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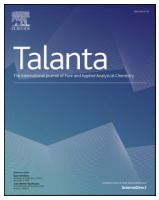

Validation of a plasmonic-based serology biosensor for veterinary diagnosis of COVID-19 in domestic animals

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ABSTRACT

The coronavirus disease 2019 (COVID-19) pandemic recently demonstrated the devastating impact on public health, economy, and social development of zoonotic infectious diseases, whereby viruses jump from animals to infect humans. Due to this potential of viruses to cross the species barrier, the surveillance of infectious pathogens circulation in domestic and close-to-human animals is indispensable, as they could be potential reservoirs. Optical biosensors, mainly those based on Surface Plasmon Resonance (SPR), have widely demonstrated its ability for providing direct, label-free, and quantitative bioanalysis with excellent sensitivity and reliability. This biosensor technology can provide a powerful tool to the veterinary field, potentially being helpful for the monitoring of the infection spread. We have implemented a multi-target COVID-19 serology plasmonic biosensor for the rapid testing and screening of common European domestic animals. The multi-target serological biosensor assay enables the detection of total SARS-CoV-2 antibodies (IgG + IgM) generated towards both S and N viral antigens. The analysis is performed in less than 15 min with a low-volume serum sample (<20 μL, 1:10 dilution), reaching a limit of detection of 49.6 ng mL^{-1}. A complete validation has been carried out with hamster, dog, and cat sera samples (N = 75, including 37 COVID-19-positive and 38 negative samples). The biosensor exhibits an excellent diagnostic sensitivity (100 %) and good specificity (71.4 %) for future application in veterinary settings. Furthermore, the biosensor technology is integrated into a compact, portable, and user-friendly device, well-suited for point-of-care testing. This study positions our plasmonic biosensor as an alternative and reliable diagnostic tool for COVID-19 serology in animal samples, expanding the applicability of plasmonic technologies for decentralized analysis in veterinary healthcare and animal research.

1. Introduction

In the last century, we have faced multiple zoonotic infection outbreaks such as the Bird flu caused by H5N1 virus [1], the Swine flu carried out by Influenza H1N1 virus [2], the Severe Acute Respiratory Syndrome (SARS) and the Middle East Respiratory Syndrome (MERS) triggered by coronaviruses [3,4], the Ebola [5], the Dengue and Zika viruses [6,7], and recently the Coronavirus Disease 2019 (COVID-19), caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) [8]. Zoonotic diseases, transmitted from animals to humans, have always occurred naturally, and it is estimated that more than 60 % of human pathogens are of zoonotic origin [9]. Nonetheless, the increasing industrial livestock farming operations, globalization, massive urbanization, and the growing social trend for acquiring domestic animals collectively pose an escalating threat to the emergence of novel and potentially hazardous zoonotic pathogens.

Coronaviruses are generally found in several domestic and wild animals, including cattle, horses, ferrets, dogs, cats, and bats. The major transmission route is among individuals of the same species, but coronavirus can also be transmitted from animals to humans and other animals and vice versa [10,11]. Due to this potential to cross the species barrier, the close contact between humans and pets, and the

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susceptibility of certain species to coronavirus infection, it is crucial to study and determine the circulation of SARS-CoV-2 virus in domestic animals, which could act either as transmission vectors or reservoirs [12,13]. Surveillance of SARS-CoV-2 circulation in domestic animals could improve the knowledge regarding the spread and susceptibility in multiple animal species, enabling anticipation and prevention of new surges of viral zoonotic diseases. Early identification of COVID-19 infection in domestic animals could provide them with specific veterinary healthcare and could help in controlling the animal transmission chain.

SARS-CoV-2 serology assays, which rely on detecting the specific immunoglobulins (Ig) in blood generated by the infected host, play an important role in the surveillance of infectious diseases and in the pandemic management, providing information about the dynamics of acquired immunity and the global incidence of the infection. Specifically, serology assays developed for COVID-19 are based on the detection of both IgG and IgM antibodies generated towards either the spike (S) protein or the nucleocapsid (N) protein of the virus [14]. In veterinary patients, traditional serology techniques include seroneutralization tests [15,16], microplate-format immunoassays, such as ELISA (enzyme-linked immunosorbent assay) [17,18], and fluorescent tests, such as Luminex assay [19,20]. These standard techniques are highly sensitive and reliable, offering multiplexed capabilities for parallel analysis of different samples. However, they are laborious, time-consuming, and require trained technicians and specific equipment to be operated in centralized laboratories [14,21]. Considering the need for massive sample screening, the immunochromatographic lateral flow assay (LFA) stands out due to its facile handling at the point-of-care and rapid time-to-result response (15 min) [22]. However, the sensitivity and specificity of LFAs tend to be moderate, and they are only validated for human diagnosis, so their reliability in animal testing cannot be assumed. Therefore, implementing new technologies that enable rapid, accurate, and decentralized serological assays in animals could greatly benefit the veterinary research and clinic community, allowing for efficient screening of animal populations and gaining relevant insights into the virus spread, potential transmission chains, and their global incidence.

