Transboundary and Emerging Diseases

SHORT COMMUNICATION

Development of a Suspension Microarray for the Genotyping of African Swine Fever Virus Targeting the SNPs in the C-Terminal End of the p72 Gene Region of the Genome

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Summary

African swine fever virus (ASFV) causes one of the most dreaded transboundary animal diseases (TADs) in *Suidae*. African swine fever (ASF) often causes high rates of morbidity and mortality, which can reach 100% in domestic swine. To date, serological diagnosis has the drawback of not being able to differentiate variants of this virus. Previous studies have identified the 22 genotypes based on sequence variation in the C-terminal region of the p72 gene, which has become the standard for categorizing ASFVs. This article describes a genotyping assay developed using a segment of PCR-amplified genomic DNA of approximately 450 bp, which encompasses the C-terminal end of the p72 gene. Complementary paired DNA probes of 15 or 17 bp in length, which are identical except for a single nucleotide polymorphism (SNP) in the central position, were designed to either individually or in combination differentiate between the 22 genotypes. The assay was developed using xMAP technology; probes were covalently linked to microspheres, hybridized to PCR product, labelled with a reporter and read in the Luminex 200 analyzer. Characterization of the sample was performed by comparing fluorescence of the paired SNP probes, that is, the probe with higher fluorescence in a complementary pair identified the SNP that a particular sample possessed. In the final assay, a total of 52 probes were employed, 24 SNP pairs and 4 for general detection. One or more samples from each of the 22 genotypes were tested. The assay was able to detect and distinguish all 22 genotypes. This novel assay provides a powerful novel tool for the simultaneous rapid diagnosis and genotypic differentiation of ASF.

Introduction

African swine fever virus is caused by a large DNA virus, which is the only member of the *Asfaviridae* family (Dixon et al., 2004). The disease is widespread in sub-Saharan Africa, but a number of devastating incursions into Europe and elsewhere have occurred over recent years (Penrith et al., 2004), most recently into Caucasus in 2007 where ASF is now endemic in domestic swine as well as in the numerous wild boar populations (Chapman et al., 2011). With a number of outbreaks reported as far north as St Petersburg (Russia) in 2009, 2010 and 2011, the threat for further westward spread via wild boar movements to the large pig-producing countries in Eastern and Central Europe is now considered an imminent risk (Gulenkin et al., 2011).

In its natural habitat in Southern and Eastern Africa, ASFV is maintained in a sylvatic cycle that involves wild *Suidae* and soft ticks within the *Ornithodoros* family. The virus has the potential to spill over to domestic swine at regular intervals from these natural hosts, which can be persistently infected without signs of disease for many years. Once present in the pig population, ASFV can spread efficiently from pig to pig or through fomites contaminated with the virus (Ravaomanana et al., 2011). Although considered a vector-borne infection, outbreaks in Europe and West Africa outside Southern and Eastern Africa have shown that once introduced, ASFV can become established and maintained in domestic and wild pig populations for prolonged periods without the presence of the vector (Costard et al., 2009). In fact, pig to pig transmission and contamination are considered the main factors in maintaining circulation of the virus in these regions. On the other hand, more than 30 years' experiences of ASF in the Iberian peninsula during the 1960s–1990s clearly demonstrated that the vector (which in the Iberian peninsula was *Ornithodoros erraticus*) significantly complicated and prolonged the eradication process; one of the reasons being the long survival of the virus in the ticks (Arias and Sánchez-Vizcaino, 2002). In addition to that, recent studies from Southern and Eastern Africa indicate that the presence of the sylvatic cycle involving the vector drives the evolution of the virus, leading to increased viral diversity with a possible impact on diagnostic performance of currently used tests (Gallardo et al., 2011). All of the genotypes have been found in Eastern and Southern Africa, with variation decreasing towards areas of more recent introduction. Outside Africa, only genotypes 1 and 2 have been reported (Boshoff et al., 2007; Chapman et al., 2011).

