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Development and validation of a TaqMan probe-based real-time PCR method for the differentiation of wild type lumpy skin disease virus from vaccine virus strains

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ABSTRACT

Lumpy skin disease (LSD) is a transboundary viral disease of cattle with severe economic impact. Immunization of cattle with homologous live attenuated vaccines poses a number of diagnostic problems, as it has been associated with adverse reactions resembling disease symptoms. The latter hampers clinical diagnosis and poses challenges in virus identification. To this end, a duplex quantitative real-time PCR method targeting the GPCR gene was developed and validated, for the concurrent detection and differentiation of wild type and vaccine *Lumpy skin disease virus* (LSDV) strains. The method was evaluated in three laboratories. The evaluation included a panel of 38 poxvirus isolates/strains and the analytical characteristics of the method were determined. Amplification efficiencies were 91.3% and 90.7%, for wild type and vaccine LSDV, respectively; the limit of detection was 8 DNA copies for both targets and the inter-assay CV was 0.30% for wild type and 0.73% for vaccine LSDV. The diagnostic performance was assessed using 163 LSDV-positive samples, including field specimens and samples from experimentally vaccinated/infected animals. The method is able to confirm diagnosis in suspect cases, it differentiates infected from vaccinated animals (DIVA) and can be regarded as an important tool for effective LSD surveillance and eradication during vaccination campaigns.

1. Introduction

The genus *Capripoxvirus* (*Poxviridae* family) is comprised of three closely related viruses, i.e. *Sheeppox virus* (SPPV), *Goatpox virus* (GTPV) and *Lumpy skin disease virus* (LSDV). Capripoxviruses (CaPVs) are responsible for economically important diseases in ruminants. Lumpy skin disease (LSD) is a re-emerging and transboundary animal disease, listed as notifiable by the World Organization for Animal Health (OIE, 2016). Since 2012, LSD spread throughout Middle East, including Israel and Turkey (EFSA, 2015; Tuppurainen et al., 2017). In 2014 it spread to Azerbaijan and Cyprus and in August 2015, LSD was laboratory-confirmed in Greece (Tasioudi et al., 2016). In 2016, LSD spread through the Balkan countries and it was also reported in the Kingdom of Saudi Arabia, Russia, Armenia, Georgia and Kazakhstan (EFSA, 2016). LSD is characterized by disseminated nodules on the skin, mucous membranes and internal organs, but also pyrexia, poor growth and lymphadenopathy. The course of the disease may be acute, subacute or chronic and tends to be more severe in milking cows in the peak of lactation and in young animals (Gari et al., 2011). Field evidence strongly suggests that mechanical virus transmission among cattle involves hematophagus arthropod vectors (Yeruham et al., 1995; Chihota et al., 2001; Tuppurainen et al., 2011), and the importance and competence of each vector species is a subject of current research.

Although CaPVs generally display a host preference (Babiuk et al.,

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2009), some GTPV and SPPV isolates were found to be pathogenic to both sheep and goats (Asagba and Nawathe, 1980; Bhanuprakash et al., 2010; Yan et al., 2012; Santhamani et al., 2015). Natural LSDV infections have only been reported in cattle and other closely related (wild) ruminants like Asian water buffalo (*Bubalus bubalis*) (Ali et al., 1990; El-Tholoth and El-Kenawy, 2016) and springbok (Le Goff et al., 2009; Lamien et al., 2011a). In contrast to GTPV and SPPV, no reports exist on LSDV infecting sheep and goats.

As all three CaPV species share common major antigens for neutralizing antibodies, it is not possible to rely on serological tests to distinguish between them (Kitching, 2003). Although they are antigenically related, genome sequencing has shown that CaPVs are phylogenetically distinct (Lamien et al., 2011a; Le Goff et al., 2009). Given that serological differentiation of CaPVs infections is not feasible, it is imperative to rely on molecular tools for their detection and differentiation. Several PCR methods have been developed for the detection of CaPVs (Ireland and Binepal, 1998; Heine et al., 1999; Mangana-Vougiouka et al., 1999, 2000; Markoulatos et al., 2000; Hosamani et al., 2004; Tuppurainen et al., 2005; Orlova et al., 2006; This full reference is not available, the article is still . Zheng et al., 2007; Balinsky et al., 2008; Bowden et al., 2008; Stram et al., 2008; Lamien et al., 2011b; Haegeman et al., 2013; Venkatesan et al., 2014; Armson et al., 2015; Sameea Yousefi et al., 2016). However, only a limited number of them are able to distinguish between the CaPV species (Orlova et al., 2006; Lamien et al., 2011a, 2011b; Sameea Yousefi et al., 2016). For the differentiation of wild type (WT) LSDV and vaccine strains, different methods have been proposed. More specifically, gel-based PCR methods combined with restriction enzymatic digestion of the products have been developed (Menasherow et al., 2014; Agianniotaki et al., 2017). In addition, a real-time PCR method was described, able to detect and differentiate both WT and live attenuated LSDV vaccines through melting curve analysis (Katsoulos et al., 2017). Finally, two TaqMan probe-based real-time PCRs were reported for the identification of WT LSDVs (Vidanovic et al., 2016).