Optical biosensors, particularly those employing plasmonic technologies, have been positioned as a powerful alternative to fill this gap. Biosensors based on the Surface Plasmon Resonance (SPR) technique can perform direct, label-free, quantitative analysis with excellent sensitivity, rapid response, and high reliability. In fact, SPR instruments are the most versatile and consolidated biosensor technology for biomedical and clinical purposes [23–25], with a myriad of applications demonstrating clinical diagnostic accuracy, especially for human diseases [14,26–28], and the suitability for miniaturisation and point-of-care operation. In the frame of COVID-19, the contribution of SPR biosensors to human diagnosis has been undeniable [14,29–33]. However, the application and implementation of SPR biosensors in veterinary healthcare and research is yet to be explored. In recent years, only a few SPR sensors have been applied for the detection of antibiotics [34] and bacteria [35] on animal milk samples or for the determination of cancer [36] and sepsis [37] biomarkers in sera. But they still need to be fully validated with real animal samples.

In this work, we report for the first time the implementation and clinical validation of an SPR serology biosensor for the rapid determination of SARS-CoV-2 total antibody levels in common European domestic animals: dogs, cats, and hamsters. The biosensor has been rationally designed and optimized to detect total SARS-CoV-2 IgG and IgM antibodies in animal sera samples, ensuring a highly sensitive and selective analysis in minimally processed samples (blood serum dilution). Furthermore, we have performed a comprehensive study of 75 clinical specimens from different species to evaluate and demonstrate the applicability of our technology for rapid COVID-19 testing in animals.

2. Materials and methods

2.1. Chemical and biological reagents

Organic solvents (acetone and ethanol) were purchased from Panreac (Barcelona, Spain). Reagents for carboxylic acid activation (N-(3-dimethyl aminopropyl)-N′-ethyl carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (sulfo-NHS), 16-mercaptohexadecanoic acid (MHDA), compounds and salts for PBS 10 mM (10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4), and MES 0.1 M (2-(N-morpholino) ethanesulfonic acid, pH 5.5), Tween 20, dextran sulfate sodium salt (DS), ethanolamine (EA 1 M, pH 8), and commercial sera were provided by Sigma-Aldrich/Merck (Steinheim, Germany). Recombinant IgG and IgM antibodies against SARS-CoV-2 Spike S1 protein (IgG-S and IgM-S), recombinant IgG and IgM antibodies against SARS-CoV-2 Nucleocapsid protein (IgG-N and IgM-N), SARS-CoV-2 Nucleocapsid protein (N-protein) and SARS-CoV-2 Spike 1 protein (S1-protein) were obtained from GenScript (Rijswijk, Netherlands). Monoclonal IgG antibody against CRP C7 (anti-CRP) was acquired from HyTest (Turku, Finland). Milli-Q water was employed for all the buffer preparation.

2.2. Plasmonic biosensor device

The biosensor device employed is a proprietary Surface Plasmon Resonance (SPR) biosensor, which is integrated into a compact platform (20 × 20 cm), and it has been described previously [38,39]. The device is based on the Kretschmann configuration and operates with a fixed incidence angle ($\theta = 70^{\circ}$). The custom-made flow cell is connected to a microfluidic system consisting of a fluidic pump to constantly deliver fluid to the gold sensor chips and injection valves coupled to a 100 μL loop to introduce samples. The SPR biosensor monitors the binding events, through an increase in the local refractive index (RI), or desorption (decrease in the local RI) in real time by tracking the SPR wavelength displacements (Δλ, nm).