Two publications reported on the p72 gene, as a reliable target for the identification and selection of the 22 different genotypes of ASFV (Bastos et al., 2003; Boshoff et al., 2007). This region remains the standard on how ASFVs are classified, although other regions are often targeted to compliment the classification (Gallardo et al., 2009, 2011; Chapman et al., 2011). The assay described in this article uses Luminex xMAP technology and SNP differentiation to classify the 22 ASFV genotypes. The Luminex 200 instrument provides an established technology for multiplex assays of proteins (Waterboer et al., 2006) and nucleic acids (Dunbar, 2006). The design is based on flow cytometry, microspheres, lasers, digital signal processing and traditional PCR chemistry for molecular assays. Nucleic acids of various sizes can be targeted, depending on nucleotide composition in cases of large targets (Dunbar, 2006). Because of its open-architecture design, assays can be designed, built and modified in-house. It has the capacity to analyse up to 100 unique xMAP microsphere sets in a single sample, that is, 100-plex capability.

Materials and Methods

The assay was tested on DNA extracted from virus isolates provided by the European Reference Laboratory for ASFV, Centro de Investigación en Sanidad Animal (CISA), Valdeolmos, Spain, and Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI), South Africa, as well as from blood samples originating from outbreaks in Moyo and Gulu districts in northern Uganda. The latter samples were obtained through ongoing research collaboration between the Swedish University of Agricultural Sciences (SLU) and Makerere University (MAK) and the Ministry of Agriculture, Animal Industry and Fisheries (MAAIF), in Uganda (Table 1). Primers used were from Bastos et al. (2003), P72-U 5′GGCACA AGTTCGGACATGT3′; P72-D 5′GTACTGTAACGCAG CACAG3′. The forward primer was modified with a 5′ phosphate and the reverse primer with a 5′ biotin. PCR was performed using the Superscript III kit (Invitrogen, Paisley, UK) with the following profile: 94°C 2 min, (94°C 15 s, 50°C 30 s, 68°C 1 min) ×40, 68°C 5 min and 25 μl reaction mix: 2× reaction buffer 12.5 μl, 10 μM forward primer 1 μl, 10 μM reverse primer 1 μl, enzyme mix 1 μl, water 7.5 μl and sample DNA 2 μl. PCR product was digested using Lambda exonuclease (Fermentas, Burlington, Canada) using the following profile: 37°C for 30 min, 80°C for 15 min and reaction mix: 25 μl PCR product, 3.2 μl 10× Lambda buffer, 3.5 μl water and 0.3 μl Lambda exonuclease. Probes of 15, 17 and 19 bp were designed based on an alignment from MegAlign software (DNASTAR, Inc., Madison, WI, USA). All available data were used in the alignment, that is, 197 ASFV sequences of approximately 420 bp at the C-terminal end of the p72 gene, with representatives from all 22

Table 1. ASFV samples originating from Spain, Africa, Armenia and North America involved in the validation of the p72 genotyping array

