Phylogenetic analysis of Moroccan sheeppox virus isolates based on P32 gene

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Abstract

Sheeppox virus (SPPV) is considered a highly contagious disease in sheep by the World Organization for Animal Health (OIE). It is classified with Goatpox virus (GTPV) and Lumpy skin disease virus (LSDV) within the *Capripoxvirus* (CaPV) genus. SPPV causes significant economic losses in endemic regions like Northern and Central Africa, Asia, India and Middle East. In Morocco, little information about the molecular characterization of SPPV is available, hence the objective of the present study is to assess the genomic relationships between Moroccan viral strains isolated from different geographic regions during several outbreaks, vaccine strains and reference strains retrieved from the NCBI Genbank, by sequencing the P32 gene. All sequences were analyzed using MEGA7 software version 7. Phylogenetic tree constructions for this gene sequences were generated using the Neighbor-Joining method. It clearly appeared that SPPV strains reported in many countries, were branched and clustered with the clade of SPPV and displayed a strong genetic relationship between them with nucleotide and amino acid identities respectively of 99-100 % and 98-100 %. These results led us to conclude that P32 gene appears highly conserved among SPPV and *Capripoxvirus*. For that, more genetic studies are required in order to control and understand the epidemiological situation of SPPV.

Keywords: Sheeppox virus, P32 gene, Phylogenetic analysis, *Capripoxvirus*, Goatpox virus.

Analyse phylogénétique d'isolats du virus marocain de la clavelée basée sur le gène P32

Résumé

La clavelée (SP) est une maladie considérée hautement contagieuse par l'Organisation Mondiale de la santé Animale (OIE). L'agent causal de la maladie (SPPV) appartient au genre des *Capripoxvirus* contenant ainsi le virus de la variole de chèvre (GTPV) et le virus de la maladie nodulaire cutanée (LSDV). Le SPPV cause des pertes économiques considérables dans les zones endémiques telles que l'Afrique du nord et centrale, l'Asie, l'inde et le moyen orient. Au Maroc, peu d'étude de caractérisation moléculaire du SPPV sont disponibles, d'où l'objectif du présent travail qui vise à évaluer la relation génétique entre les souches virales marocaines isolées à partir de différentes régions du Maroc durant les flambées épizootiques, des souches vaccinales et des souches de références publiées sur Genbank, et ce, par le séquençage du gène P32. Toutes les séquences sont analysées par le logiciel MEGA 7.0, l'arbre phylogénétique est généré par la méthode Neighbour-Joining. Il apparaît clairement que tous les SPPV rapportés dans la plupart des pays sont branchés et groupés dans le clade du SPPV, et ont montré une forte relation génétique entre eux avec une identité d'acides nucléiques et d'acides aminés de 99-100 % et 98-100 % respectivement. Ces résultats nous mènent à conclure que le gène P32 apparaît hautement conservé chez tous les SPPV et les *Capripoxvirus*. Pour cela, plus d'études génétiques sont nécessaires afin de contrôler et expliquer la situation épidémiologique du SPPV.

Mots-clés: Sheeppox virus, gène P32, analyse phylogénétique, *Capripoxvirus*, Goatpox virus.

INTRODUCTION

The *Capripoxvirus* genus, one of the eight members of the subfamily Chordopoxvirinae, is comprised of three important pathogens that infect only ungulates, Sheeppox virus (SPPV), Goatpox virus (GTPV) and Lumpy skin disease virus (LSDV), which are respectively causing disease in sheep, goats and cattle (Diallo and Viljoen, 2007).

Sheeppox, considered as notifiable animal diseases by the World Organization for Animal Health (OIE), is mainly endemic in central and northern Africa, the Middle East, India and other Asian countries from Central Asia to China (Diallo and Viljoen, 2007; Babiuk *et al.*, 2008). SPPV is responsible for one of the most economically significant diseases of domestic ruminants in Africa and Asia (Carn, 1993; Esposito and Fenner, 2001). In Morocco, SPPV has been reported in enzootic form (CFSPH, 2017) and is still responsible for serious economic damage in sheep (FAO, 2010). It seems that the sheeppox disease appears only in sheep and no case affecting goats has been reported, which means that the entire circulated virus for several outbreaks in different regions of Morocco affects solely sheep (Zro *et al.*, 2014).

