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Short communication

Re-emergence of Peste des Petits Ruminants virus in 2015 in Morocco: Molecular characterization and experimental infection in Alpine goats

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ABSTRACT

Peste des Petits Ruminants (PPR) is a transboundary viral disease of small ruminants that causes huge economic losses in Africa, The Middle East and Asia. In Morocco, the first PPR outbreak was notified in 2008. Since then no cases were reported for seven years, probably due to three successive vaccination campaigns during 2008–2011 and close surveillance at the border areas. In June 2015, the disease re-emerged in Morocco, raising questions about the origin of the virus. The PPR virus was confirmed by qRT-PCR and virus was isolated from clinical samples on VeroNectin-4 cells. The disease was experimentally reproduced in Alpine goats using both sheep and goat derived outbreak isolates. Molecular characterization of the 2015 Moroccan PPR isolate confirmed the identity of the virus as lineage IV, closely related to the 2012 Algerian (KP793696) and 2012 Tunisian (KM068121) isolates and significantly distinct from the previous PPRV Morocco 2008 strain (HQ131927). Therefore this study confirms a new incursion of PPR virus in Morocco during 2015 and highlights the urgency of implementation of a common control strategy to combat PPR in Maghreb region in North Africa.

1. Introduction

Peste des Petits Ruminants (PPR) is one of the most contagious viral diseases of small ruminants. The disease is reported to be one of the major limitations of small ruminant farming (Albina et al., 2013; Brown, 2011; FAO and OIE, 2015; Parida et al., 2015). The causative agent, PPR virus (PPRV) belongs to the genus *Morbillivirus,* in the family *Paramyxoviridae*. The genome encodes for 6 structural proteins: the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the large protein (L), and two envelope glycoproteins, the fusion (F) and haemagglutinin (H) proteins. In addition, two non-structural proteins (C and V) are produced from alternative reading frames in the phosphoprotein transcription unit (Mahapatra et al., 2003). Based on phylogenetic analysis of partial N or F gene sequence four distinct lineages (I–IV) of PPRV have been identified; the first two lineages are mainly circulating in West Africa, lineage III is circulating in East Africa and the Middle East, while lineage IV is present in Asia, the Middle East and Africa (Kwiatek et al., 2011; Parida et al., 2015). In recent years PPRV has extended its boundaries southwards in Africa as far as southern Tanzania (2008), Zambia (2015) and the Democratic Republic of Congo and Angola (2012). PPR outbreaks have also been reported across North Africa including within Tunisia (2006), Morocco (2008 and 2015) and Algeria (2011 and 2016) (Libeau et al., 2014; Parida et al., 2016). The first occurrence of PPR was reported in Georgia in the month of February 2016 (Parida et al., 2016). In East Asia, the virus spread to Tibet (2007) and has recently been reported all over China (2013–2014) (Banyard et al., 2014; Parida et al., 2016).

In 2008, a PPR outbreak was reported in Morocco, North Africa for the first time, causing huge economic losses because of the high mortality of young animals and the restriction of movement of animals within the country. The last case was recorded in November 2008. Following the devastating outbreak the national government organized three successive vaccination campaigns that covered all the small ruminant population in the country, leading to successful disease control. Vaccination was then stopped and replaced by close surveillance mainly in the border areas. Seven years later, in June 2015, PPR was again notified in

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Morocco (World Organisation for Animal Health (OIE), 2016) in the North-west and also in the central part of the country in sheep and goats (Fig. 1). Although the first case was confirmed in sheep in Nord Quest, subsequently there was spread of the disease to the central region (Centre) of the country where goats were severely affected and high mortality rate was observed in goats than sheep. This re-emergence of the disease raised questions on the source of infection: (i) continued circulation of the 2008 viral strain in the field without being detected or (ii) a new introduction because of unrestricted movement of the animals across the border. Further to compare the virulence of the sheep and goat derived viruses, the isolated viruses were used to infect Alpine goats experimentally. Finally this study reports the molecular characterization of the 2015 outbreak virus, and addresses the source of infection and future PPR control strategy for the region.

2. Material and methods

2.1. Sample collection

Biological samples (ocular, nasal and rectal swabs, and blood) were collected during the acute phase of the disease from animals that were reportedly ill with suspected signs of PPR and presenting hyperthermia (>40 °C) in the province of Benslimane in late July 2015. Farms specialized in sheep fattening or goat rearing were selected. For both species, animals were aged less than one year living in high promiscuity conditions. Most of sheep in this area were of Timahdit breed known to be highly sensitive to PPRV (Fakri et al., 2016).

