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

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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

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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

mix), 95 °C for 15 s, 58 °C for 1 min, 40 cycles.

2.7. Analytical sensitivity of qPCR

To determine the LOD of qPCR, the plasmid was serially diluted in the range 1–10^6 copies/μL. Each dilution was tested in triplicate. The limit of detection LOD was defined as the lowest amount of DNA that can be detected in all three replicates.

2.8. Design of the nucleic acid capture probes for biosensor surface functionalization

Based on the LNA-induced improvement in the discrimination ability between highly similar sequences, and on the acquired ability of the modified constructs to bind to duplex DNA *via* triple helix formation [17] at neutral pH [18], we designed a ssDNA/LNA chimeric probe presenting 7 LNA-to-DNA substitutions. The sequence of the 25-mer construct capture probe was the same used by King et al. (2003), selected to be complementary to a highly conserved region of vp72 gene of ASFV, thus being able to match different genotypes.

Poly-A tail was added at 5' end of the probe to reduce potential hindering effects likely to hamper recognition events. The probe was 5'-biotinylated to bind to streptavidin-coated surface (probe sequences, 5' modifications, and LNA-to-DNA substitutions are reported in Table 1).

2.9. Assembly of capture probes on the sensor surface

A biosensor was developed exploiting a ssDNA/LNA probe. The sensing surface was prepared *via* streptavidin-biotin coupling [19]. Briefly, carboxylate surfaces were sequentially washed/equilibrated with PBS-T (10 mM NaH_2PO_4, 2.7 mM KCl, 138 mM NaCl, Tween-20 0.05%(v/v), pH 7.4), and detergent-free PBS at 25 °C. Streptavidin (200 μg/mL) was covalently immobilized *via* EDC/NHS coupling procedure [20]. Readout in the range 700–800 arcsec were generally obtained upon covalent immobilization of streptavidin (Fig. 1, inset A). Responses in this range indicated the achievement of partial Langmuir monolayers (≈66–76% surface occupancy) for a 53 kDa protein, corresponding to a surface density of 1.20–1.30 ng/mm^2, approximately equivalent to 90–100 μM.

Under these experimental conditions, we obtained surfaces with a significant number of available binding sites for biotinylated ssDNA/LNA, at the same time minimizing hindering effects.

Finally, independent stepwise additions of biotinylated chimeric ssDNA/LNA (40 μM) probes were performed until no further increase in instrumental response was observed (Fig. 1, insets B and C). The 4:1 binding stoichiometry of biotin (and biotinylated probes) to streptavidin allowed the estimation of theoretical maximum concentration of the capturing ligands (360–400 μM, in large excess with respect to target DNA).

2.10. Biosensor measurements

dsDNA extracts were tested directly with the chimeric ssDNA/LNA probe without any thermal denaturation required, by virtue of the ability of LNA nucleotides to form triplex [21]. Experimental protocols for this biosensor assay are schematically illustrated in Fig. 2. Between consecutive additions of different DNA samples, ssDNA/LNA baseline was always recovered with serial washes with PBS-T. The time *per*

