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Development of an avian avulavirus 1 (AAvV-1) L-gene real-time RT-PCR assay using minor groove binding probes for application as a routine diagnostic tool

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ABSTRACT

Newcastle disease is a devastating disease of poultry caused by Newcastle disease virus (NDV), a virulent form of avian avulavirus 1 (AAvV-1). A rapid, sensitive and specific means for the detection of NDV is fundamental for the control of this notifiable transboundary virus. Although several real-time RT-PCR assays exist for the detection of AAvV-1, diagnostic sensitivity and specificities can be sub-optimal. In this study, we describe a modification to an existing AAvV-1 L-gene RT-PCR screening assay, where the original probe set was replaced with minor groove binding (MGB) probes, to create the MGB L-gene assay. The diagnostic sensitivity and specificity of this assay was evaluated against a broad panel of both Class I and Class II AAvV-1 viruses of diverse and representative lineages/genotypes in both clinical samples and amplified viruses, and compared with a number of previously published real-time RT-PCR screening assays for AAvV-1. The MGB L-gene assay outperformed all other assays in this assessment, with enhanced sensitivity and specificity, detecting isolates from a broad range of virus lineages/genotypes (including contemporaneously-circulating strains). The assay has also proved its value for screening original clinical samples for the presence of AAvV-1, thus providing an improved screening assay for routine detection of this notifiable disease agent.

1. Introduction

Newcastle disease (ND) is one of the most prevalent and economically important diseases of poultry worldwide (Alexander, 2001, 2011) caused by Newcastle disease virus (NDV), a virulent form of avian avulavirus 1 (AAvV-1). NDV is an *Avulavirus* (genus) belonging to the family *Paramyxoviridae*, and is classified as a notifiable disease by the World Organisation for Animal Health (OIE) resulting in statutory control measures and trade restrictions to prevent the spread of the virus (OIE (World Organisation for Animal Health), 2015). The virus has high genetic diversity and is spread worldwide (Dimitrov et al., 2016). A new classification system for NDV was recently introduced which has now accepted by the International Committee on Taxonomy of Viruses (Amarasinghe et al., 2017).

The International Reference Laboratory (IRL - EU/OIE/FAO) for ND at the Animal and Plant Health Agency (APHA) distributes ring trial samples annually to National Reference Laboratories concerned with ND diagnosis both in Europe but also the wider region. This comprises part of the ongoing proficiency assessments made by the IRL and is mandated by the European Commission for those laboratories in the European Union (EU). The data derived from the ring trial is important in identifying diagnostic gaps and the need to develop new or improved molecular diagnostic tests. Twenty-nine molecular tests covering real-time and conventional RT-PCR protocols were used by participant laboratories in 2018 to detect AAvV-1 (Steve Essen, IRL, personal communication). These included screening assays detecting the M-gene (Wise et al., 2004; Cattoli et al., 2009; Fratnik Steyer et al., 2010), the L-gene (Fuller et al., 2010), or an L-gene/M-gene multiplex assay (Kim et al., 2008), along with many assays targeting the F-gene also enable sequencing or pathotyping (Collins et al., 1993; Seal et al., 1995; Kant et al., 1997; Oberdorfer and Werner, 1998; Huovilainen et al., 2001; Wang et al., 2001; Creelan et al., 2002; Aldous et al., 2003; Li and

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Zhang, 2004; Wise et al., 2004; Fuller et al., 2009; Cattoli et al., 2010; Fratnik Steyer et al., 2010; Liu et al., 2011; De Battisti et al., 2013) and other non-accredited in-house tests. The results from the most recent and past ring trials have not only highlighted that the multitude of rRT-PCR assays in use (especially within the EU) have varying sensitivities and specificities (EURL, 2017) but that harmonisation of protocols is still needed to optimise the diagnostic performance across more laboratories.