Swabs were collected in 2 ml PBS supplemented with 2% antibiotic-antimycotic solution. In post mortem cases, lungs and mesenteric lymph nodes were collected. A total of 136 samples consisting of 85 swabs, 44 tissues and seven blood samples were collected. The samples were transported to the laboratory on ice and used directly for viral genome detection and virus isolation.

2.2. Real time RT-PCR (qRT-PCR)

All the 136 samples were screened for detection of viral genome by qRT-PCR. RNA extraction was carried out using an RNA extraction kit (Bioline BIO-52075, isolate II RNA Mini kit). Viral genome detection was performed as described by Batten et al. (2011). Superscript III Platinum R one step qRT-PCR system kit (Invitrogen) was used for qPCR.

2.3. Virus isolation

Selected qRT-PCR positive samples (n = 22) were used for virus isolation. The swab samples were centrifuged at 2000 rpm for 20 min at 4 °C. Tissues were homogenized and centrifuged at 2000 rpm for 20 min at 4 °C. The supernatant (500 μl) was used for infecting fresh monolayer of VeroNectin-4 cells (Birch et al., 2013; Fakri et al., 2015). Following appearance of PPRV-specific cytopathic effect (CPE) the virus was harvested and stored at −80 °C until use. Virus titration was performed on VeroNectin-4 cells following the method described in the OIE Terrestrial Manual.

Fig. 1. Map showing the 2015 PPR outbreak locations in Northern Morocco. (Source: OIE, 2016).

2.4. Pathogenicity of the isolates

Two isolates (MOR OV 15-sheep origin and MOR CP 15-goat origin) were selected for experimental infection. The animal experiment was performed under BSL3 containment animal housing, in accordance with the guidelines (EU Commission, 2010; OIE and Terrestrial Animal Health Code, 2016) described for the care and handling of experimental animals by the Laboratory Committee for Control and Supervision of Animal Experimentation. Two groups of Alpine goats (containing two animals in each group) were used in this study. The goats were aged 6–8 months and tested negative for PPRV specific antibody by virus neutralization test (VN) and ELISA [kit "ID Screen PPR Competition" (PPRC-4P ID-VET)]. The goats were infected with 2 ml of inoculum containing $10^{5.4}$ TCID 50/ml by intravenous (IV) injection (n = 1) and intra-nasal (IN) spray (n = 1), according to the protocol described by El Harrak et al. (2012). The first group (Goat N 1 and Goat N 2) was infected with MOR OV 15 virus whereas the second group (Goat N 3 and Goat N 4) was infected with MOR CP 15 virus. The animals were monitored daily for the presence of PPRV specific symptoms. Body temperature and clinical scoring were recorded daily from day 1–12 days post infection (dpi). Rectal, nasal and ocular swabs were taken on alternate days and analyzed by qPCR to monitor viral excretion. All surviving animals were humanely euthanized at the end of the study. During post-mortem, tissue samples from lung, mesenteric and pulmonary nodes and trachea were collected for the determination of viral load by qPCR.

2.5. Polymerase chain reaction and sequencing

The C-terminus of the N-gene was amplified from the viral RNA extracted from infected animal tissues using primer pairs NP3/NP4 (Couacy-Hymann et al., 2002). The PCR amplicons were purified using the QIAEXII PCR purification kit (Qiagen) according to the manufacturer's instructions. The purified products were sent to GATC Biotech Company, Konstanz, Allemagne for sequencing. The obtained sequences were assembled and analyzed using Bioedit Software. The generated sequences were aligned and compared with the published sequences representing all four lineages of PPRV available in GenBank. The alignments were used to construct distance matrices and phylogenetic trees using the Kimura 2-parameter nucleotide substitution model as implemented in the program MEGA 6.0 (Tamura et al., 2013).

3. Results

3.1. Field observations

The re-emergence of PPR virus was recently notified in Morocco in the North (Nord Quest) and also in the central part of the country (Centre and Centre Nord) in sheep and goats (Fig. 1). The first case was confirmed in sheep in Nord Quest by laboratory diagnosis (RT-PCR and antigen ELISA) on 29th June and reported to OIE on 3rd July 2015. Subsequently there was spread of the disease to the central region (Centre) of the country and by latter part of August outbreaks in goats were also reported in Centre Nord. Out of five outbreaks in sheep in North West and Centre, 83 PPR cases were confirmed with only 1 death. However the disease was more severe in goats, a total of 14 cases with 11 deaths were recorded. An apparent morbidity rate of 7.4%, an apparent mortality rate of 5.8%,