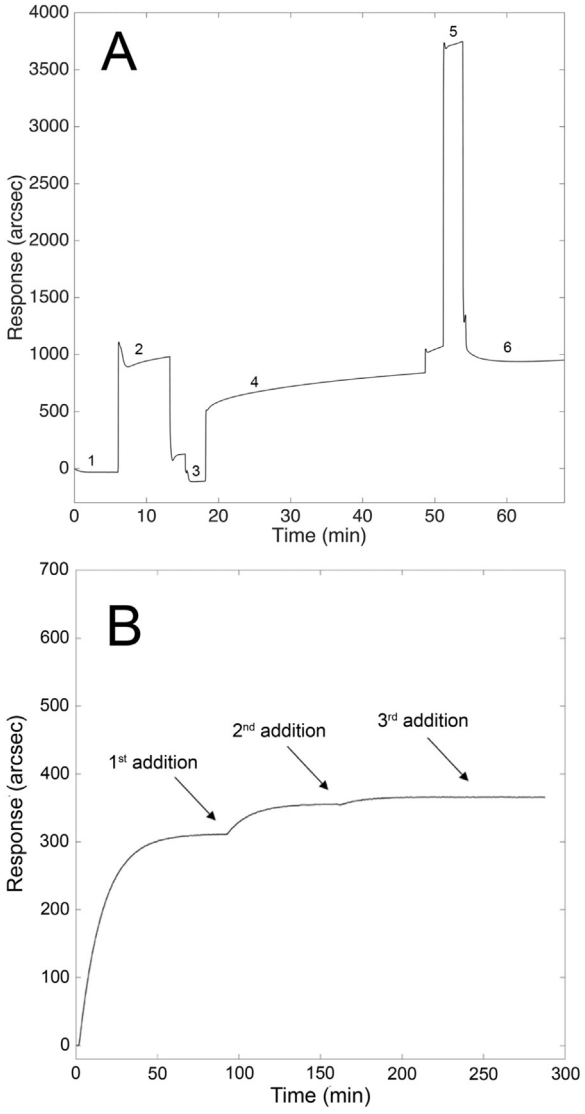

Fig. 1. Preparation of the biosensing surfaces. Immobilization of streptavidin (Panel A). Principal steps of blocking procedure are indicated: PBS wash and baseline (1); EDC/NHS activation (2); 10 mM CH_3COONa wash (3); addition of streptavidin (4); inactivation of unreacted COOH groups with ethanolamine (5); streptavidin baseline (6). Saturation of surface-bound streptavidin by stepwise additions of ssDNA/LNA (Panel B).

single analysis ranged between 3 and 5 min.

Additionally, the number of regeneration cycles that the sensor surface could withstand without significant loss of assay sensitivity and accuracy, and the stability of the sensing surface throughout multiple measurements were evaluated and assessed.

Each experiment was replicated in triplicate. Data are presented throughout as the mean ± standard deviation.

2.11. Limits of detection and quantitation of the DNA/LNA biosensor

Complying with the IUPAC rules [22], the limits of detection (LOD) and quantification (LOQ) of the proposed method were calculated as

Table 1
Comparison of the qPCR [16] and ssDNA/LNA 5'-3' probes sequences (LNA nucleotides are labelled as +***N***).

Probe ID	Probe sequence
qPCR	CCACGGGAGGAATACCAACCCAGTG
ssDNA/LNA	Biotin–AAAAA–CCA(+***C***)GGG(+***A***)GGA(+***A***)TAC(+***C***)AAC(+***C***)CAG(+***T***)G

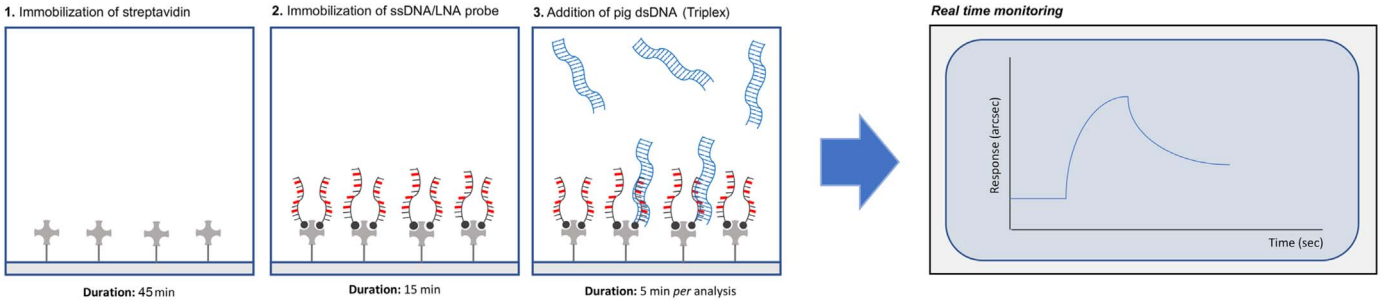

Fig. 2. Schematic representation of the proposed ss-DNA/LNA nucleotide based biosensors.

three and ten times the standard deviation of the blank measurements, respectively (DNA extracted from healthy pigs was used as a blank reference).

3. Results and discussion

3.1. Analytical sensitivity and specificity of qPCR

All ASFV-infected samples (both blood and isolate) were positive to qPCR, and the limit of detection was determined to be 10 copies/μL. Additionally, the method showed excellent specificity, as ASF-negative samples did not give any signal (Table 2).