The LSDV genome contains 156 putative genes (Tulman et al., 2001). The GPCR gene (ORF 011) is a host range gene that encodes the G-protein-coupled chemokine receptor, a membrane bound protein which is involved in host immunomodulation (Lalani et al., 1999; Le Goff et al., 2009). GPCR has been extensively analyzed for worldwide genetic characterization of CaPV field isolates in Turkey (Sevik and Dogan, 2017), Egypt (El-Tholoth and El-Kenawy, 2016), Ethiopia (Gelaye et al., 2015), India (Santhamani et al., 2014) and China (Zhou et al., 2012; Su et al., 2015). Phylogenetic analysis based on the GPCR gene has also been proved valuable for vaccine molecular characterization (Tuppurainen et al., 2014). More recently, a recombinase polymerase amplification (RPA) assay for the detection of LSDV genome targeting the GPCR gene was developed (Shalaby et al., 2016). An important difference in the GPCR gene (12 bp deletion) between WT LSDV isolates and the vaccine strains has been suggested to be a stable molecular signature in WT LSDV genome (Le Goff et al., 2009; Gelaye et al., 2015; El-Tholoth and El-Kenawy, 2016). Moreover, recent sequence analysis indicates that the GPCR gene is a candidate target for the development of a DIVA (differentiation of infected from vaccinated animals) assay, which can be used for the monitoring of WT LSDV infections within immunized herds (Agianniotaki et al., 2017).

Cattle immunization against LSD with live attenuated vaccines is sometimes associated with the appearance of skin nodules following vaccine administration (Ben-Gera et al., 2015; Abutarbush et al., 2016), a condition which has been described as “Neethling disease” (Ben-Gera et al., 2015). More recently, development of small-sized skin nodules in a number of vaccinated animals, between days 8–18 post-vaccination has been observed (Katsoulos et al., 2017). Although the incidence of these reactions has not been evaluated in the population of vaccinated cattle, these constitute an issue of major importance in LSD surveillance and eradication, when stamping-out is applied (Agianniotaki et al., 2017). The development of DIVA assays for the accurate differentiation of the vaccine and WT LSDV strains is of major importance, in order to confront the problem of animals presenting adverse reactions due to vaccination. In this regard, both WT and vaccine virus targets need to be amplified. Therefore, a highly specific and sensitive duplex real-time PCR is of extreme importance for effective surveillance in LSD eradication programs and a prerequisite during a vaccination campaign. It can serve for the definition of cases attributed to the vaccine adverse reactions and for the early detection of infected animals, due to infection prior to vaccination or prior to the establishment of vaccine immunity.

The aim of the present study was the development and preliminary validation of a duplex real-time PCR method for the specific detection and differentiation of both WT LSDV and Neethling/SIS vaccine viruses currently used for cattle immunization in Greece. The GPCR gene was targeted, in order to maximize the specificity in LSDV detection.

2. Material and methods

2.1. Viruses and vaccines

For the evaluation of the analytical specificity of the developed method, 33 WT and vaccine CaPV isolates/strains (19 SPPV, 7 GTPV and 7 LSDV isolates), including the recent Greek Evros/GR/15 field LSDV isolate, were tested (Table 1). Two samples of the live attenuated Neethling strain-based vaccine (Lumpy Skin Disease Vaccine for Cattle,

Table 1
Isolates/strains of the *Poxviridae* family, which were tested for the evaluation of analytical specificity of the developed assay.

Genus	Species	Strain/Isolate/Origin
Capripoxvirus	*Sheeppox virus*	Greece 1 – Pieria 1996a
		Greece 2 – Pieria 1996b
		Greece 3 – Samothraki 1997
		Greece 4 – Thessaloniki 2013
		Greece 5 – Evros 2013
		Greece 6 – Rodopi 2015
		SPPV 545
		Morocco – field isolate 2010
		Kenyan
		Nigeria
		Pakistan
		Romania
		Sudan
		Turan
		Turkish
		Yemen
		Arbel 2000
		Abu-Ghraib[a]
		Romania 65 – Morocco (Vaccine)
	Goatpox virus	Gorgan
		Indian
		Bangladesh
		Isiolo
		Kano
		Kedong
		GTPV[a]
	Lumpy skin disease virus	Evros/GR/15 (WT)
		LSDV 2 (WT)
		Massalamia – Sudan (WT)
		Neethling LSDV (Vaccine)[a]
		Deltamune (Vaccine)
		Neethling (Onderstepoort Vaccine)
		SIS (Lumpyvax Vaccine)
Orthopoxvirus	*Vaccinia virus*	N/A[a]
	Cowpox virus	N/A[a]
Parapoxvirus	*Orf virus*	Greece isolate 2014
Leporipoxvirus	*Myxoma virus*	Greece isolate 2013
Suipoxvirus	*Swinepox virus*	Greece – Thrace 2010

N/A: Not applicable.
[a]Indicates isolates provided by The Pirbright Institute (Pirbright, UK) to the Hellenic NRL for CaPVs.