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RESEARCH

Some molecular assays used for identity of Peste des Petits Ruminants (PPR) vaccines

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ABSTRACT

Background: Evaluation of Peste des petits ruminants (PPR) vaccine is a matter of concern to ensure releasing of safe, effective and potent product.
Objective: To use of some molecular techniques as conventional reverse transcriptase polymerase chain reaction (RT-PCR) and real time RT-PCR in identity test of PPR vaccine.
Methods: Four batches of PPR vaccines that produced in Egypt by Veterinary Serum and Vaccines Research Institute (VSVRI) were collected and tested for identification of PPRV by conventional RT-PCR using two primer sets targeting F-gene and N-gene and real time RT-PCR.
Results: By F-gene based RT-PCR, PPRV could be detected in only 2 samples out of 4samples (50%). By N-gene based RT-PCR, PPRV could be detected in all samples (100%). By real time RT-PCR, PPRV was detected in all the 4 samples.
Conclusion: Molecular techniques could be used for rapid evaluation of PPR vaccine including RT-PCR and real time RT-PCR for identity testing. However, the one-step real-time RT-PCR is proved to be the most rapid, sensitive and specific assay for identification of peste des petits ruminants virus in PPR vaccines. In the meanwhile, the conventional RT-PCR using primer set that targets N gene is more sensitive in identification of PPRV than RT-PCR using primer set that targets F gene which could give false negative results.

Key words: Identity test; PPR vaccine; real time RT-PCR; RT-PCR

BACKGROUND

A Peste des petits ruminants (PPR) is a highly contagious viral disease of sheep and goats characterized by high morbidity and mortality **(Khan et al., 2007; Asim et al., 2009 and Dhar et al., 2002).** The disease was first reported in Ivory Coast in West Africa in 1942 (**Gargadennec and Lalanne, 1942**) and later found in Senegal **(Gilbert and Monnier, 1962)**, Central Africa **(Scott, 1981)**, Sudan **(Taylor, 1984)**, India **(Shaila et al., 1989)**, Saudi Arabia **(Abu-Elzein et al., 1990)**, Jordan and Middle East **(Lefevre at al., 1990)**, in Egypt **(Karim et al., 1988 and Ismail and House 1990; Mouaz et al., 1995 and Abd El-Rahim et al., 2010)** and East Africa **(Wamwayi et al., 1995)**. It is even considered a threat to Europe after reaching Turkey, Morocco **(Yesilba et al., 2005 and Banyard et al., 2010)** Algeria and Tunis **(Ayari-Fakhfakh et al., 2010 and De-Nardi et al., 2012)**. The causative agent of the disease; PPRV; is a member of genus morbillivirus in the family Paramyxoviridae **(Barrett et al., 2005)**. It has a single strand negative sense RNA genome that encodes eight proteins in the order of 3'- N-P/C/V-M-F-H-L-5' **(Bailey et al., 2005)**. Among them, the nucleocapsid protein (NP) is the major viral protein. It has been the target for developing diagnostic tests that can be used to identify PPRV **(Couacy-Hymann et al., 2002)**. For control of PPR disease in its endemic areas, a live attenuated PPR vaccine is produced. Since 1995, several reverse transcription-PCR (RT-PCR) assays have been developed for the rapid and specific detection of PPRV using different sets of primers targeting F, M or N proteins **(Couacy-Hymann et al., 2002 and Balamurugan**

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et al., 2006). However, these conventional RT-PCR assays are time and labor consuming, as they require gel electrophoresis for the detection of PCR products. On the other hand, one step real-time RT-PCR, has many advantages over conventional RT-PCR assays as it minimize the chance of contamination as it completes both amplification and analysis in closed system, allows quantitative measurement of RNA, and is more rapid to perform with a much higher sensitivity. This study aims to compare between conventional RT-PCR targeting F and N proteins and TaqMan-based, one-step real-time RT-PCR as possible molecular assays used for identity testing in the evaluation of PPR vaccines.