However, even within the more conserved genes of AAvV-1 targeted in screening rRT-PCR assays, there is still considerably high genetic variation within AAvV-1 to challenge primer/probe combinations to detect all contemporaneously circulating strains with sufficient diagnostic specificity/sensitivity. For example, the two most recent outbreaks of ND in the United Kingdom (UK) were associated with a sublineage 5b (Class II, genotype XIII) virus in pheasants (*Phasianus colchicus*) in 2005 (Aldous et al., 2007) and a sublineage 4b (Class II, genotype VI) virus in grey partridges (*Perdix perdix*) in 2006 (Irvine et al., 2009). These two virus strains were poorly detected by the 'Fuller' L-gene rRT-PCR assay (Fuller et al., 2010), suggesting limitations to its sensitivity. In order to improve the sensitivity of the 'Fuller' L-gene assay, the probes used in the assay have been replaced with novel minor groove binding (MGB) probes, which have been shown to increase sensitivity (Kutyavin et al., 2000; Farkas et al., 2009; Fratnik Steyer et al., 2010). The updated assay is described as the 'MGB L-gene assay'. This assay has been evaluated against a selection of rRT-PCR assays commonly used by participating countries during the annual IRL ring trials, along with a commercially-available NDV rRT-PCR kit, in order to compare robustness/reproducibility, sensitivity and specificity to detect AAvV-1 representing a diverse range of lineages/genotypes in clinical samples as well as amplified virus from egg fluid.

2. Materials and methods

2.1. Design of the MGB probes

In order to design the MGB probes, a set of over 220 published virus sequences coding for the full L-gene were downloaded from Genbank (National Centre for Biotechnology Information [NCBI] (Benson et al., 2015)) and aligned using Megalign (DNASTAR, Madison, Wisconsin, United States). A panel of AAvV-1 isolates representative of the lineages/genotypes defined by both major classification systems were assessed in this data set. Identical sequences were removed, and a subset of 200 isolates was analysed using Allele ID software (PREMIER Biosoft | 3786 Corina Way, Palo Alto, CA 94303-4504 USA). The selected target sequence was identical to that detected by the Fuller L-gene assay (Fuller et al., 2010), and a small number of MGB probe-binding sites were located within this target region, leading to the production of two TaqMan® MGB probes (LproMGB and LproMGB2) (Table 1).

2.2. MGB L-gene rRT-PCR assay

The Qiagen® QuantiFast® Probe RT-PCR kit (Qiagen, Manchester, UK) at a final reaction volume of 25 µl was used. Each reaction comprised 2x QuantiFast® Probe RT-PCR Master Mix (12.5 µl), 50x ROX dye solution (0.5 µl), 12.5 µM primer NDF1 (1 µl), 12.5 µM primer NDR2 (1 µl), 5 µM probe LproMGB (1 µl), 5 µM probe LproMGB2 (1 µl), QuantiFast® RT enzyme mix (0.25 µl), molecular biology grade water (2.75 µl) and RNA (5 µl). In all rRT-PCR runs, molecular biology grade water 'no template controls' (NTC) were included to detect any viral RNA contamination between wells or non-specific probe breakdown. NDF1 and NDR2 primers were used as previously published (Fuller et al., 2010), and all the L-gene MGB primers and probes are detailed in Table 1. Cycling parameters for the rRT-PCR using the MX3000 P or MX3005 P (Agilent Technologies, California, USA) were one cycle at 50 °C for 10 min, one cycle at 95 °C for 5 min and 40 cycles at 95 °C for 10 s, 50 °C for 30 s, and 72 °C for 30 s. Data analysis was undertaken using MxPro QPCR software version 4.10. (Agilent Technologies, California, USA).

Table 1
Nucleotide sequences and genomic locations of the primers and probes used in the MGB L-gene assay.

Primer/probe	*Nucleotide sequence (5'-3')*	*Full-genome position*
NDF	GAGCTAATGAACATTCTTTC	12611-12630
NDR	AATAGGCGGACCACATCTG	12753-12771
LproMGB	[FAM]CCAATCAACTTCCC[MGB]	12641-12654
LproMGB2	[VIC]AATAGTGTATGACAACAC[MGB]	12706-12723