Their genomes consist of double-stranded DNA of around 150 kb with terminal repeated sequences at each end. SPPV and GTPV genomes are very similar to that of lumpy skin disease virus (LSDV), sharing 97 % nucleotide identity (Tulman *et al.*, 2002).

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The usual criteria for classifying CaPVs is based upon the animal species from which the viruses are isolated, that is, SPPV from sheep, GTPV from goats and LSDV from cattle. But now, research has shown that some strains of SPPV and GTPV could infect both sheep and goats (Diallo and Viljoen, 2007; Bhanuprakash *et al.*, 2010). Thus, strains identification based on the host animal species from which the strain was first isolated is not valid (Le Goff *et al.,* 2009; Lamien *et al.*, 2011).

Serological tests cannot differentiate SPPV from GTPV, because of the very close antigenic relationship among CaPVs (Balisky *et al.*, 2008). So, the identification of these pathogens needs molecular methods. Fortunately, some molecular techniques for differentiation of CaPVs targeting specific genes had been developed, viz., the P32 gene (Hosamani *et al.,* 2004), the RPO30 gene (Lamien *et al*., 2011), and the GPCR gene (Le Goff *et al*., 2009).

P32 is highly conserved among capripoxviruses. Its sequence information can them be used to differentiate SPPV, GTPV and LSDV, presenting the genetic relationship among different virus strains (Lamien *et al.*, 2011; Hosamani *et al.,* 2004). Moreover, it corresponds to an envelope protein homologous to P35 protein encoded by *Vaccinia* virus H3L gene and located on the membrane surface of the mature intracellular viral particle (Tulman *et al.*, 2002).

This gene was targeted in this work, reported for the first time in Morocco, in order to carry out a phylogenetic analysis and to assess the genomic relatedness between Moroccan viral strains isolated from different geographic regions, vaccine strains and other reference strains retrieved from Genbank.

For this study, 24 Moroccan Sheeppox isolates mentioned above, 5 SPPV and GTPV vaccine strains were used. Furthermore, the sequencing results were used for phylogenetic analysis by comparing them with different *capripoxvirus* isolates retrieved from GenBank to evaluate the genetic relationship between them.

MATERIAL AND METHODS

Viruses

24 Cell-cultured strains isolated in Morocco from 1981 to 2010, 3 reference strains, 2 vaccine strains and 1 goat pox virulent strain were used for the study (Table 1).

These field isolates were compared to the corresponding sequences of 7 SPPV, 2 GTPV and 1 LSDV isolates retrieved from GenBank (Table 2).

DNA extraction, amplification and sequencing

DNA extraction

For viral DNA isolation from the samples, the Purelink viral DNA/RNA kit, INVITROGEN extraction kit was used according to the manufacturer's instructions and eluted in 50 ml of elution buffer.

Table 1: Moroccan isolates, vaccine and reference strains

Access Number	ID sample	Host	Date of isolation	Origin
MG201788	RM3 TA	Sheep	05/81	Unknown,
MG201815	TA H3	Sheep	18/06/82	Unknown,
MG201789	SPLT2	Sheep	21/04/1982	Unknown
MG201790	ALG	Sheep	24/11/1989	Morocco-Algerian borders
MG201791	CFES88	Sheep	15/03/1989	Fès
MG201792	CRM59	Sheep	07/01/1992	Marrakech
MG201794	BLMP	Sheep	22/01/1992	INAV (Rabat)
MG201795	CHEFV	Sheep	20/11/92	Chefchaouen
KY769282	OJ1P4	Sheep	20/10/1993	Oujda
KY769277	BerP5	Sheep	24/10/93	Berkane (Beni Chiker)
KY769279	OJ2P4	Sheep	15/11/1993	Oujda
MG201796	CRP1H	Sheep	25/11/94	Haouz (Sraghna)
KY769280	AziP3	Sheep	30/12/1994	Azilal
MG201797	CR93I	Sheep	25/01/1995	Ifrane
MG201798	CR95I	Sheep	25/01/1995	Ifrane
MG201799	BRCRL	Sheep	26/11/1999	Larache
MG201800	CBK1 P1	Sheep	04/07/2000	El Kelaâ
MG201801	SP K5 P1	Sheep	20/12/2000	El Kelaâ
MG201803	P3S	Sheep	11/02/2002	Settat
MG201804	SP1C	Sheep	04/04/2002	Chichaoua
MG201805	MP3S	Sheep	18/03/2002	Marrakech
MG201806	CRT4G	Sheep	08/12/2006	Guelmim
MG201807	CR3S	Sheep	15/02/2007	Sefrou
MG201808	CRH1	Sheep	04/11/2010	Haouz
MG201810	Bertville Strain	Reference strain	18/04/1978	Mérial
MG201811	Perego Strain	Vaccinal Strain	1986	Mérial
MG201812	RM65 Strain	Reference strain	1986	Mérial
MG201813	KEN/ 2014	Reference strain	1976	Kenya
MG201814	GTPV N 35 NIGER	Vaccinal strain GTPV	1988	CIRAD