2.3. Plasmonic sensor chip preparation

The gold sensor chips (glass substrates (n = 1.52) coated with 1 nm of titanium and 49 nm of gold) were fabricated by metal evaporation using electron beam deposition (AJA International Inc. ATC-8E, Orion, USA). After cleaning, the sensor chips were chemically modified by forming a self-assembled monolayer (SAM) of carboxyl groups with 16-mercaptohexadecanoic acid (MHDA) at 1 mM. The sensor chip was then rinsed with ethanol and water, dried with N_2 flow, and placed on the SPR setup to immobilize the receptor proteins *in-situ*. The biofunctionalization procedure consisted of the covalent binding of viral proteins (N protein, S1 protein, or multianalyte (N + S1 1:1)) to carboxyl groups present in the SAM through EDC/NHS reaction. The unreacted carboxylic groups on the sensor surface were blocked with an EA solution injection for 2 min. Finally, the sensor chips were kept under a continuous flow of PBST + DS (PBS 10 mM + 2 % Tween 20 + 2 mg mL^{-1} DS) at 15 μL min^{-1}. Fig. 1 shows a scheme for the SPR biosensor employed and an interaction between the proposed biosensor and antibodies IgM and IgG on the chip surface.

2.4. Biosensor assays for the antibodies detection

The biofunctionalized plasmonic sensor chips were employed to detect COVID-19 antibodies directly. The experiments were performed and optimized using the sensor chips functionalized with viral antigens (N and S1 protein), and two different types of antibodies (IgG and IgM) specific for each of them (anti-N IgG and IgM, and anti-S IgG and IgM, respectively) as detection targets. Biosensor assays for the antibody detection were performed by injecting several antibody solutions at different concentrations (from 1000 ng mL^{-1} to 9000 ng mL^{-1}) and monitoring the binding to the immobilized viral antigens in real-time

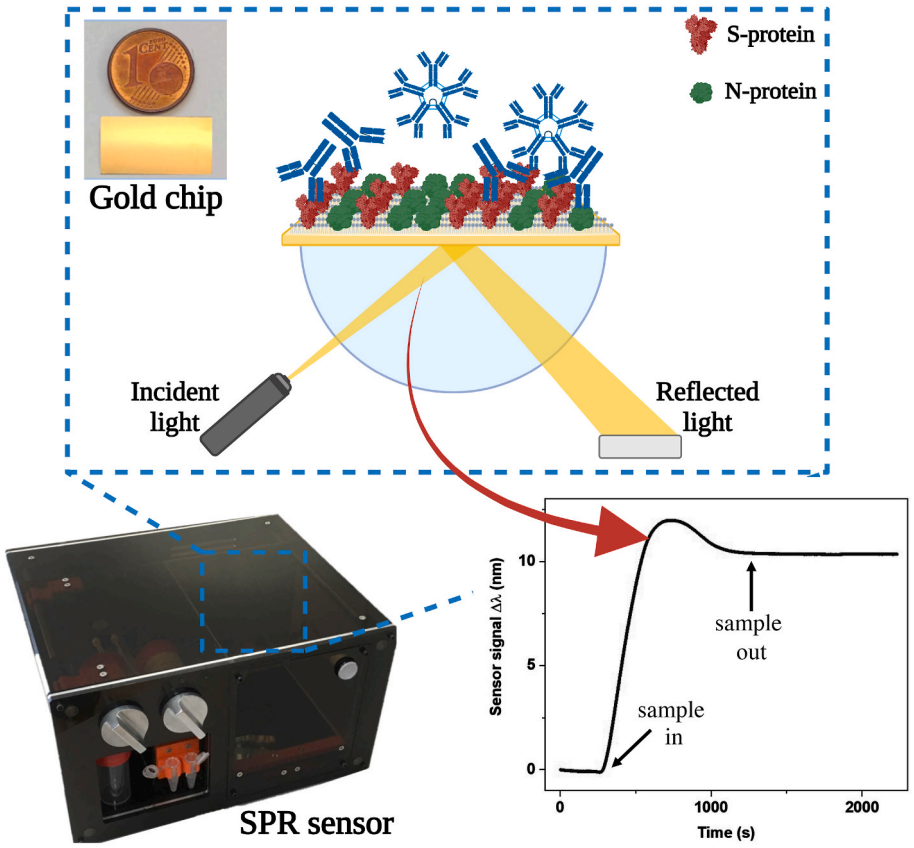

Fig. 1. Scheme of the experimental SPR device, including the plasmonic gold chip (top) and the real-time monitoring of the wavelength displacement (Δλ) related to antibodies concentration in the sample.

