Evaluation of six molecular assays for the detection of Aigai virus

Anna Papa[1], Styliani Pappa[1]

AFFILIATION
1 Aristotle University of Thessaloniki, Thessaloniki, Greece

CORRESPONDENCE TO
Anna Papa. Aristotle University of Thessaloniki, 54124, Thessaloniki, Greece. E-mail: annap@auth.gr ORCID ID: https://orcid.org/0000-0002-6643-3322

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ABSTRACT

INTRODUCTION Aigai virus (AIGV) is the prototype strain of the novel Orthonairovirus parahaemorrhagiae species (*Nairoviridae* family), which contains the strains of the previous CCHFV genogroup VI (or Greece/Europe-2 or AP-92-like). The reclassification was done due to the genetic distance of AIGV from all genotypes of CCHFV. The aim of the present study was to evaluate the performance of six molecular assays to detect AIGV.

METHODS Undiluted and serial dilutions (1:10 to 1:10000) of culture supernatant of AIGV strain Pentalofos were used for the comparative study. The strain was isolated from *Rhipicephalus bursa* ticks removed in 2015 from a goat in Pentalofos village, Greece. Following RNA extraction, six different molecular assays were applied: two nested RT-PCRs, one RT-PCR, and three real-Time RT-PCRs (one commercial).

RESULTS All assays detected AIGV up to the 1:1000 dilution, while even higher sensitivity (detection of the 1:10000 dilution) was seen in the nested PCRs designed/modified based on the AP92 sequence, and in two of the real-time RT-PCRs. Lowest Ct values were taken using the commercial assay.

CONCLUSIONS All assays performed well for the detection of AIGV, suggesting that the risk for underdiagnosis of AIGV infections is low using these assays. However, mismatches in the primers/probes affected the sensitivity of the assays. Genetic surveillance is needed to monitor the mutations in the virus which might affect the efficacy of the diagnostic tools, while a sensitive real time RT-PCR capable to differentiate AIGV and CCHFV will be extremely helpful to estimate the exact burden of AIGV infections.

INTRODUCTION

Aigai virus (AIGV) is the prototype strain of the recently established Orthonairovirus parahaemorrhagiae species which contains the previously Crimean-Congo hemorrhagic fever virus (CCHFV) strains of genogroup VI (or Greece/Europe-2 or AP-92-like); the name was given after the place of discovery of the original AP-92 strain[1]. The reclassification was done by the International Committee on Taxonomy of Viruses (ICTV) based on the sufficient genetic distance of genogroup VI from genotypes I–V/VII[2,3].

AIGV has been detected in several tick species, mainly in *Rhipicephalus bursa* ticks, collected in several regions of the Balkans and Turkey[4-8]. However, only few human cases have been associated with the virus[9,10]. The rarity of AIGV human infections could be due to low pathogenicity of the virus or to low sensitivity of the diagnostic methods related to primer mismatches. Gruber et al.[11] reported that due to the high genetic variability of CCHFV strains in different geographical regions, the diagnostic potential of the molecular tests may be decreased, and they suggested the application of combined protocols. The problem is higher as AIGV presents the highest genetic diversity from all CCHFV genogroups. Currently there is no commercial molecular assay for the specific detection of AIGV, and the diagnosis is based on assays designed for CCHFV. Therefore, the aim of the present study was to evaluate the performance of six molecular assays for detection of AIGV.

METHODS

The culture supernatant of AIGV strain Pentalofos was used for the comparative study. The strain was isolated from a pool of two adult *R. bursa* ticks collected in 2015 from a goat in Pentalofos village, Greece[4]. Viral RNA was extracted using the QiaAmp Viral RNA mini kit (Qiagen, Hilden, Germany)

Table 1. Results of the six molecular protocols applied in this study

Culture supernatant AIGV	Nested RT-PCR12			Nested RT-PCR9		RT-PCR13	Ct in real-time RT-PCR		
	1st round	2nd round	2nd round with the new primer	1st round	2nd round		Wölfel et al.[14]	Atkinson et al.[15]	altona
undiluted	Positive	Negative	Positive-faint	Positive	Positive	Positive	23.91	19.18	18.16
1:10	Positive	Positive	Positive-faint	Positive	Positive	Positive	25.43	23.21	19.83
1:100	Positive	Positive	Positive	Positive	Positive	Positive	29.28	27.11	23.34
1:1000	Negative	Positive	Positive	Positive	Positive	Positive-faint	35.99	31.77	28.21
1:10000	Negative	Negative	Positive	Positive	Positive	Negative	Negative	35.48	31.37

Table 2. Alignment of forward primer, reverse primer, and probe sequences of the 6 diagnostic assays to four AIGV strains and to the prototype CCHFV strain IbAr10200