Virus isolates	Year	Country	Source	p72 Genotype	Lab	Array Detection	Array Typing
Ang72	1972	Angola	Pig	1	CISA	Yes	1
Ba71V	1971	Spain	Verocell adapted	1	CISA	Yes	1
CV97	1997	Cape Verde	Pig	1	CISA	Yes	1
E70	1970	Spain	Pig	1	CISA	Yes	1
E75	1975	Spain	Pig	1	CISA	Yes	1
Haiti	1981	Haiti	Pig	1	CISA	Yes	1
L60	1960	Portugal	Pig	1	CISA	Yes	1
Ss88	1988	Italy	Pig	1	CISA	Yes	1
BF07	2007	Burkina Faso	Pig	1	CISA	Yes	1
Armenia07	2007	Armenia	Pig	2	CISA	Yes	2
Bot/P/1/99/+	1999	Botswana	Pig	3	OVI	Yes	3
RSA/W/1/99/+	1999	South Africa	Warthog	4	OVI	Yes	4
Moz64	1964	Mozambique	Pig	5	CISA	Yes	5
Spec 265	1994	Mozambique	Pig	6	OVI	Yes	6
RSA/P/1/98/+	1998	South Africa	Pig	7	OVI	Yes	7
MwLil 20/1	1983	Malawi	Tick, pig pen	8	CISA	Yes	8
Ken06.B1	2006	Kenya	Pig	9	CISA	Yes	9
Ken07.Eld1	2007	Kenya	Pig	9	CISA	Yes	9
Ug03H	2003	Uganda	Pig	9	CISA	Yes	9
Ken05.Tk1	2005	Kenya	Pig	10	CISA	Yes	10
KAB6/2	1983	Zambia	Tick (Warthog)	11	OVI	Yes	11
MZI 92/1	1992	Malawi	Pig	12	OVI	Yes	12
SUM 14/11	1983	Zambia	Tick (Warthog)	13	OVI	Yes	13
NYA 1/2	1988	Zambia	Tick (Warthog)	14	OVI	Yes	14
TAN/2001/1	2001	Tanzania	Pig	15	OVI	Yes	15
Tan 2003/1	2003	Tanzania	Pig	16	OVI	Yes	16
ZIM 92/1	1992	Zimbabwe	Pig	17	OVI	Yes	17
NAM/P/1/95/+	1995	Namibia	Pig	18	OVI	Yes	18
RSA/P/3/96/+	1996	South Africa	Pig	19	OVI	Yes	19
RSA/P/1/95/+	1995	South Africa	Pig	20	OVI	Yes	20
RSA/P/1/96/+	1996	South Africa	Pig	21	OVI	Yes	21
Spec 245	1992	South Africa	Pig	22	OVI	Yes	22
Field samples							
Moyo 4	2009	Uganda	Pig	9	MAK	Yes	9
Moyo 8	2009	Uganda	Pig	9	MAK	Yes	No
Gulu 3-1	2009	Uganda	Pig	9	MAK	Yes	9
Gulu 4-2	2009	Uganda	Pig	9	MAK	Yes	9

genotypes. Of the 197 sequences, many were identical in this region. As a result, 61 unique sequences provided the template for the assay design and the alignment of these sequences can be found in the Supporting information (Figure S1). All genotyping probes were designed in complementary pairs with a SNP in the middle of the tag. In addition to genotyping probes, general detection probes were designed. M-fold (m-fold web server: 1995–2010, State University of New York at Albany) and Visual OMP software (version 6.6.0, DNA Software, Inc., Ann Arbor, MI, USA) were used initially in the design process to determine suitability of some probes; however, most probes were selected based solely on the sequence(s). Amino-functionalized ssDNA oligonucleotide probe sequences (Biomers) were coupled to carboxylated microspheres (Luminex Corporation, Austin, TX, USA) according to the protocol recommended by the manufacturer. The final probes selected for use in the assay are provided in Supporting information (Table S1). The bead stocks (50 000 beads/ml) were diluted in 1.5× TMAC hybridization buffer such that 3000 of each probe bead were present in each sample well. Hybridization reactions were carried out in a total volume of 75 μl/well consisting of 5 μl of the PCR product, 12 μl TE buffer and 33 μl of the bead mix in duplicate in 96-well microtiter plates (Fisher Scientific, Waltham, MA, USA). For a background control, no template control from the PCR assay was used. The microtiter plates were incubated on a shaker plate at

50°C for 15 min at 600 rpm. A 25 μl volume of streptavidin-R-phycoerythrin (Caltag Laboratories, Burlingame, CA, USA) in 1× TMAC hybridization buffer, at a concentration of 0.01 mg/ml, was added and mixed with the reactants, and incubation was continued at 50°C for 5 min. The median fluorescent intensity (MFI) of each reaction was measured in Luminex LX200 instrument at 50°C. A minimum of 100 beads per probe were analysed to calculate the MFI of each reaction.