3.2. Optimization of biosensor experimental conditions

ASFV DNA samples extracted from FTA® cards previously impregnated with the blood of experimentally infected animals (isolate 49/08, genotype I) were tested against DNA/LNA sensing surfaces on a resonant mirror IAsys *plus* biosensor system. First, different dilutions of the DNA samples were evaluated. The response of ASFV-positive samples upon the addition of 1:8 diluted sample showed the lowest matrix effect. Lower dilution factors were excluded as they were associated to poor discriminating capacity among different samples: in fact, the responses associated to ASFV-DNA in non-diluted, 1:2 and 1:4 diluted samples fell in the plateau region of the calibration curve, largely outside the linear region. Additionally, 1:8 dilution significantly reduced the time requested for surface regeneration (under the adopted dilution, regeneration procedures always required less than 5 min). Therefore, this dilution factor was adopted throughout.

3.3. Calibration curve

The sensing surface bearing the chimeric ssDNA/LNA construct was used to generate a calibration curve. Specifically, ASFV dsDNA (isolate

Table 2
Comparison of ssDNA/LNA biosensor and qPCR responses.

Sample ID	ASF isolate	Country	Matrix	Genotype	Biosensor		qPCR		
					Response (arcsec)	Copies/μL	C_t	Copies/μL	Response
Control-1	–	Italy	Blood	–	15.3 ± 0.6	–	–	–	Negative
Control-2	–	Italy	Blood	–	16.3 ± 0.6	–	–	–	Negative
Control-3	–	Italy	Blood	–	17.5 ± 0.7	–	–	–	Negative
Control-4	–	Italy	Blood	–	16.9 ± 0.5	–	–	–	Negative
Control-5	–	Italy	Blood	–	15.5 ± 0.6	–	–	–	Negative
Control-6	–	Italy	Blood	–	16.5 ± 0.7	–	–	–	Negative
Control-7	–	Italy	Spleen	–	17.9 ± 0.7	–	–	–	Negative
Control-8	–	Italy	Lymph nodes	–	17.3 ± 0.6	–	–	–	Negative
PCV2	–	Italy	Blood	–	15.3 ± 0.5	–	–	–	Negative
CFSV-1	–	Italy	Blood	–	15.9 ± 0.7	–	–	–	Negative
CFSV-2	–	Italy	Blood	–	14.8 ± 0.7	–	–	–	Negative
ASF-1	49/08#	Italy	Blood	I	22.5 ± 2.1	< LOQ	34.97 ± 0.1	198 ± 15	Positive
ASF-2	49/08#	Italy	Blood	I	38.0 ± 1.6	558 ± 13	33.58 ± 0.03	543 ± 12	Positive
ASF-3	49/08#	Italy	Blood	I	46.0 ± 2.6	905 ± 21	32.91 ± 0.02	889 ± 16	Positive
ASF-4a*	49/08#	Italy	Blood	I	48.0 ± 1.3	1032 ± 10	32.76 ± 0.02	992 ± 15	Positive
ASF-4b*	49/08#	Italy	Blood	I	16.8 ± 0.5	< LOD	38.12 ± 0.21	20 ± 3	Weak positive
ASF-4c*	49/08#	Italy	Blood	I	15.9 ± 0.7	< LOD	39.07 ± 0.27	10 ± 2	Weak positive
ASF-5a*	Ukr12/Zapo	Ukraine	Isolate	II	35.7 ± 2.3	490 ± 19	33.75 ± 0.05	482 ± 16	Positive
ASF-5b*	Ukr12/Zapo	Ukraine	Isolate	II	22.5 ± 1.8	< LOQ	35.01 ± 0.08	192 ± 14	Positive
ASF-6a*	E70	Spain	Isolate	I	35.5 ± 1.6	483 ± 13	33.76 ± 0.04	479 ± 14	Positive
ASF-6b*	E70	Spain	Isolate	I	23.0 ± 1.5	< LOQ	34.93 ± 0.08	203 ± 13	Positive
ASF-7a*	Lisbon 60	Portugal	Isolate	I	37.5 ± 1.2	542 ± 10	33.61 ± 0.02	535 ± 10	Positive
ASF-7b*	Lisbon 60	Portugal	Isolate	I	31.2 ± 1.7	373 ± 14	34.16 ± 0.05	357 ± 12	Positive
ASF-8a*	Arm 07	Armenia	Isolate	II	35.1 ± 2.0	470 ± 16	33.82 ± 0.04	456 ± 15	Positive
ASF-8b*	Arm 07	Armenia	Isolate	II	32.2 ± 2.2	395 ± 18	34.07 ± 0.04	382 ± 11	Positive
ASF-9	TO/83	Italy	Blood	I	36.5 ± 2.4	530 ± 21	33.63 ± 0.03	525 ± 13	Positive
ASF-10	53/78	Italy	Blood	I	24.4 ± 1.9	< LOQ	34.44 ± 0.08	292 ± 17	Positive
ASF-11	60/79	Italy	Blood	I	36.2 ± 2.1	488 ± 14	33.72 ± 0.03	491 ± 13	Positive
ASF-12	29106/15	Italy	Blood	I	23.3 ± 1.7	< LOQ	34.55 ± 0.06	268 ± 13	Positive
ASF-13	364868/15	Italy	Blood	I	49.0 ± 5.3	1058 ± 53	32.66 ± 0.02	1063 ± 15	Positive
ASF-14	3648620/12	Italy	Blood	I	39.2 ± 1.3	684 ± 18	33.30 ± 0.06	669 ± 28	Positive