2.3. Viruses and sample preparation

In order to assess the revised MGB probe, 76 AAvV-1 and 18 non-AAvV-1 strains were provided by the IRL for ND at APHA. The isolates included virulent and avirulent AAvV-1 strains (n = 76; Supplementary Table 1), selected to reflect the genetic diversity of contemporary AAvV-1. Viruses were amplified in specific pathogen free embryonated fowls' eggs and characterised as AAvV-1 (OIE (World Organisation for Animal Health), 2015). For assessment of assay specificity, the following non-AAvV-1 isolates (n = 18) were also included in the analysis: eight AAvV serotypes 2–9, four strains of avian influenza virus (H1N1, H5N1, H7N7 and H9N2), three strains of infectious bronchitis virus (IBV-2119, IBV-H120 and IBV-CC220), and a strain from all avian metapneumovirus (aMPV) groups A to C, as detailed (Fuller et al., 2010). Total nucleic acid extraction was performed using the QIamp viral RNA mini kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. Prior to testing, extracts were diluted 1:100 in molecular biology grade water, in order to minimise cross-contamination when handling large numbers of isolates in multiple PCR operations.

The MGB L-gene assay was routinely used to screen original clinical material comprising oropharyngeal and cloacal swabs, and four standard tissue samples (comprising 1. brain, 2. lung and trachea, 3. mixed viscera (namely, heart, liver, kidney and spleen) and 4. mixed intestines) submitted to APHA-Weybridge for statutory notifiable disease (i.e. avian influenza and ND) investigation according to internationally-recognised standards (OIE (World Organisation for Animal Health), 2015). In addition to these official samples, original clinical material from four experimentally-infected samples was also tested (Supplementary Table 1). Eight further lineage 6/Class I isolates were available for testing. RNA was extracted from the original clinical material by an automated extraction process (Slomka et al., 2009).

3. Assessment of the performance of the MGB L-gene assay compared to published AAvV-1 screening assays

The performance of the MGB L-gene assay was evaluated through comparison with a number of published AAvV-1 screening assays (Wise et al., 2004; Kim et al., 2008; Cattoli et al., 2009; Fuller et al., 2010), and the commercially available Ambion® M-gene assay (Ambion® TaqMan® NDV Reagents [Ambion, Paisley, UK]). Additionally, the MGB L-gene assay was compared with two assays detecting the partial F-gene (Wise et al., 2004; Fuller et al., 2009). In each case, the Qiagen® QuantiFast® Probe RT-PCR kit was used as described in section 2.2. Positive PCR controls were included in each assay run; an AG68 RNA standard and an Ulster RNA standard, derived from virulent and avirulent AAvV-1, respectively. All assays were evaluated as previously published except for the Fuller F-gene assay (current laboratory practice uses probes at 5µM concentration).

Table 2
2 × 2 frequency table comparing the diagnostic sensitivity and specificity of four rRT-PCR assays with gold standard (virus isolation in embryonated fowls' eggs and sequencing of cleavage site motif).

	Gold standard				Gold standard		
MGB L gene	+	–	Total	Fuller et. al 2010	+	–	Total
PCR result				PCR result			
+	75	1	76	+	53	7	60
–	0	18	18	–	0	18	18
Total	75	19	94	Total	53	25	78
	Sensitivity (%)	Specificity (%)			Sensitivity (%)	Specificity (%)	
	99	**100**			**88**	**100**	
95% CI	92.89-99.97	81.47-100		95% CI	77.43-95.18	81.47-100	
Cohens κ	0.966			Cohens κ	0.777		

	Gold standard				Gold standard		
Wise/Kim multiplex	+	–	Total	Ambio®	+	–	Total
PCR result				PCR result			
+	56	4	60	+	52	8	60
–	0	18	18	–	0	18	18
Total	56	22	78	Total	52	26	78
	Sensitivity (%)	Specificity (%)			Sensitivity (%)	Specificity (%)	
	93	**100**			**87**	**100**	
95% CI	83.8-98.15	81.47-100		95% CI	75.41-94.06	81.47-100	
Cohens κ	0.866			Cohens κ	0.750		

3.1. Preliminary evaluation of the rRT-PCR assays

Initial evaluation assessed the ability of each assay to detect 16 AAvV-1 isolates (denoted * in Supplementary Table 1), on a minimum of three separate occasions by the same operator. Correct detection and average threshold cycle (Ct) values were assessed as indicators of assay performance, with the four best performing assays subsequently evaluated further for diagnostic sensitivity and specificity (Table 2).