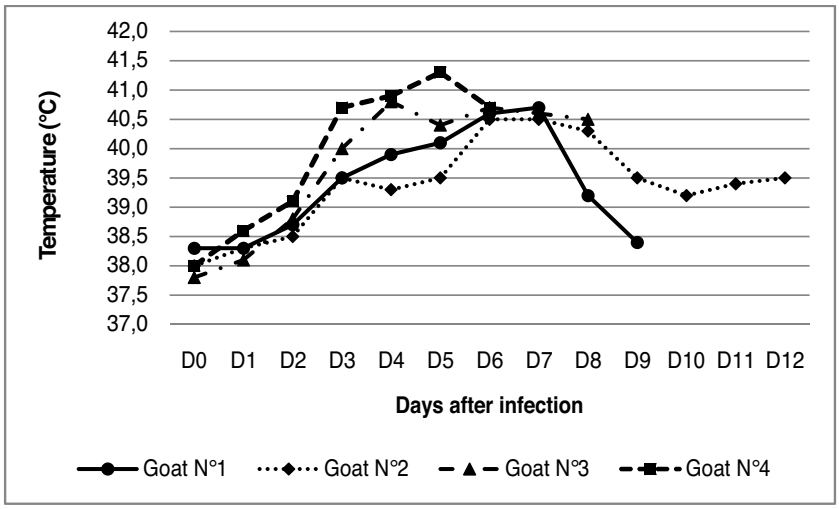

Fig. 2. Temperature monitoring of goats: Group 1 infected by MOR OV 15 (goats No 1 and 2) and Group 2 infected by MOR CP 15 (goats No 3 and 4). Goat No 1 died at 9 dpi and goat No 2 was euthanized at 12 dpi; goats No 3 and 4 died at 8 dpi and 6 dpi respectively.

an apparent case fatality rate of 78.6%, and a proportion susceptible animals loss of 5.8% in goats were estimated (World Organisation for Animal Health (OIE), 2016).

The observed clinical symptoms and epidemiological characteristics of the 2015 outbreak were similar to the 2008 epidemic. The affected animals exhibited lesions in the mouth with lacrimation, dyspnea, muco-purulent nasal discharge and severe diarrhea. The morbidity rate was much higher in young animals compared to the adult animals. Similarly, morbidity rate was much higher in goats compared to sheep. In case of Alpine goats and the Timahdit sheep breed (normally habiting the Atlas Mountains) a severe disease with relatively higher mortality rate was observed. In response to the outbreak, emergency vaccination with Nigeria 75/1 vaccine (locally produced by government laboratory) was carried out to control the disease. The economic impact of the 2015 outbreak was significantly lower compared to the 2008 outbreaks in Morocco.

3.2. Viral genome detection and virus isolation

Among the 136 samples analyzed in this study 42% were positive by q RT-PCR. Out of 22 (15 swabs and 7 tissues) qRT-PCR positive samples used for virus isolation, six samples (2 swabs and 4 tissues) were positive. The viruses of goat origin were observed to grow much faster (by 3 day post-infection) than viruses of sheep origin (by 9 day post infection) on VeroNectin-4 cells. The titer for MOR CP 15 and MOR OV 15 (two viruses used for animal experiment) was found to be 5.2 TCID 50/ml and 5.8 TCID 50/ml, respectively.

3.3. Experimental infection

Two naïve Alpine goats, inoculated with the sheep isolate (MOR OV 15), showed typical PPR symptoms from 4 dpi, i.e. nasal and lacrimal discharges, coughing and dyspnoea. The temperature is reported in Fig. 2; the clinical scoring (El Harrak et al., 2012) for one goat reached 12 and was euthanized on 12 dpi and for the other reached 14 and was dead on 9 dpi (Table 1). Post-mortem investigations showed typical PPR lesions, with a Ct value of 22 for the lungs and 19 for the mesenteric lymph nodes in qPCR. Two naïve Alpine goats inoculated with MOR CP 15 isolate, exhibited severe PPR symptoms from 3 dpi. The temperature was found much higher than MOR OV 15 infected goats (Fig. 2). The MOR CP 15 inoculated goats died on 6 dpi and 8 dpi, with very high clinical score ranging from 17 to 18. Post-mortem investigations showed typical PPR lesions, with a Ct-value of 17 for lung and 15 for mesenteric lymph nodes in qPCR.