Biosensor data are presented as instrumental response (arcsec) and number of copies/μL of ASFV DNA. qPCR data are presented as number of copies/μL of ASFV DNA. All results are the mean value of three additions ± standard deviation (#samples from experimentally infected pigs; *samples from the same ASFV swine material but with different viral concentration).

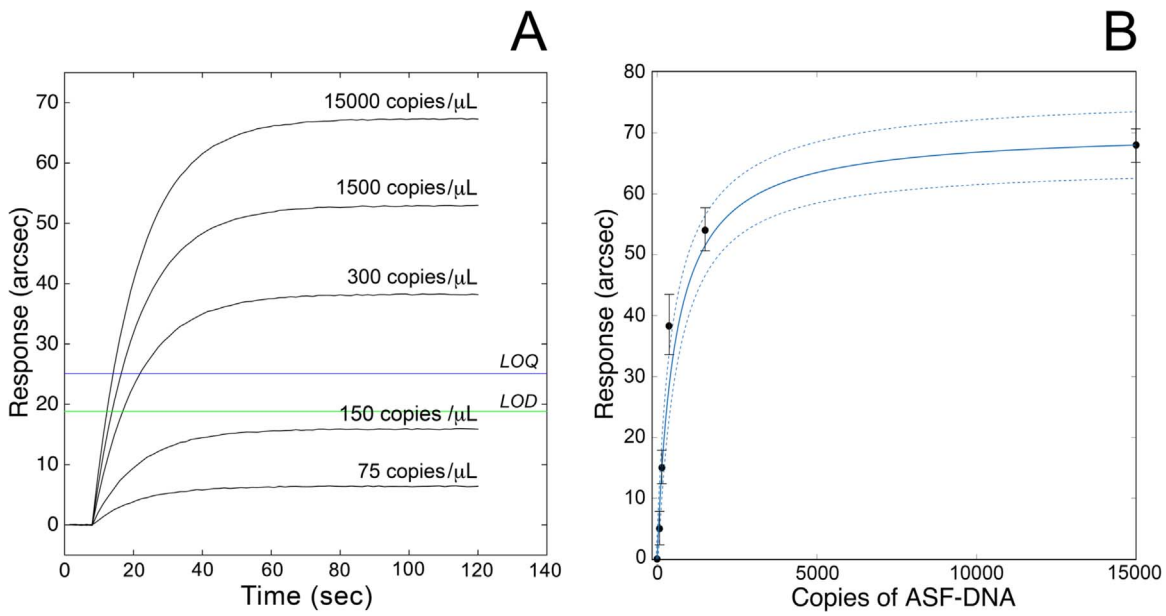

Fig. 3. Superimposition of responses obtained upon independent additions of serial dilutions of ASFV DNA extracted from pigs experimentally infected with virulent isolate 49/08 onto DNA/LNA chimeric biosensor; LOD and LOQ values are visualized (Panel A). Maximal response *vs* corresponding ASFV DNA levels plot: fit to Eq. (1) (solid line) and 95% confidence bound (dashed lines) are reported. Each experimental point was the average of three replicates (standard errors of the mean are shown) (Panel B).