3.2. Assessment of diagnostic sensitivity and specificity of the four best performing rRT-PCR assays

The four best performing assays were evaluated for AAvV-1 sensitivity and specificity using all of the AAvV-1 isolates (Supplementary Table 1) (n = 60), together with non-AAvV-1 (AAvV 2-9) isolates, AIV, IBV and aMPV isolates (n = 18). The Ct value above which isolates were not considered positive was set at 37.0, consistent with the validated and accredited (quality assured to ISO17025 standards) assays currently in use at the APHA. Therefore any nucleic acid sample that yielded a Ct value result > 37.0 was repeat tested by the corresponding PCR method. If a Ct value result of ≥37.0 was obtained for two consecutive runs with any isolate, it was considered negative for the purposes of this assessment (the negative results were likely a result of sample degradation). A 2 × 2 frequency table was generated for each assay to assess correlation with sequence data determined previously for these isolates (Table 2).

3.3. Assessment of diagnostic sensitivity (linear range/limit of detection)

Diagnostic sensitivity testing to determine the limit of detection was undertaken using six isolates of known median egg infectious dose (EID_{50}) encompassing a diverse spectrum of genotypes/lineages (denoted ** in Supplementary Table 1). These were serially-diluted tenfold until a quantitative threshold (endpoint) level was exceeded. A standard curve was generated for the detection of each isolate using the MGB L-gene assay, for which the linear range and limit of detection was determined. The values obtained for diagnostic sensitivity, linear range and limit of detection of the MGB L-gene assay were then directly compared to those for the Wise/Kim M/L-gene multiplex assay (Kim et al., 2008), Fuller L-gene assay (Fuller et al., 2010) and the Ambion® M-gene assay.

4. Results

4.1. Preliminary evaluation of the rRT-PCR assays

The nine assays selected for the initial screening evaluation were assessed for ability to detect a range of AAvV-1 isolates (n = 16), with emphasis on early detection (i.e. low Ct value), and reproducibility in repeated (≥3) assays. Mean Ct values, calculated for each assay, were compared as an initial indicator of assay sensitivity (Supplementary Table 2). The Ambion® TaqMan NDV control (supplied with the Ambion® assay kit) was correctly detected in all repeated assays, with a mean Ct value of 25.78.

Amongst the nine assays assessed, only the Wise/Kim M-gene/L-gene multiplex assay was 100% sensitive for all 16 isolates in the control screening panel, so was selected for further diagnostic specificity and sensitivity assessment against the complete panel of AAvV-1 isolates. Of the remaining assays, the Fuller L-gene, the Ambion® M-gene and the MGB L-gene assays were also selected for further specificity and sensitivity assessment on the basis of a subjective aggregate score of specificity, average Ct value and reproducibility. The remaining assays (Wise M-gene, Cattoli M-gene, Wise F-gene and Fuller F-gene) were not selected for further evaluation, as each test failed to detect at least one isolate or had higher aggregate Ct values (25.99, 27.61, 31.61 and 31.24, respectively).

4.2. Diagnostic specificity assessment

Using an enlarged panel of 60 isolates, four screening assays were again compared for specificity (MGB L-gene assay, Wise/Kim Multiplex assay, Fuller L-gene assay and the Ambion® M gene assay). This extended evaluation revealed that the MGB L-gene assay had the greatest specificity, with 98% of isolates positive defined by a Ct value < 37.0 (Table 3). However, none of the assays detected all of the 60 AAvV-1 isolates defined in the specificity panel (Supplementary Table 1). The isolates not detected by each assay were as follows:

MGB L-gene assay, isolate 16 (Lineage 3c/Class II genotype V); Wise/Kim assay, isolates 29 (Lineage 4b/Class II genotype VI), 54 and 57 (Lineage 5/Class II genotype VII and Lineage 4/Class II genotype

Table 3
Diagnostic sensitivity assessment demonstrating the linear range (LR) and limit of detection (LoD) of the MGB L-gene rRT-PCR and other screening assays against a representative isolate (n = 6) of each AAvV-1 lineage (genotype shown in brackets). LR, linear range; LoD, limit of detection; *nd*, not detected.