Polymerase chain reaction (PCR)

The pair of primers P32-1 (5'-ATG GCA GAT ATC CCA TT-3') and P32-2 (5'-TTA CCA CAG GCT ATT AGA AG-3') amplify the 1181 bp fragment containing the complete P32 ORF (Zhou *et al.*, 2012) were used in PCR.

The PCR mixture contained 120 ng of extracted DNA, 5 µl5×PSbuffer, 0,2 µl Taq DNA polymerase 5U/ul, 8 µl 2.5 mM dNTP, 25 µl H2O, 1 µl of primer. DNA amplification was performed by ABI Verity Thermal Cycler. Thermal cycling parameters were: initial denaturation at 95°C for 1 min, then 35 cycles of: denaturation at 95°C for 15 sec, annealing at 49°C for 20 sec, and extension at 72°C for 3 min, followed by final extension at 72°C 20 min. The PCR product sizes were checked by the Qiaxcel bio analyzer, which generates virtual gels on the device software.

Sequencing

All obtained amplicons were purified using ExoSAP-IT treatment (USB Corporation, Cleveland, OH, USA) in order to achieve the Bi-directional sequencing which was performed by using a BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3130XL automated DNA sequencer. Purified DNA was used at a concentration of 100 ng/µl for each sample and each reaction in a final volume of 10 µl. The thermal cycler program was: initial denaturation at 96°C for 1 minute and denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds and 4 minutes of elongation at 60°C for 25 cycles.

Sequence alignment and phylogenetic analysis

For phylogenetic analysis, nucleotide sequences of related strains for P32 gene were retrieved from GenBank using online BLAST program on NCBI database. Multiple-alignment of these sequences was performed by MEGA 7 with ClustalW method. Once aligned, Neighbor-Joining tree (Saitou *et al.*, 2016; Tamura *et al.*, 2004) were constructed using MEGA 7 software (Kumar *et al.*, 2016) with the Tamura 3-parameter model and Gamma distribution. The statistical significance of the nodes was assessed by bootstrap resembling analysis (1000 replicates) (Felsenstein, 1985). Sequences obtained in the current study were deposit in the GenBank database under the following accession numbers: MG201788 to MG201815 and KY769277 to KY769282.

RESULTS

Sequence analysis of P32 gene

The complete open reading frame (ORF) of P32 gene, of SPPV Moroccan isolates were sequenced and subjected to similarity analysis.

The results showed that all Moroccan SPPV samples shared a closed relationship between them, the nucleotide sequence identities varied between 91 and 100 %. The homology percentages of the amino acid sequence reached 91.5 to 100 % for Moroccan strains and varied from 98 to 100 % in comparison with the SPV reference strains.

Fortunately, BLAST results based on NCBI database showed that all of them had a high identity with SPPV strains (99-100 %) but the homology with GTPV and LSDV was 98 %.

Phylogenetic analysis

Using the phylogenetic analysis of the P32 gene performed on MEGA7, we concluded that CaPVs comprise 3 groups: GTPV, SPPV, and LSDV (Figure 1) and that SPPVs are more related to GTPVs than LSDV.