49/08, genotype I) was added at different levels in the range 0–15000 copies/µL (Fig. 3, Panel A), and binding responses were monitored up to saturation point (*Req*). The plot of *Req versus* the number of copies/µL of ASFV dsDNA showed the hyperbolic correlation:

$$R_{eq} = \frac{a\,[copies\ of\ ASF\ DNA/\mu L]}{b + [copies\ of\ ASF\ DNA/\mu L]} \quad (1)$$

where *a* is the response at asymptotically high concentrations of ASFV DNA, and *b* is the number of copies/µL of ASF DNA yielding to half-maximal response.

Fit (solid line) and 95% confidence bound (dashed lines) are reported in Fig. 3, Panel B, (R^2 = 0.9952; RMSE = 2.444). Best fitted values for *a* and *b* were 71 ± 8 arcsec and 555 ± 286 copies/µL of ASFV DNA. Calibration data were expressed throughout as copies/µL of ASFV DNA. Conversely, linear dynamic range was much narrower (0–300 copies/µL of DNA).

3.4. Quantification of ASFV DNA

LOD and LOQ values of the chimeric DNA/LNA biosensor for ASFV DNA (determined as described in the Materials and Methods section) were calculated to be 178 and 245 copies/µL, respectively (Fig. 3). dsDNA samples isolated from 20 ASFV samples (belonging to 11 different isolates) were tested, 18 out 20 being significantly higher with respect to negative control (mean response of controls = 16.3 arcsec). Specifically, these ASFV dsDNA samples generated responses in the range 22–49 arcsec corresponding to concentration values in the range 373–1058 copies/µL of ASFV DNA (five samples were below the LOQ, but still being significantly higher than LOD) (Table 2). Conversely, this biosensor method could not distinguish between weak positive ASFV samples (close to the LOD of the qPCR, obtained from the dilution of sample ASFV-4, Table 2) and controls.

Interestingly, similarly to qPCR DNA extracts from swine infected by other pathogens (PCV2, CSF) did not generate responses significantly different from controls (Table 2).

Nevertheless, any statistical intra- or inter-group interpretation of data was of limited significance due to the small number of samples *per* group.

3.5. Reusability and efficiency of the biosensor

The response upon binding of both ASFV DNA from blood of infected pigs and DNA from controls to surface-blocked ssDNA/LNA construct was reproducible when measurements were repeated after reiterated surface regenerations (coefficient of variation < 5% at 300 copies/µL, n = 5)

Next, we evaluated ssDNA/LNA surface longevity, another critical characteristic of re-usable biosensors performance. Surface stability benefited from regeneration procedures based on serial washes with PBS-T buffer, baseline recovery being achieved in few min (> 5 min): in fact, no significant loss in surface stability, and consequently in both assay sensitivity and reproducibility (CV < 5%), was observed after 40 association/regeneration cycles (Fig. 5).

Detection procedures were replicated on different days (n = 3) both on the same and on different DNA/LNA-functionalized surfaces (n = 3) to assess the inter-day and the “surface-to-surface” variability. Analyses of ASF-DNA samples were always performed in triplicate. In line with the results of surface stability evaluation, a single surface (stored at 4 °C in the presence of sodium azide containing PBS, and re-used on different days) could withstand a total number of approximately 40 binding events before experiencing a significant loss of signal (> 5%). Concerning the inter-day variation of the same surface, the maximal coefficient of variation (5.5% at 300 copies/µL) were within the confidence limits of the calibration curve. Additionally, the “surface-to-surface” variation of assay sensitivity was assessed by comparing the response upon binding of 300 copies/µL of ASFV DNA to different DNA/LNA surfaces, the coefficient of variation always being within the experimental error (CV < 10%).