Assay	*EID_{50}/ml (LR/LoD)*					
	Lin. 1 (Gen. I)	*Lin. 2 (Gen. II)*	*Lin. 3c (Gen. V)*	*Lin. 4b (Gen. VIb)*	*Lin. 5b (Gen. XIII)*	*Lin. 6 (Class I)*
MGB L-gene	$10^{2.1}/10^{1.1}$	$10^{3.6}$	$10^{0.6}$	$10^{1.1}$	$10^{0.1}$	$10^{5.3}/10^{4.3}$
Kim et al., 2008	$10^{3.1}/10^{2.1}$	$10^{5.6}$	$10^{2.6}$	$10^{2.1}$	$10^{1.1}$	$10^{3.3}$
Fuller et al., 2010	$10^{3.1}$	$10^{4.6}$	$10^{1.6}$	$10^{2.1}$	$10^{2.1}$	*nd*
Ambion®	$10^{2.1}/10^{1.1}$	$10^{8.6}$	$10^{2.6}/10^{1.6}$	$10^{2.1}/10^{1.1}$	$10^{1.1}/10^{0.1}$	*nd*

VIf) and 55 (Lineage 5b/Class II genotype XIII); Fuller L-gene assay, isolates 29–31 (Lineage 4b/Class II genotype VI), 55 (Lineage 5b/Class II genotype XIII) and 58–60 (Lineage 6/Class I); Ambion® M-gene, isolates 27 and 29 (Lineage 4b/Class II genotype VI), 54 and 57 (Lineage 5/Class II genotype VII and Lineage 4/Class II genotype VIf), 55 (Lineage 5b/Class II genotype XIII) and 58–60 (Lineage 6/Class I).

Additionally, a 2 × 2 frequency table (Table 2) was generated for each assay to assess correlation with previous sequence data obtained for the specificity panel (Aldous et al., 2003). Statistical significance was defined by the Kappa agreement where values ranging from 0.61-0.80 reflected substantial agreement with sequence data, whilst 0.81-0.99 reflected almost perfect agreement with sequence data. This demonstrated that although all four assays were highly specific, the MGB L-gene assay demonstrated a higher degree of specificity in terms of correlation with previous sequence data, when compared to the other three assays.

In order to demonstrate the improved sensitivity of the MGB L-gene assay, the difference in Ct values obtained for this assay when compared to those obtained for the Wise/Kim, Fuller L-gene and Ambion® assays, are shown in Fig. 1. A 'No Ct' result was assigned a value of zero for comparison purposes. Mean Ct value differences in comparison to the MGB L-gene assay were 5.9, 4.1 and 1.1 for the Fuller L-gene, Ambion® and Wise/Kim assays, respectively. Lower Ct values were achieved with the Kim multiplex assay than the Wise M-gene assay which was possibly due to the different chemistry being used with the respective assays. Of the 60 isolates assessed using the MGB L-gene assay, differences of > 2 Ct value were observed in 58/60, 37/60 and 17/60 isolates for the Fuller L-gene, Ambion® and Wise/Kim assays, respectively. Similarly, differences of < 2 Ct value were observed for the Fuller L-gene (1/60), Ambion® (3/60) and Wise/Kim assays (9/60).

4.3. Diagnostic sensitivity assessment

The linear range (LR) and limit of detection (LoD) for each assay in relation to infectious doses was determined (Table 3). A single figure denotes that the limit of detection falls within the linear range of the standard curve. The sensitivity of the MGB L-gene assay was shown to be equal or superior to the other assays, with the exception of the lineage 6 /Class I isolate where the sensitivity of the Wise/Kim multiplex assay was greater by 1 $\log EID_{50}$/ml. This observation was as expected, since the Kim assay used in the multiplex is known to detect lineage 6/Class I as well as Class II AAvV-1 isolates.