MATERIALS AND METHODS

Vaccines:

Four batches of PPRV vaccines that were produced in Veterinary Serum and Vaccine Research Institute (VSVRI) from the isolate Nigeria 75/1 are reconstituted and used in the current study.

Nucleic acid extraction:

The nucleic acid extraction was carried out using pathogene spin viral extraction kit following the manufacturer's guidelines for detection of PPRV. Extracted nucleic acid was kept briefly at 4°C pending molecular assays.

Primers and probe:

For conventional RT-PCR, two sets of primers used for identification of PPRV in the vaccine samples (Table 1).

Table 1: Details of the primers used for conventional RT- PCR for identifying PPRV.

Primer	Primer sequence	Expected amplicon size	Reference
NP3/NP4	5'-TCT CGG AAA TCG CCT CAC AGA CTG-3'	351bp	Couacy-Hymann et al, 2002
	5'-CCT CCT CCT GGT CCT CCA GAA TCT-3'		
F1/F2	5'-ATC ACA GTG TTA AAG CCT GTA GAG G-3'	372 bp	Luka et al., 2012
	5'-GAG ACT GAG TTT GTG ACC TAC AAG C-3'		

For real-time RT-PCR, primers and probe were designed as per Bao et al., 2008. The forward primer was PPRN:
(5'-CACAGCAGAGGAAGCCAAACT-3'), the TaqMan probe was PPRN: (FAM-5'-CTCGGAAATCGCCTCGCAGGCT-3'-TAMRA), where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxy-N,N,N_,N_-tetramethylrhodamine; and the reverse primer was PPRN: (5'-TGTTTTGTGCTGGAGGAAGGA-3'.

Conventional RT-PCR:

RT-PCR was performed according to iScript one step qRT-PCR kit, Biomatik. 5µl of RNA template were added to 15 µl of PCR master mix containing 2.6 µl nuclease free water, 10 µl of igreen master mix, 0.4 µl of qRT-PCR enzyme master mix(50X), 1µl of forward primer (6µM) and 1 µl of reverse primer (6µM) to obtain a final volume of 20 µl. The amplification was carried out according to the following programs (Table 2).

Table 2: RT-PCR conditions for different primer sets

	Primer NP3/NP4	**Primer F1/F2**
Reference	Couacy-Hymenn et al., 2002	Luka et al., 2012
Initial denaturation	94 C / 4 min	95 C/ 5 min
Number of cycles	34	35
Denaturation	94 C/ 30 sec	94 C/ 30 min
Annealing	55 C/ 30 sec	50 C/ 30 sec
Extension	72 C/ 30 sec	72 C/ 2 min
Final extension	72 C/ 10 min	72 C/ 25 min
Amplicon size	351 bp	372 bp

PCR product was analyzed by electrophoresis on 1.5% agarose gel that was stained with ethidium bromide and visualized by UV fluorescence.

Real-time quantitative RT-PCR:

qRT-PCR amplification and detection was performed using the Superscript III/Platinum Taq One-step qRT-PCR kit (Invitrogen). The 25 µl reaction mixture contained 5µl extracted RNA, 12.5µl Superscript III/Platinum Taq One-step qRT-PCR reaction mix, 1 µl Superscript III/Platinum Taq One-step qRT-PCR enzyme mix, 5 pmol Taqman probe, and 10 pmol of forward and reverse primers. The following thermal profile was used: an initial reverse transcription at 45 ◦C for 30 min, followed by reverse transcriptase inactivation and DNA polymerase activation at 95 ◦C for 5 min and 50 cycles of amplification (15 s at 94 ◦C and 30 s at 60 ◦C).