Strain	Nested RT-PCR[12]				
	1st round		2nd round		
	Primer F2	Primer R3	Primer F3	Primer R2	Primer R2 (this study)
	TGGACACCTTCACAAACTC	GACAAATTCCCTGCACCA	GAATGTGCATGGGTTAGCTC	GACATCACAATTTCACCAGG	TCATGTCTGACAGCAT
NC_005302, IbAr10200	----------	------C----------	--G--C--A----------	----------C-----A--	----------
DQ211638, AP92	----------	--TG--C----------	--G-----C-----C-----	-G--C--AG--C--T--T--	----------
MG516211, Pentalofos	----------	--TG--C----------	--G-----T----------	-G--C--AG--C--T-----	----------
MN811033, CAP14	----------C---	--T---C-----A-----	--G-----C----------	-G--C--AG--C--T-----	----------
MK299344, MT	----------	--TG--C--T--------	--G-----T----------	-G--C--AG--C--C-----	----------
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Strain	Nested RT-PCR[9]			
	1st round		2nd round	
	Primer Gre-F1	Primer Gre-R1	Primer Gre-F2	Primer Gre-R2
	AATGTGCCGAACTTGGACAG	TGCGACAAGTGCAATCCCG	ATCAGATGGCCAGTGCAACC	ACTCCCTGCACCACTCAATG
NC_005302, IbAr10200	-G---T--C--T--------	---A--------T-----T	T----------C---	----------G--T-CA
DQ211638, AP92	----------	----------	----------	----------
MG516211, Pentalofos	-----A--A----------	----------A	----A----------	----------
MN811033, CAP14	-----A----------	----------A	-C--A----------	-------A-----T------
MK299344, MT	--------A----------	----------A	-C--A----------T	----T----------
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Table 2. Continued

Strain	RT-PCR[13]	
	Primer 6942 +	Primer 7385 -
	ATGATTGCIAAYAGIAAYTTYAA	ACAGCARTGIATIGGICCCCAYTT
NC_005301, IbAr10200	-----A----------	----------
DQ211612, AP92	----------	----------
MG516213, Pentalofos	----------	----------
MN811030, CAP14	----------	----------
MK299346, MT	----------	----------
	***** **********	**********

Strain	Real Time RT-PCR[14]	
	Primer CCForSEO1	Primer CCRevSEO2
	CAAGGGGTACCAAGAAAATGAAGAAGGC	GCCACAGGGATTGTTCCAAAGCAGAC
NC_005302, IbAr10200	----A----------	----------C----------
DQ211638, AP92	-------C----------A--	--T-----A-----C----------
MG516211, Pentalofos	-------C----------A--	--T-----A----------
MN811033, CAP14	----------	--------A----------
MK299344, MT	----------A--A--	--T-----A----------
	***** ** ********** ** **	** ***** ***** **********

Strain	Real Time RT-PCR[14]		
	Probe SEO1	Probe SEO3	Probe SEOA
	ATCTACATGCACCCTGCTGTGTTGACA	ATTTACATGCACCCTGCCGTGCTTACA	AGCTTCTTCCCCCACTTCATTGGAGT
NC_005302, IbAr10200	--T--------T-----C---C-T---	----------T----------	----------G--
DQ211638, AP92	----------C-----	--C----------T-----G---	--------T----------G-----
MG516211, Pentalofos	--C----------C--C---------	--C----------C------T-G---	--------T-----T-----G-----
MN811033, CAP14	----------C---------	--C----------T-G---	--------T-----T-----G-----
MK299344, MT	----------	--C----------T---T-G---	--------T-----T-----G-----
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Table 2. Continued

Strain	Real Time RT-PCR[15]		
	Primer CCHF S1	Primer CCHF S122	Probe CCHF-probe
	TCTCAAAGAAACACGTGCC	CCTTTTTGAACTCTTCAAACC	ACTCAAGGKAACACTGTGGGCGTAAG
NC_005302, IbAr10200	----------	-T----------	-------AG----------
DQ211638, AP92	----------	-T--C--------C-------	-------AT----------G--
MG516211, Pentalofos	----------	-T--C--------C-------	-------AT----------G--
MN811033, CAP14	??????????????????	-T--C----------	-------AT----------?????
MK299344, MT	?----------	-T--C----------	-------AT----------G--
		* ** ******** *******	******** **********

?: not available sequence for this site of the genome.

and eluted in 50 μL elution buffer. cDNA was synthesized with SuperScript III Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific) in accordance with the instructions of the manufacturer. Virus dilutions of 1:10, 1:100, 1:1000 and 1:10000 in nuclease-free water were prepared and six different molecular assays were used to evaluate the detection of AIGV: 1) one of the first described and often used nested RT-PCR, which amplifies a fragment of the S RNA segment of CCHFV[12]; a modified version was also applied by replacing the reverse primer of the second round PCR with a newly designed primer (TCATGTCTGACAGCAT) to decrease the number of mismatches; 2) an RT-PCR targeting a 443bp region of the highly conserved polymerase domains within the L-polymerase encoding region[13]; 3) a real-time RT-PCR assay which amplifies a 181bp region near the 5′-end of the S segment[14]; 4) a real-time RT-PCR assay which amplifies a 122bp region of the 5′ untranslated region of the S segment[15]; 5) a commercial Real Time RT-PCR assay (RealStar, CCHFV RT-PCR kit altona, Hamburg, Germany); and 6) a nested RT-PCR which was specifically designed to amplify a fragment of the S segment of AP92 strain (U04958)[9].