4.4. Assessment of the MGB L-gene assay on original clinical samples

Since 2014, the MGB L-gene assay has been used as a front-line diagnostic tool along with the other internationally-recognised methods of attempted virus isolation in embryonated fowls' eggs and nucleotide sequencing for detection of AAvV-1 in more than 3700 original clinical samples submitted to APHA-Weybridge from poultry for statutory avian notifiable disease investigation (OIE (World Organisation for Animal Health) 2015; data not shown). Positive results from the MGB L-gene assay, followed by statutory nucleotide sequencing and isolation of virus in eggs led to the identification in 2015 of an avirulent AAvV-1 virus of lineage 1 origin followed in the same year by two other lineage 1 viruses (Reid et al., manuscript in preparation). Aside from these AAvV-1 positive cases, all other official samples submitted for statutory testing were negative ('no Ct value') by the MGB L-gene assay and restrictions were lifted on the premises following no isolation of virus in eggs.

Furthermore, the MGB L-gene assay detected AAvV-1 in a range of tissues from experimentally-infected birds (data not shown).

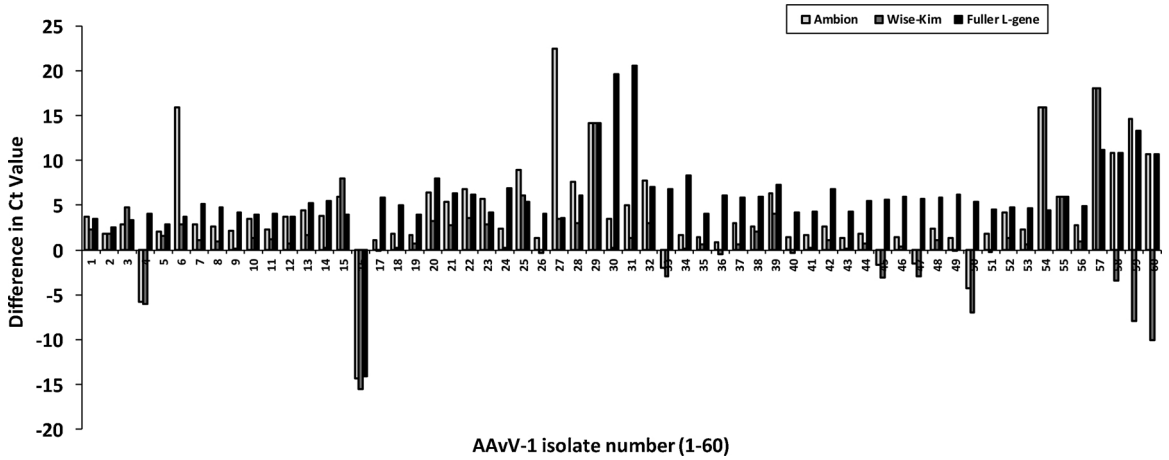

Fig. 1. Comparison of diagnostic specificity demonstrated by differences in Ct values obtained using the Ambion®, Wise-Kim and Fuller L-gene assays, when compared to the MGB L-gene assay. For each AAvV-1 isolate tested, a positive value (> 0) for the Ambion®, Wise-Kim and Fuller L-gene assays reflects decreased diagnostic sensitivity when compared to the MGB L-gene assay.

5. Discussion

Newcastle disease is a notifiable disease which can have a devastating impact in poultry populations, therefore sensitive, rapid and accurate methods of diagnosis during an outbreak are essential. Additionally, an assay must be able to detect a wide range of contemporaneously circulating lineages/genotypes, including new and emerging strains. However, the considerable genetic diversity within AAvV-1 means that the development of a single molecular assay for the detection of any AAvV-1 is both complex and challenging. One strategy to overcome this genetic diversity has been to multiplex together separate rRT-PCR assays (Kim et al., 2008). However, an alternative approach is to optimise existing assays through the modification of primers and probes, taking into account any genetic or heterogeneity observed, and potentially utilising alternative technologies. One such technology is the use of MGB probes (Kutyavin et al., 2000). These probes bind to the minor groove of DNA with strong specificity and affinity and an increase in the probe melting temperature (T_m), thus allowing shorter, more specific probes to be constructed. We have assessed the use of MGB probes as a replacement for existing probes in the previously published Fuller L-gene screening assay (Fuller et al., 2010), thus producing the MGB L-gene assay. This modification has been shown to improve not only the diagnostic sensitivity of the assay, but also the test specificity, enabling the detection of lineage 6/Class I isolates which have previously been difficult to detect using the Fuller L-gene assay and others without modification. The reduced diagnostic specificity for lineage 6/Class I isolates observed with the MGB L-gene assay, when compared to Class II isolates, does not have significant diagnostic implications, as Class I encompasses viruses of low virulence with only one virulent virus field sample being reported (Alexander et al., 1992).

