Talanta 184 (2018) 35–41

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Chimeric DNA/LNA-based biosensor for the rapid detection of African swine fever virus

Massimo Biagetti[a,1], Massimiliano Cuccioloni[b,*,1], Laura Bonfili[b], Valentina Cecarini[b], Carla Sebastiani[a], Ludovica Curcio[a], Monica Giammarioli[a], Gian Mario De Mia[a], Anna Maria Eleuteri[b], Mauro Angeletti[b]

[a] Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, Perugia, Italy
[b] School of Biosciences and Veterinary Medicine – University of Camerino, Camerino, Italy

ARTICLE INFO

Keywords:
Optical biosensor
Biomimetic nucleotide
DNA virus
African swine fever

ABSTRACT

African swine fever (ASF) virus is a DNA virus responsible for a severe haemorrhagic fever in pigs, which (still in the absence of vaccination strategies) results in high mortality rates. Herein, we present a biosensor-based method for the detection of ASF viral DNA in the blood of pigs. The biosensor exploits a single-strand DNA probe with locked nucleic acid nucleotides (LNA) substitutions as the complementary recognition element for the conserved region of vp72 gene of ASF virus.

The biosensor was calibrated using qPCR-quantified ASF viral DNA extracted from the blood of pigs experimentally infected with the virulent Italian isolate 49/08, genotype I. Globally, the proposed biosensor showed good sensitivity and specificity, with the limits of detection (LOD) and quantification (LOQ) being 178 and 245 copies/μL of genomic ASF viral DNA, respectively. The reversible nature of the interaction between the DNA/LNA probe and the target DNA sequence granted multiple rapid analyses, with up to 40 analyses *per* single surface possible, and a single test requiring approximately 5 min.

When applied to non-amplified DNA extracts from the blood of field-infected pigs, the assay discriminated between ASFV-infected and ASFV non-infected animals, and allowed the rapid quantification of ASF viral DNA, with values falling in the range 373–1058 copies/μL of genomic ASFV DNA. In this range, excellent correlation was observed between the results of this biosensor and OIE-approved qPCR.

This method represents a promising screening assay for preliminary ASF diagnosis, having the major advantages in the relative rapidity, ease-of-use, the reusability of the sensing surface, and low cost *per* single test.

1. Introduction

African swine fever (ASF) is an extremely infectious and deadly disease affecting domesticated and wild suids, and as such it represents a major economic threat for all countries in which pig breeding is developed. The causative agent, namely the African swine fever virus (ASFV), is the only member of the *Asfarviridae* family, genus *Asfivirus* [1]. ASFV is a large, icosahedral, double-strand DNA virus, which contains a variable number of genes (151–167), depending on the different isolate [2]. The virus has a high survival capacity and can be transmitted rapidly by direct and indirect contact, as well as by natural transmission by soft ticks of the genus *Ornithodoros*. To date, 23 different genotypes are identified based on the sequencing of the p72 gene [3].

ASFV circulates in several countries of Sub-Saharan Africa, where the disease is considered endemic, and constantly reaches new areas driven by the non-controlled growth of local pig farms. Outside of African continent, the virus was reported in Europe during the period 1950–1980 (finally being controlled with the only exception of Sardinia island in Italy, where it persists since its introduction in 1978) and in Latin America [4]. The disease emerged in Eastern Europe in 2007 with its first entrance in Georgia, from where it spread rapidly across the Caucasian countries to the Russian Federation [5]. In recent years, it has expanded to the west, first in Ukraine in 2012, in Belarus in 2013, in the European Union (Lithuania, Poland, Estonia and Latvia) in 2014, in Moldova in 2016 and finally in Czech Republic and Romania in 2017 [6].

To date no vaccine strategy and effective control are available

* Corresponding author.
E-mail address: massimiliano.cuccioloni@unicam.it (M. Cuccioloni).
[1] These authors contributed equally.

https://doi.org/10.1016/j.talanta.2018.02.095
Received 8 January 2018; Received in revised form 22 February 2018; Accepted 24 February 2018
Available online 26 February 2018
0039-9140/

against this infection, therefore the rapid detection is fundamental to prevent the spread of disease. ASFV antibodies are reliable indicators of infection, the detection of seropositive animals being one of the most consistent and cost-effective method for the control of the disease, particularly when chronic or apparently asymptomatic pigs are present in the field. Nevertheless, this serological diagnosis is useless in the case of animal death prior to the appearance of antibodies.

OIE-recommended tests for virus detection include virus isolation, fluorescence antibody test (FAT), and both conventional and real-time PCR [7,8]. Recently, high sensitive real-time PCR protocols have been developed, one of which is based on universal probe library (UPL), short hydrolysis probes substituted with locked nucleic acids [7,9]. However, these methods require well equipped laboratory and qualified personnel, and samples need be moved from field to laboratories to obtain the final diagnosis.

To overcome both time and logistic problems, many efforts have been focused on the development of simple and rapid diagnostic tests for ASF to be used during the early containment of the disease among pigs, such as lateral flow assay (LFA) [10], portable real-time PCR [11], cross priming amplification (CPA) [12] and polymerase cross linking spiral reaction (PCLSR) [13].

In this perspective, biosensor-based methods gained great interest for possible application in nucleic acids analysis, with the combination of different capturing agents and physical transducers being exploited to customize biosensor and enhance detection performance, and eventually address the analytical needs in pathogen diagnostics. In particular, genosensors [14], biosensors that combine the specificity of nucleotide hybridization with the sensitivity of electrical, optical or piezoelectric transducers, represent a promising alternative diagnostic tool to the above mentioned molecular methods. In fact, biosensors offer major advantages, including real-time and label-free detection that provides the possibility for fast screening, nanogram-to-picogram sensitivity, with small sample volume required (in the microliter to nanoliter range).