RESULTS

Identification of PPRV in PPR vaccine by amplification of F and N gene based RT-PCR:

Analysis of different PPR vaccine batches with the NP3 and NP4 primers yield an amplicon of the expected size of 351 bp in all four batches "Fig. 1" while amplification using F1/F2 primers yield an amplicon of the expected size of 372 bp only in batch (1) of tested PPR vaccine "Fig. 2"

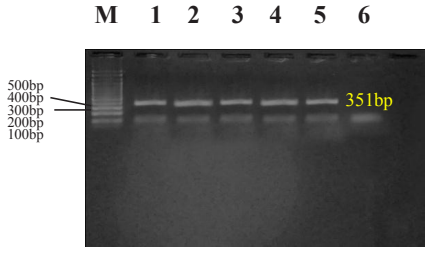

Fig. 1 PCR amplicon of the N- gene showing the band size of 351 bp. Lane M (molecular ladder), lane 1 (positive PPRV), lanes 2-5 (PPR tested vaccines), lane 6 (negative control).

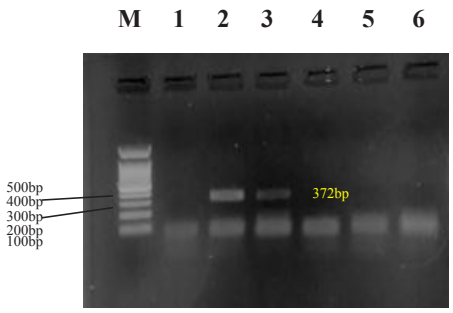

Fig. 2: PCR amplicon of the F- gene showing the band size of 372 bp. Lane M (molecular ladder), lane 1 (negative control), lane 2 (positive PPRV), lane 3 (batch 1 of PPR tested vaccine), lanes 4-6 (PPR tested vaccines batches 2,3 and 4).

Identification of PPRV in PPR vaccine using real time RT-PCR:

Real time PCR amplification was able to detect PPRV in different batches of PPR vaccines.

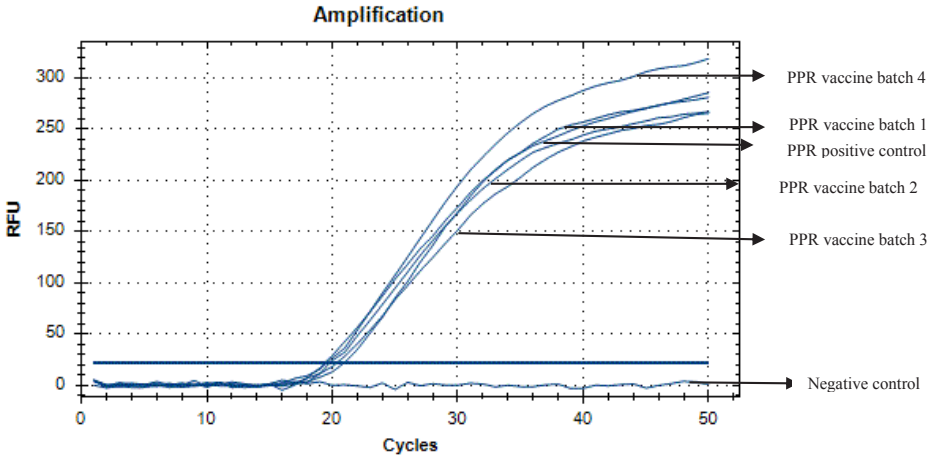

Fig. 3: real-time qRT-PCR for detection of PPRV N gene. Amplification curves from left to right are: tested vaccine batch 4, tested vaccine batch 1, PPRV positive control, tested vaccine batch 2, tested vaccine batch 3.

DISCUSSION

Peste des petits ruminants is a major transboundary disease of small ruminants in sub Saharan Africa that causes significant morbidity and socio-economic losses. Its expansion to Asia and other African countries is a great concern and challenge to the whole world.

Effective control strategies need to be contemplated in countries that rely on sheep and goat rearing and also to protect their marginal countries.