In this study we evaluate the MGB L-gene assay as a diagnostic tool by direct comparison to other established AAvV-1 molecular assays. It demonstrated improved diagnostic sensitivity, assessed using a genetically diverse and representative AAvV-1 viruses. The isolates utilised for diagnostic specificity testing were chosen on the basis of their potential threat to Europe, selected from outbreaks in Africa, Asia and the Middle-East, or those that had already been identified as circulating in Europe. The MGB L-gene assay demonstrated improved diagnostic performance against existing assays, and only failed to detect one isolate, while also exhibiting more sensitive detection by lower Ct value threshold values for the majority of isolates, when compared to the other three evaluated assays. The single isolate not detected by the MGB L-gene assay was a lineage 3c (Class II genotype V) isolate from Sudan, a lineage/genotype which had demonstrated only low reactivity in a previously-published MGB rRT-PCR assay targeting the AAvV-1 M-gene (Fratnik Steyer et al., 2010). Overall, the MGB L-gene assay exhibited greater diagnostic sensitivity and specificity compared to the three other established tests, including the limit of detection (Table 3) which was equal to, if not greater than, that of the other three assays aside from lineage 6 viruses. The MGB L-gene assay is routinely used at the APHA for the screening of original clinical material submitted from statutory avian notifiable disease investigations. Where original clinical material was unavailable for testing, sequences of untested genotypes were aligned (Megalign) against the MGB L-gene primer probe region. This identified two mismatches in the probe-binding site with the lineage 3c virus AAvV1/chicken/Sudan/trachea1/2006 and therefore the likely cause of the false negative results from this strain. The nucleotide sequences of the primers and probe binding sites are routinely aligned against the corresponding sequences of currently-circulating AAvV-1 isolates submitted to the IRL for virological investigation. Together with the downloading of available sequences of AAvV-1 s not available for testing in this study, these ongoing analyses provide definitive information towards predicated test utility and whether the incorporation of degenerative bases in the primers/probe design was ultimately needed to cover potential mismatching. The Wise/Kim multiplex assay also performed effectively, however, the MGB L-gene assay was more sensitive, despite the requirement for less RNA template (5 µl compared to 8 µl). The diagnostic specificity evaluation of the screening assays (MGB L-gene, Fuller et al., 2010; Kim et al., 2008 and Ambion®) highlighted that, due to the genetic diversity of AAvV-1 isolates, no single assay may be capable of detecting all AAvV-1 isolates. However, unlike the Fuller at al. L-gene assay (2010, which utilised only one probe), the MGB L-gene assay utilised two probes targeting different regions within the gene, and was the most successful screening tool, detecting 75 out of 76 isolates. As for the other AAvV-1 screening assays, the MGB L-gene assay does not provide virulence predication or differentiate between the different virulences of AAvV-1.

In conclusion, the MGB L-gene assay demonstrated rapid and specific detection of AAvV-1, with a higher degree of diagnostic sensitivity and specificity when compared to existing screening assays. This study highlights the importance of continuously monitoring the performance of currently-used diagnostic assays. Validated molecular tests, particularly those used for detection of notifiable diseases, should be routinely monitored at regular intervals, to ensure that each remain fit-for-purpose for sensitive and specific detection of currently-circulating viruses. Regular assessment against newly-emerging strains with relevance to poultry health would facilitate the modification of primers and probes, to account for any important heterogeneity not previously detected, but without a loss of assay performance for the detection of other contemporary AAvV-1 s. This approach ensures that assays remain sufficiently sensitive to detect virulent strains with the potential to cause ND outbreaks in poultry, and is an important foundation of international reference laboratories.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2018.12.001.

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