4. Conclusion

Currently, OIE recommends molecular methods for the diagnosis/containment of ASFV. Some of the best-established methods include real-time PCR and UPL real-time PCR, both methods offering optimal sensitivity (10–100 copies/µL [23] and 2–10 copies/µL of ASFV DNA [9], respectively) and the LAMP method (sensitivity: 300 copies/µL of ASFV DNA [24]). On the other hand, PCR based techniques are technically demanding, require expensive equipment, and are hardly adaptable to on-site screening.

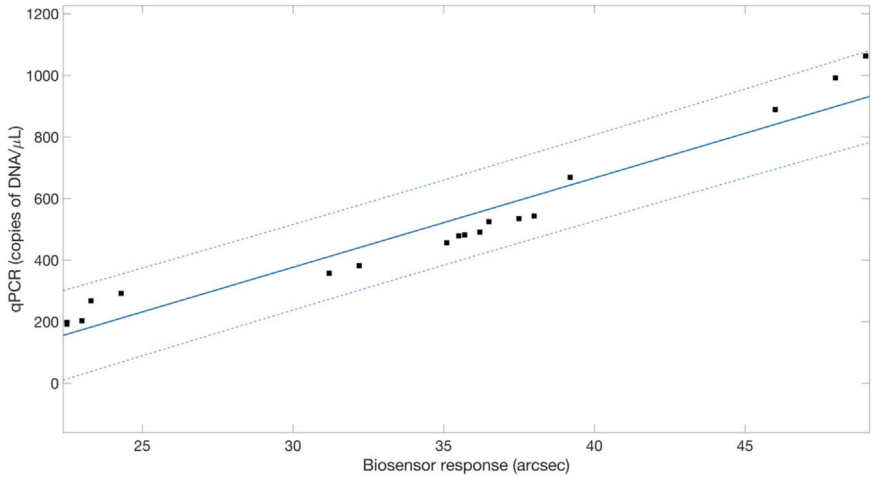

Fig. 4. Correlation between reference qPCR and biosensor for ASFV detection in field-infected animals. Linear fit (solid line, $r^2 = 0.9176$) and 90% confidence limits (dashed lines) are reported.

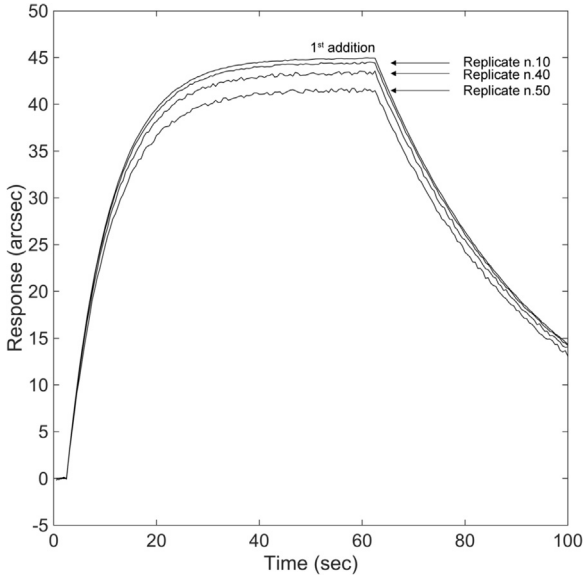

Fig. 5. Superimposition of representative sensorgrams corresponding to replicates of a given ASFV DNA sample (300 copies/μL of ASFV DNA) after different regeneration cycles.

Point-of-care diagnostic tools should work simply and robustly also in conditions with few resources, should be easy to use and provide clearly interpretable results. In this perspective, isothermal amplification-based assay, among these CPA [12] and PCLSR [13] were developed, with a sensitivity of 7.2 and 720 copies/μL, respectively. As declared by the authors [13], this latter showed higher diagnostic specificity than other isothermal assays reviewed by Yan [25]. On the other hand, in a comparative study [13], the results obtained by PCLSR resulted more robust and reliable than LAMP and CPA.

Among emerging alternative analytical techniques, biosensors (both single-use and re-usable) are having great impact on human and animal clinical diagnosis. Thanks to their sensitivity, target-specific biosensors are becoming established tools for monitoring biological threats in both human [26] and animal health care [27], and in food safety industries [28]. Nevertheless, in the context of swine fever, only a few biosensor-based methods have been developed to date for the diagnosis of either ASF [29–31] or classical swine fever [32], all exploiting the antigen-antibody recognition processes (immunosensors). Despite always offering good sensitivity, only indirect ASF immunosensors using labelled species presented also good longevity (in line with other indirect immunosensors [33–35]), whereas direct counterparts were characterized by a limited number of possible regeneration cycles due to the highly aggressive regeneration procedures necessary to break antigen-antibody complexes.