The primer (forward and reverse) and the probe sequences of all assays were aligned to the respective sequences of four representative AIGV strains and the prototype CCHFV strain IbAr10200. The AIGV strains were AP92 (isolated from *R. bursa* ticks collected in 1975 from a goat in Vergina village in Greece) and Pentalofos from Greece[4,16], strain MT-1362 (detected in 2012 in a *R. bursa* tick collected from a cow in Malko Tarnovo in Bulgaria)[8], and strain CAP14 (detected in 2017 in a female *Rhipicephalus sanguineus* s.l. tick collected from a dog in Mersin in Turkey)[7].

RESULTS

The diagnostic performances of the six applied assays are shown in Table 1, while the sequence differences between the primers/probes of each molecular assay in various strains of AIGV and the prototype strain of CCHFV IbAr10200 are seen in Table 2. The nested RT-PCR of Schwarz et al.[12] worked well up to the dilution of 1:1000, while by replacing the reverse primer of the second round PCR (which presented many mismatches) by the newly designed primer, a positive result was taken also at the dilution of 1:10000. As expected, the highest sensitivity (positive result up to the 1:10000 dilution) was seen in the protocol of Midilli et al.[9], since the primers had been designed based specifically on the sequence of the AP92 strain and although some mismatches were present, they were less than 2. The application of the RT-PCR protocol with the degenerate primers described by Honig et al.[13] gave a positive result up to the dilution of 1:1000. Similarly, the real-time RT-PCR described by Wolfel et al.[14] gave a positive result up to the dilution of 1:1000; there were 0–3 mismatches in the primers and 0–4 mismatches in the probes. The protocol of Atkinson et al.[15] performed better, as a positive result was taken also at the dilution of 1:10000 (Ct value 35.48). For this assay, it was not feasible to perform a robust variant analysis because its target region includes the CCHFV 5′ end of the S segment that is rarely sequenced and reported. The commercial RT-PCR successfully detected the 1:10000 dilution (Ct value 31.27); in general, all Ct values were lower than the in-house real time RT-PCRs.

DISCUSSION

A wide range of molecular methods for CCHFV diagnostics have been reported. An *in silico* study analyzed 22 molecular assays and detected up to 28 mismatches between primers/probes and CCHFV sequences (that time AIGV was considered as genogroup Europe 2) and suggested that combination of assays have to be applied depending

on the geographical region[11]. In this study, we compared the performance of six different molecular assays for the detection of AIGV. The results of the study showed that mismatches in the primers and probes were seen in all six assays. Despite the high number of mismatches in some assays, all performed well and detected the virus at least up to the 1:1000 dilution. The highest sensitivity was seen in the protocol of Midilli et al.[9], as the primers were specifically designed on the basis of AP92 sequence, but also in two real time RT-PCRs with lowest Ct values taken using the commercial assay, which is one of the most commonly used in the European laboratories for CCHFV diagnostics.

The pathogenicity of AIGV is currently unknown. The number of human cases associated with AIGV is extremely low and there is no information about the viral load. However, it seems that if the viral load is moderate or high, the virus can be easily detected at least with the assays included in the present study. A well-designed study is needed to test febrile cases, especially in patients who report a tick bite, to estimate the real burden of AIGV. A real time RT-PCR able to differentiate CCHFV and AIGV will be extremely helpful both for human diagnostics and for screening ticks. Similarly, identification of epitopes that could be used for serological assays able to differentiate IgG antibodies against CCHFV and AIGV will provide useful information, since neutralization assays for CCHFV are difficult and there is a need for BSL-4 facilities.

CONCLUSIONS

All assays of the study performed well for the detection of AIGV. However, mismatches in the primers/probes affected the sensitivity level of the assays. Genetic surveillance is needed to monitor the mutations in the virus, which might affect the efficacy of the diagnostic tools, while a sensitive real time RT-PCR able to differentiate AIGV and CCHFV will be extremely helpful to estimate the exact burden of AIGV infections.

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CONFLICTS OF INTEREST
The authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest and none was reported.

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Ethical approval and informed consent were not required for this study.

DATA AVAILABILITY
The data supporting this research are available from the authors on reasonable request

AUTHORS' CONTRIBUTIONS
SP: laboratory experiments and writing of the manuscript. AP: conceptualization, design of the work, interpretation of data, and writing the final version of the manuscript.

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