Results and Discussion

The array is interpreted first by comparing the intensity of fluorescence between each probe pair. Complementary paired DNA probes are identical except for a SNP at the centre position. The probe in the pair with the higher fluorescence indicates the SNP that is present in the sample (Drago et al., 2009; Ivanova et al., 2010). Therefore, this array identifies 24 SNPs for each sample. The SNP profile is then used to identify the genotype. For several genotypes (1, 2, 6, 7, 8, 9, 10, 12), SNP probes were developed in regions where the SNP was conserved for the specific genotype and differed from all other genotypes. For example, in genotype 2, the base at position 9 of the PCR-amplified sequence (based on GenBank AF270714) is a cytosine, while for all other genotypes, it is a thymine. For the eight genotypes listed earlier, looking at the one complementary probe pair for the respective genotype reveals whether or not that genotype is present. The rest of the probes verify this by providing supporting data. For the remaining genotypes, two or more probe pairs are needed in combination to identify the exact genotype. This is due to the fact that in the amplified P72 region, there is not a SNP that is exclusively conserved in these genotypes. Therefore, a unique pattern of SNPs is needed to identify them. Figure 1 shows an abridged graphical display of results from the Luminex 200, with results from six pairs of probes. It is impractical to display all the complementary probe pairs; however, the results shown demonstrate the process for determining the genotype. For all the testing performed, both net and raw MFIs provided comparable results (i.e. background in the assay was low). The process of using Lambda exonuclease to produce single-stranded PCR product for hybridization to Luminex microspheres has been shown to improve molecular assays on Luminex 200 instruments (LeBlanc et al., 2009, 2010). The assay in this study was also significantly improved by this process.

All the probes used in the final assay and their usefulness in identifying genotypes can be seen in the Supporting information provided (Table S1). The array was tested on a total of 36 different ASFV samples; 32 viral isolates from the collections at CISA and OVI and four

