.9	80.5	25.2	76.0
Fr-AMPV C	Duck	swab 1	20.5	82.0	18.6	77.0
		swab 2	22.3	82.0	18.9	77.0
AMPV D	Turkey	swab 1	26.2	80.5	22.1	78.5
		swab 2	28.4	80.5	24.0	78.0
None (SPF)	Duck	swab 1	26.6	82.0	ND	–
		swab 2	28.0	82.0	ND	–
None (SPF)	Turkey	swab 1	26.7	80.5	ND	–
		swab 2	30.7	80.5	ND	–

ND = No detected.

compared to the results obtained for the rest of the MPVs tested. These later responses may be explained by the presence of a mismatch at the seventh nucleotide of the primer PanMPV/N1Rev from the 3′ end with all HMPV B sequences. It is also possible that the HMPV B clinical samples contained more PCR inhibitors than HMPV A samples although the similar results obtained for the GAPDH house keeping gene in both A and B lineages would suggest otherwise.

This method which was validated using AMPV RNA transcripts or reference viruses for the four AMPV subgroups showed high linearity and efficiency with the lowest R^2 equal to 0.9971 for the RT-PCR. In addition these signals had a high repeatability for the four AMPV subgroups within the concentration ranges tested (10^7 to 10^1, 10^7 to 10^2, 10^6 to 10^1 and 10^8 to 10^3 for AMPV A, B, C and D respectively, Table 3a). Finally, the LoDs which ranged from 1000 to10 copies/µL demonstrate that this “broad range” RT-PCR was equally sensitive in detecting all AMPVs as the qRT-PCR designed specifically for each AMPV subgroup by Guionie et al., 2007.

In summary, a SYBR Green I RT-PCR pan MPV has been developed and validated with a high specificity, sensitivity, efficiency and repeatability. This method with its broad-spectrum detection format will be useful for application in screening MPV infections and particularly for AMPV in large scale on-farm epidemiological studies, or for the detection with minimal diagnostic costs of MPVs with undetermined subgroup assignment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2017.10.010.

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