3.4. Phylogenetic analysis

The partial N-gene sequences of two outbreak viruses (one each from goat and sheep) were generated in this study. Both of them were identical, therefore only one sequence has been used for further analysis. Twenty seven partial N-gene sequences available in GenBank were also included in the analysis. Phylogenetic analysis revealed that Morocco 2015 isolates belong to lineage IV (Fig. 3). This virus along with the sequence of 2012 Algeria (Ghardaia) and 2012 and 2013 Tunisia formed a distinct cluster (Fig. 3). It had the highest nucleotide identity with the Algerian (97.2%) (Ghardaia-5-2012, KP793696) isolate followed by Tunisian (96.7%) (Sidi Bouzid 2012, KM068121) isolate. Surprisingly the 2015 Morocco isolate seems significantly distinct (4.1% nucleotide diversity) from the previous PPRV Morocco 2008 isolate (HQ131927).

4. Discussion

Until 2008, PPRV was considered as an exotic disease in the Maghreb region in North Africa. For decades, the virus was endemic in sub-Saharan African countries; however it never succeeded in the past to cross the Sahara barrier andenter into

Table 1
Results of experimental infection on Alpine goats using two Moroccan PPRV 2015 isolates. (*) Euthanized.dpi: days post infection.

Virus isolate	Goat	First symptoms	Clinical score	Mortality	Lung threshold value (Ct)	Mesenteric node Ct
MOR OV 15	1	4 dpi	12	12 dpi*	24	21
	2	4 dpi	14	9 dpi	20	17
MOR CP 15	3	3 dpi	17	6 dpi	17	14
	4	3 dpi	18	8 dpi	17	16