In this work, we focused on the establishment of a surface plasmon resonance (SPR) test for detection of ASF viral DNA from pig blood samples, and we designed and developed a label-free biosensor that uses a 25-mer single strand DNA/LNA chimeric probe (DNA:LNA nucleotide ratio = 18:7) as the “molecular bait” for the complementary sequence of vp72 gene of ASFV genome. We describe herein this analytical method able to rapidly and reliably screen pig blood withdrawals for ASF infection, and we compared the results in terms of sensitivity, specificity and rapidity with the OIE-recommended real-time PCR, this latter being used as reference method.

2. Materials and methods

2.1. Reagents, biological samples and devices

NaH_2PO_4, KCl, NaCl, Tween-20, CH_3COONa, ethanolamine, streptavidin, N-hydroxysuccinimide (NHS) and 1-3-etil-(3-dimetil-aminopropil)-carbodiimide (EDC) were obtained from Sigma Aldrich (Milan, Italy). The ssDNA/LNA probe was purchased from TIB MolBiol (Genova, Italy).

DNA samples were extracted from the blood of experimentally infected pigs (ASF isolate 49/08-genotype I), from the blood of field-infected pigs from different Italian outbreaks (ASF isolates TO/83, 53/78, 60/79, 29106/15, 364868/15, 3648620/12 - genotype I) and from other isolates (ASF isolates E70, Lisbon 60 - genotype I; Arm 07 and Ukr12/Zapo - genotype II). Analogously, DNA samples extracted from the blood of ASFV negative pigs (n = 6), negative spleen (n = 1) and lymph node (n = 1), as well as porcine circovirus type 2 (PCV2) (n = 1) and classical swine fever virus (CSFV), strain Alfort 187 (n = 2) were used to test the analytical specificity of the DNA/LNA probe. All these samples were provided by the National Swine Fever Reference Laboratory (Istituto Zooprofilattico Sperimentale dell’Umbria e delle Marche - IZSUM).

Quantification of viral DNA was performed with a 7500 Fast Real Time PCR system (Life Technologies). TaqMan universal master mix were purchased from Life Technologies. Binding test was designed, developed and optimized on an evanescent wave/resonant mirror [15] optical biosensor (IAsys *plus* - Affinity Sensors Ltd, Cambridge, UK) equipped with planar carboxylate-functionalized surfaces (Neosensors - Sedgefield, UK).

2.2. Experimental infection with ASFV 49/08 isolate

Four commercial hybrid pigs ((Landrace × Large White) × Duroc) were infected with $10^{7.69}$ HAD_{50}/mL of the 49/08 field ASFV isolate by intramuscular injection (volume: 3 mL) within BSL-3 animal facilities at the Istituto Zooprofilattico Sperimentale dell’Umbria e delle Marche, Perugia (Italy). Blood samples from these experimentally infected pigs were collected in EDTA tubes 5 days after inoculation and used for the calibration and the optimization of the assay.

2.3. Compliance with ethical standards

Animal experiments were carried out in accordance with the European Directive 2010/63/UE on the protection of animals used for scientific purposes, under the approval of the Italian Ministry of Health (no. 825/2015-PR).

2.4. Extraction of DNA from ASFV infected samples

100 µL of either blood samples of experimentally infected (n = 4) and field infected pigs from Sardinia outbreaks (n = 6) or supernatants from cell cryolysates (n = 8) were adsorbed onto FTA® Mini cards. Constant radius circles (3 mm) were cut from each card, then rinsed and incubated with nuclease-free H_2O at 100 °C for 30 min. The water extract was analysed by real-time PCR [16] to assess the efficacy of the extraction procedure. Blood samples from ASF negative control pigs from the Italian National reference centre for ASF (n = 6) were processed according to the same protocol.

2.5. Quantitative real-time PCR

DNA from the blood of a pig experimentally infected with 49/08 isolate (ID: ASF 4a, Table 2) was amplified by end-point PCR using primer pairs designed for the qPCR assays [16]. Amplification was carried out on a Mastercycler Ep Gradient S (Eppendorf) with the following thermal cycling conditions: an initial denaturation at 95 °C for 5 min, followed by 30 cycles at 95 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min and a final elongation step at 72 °C for 7 min. Purified PCR products were cloned in pCR2.1® TOPO® TA vector using a TOPO® TA Cloning Kit. The identity of clones was tested both by restriction analysis with EcoRI enzyme (New England Biolabs) and by sequencing analysis using BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and a 3500 Genetic Analyzer (Thermo Fisher Scientific). Plasmid serial dilutions (each analysed in triplicate), in the range 10–10^6 number of copies/µL, were used to create a standard curve.

2.6. Quantitative determination of ASFV DNA

Quantification ASFV DNA from both experimentally- and field-infected animals was performed according to OIE-recommended quantitative real-time PCR assay [16]. The PCR mix (20 µL of total volume) consisted of 50 pmol/µL of sense and antisense primers, 10 pmol/µL of probe, 1 × of TaqMan Master mix UNG. 5 µL of extracted DNA/plasmid were added to each well. The samples were amplified in a 7500 HT Fast Real Time PCR System (Life Technologies) with the following thermal profile: 50 °C for 2 min, one cycle, (uracil N-deglycosylase digest), 95 °C for 10 min, one cycle (activation of Taq DNA polymerase in the master