DETECTION AND QUANTITATION OF PPR RNA USING REAL-TIME RT-PCR AFTER MANUAL AND AUTOMATED SAMPLE PROCESSING

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Introduction

Peste des Petits Ruminants (PPR) results from the infection by an enveloped negative RNA virus (16 kb) belonging to the family *Paramyxoviridae*, genus *Morbillivirus*. PPR is highly contagious for small ruminants and the high mortality rates makes it necessary to speed up the diagnostic procedure in order to quarantine infected sheep and goats and start appropriate control measures. In this study we developed a real-time RT-PCR (RRT-PCR) method that can be performed in a 96-well format, and this feature opens the possibility of using this methodology in the high-throughput screening of field samples or to evaluate antiviral therapy.

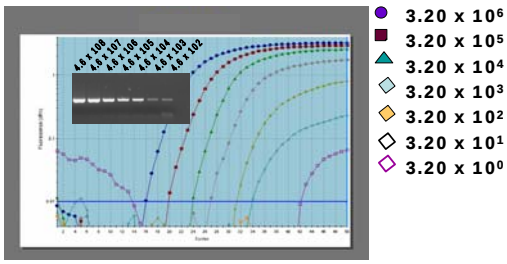

Fig 1: Sensitivity of the RRT-PCR based on gene N transcript (cRNA) of PPRV. Comparison to conventional RT-PCR

Material and Methods

We determined the primer and probe sequence requirements (Beacon Design) for specific detection of PPR lineages by comparing the nucleoprotein (N) gene of all morbilliviruses. The TaqMan probe was labeled at the 5' end with the fluorescent reporter dye 6-carboxy-fluorescein (FAM) and at the 3' end with a nonfluorescent quencher, black hole 1 (BHQ1).The assay could amplified a fragment of 54 bp targeting the 3' end of the N-protein gene. Sensitivity was assessed on transcripts from the NPPR gene (cRNA) containing initially 3.2×10^6 copies per reaction mixture. Comparison was made to conventional RT-PCR as described previously [1]. Tenfold dilutions of each culture supernatants of the different lineages as well as samples collected from sick animals in outbreaks were subjected to nucleic acid extraction (Nucleospin Plasmid, Macherey Nagel, USA) and analyzed by RRT-PCR.

Results

Sensitivity of the method was measured using 10-fold dilutions (undiluted to 10^{-6}) of the cRNA and detection limit was a viral RNA load of 32 copies per reaction with a corresponding Ct value of 34. For the conventional method it was of 4.6 10^2 copies per reaction (Fig. 1).

Specificity was demonstrated on PPRV isolates from all 4 lineages, as well as on the vaccine strain. We also confirmed that the method did not cross-react with other Morbilliviruses, especially RPV and MV. Ct values were found to be within a range of 8 (vaccine strain used to delineate the primers and probe) and 27. Viral RNA loads were in the range of 10^4 to 10^8 copies per reaction mixture (Fig. 2)

Field samples (15) collected from PPRV-suspected animals a recent outbreak were analyzed. All the samples (4) identified as positive by conventional RT-PCR were confirmed by RRT-PCR. Five additional samples from lung, spleen, and intestine were identified as positive with this last method. The viral RNA loads were found to be in the range of 10^2 to 10^5 copies per reaction mixture (Fig. 3).

Suitable for high-throughput testing and automation : We implemented this assay on blood specimens with automated extraction method. The rate could reach 440 RNA extractions on the same day. After intra-venous challenge of goats to detect PPR RNA at different time points, RNA loads varied between 55 and 2.510^3 (Fig. 4) .

Virus Lineage	Name of the Strain	Dilution	Ct observed	Copy number
PPRV I	COTE D'IVOIRE	10^{-4}	24	8.6×10^5
		10^{-5}	26	2.0×10^4
		10^{-6}	27	1.7×10^4
PPRV II	NIGERIA 75 1 vac	neat	8	4.6×10^8
		10^{-1}	11	4.3×10^7
PPRV II	NIGERIA 75 1 wt	neat	17	9.0×10^5
		10^{-1}	20	1.7×10^5
		10^{-2}	23	2.6×10^4
PPRV III	ETHIOPIA 94	neat	17	1.1×10^6
		10^{-1}	18	4.4×10^5
		10^{-2}	22	4.6×10^4
PPRV III	SUDAN 72	neat	15	5.6×10^6
		10^{-1}	18	5.9×10^5
		10^{-2}	20	1.3×10^5
PPRV IV	INDIA 94	neat	12	2.7×10^7
		10^{-1}	15	5.0×10^6
		10^{-2}	18	5.6×10^5
MV	Onderstepoort	neat	No Ct	Negative
RPV	RBOK vaccine	neat	No Ct	Negative

Fig 2: Specificity of the RRT-PCR assay for PPRV. Analysis of PPRV cell culture supernatants of representative strain, of vaccines, and other viruses to assess specificity.

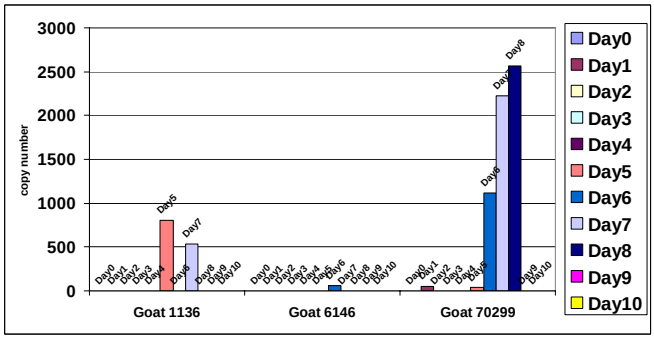

Fig. 4: Real-time quantitative detection of PPR RNA in blood of goats after intra-venous challenge.

		RT-PCR	QRT-PCR		
Vial N°	Sample	Status	Status	Ct number	Copy number
17	intestine	-	-	-	-
18	lung	-	-	-	-
19	intestine	-	positive	29	4.8x103
21	intestine	-	positive	34	75
22	lung	-	-	-	0
23	intestine	-	positive	33	2.6x102
24	lung	-	positive	31	4.6x103
25	lung	positive	positive	20	1.1x105
26	spleen	positive	positive	16	4.4x105
27	intestine	positive	positive	15	6.6x105
28	spleen	positive	positive	18	2.9x105
29	Lymph node	-	-	49	3.4
30	spleen	-	positive	28	6.5x103
31	Lymph node	-	-	49	3.5
32	spleen	-	-	-	-

Fig 3: Diagnostic of samples tested positive by conventional RT-PCR or RRT-PCR

Conclusion and discussion

Amplification based on the N-protein gene developed in this study proved to be specific and no cross-amplification of other morbilliviruses was observed. All lineages of PPR isolates, those currently circulating in Africa, Middle East and Asia were detected. The minor nucleotide changes within the primer-binding and the probe regions were not detrimental to amplification. Viral load evaluation in clinical specimens was also performed. When adapted to high throughput format (96-well plates), the RRT-PCR method provides the basis to evaluate the efficacy of antiviral drugs in vivo and in vitro.

Acknowledgements

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References

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