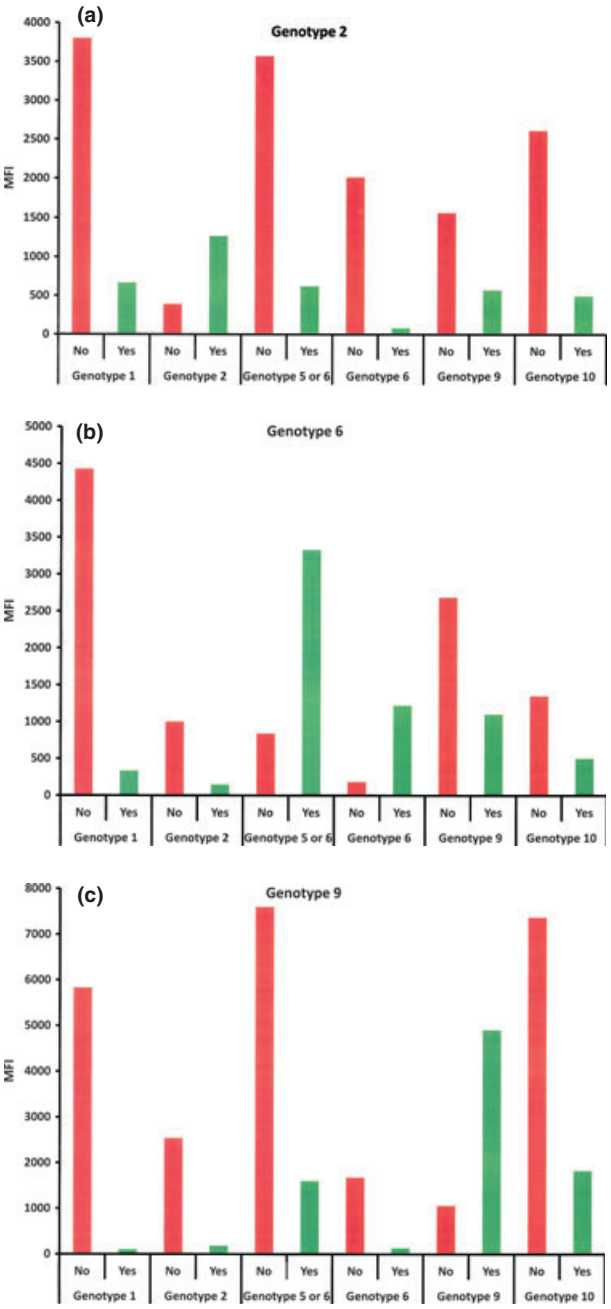

Fig. 1. Median fluorescence intensity (MFI) from the Luminex 200 for six complementary probe pairs. The results are shown for two isolates, (a) Genotype 2 (Armenia07) and (b) Genotype 6 (Spec 265) as well as one field sample, (c) Genotype 9 (Moyo 4). See Table 1 for details about the samples. Interpretation is made through deductive analysis of the probe pairs. Taking into account that all the probe pairs not shown support the probes in the charts, the comparative MFI values reveal what genotype is present.

blood samples from two outbreaks in Moyo and Gulu, Uganda (Table 1). In the sample collection tested, all 22 genotypes are represented. The assay correctly identified

Table 2. Sensitivity of the array tested on three genotypes, net median fluorescence intensity (MFI) of the four detection probes is shown. Results show sensitivity was comparable to the real-time PCR run at the National Veterinary Institute, Uppsala, Sweden, which is based on ASF EU Reference Laboratory recommended method, King et al., 2003;. Ct results shown are means of three replicates.

Sample Name	Genotype	MFI PanASFV1 17mers	MFI PanASFV1 15mers	MFI PanASFV2 17mers	MFI PanASFV3 17mers	Array result	SVA routine real-time PCR Ct
Spain 70	1	2905	4538	2380	1452	+	26
Dilution 10^{-1}	1	3539	4813	1608	1135	+	25
Dilution 10^{-2}	1	3554	4063	798	716	+	28
Dilution 10^{-4}	1	906	519	−55	−70	+	42[a]
Armenia 07	2	4293	6094	2085	1252	+	19
Dilution 10^{-3}	2	4142	4525	774	607	+	31
Dilution 10^{-4}	2	3337	2281	159	233	+	36
Dilution 10^{-5}	2	−10	−20	−51	−68	–	No ct
Ken 06.Bus	9	5567	6743	4394	3266	+	18
Dilution 10^{-2}	9	5720	6124	2459	2500	+	25
Dilution 10^{-3}	9	4899	4116	1010	1558	+	28
Dilution 10^{-4}	9	2350	769	206	425	+	32

[a]1 of 3 replicates had no ct, mean of 2 positives presented.

all genotypes. In terms of sensitivity, the assay was found to be equivalent to the real-time PCR used for routine ASF diagnosis at the National Veterinary Institute (SVA) in Uppsala, Sweden, which is based on the EU reference laboratory recommended method, King et al. (2003) (Table 2). In one case, the detection sensitivity was found to be higher than the typing capability. One field sample from Moyo was detected with the detection probes on array but not with the typing probes (Table 1). In terms of robustness, the assay proved sound in terms of repeatability. As with conventional PCR, there is the requirement for PCR tubes to be opened, so good laboratory practices are required to prevent contamination. The process developed here appears no more contamination prone than other conventional PCR methods, as no contamination issues arose during the course of the development and testing.

In summary, in this study, a genotyping array was developed based on the C-terminal region of the p72 genome region, to increase the speed and to improve the capacity to detect and characterize ASFVs. The assay can be used as a complement to real-time PCR techniques and other surveillance methods, or as a primary diagnostic tool, given its high sensitivity. This new diagnostic approach provides a great advantage compared to nucleotide sequencing in terms of throughput and costs, with the time and expense to run the array much more comparable to real-time PCR. A considerable strength of the new approach is that it allows the simultaneous detection of different variants of the virus on a single diagnostic platform, saving time, labour and verifying the diagnosis. Aside from surveillance, the new assay also can be used for quality assurance of sample collections to verify sample identity and integrity (i.e. free of cross-contamination with other ASFVs). It is likely that the novel assay will provide a high throughput, robust and highly economical novel tool for the improved diagnosis of ASFV and will support the eradication and control programmes.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Figure S1. Alignment of 61 unique sequences from the c-terminal end of the p72 gene of African swine fever virus.

Table S1. Probes used in the suspension microarray for ASFV genotyping.

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