As shown, all of Moroccan isolates were clustered together and formed a single cluster with SPPVs strains isolated from distant countries like India, China, Russia, Iraq and Saudi Arabia; and from nearest countries like Tunisia. Furthermore, vaccine strains used in Morocco and Algeria were also clustered together with viral isolates.

According to the result obtained, Moroccan isolates are closely related despite the date of isolation and the geographical origins. Furthermore, we confirmed that the P32 gene is highly conserved region among the genome of capripoxviruses, the conservatism percentage per sites was 100% (Figure 2).

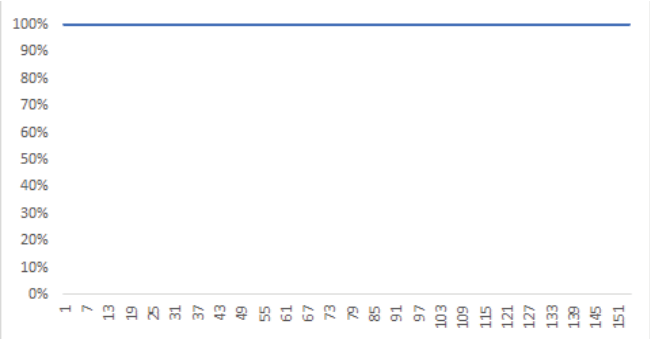

Figure 2: Histogram of conservatism percentage per sites

Table 2: *Capripoxvirus* strains used for phylogenetic analysis

Accession Number	Species of CaPV	Country of isolation	Reference
JN596274	SPPV	China	Zhou *et al.,* 2012
KT964233	SPPV	Tunisia	Unpublished
EU314721	SPPV	India	Unpublished
KF661977	SPPV	China	Unpublished
KP342531	SPPV	Saudi Arabia	Unpublished
KC847056	SPPV	Russia	Maksyutov *et al.*, 2013
MF572295	SPPV	Iraq	Unpublished
HM572329	GTPV	China	Unpublished
EU625262	GTPV	Yemen	Babiuk *et al.*, 2009
KX683219	LSDV	Kenya	Unpublished

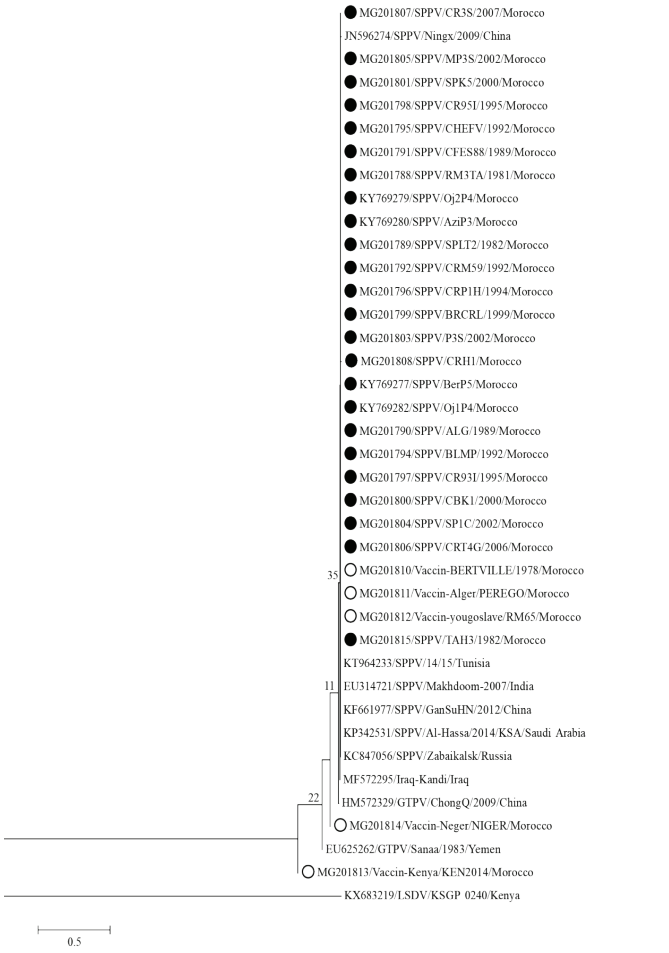

Figure 1: Phylogenetic tree based on P32 of Moroccan isolates

DISCUSSION

Sheeppox (SP) is an infectious viral disease, highly contagious, classified by the World Organization for Animal Health in the list of reportable diseases (Diallo and Viljoen, 2007). Clinically, sheeppox is characterized by hyperthermia and skin lesions (Hajjou *et al.*, 2017). By its gravity, it is considered the most lethal animal pox. It develops either in a classic form (vesicular or nodular) or in a complicated form (Babiuk *et al*., 2008). It's responsible of highest economic losses on meat and wool in Africa and worldwide (Diallo and Viljoen, 2007).