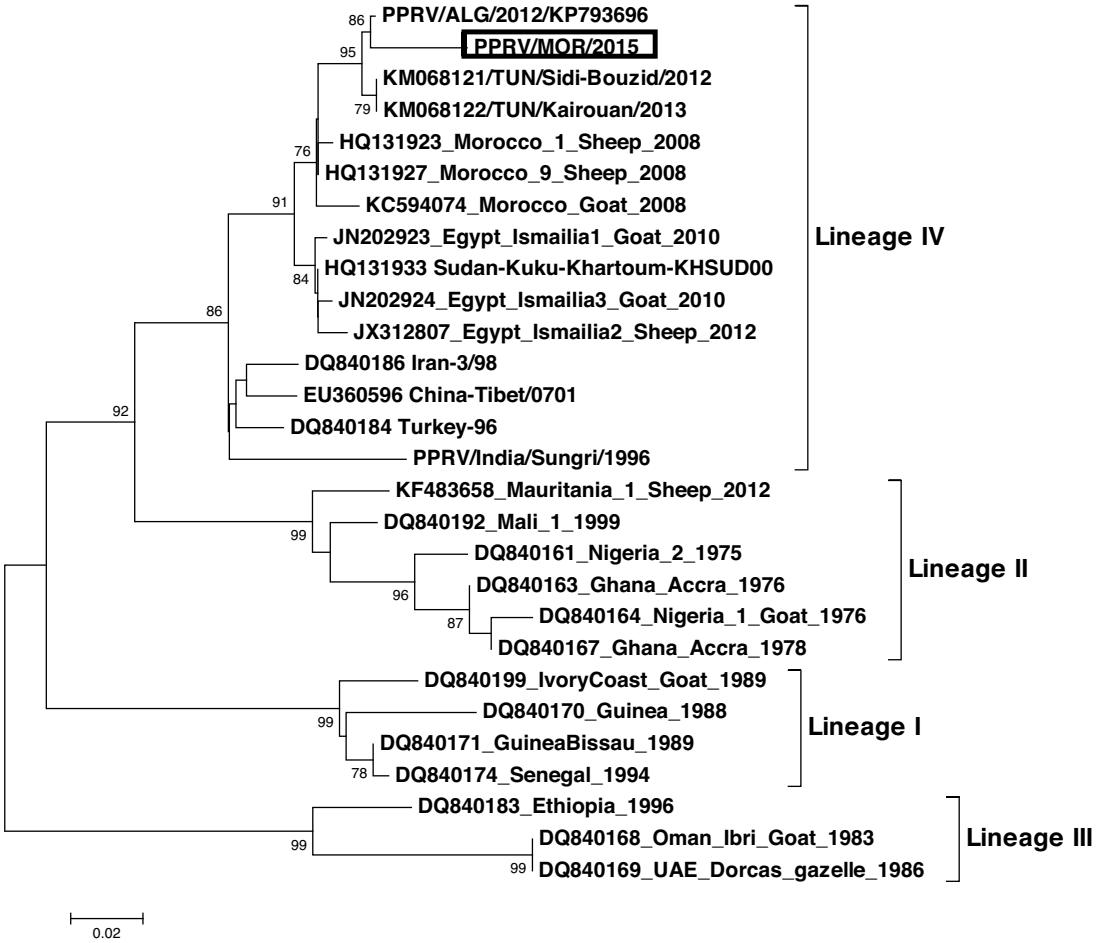

Fig. 3. Neighbour-joining tree based on the partial PPRV N gene sequence of Peste des Petits Ruminants virus isolated from 2015 Morocco outbreak (black rectangle) and selected sequences from GenBank. The Kimura 2-parameter model with percentage of replicate trees in which the associated taxa clustered together in the 10000 boot strap replicates is shown next to the branches. The GenBank accession numbers are given against each sequence. Scale bar indicates nucleotide substitutions per site. The sequence generated in this study has been submitted to GenBank and the accession number is Mor-2015_PPRV.sqn PPRV/MOR/2015 KY197740.

North Africa. In 2008, Morocco was the first country to notify the presence of the PPRV in the region (FAO EMPRES, 2008). The introduction of PPRV was thought to be from the sub-Saharan region across the southern border of Morocco. However, laboratory investigations showed that the causative agent belongs to the Asiatic lineage IV, that is also circulating in the Middle East (Boshra et al., 2015) and recently in Egypt (Mahmoud et al., 2015; Soltan and Abd-Eldaim, 2014). The introduction of the virus was thought to be through animal movements in North Africa and/or trades with the Middle East countries. The disease was recorded later on in Tunisia (Ayari-Fakhfakh et al., 2011) and in Algeria (Kardjadj et al., 2015). Following the 2008 outbreak the Morocco government implemented a mass vaccination program for control and eradication of the disease from the country.Three successive mass vaccination campaigns were conducted in 2008, 2009 and 2010. Since 2011, vaccination and surveillance was maintained only at the eastern borders of the country and no laboratory confirmed case was reported until June 2015. Indeed in June 2015, the first cases of PPR were observed during the breeding season similar to the 2008 outbreak. In farms specialized in young sheep fattening with high animal density, characteristic PPR symptoms were observed. After laboratory confirmation, the disease was notified to the OIE. This new outbreak was observed after 7 years of epidemiological silence, PPR was thought to be eradicated from the country.

No regular vaccination campaigns are organized in neighboring countries such as Tunisia, Algeria and Mauritania, where several outbreaks have been recorded in recent years (Ayari-Fakhfakh et al., 2011; Kardjadj et al., 2015). The small ruminant population in northern Africa estimated to be around 70 million, is dominated by sheep breeds that are not very sensitive to the PPRV and this ovine species may play a role as a reservoir, where the virus may circulate silently-especially in adult animals over one year of age who can amplify the virus without showing any clinical symptoms. For this reason, PPRV is not considered clinically as a severe pathogen in North Africa and vaccination has not been implemented in all the countries in the region despite the proposed global program of PPR eradication by the FAO and OIE (2015).

The experimental infection showed typical symptoms and lesions as seen in the field outbreak. The virus of goat origin was more virulent in Alpine goats whereas the virus of sheep origin took a longer time to produce similar disease in Alpine goats. This is not surprising, as sheep originated viruses usually takes more time to be adapted in goats and vice versa.

The phylogenetic analysis confirmed the circulating virus in the Morocco 2015 outbreak as Lineage IV and is closer to the 2012 Algerian and 2013 Tunisian viruses. The quick turnover of the small ruminant population allows replacement of vaccinated animals with a naïve population highly susceptible to the virus. There are approximately 25 million sheep and goats in Morocco. The disease free status could not be maintained for long without repeated

vaccination including vaccination of lambs and kids, due to the endemicity of the disease in the region, which poses a high risk of virus circulation, without proper vaccination strategy. Therefore, the 2015 virus could have come through transboundary movements of infected animals from neighboring countries where mass vaccination has never been implemented.

In conclusion, this study highlights the urgency of implementation of mass vaccination strategy for controlling and eradicating PPR in the North African region. The vaccination strategy needs to be implemented in the North Africa at regional level, as controlling disease in individual countries may not eradicate the disease and new born naïve animals may bring the disease back through transbounday movement of infected animals. The Moroccan outbreak in 2008 and its control by mass vaccination is an excellent example of the disease control strategy by vaccination. With the global PPRV eradication program targeted to be achieved by 2030 (FAO and OIE, 2015), Northern African countries should establish a common strategy, based on a minimum of three successive vaccination campaigns of the whole small ruminant population. Further, regular vaccination of new born lambs and kids immediately after weaning of the maternal antibody and annual sero-monitoring are essential to eradicate the disease in the region.

Competing interests

The authors declare that they have no competing interests.

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