For the purpose of control of any viral disease, an effective vaccine is essential prerequisites. In Egypt, a specific vaccine against PPR is developed by attenuating the isolate Nigeria 75/1 in VERO cell culture system (Diallo et al., 1989a,b).

As identity test is a mean of evaluation of any produced vaccine, several conventional RT-PCR systems have been described for detection and identification of PPRV. However, real-time RT-PCR has several advantages over conventional RT-PCR as it is rapid and sensitive and performed in closed one tube avoiding potential cross contamination during sample preparation for gel electrophoresis (Aguero et al., 2007; Shaw et al., 2007 and Bao et al., 2008).

Four batches of the locally produced attenuated PPR vaccine were tested for identity as per Office International des Epizooties (OIE) guidelines (OIE, 2013) and according to Couacy-Hymann et al.,2002 and Luka et al., 2012 using molecular biological techniques in agreement with Albayrak et al., 2009 who determined that RT-PCR is sensitive and reliable method for identifying PPRV. Moreover, all the vaccine samples were tested for identity using one step real time PCR as per Bao et al., 2008 in agreement with Kwiatek et al., 2010 who stated that real-time RT-PCR was capable of detecting 20% more positive results with low viral RNA loads compared to conventional RT-PCR and Batten et al., 2011 who described real time PCR as ideal, high throughput, rapid, sensitive and specific method for detection of PPRV.

All vaccine samples were positive for detection of N-gene amplification and this agreed with Saravanan et al., 2010 who used RT-PCR for identity testing of different vaccines against PPRV using primers against F, M, and N proteins.

Using primers to amplify N-gene for detection of PPRV was also supported by the results of Kwiatek et al., 2010 who mentioned that the N protein is a good candidate for differential diagnosis between PPRV and RPV. These results also agreed with Kerur et al., 2008 who used N gene sequences in classification of PPRV into linages as they yield better pictures of molecular epidemiology of PPRV. The used NP3 and NP4 primers used in this study were also proved to be the most sensitive and specific primers for detection of PPRV as per Mahajan et al., 2014 who used kappa value to compare different primers based RT-PCR and found that NP3, NP4 primers show almost 100% agreement.

One vaccine sample out of four gave positive result when detected using F1/F2 primer set while the other three samples failed to produce the desired amplicon of 372 bp. These results are in line with those reported by Kerur et al., 2008 who mentioned that F gene target is less sensitive for detection of PPRV and the classification of PPRV based on F gene sequence into linages give a worse epidemiological picture about PPRV than classification based on N gene sequence. Results also run in agreement with Mahajan et al., 2014 who stated that F gene primers could give false negative results and suggested that F gene primers could be easily replaced by the highly sensitive and specific N gene primers for detection of PPRV nucleic acid. Balamurugan et al., 2006 and

There was difference in the intensity of the bands observed in F gene amplification between samples and this may be due to various amount of templates found in each vaccine preparation subjected to the identity test in agreement with Saravanan et al., 2010 who mentioned the difference in bands intensity with using different sets of primers .

In this study, one-step real time RT-PCR was developed for detection of PPRV in PPR vaccine samples. Real time RT-PCR proved to be sensitive and specific in identification of PPRV in the four tested batches of PPR vaccine samples. These results are in agreement with Bao et al., 2008 who compared it to conventional RT-PCR and proved that real-time RT-PCR is rapid tool for identification of PPRV as it can generates the result in 3 hours and its sensitivity is one log over that of conventional RT-PCR.

Results of real-time RT-PCR are also in line with those provided by Kwiatek et al., 2010 who reported that it is a sensitive and specific detection of all PPRV lineages, including those currently circulating in Africa, the Middle East, and Asia.

In conclusion, the conventional RT-PCR using primer set that targets N gene is more sensitive in identification of PPRV than RT-PCR using primer set that targets F gene which could give false negative results. However, the one-step real-time RT-PCR is proved to be the most rapid, sensitive and specific assay for identification of PPRV in PPR vaccines.

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