According to the animal health information published by the National Office for Food Safety, SP is enzootic in Morocco. Recently, 5 outbreaks (71 cases) were reported in the eastern and northern regions in January 2017 and caused the death of 12 affected animals. This situation leads us to understand why the disease still exists in Morocco despite vaccination programs and controls adopted by veterinary services.

In the present study, 24 isolates were collected from different regions of Morocco and spread over different years from 1981 to 2010 during several outbreaks. These isolates have undergone routine diagnostic tests, such as cell culture isolation and virus-neutralization (VNT) and enzyme-linked immunosorbent assay (ELISA), but these methods can not differentiate between SPPV, GTPV and LSDV. For this reason, and because of the few molecular studies of SPPV in Morocco, we opted for the sequencing of the viral envelope protein gene P32 of these isolates. Firstly, this gene was amplified by using polymerase chain reaction and sequenced to analyze the nucleotide divergence and to study the relationship between Moroccan isolates and other CaPVs strains retrieved from Genbank.

Sequencing of the P32 envelope protein gene, which is a structural protein containing the most important antigenic determinants and is present in all species of CaPVs (Heine *et al*., 1999; Tian *et al*., 2010), showed after nucleotide and protein sequence analysis that there exists a stronger relationship between the Moroccan strains of SPPV. They are clustered in the same clade of SPPV on the phylogenetic tree, with homology percentages ranging from 91 to 100 %. Furthermore, some restriction analysis of virus's genomes isolated from different countries revealed that SPPVs are closely related (Black *et al*., 1986).

However, BLAST results on the NCBI database showed that all of local isolates of SPPV had a high identity (99-100 %) with SPPV strains but the homology with GTPV and LSDV was 98 %, which confirms the hypothesis that the GTPV and the LSDV have the same common ancestor that is close to the SPPV. The obtained results are in agreement with the studies carried out by Hosamani (Hosamani *et al.,* 2004) and Stram (Stram *et al*., 2008), they have performed their phylogenetic studies on different segments of the genome. Another study concluded that CaPVs may be derived from an ancestor similar to LSDV (Tulman *et al*., 2002).

Using the phylogenetic analysis of the P32 gene, we concluded that CaPVs comprise 3 major groups: SPPV, GTPV and LSDV. Furthermore, we confirmed that SPPV is more related to GTPV than LSDV (Tulman *et al.*, 2002; El-Kenawy et El-Tholoth 2010; Zhou *et al*., 2012; Hasoksuz *et al*., 2014; Su *et al*., 2015).

Phylogenetic studies, especially those comparing the nucleotides of membrane protein P32 showed that these viruses are host-specific groups (Hosamani *et al.*, 2004), which is the case for our study. Contrariwise, Le Goff (Le Goff *et al.*, 2009) and Lamien (Lamien *et al*., 2011) have shown that the classification based on host from which the virus has been isolated is not reliable, by sequencing and analyzing the GPCR and RPO30 genes respectively.

The gene encoding the P32 envelope protein is considered the most appropriate for epidemiological research of CaPV isolates because of the considerable amount of information available on this gene. It is absolutely indispensable to study different genes and even the whole genome to have more precision to contribute to a reliable epidemiological study on CaPVs (Zhou *et al.*, 2012).

CONCLUSION

This study presents the first molecular characterization of SPPV isolates that emerged in Morocco. Based on sequence and phylogenetic analysis of P32 gene, it elucidated genetic relationship between local SPPV and other viruses reported in other countries, and it confirms that this gene is very conserved among CaPVs. These results supply new information on the epidemiology of sheeppox in Morocco, but further studies of other genes are required.

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