On the strength of the growing number of genosensors developed for the detection of pathogens [36], we designed a label-free re-usable nucleic acid based biosensor functionalized with a ssDNA/LNA probe targeting a region of the vp72 coding gene of ASF virus to screen blood samples from pigs for the presence of ASFV infection.

Prior to each biosensor analysis, a rapid preliminary extraction step of DNA from blood samples was mandatory, since the addition of whole blood onto the sensing surface produced non-specific responses and non-interpretable results.

Given the reversibility of the interaction between the capture probe and the target ASFV DNA under mild conditions, the proposed biosensor can rapidly process multiple samples (up to 40, one sample at a time) with adequate sensitivity (time *per* analysis = 5 min; LOD = 178 copies/μL, LOQ = 245 copies/μL of viral DNA). Additionally, once functionalized with ssDNA/LNA, the removable sensing cuvette can be stored at 4 °C and (re-)used at necessity for the same total number of analyses. In general, the self-imposed stricter requirements in terms of the maximum coefficient of variation allowed (5%) in part justified the lower number of analyses *per* surface with respect to analogous biosensor-based methods [37,38].

Indeed, the real-time detection of non-amplified ASF dsDNA *via* triplex formation represents a major advantage of the proposed biosensor, as neither any denaturation pre-treatment nor amplification step of pig dsDNA are required. Nevertheless, the ease of use and the rapidity of this technique is balanced by the approximately 18-fold lower analytical sensitivity with respect to the reference qPCR method [16]. In fact, due to the different principles of detection (the biosensor monitors the change in the mass on the sensing surface upon addition of non-amplified ASFV DNA, whereas in qPCR the amount of ASFV DNA is fluorometrically determined upon amplification), samples with a very low viral load but still positive to qPCR (between 178 and 10 copies/μL) fell below the LOD of our biosensor (Table 2). Conversely, an excellent agreement existed between the proposed biosensor and reference qPCR for samples with viral load higher than 178 copies/μL (Fig. 4).

Moreover, in line with qPCR results, our biosensor showed optimal analytical specificity, as the responses upon analysis of other negative swine material (spleen and lymph nodes) as well as other swine pathogens (namely, classical swine fever and porcine circovirus type 2) were always non-significantly different from controls (Table 2).

Analogously, the label-free nature of our assay, although requiring no additional chemical, time and costs, presented lower sensitivity with respect to (relatively) more elaborate label-based fluorescence

Table 3
Comparison of costs and time associated with qPCR and with the proposed biosensor assay.

	qPCR	DNA/LNA Biosensor
Time for sample pre-treatment	45 min	45 min
Time for device (plate/cuvette) preparation	45 min	60 min
Time *per* single analysis	100 min	5 min
Maximum number of simultaneous analyses	96	1
Number of analyses *per* device	96	40
Total costs	580 €	280 €
Cost *per* single analysis	6 €	7 €

biosensors [36].

Globally, the proposed target-specific biosensor achieved promising analytical sensitivity and optimal analytical specificity, combined with the low cost *per* test (comparable to reference qPCR - Table 3) and the relatively short time of analysis under optimized conditions (total time *per* sample: 5 min, 2 min for the analysis and 3 min for the regeneration of the sensing surface).

Importantly, the assay can be performed even by non-specialized lab users. In fact, after an easy protocol extraction of DNA with FTA® Mini cards, the operator just has to inject a fixed volume of DNA extract onto a pre-functionalized ssDNA/LNA cuvette, monitor the response for 2 min, then wash away the DNA sample.

Future study will focus on testing this analytical tool on other strains/genotypes of ASFV, and eventually transferring/adapting the proposed method to portable biosensors (*i.e.* Spreeta – Texas Instruments) to develop an "on-site" tool for rapidly screening of ASF in infected animals.

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Competing interest statement

None of the authors have conflicts of interest to declare.

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