(shift in the resonance peak position (Δλ, nm)). Calibration curves were obtained by analysing the response of the antibody concentrations in duplicate in standard phosphate buffered saline (PBS) or in commercial serum (diluted to 10 % in the antifouling buffer: PBS + Tween 20 + Dextran Sodium Sulfate (PBST-DS)) and fitting them into a linear regression equation. Limits of Detection (LOD) were calculated by interpolating the signal value corresponding to three times the standard deviation (3xSD) of the blank signal, and Limits of Quantification (LOQ) were determined as the concentration corresponding to 10 times the standard deviation. All the antibody solutions were injected at a constant flow rate of 15 μL min^{-1}. A solution of NaOH 20 mM was injected for 1 min at a constant flow rate after each antigen-antibody interaction to completely dissociate it, allowing the reuse of the biosensor 10–15 times without modifying the immobilized proteins and the biosensor performance.

2.5. Animals' samples

A total of 75 animal sera samples were collected by the Ecole Nationale Vétérinaire d'Alfort (Maisons-Alfort, France), the Nancy Laboratory for Rabies and Wildlife (Malzéville, France), and the Department of Veterinary Medicine of the University of Bari Aldo Moro (Bari, Italy) following their studies [40,41]. The collection included cats, dogs, and hamster samples, and SARS-CoV-2 infection was determined by ELISA (N-prot), seroneutralization, or Luminex, as available standard reference method in each laboratory (see Table S1 in Supporting Information (SI)). This collection consisted of 42 sera samples from hamsters (12 negative samples collected and 30 COVID-19 positive samples), 14 sera samples from dogs (11 negative pre-pandemic samples collected and 3 COVID-19 positive samples), and 19 sera samples from cats (10 negative pre-pandemic samples, 5 negative samples collected in 2020, and 4 COVID-19 positive samples). All the data analysis and the determination of relevant parameters can be found in the SI.

3. Results and discussion

3.1. Serology biosensor development

For developing an accurate and highly sensitive serology biosensor, the most immunogenic antigens from the SARS-CoV-2 should be considered as the biorecognition elements, granting for the capture and detection of all antibodies generated by the host during its immune response. In the SARS-CoV-2 infection, both the N protein (contained inside the viral capsid) and the receptor binding domain (RBD) on the S1 sub-unit of the Spike (S) protein have been identified as immunogenic antigens, and thereby, antibodies targeting the two proteins may be found circulating in the host blood. In a previous work [14], we developed an SPR COVID-19 serology biosensor employing a combination of both the N and the RBD antigens as a biorecognition interface, demonstrating that the multi-target strategy maximizes the sensitivity and specificity for human diagnostics. However, the RBD antigen is known to be prone to undergo mutations during viral spread, especially during cross-species transmission. Therefore, to ensure the capture of all immunoglobulins in different animal samples, we selected both the N protein and the whole S1 protein as our biorecognition elements in this new biosensor.

Another pivotal aspect for maximizing the accuracy and reliability of the serology biosensor assay is to consider the detection of both IgG and IgM, as they can be commonly found in infected individuals depending on the sample collection time from infection. IgMs appear first during the acute infection phase and disappear after a few days or weeks and IgGs appear after a few days and remain in the bloodstream for months.

Thus, detecting both immunoglobulins can deliver valuable data about present and past infections and the immunological host response. Therefore, we employed both types of immunoglobulins to assess the performance of the biosensor assay regarding sensitivity. We employed commercial antibodies (IgG and IgM) for both N and S proteins and evaluated the two types individually over a multianalyte surface (N + S1): a mixture of anti-N and anti-S IgGs (1:1) and a mixture of anti-N and anti-S IgMs IgM (1:1). Fig. 2A and B shows the calibration curves for IgGs and IgMs detection, respectively, over a range from 1000 to 9000 ng mL^{-1}. A linear relationship was obtained in both cases (slope = 0.151 nm mL ng^{-1}, R^2 = 0.929 for IgG, and slope = 0.161 nm mL ng^{-1}, R^2 = 0.986 for IgM). From these calibration curves, we determined the Limit of Detection (LOD), for each biosensor assay, which resulted in 94.2 ng mL^{-1} for IgG and 88.4 ng mL^{-1} for IgM, respectively. These values confirm the capability of our multi-target sensor to effectively capture and detect both types of antibodies with high sensitivity.

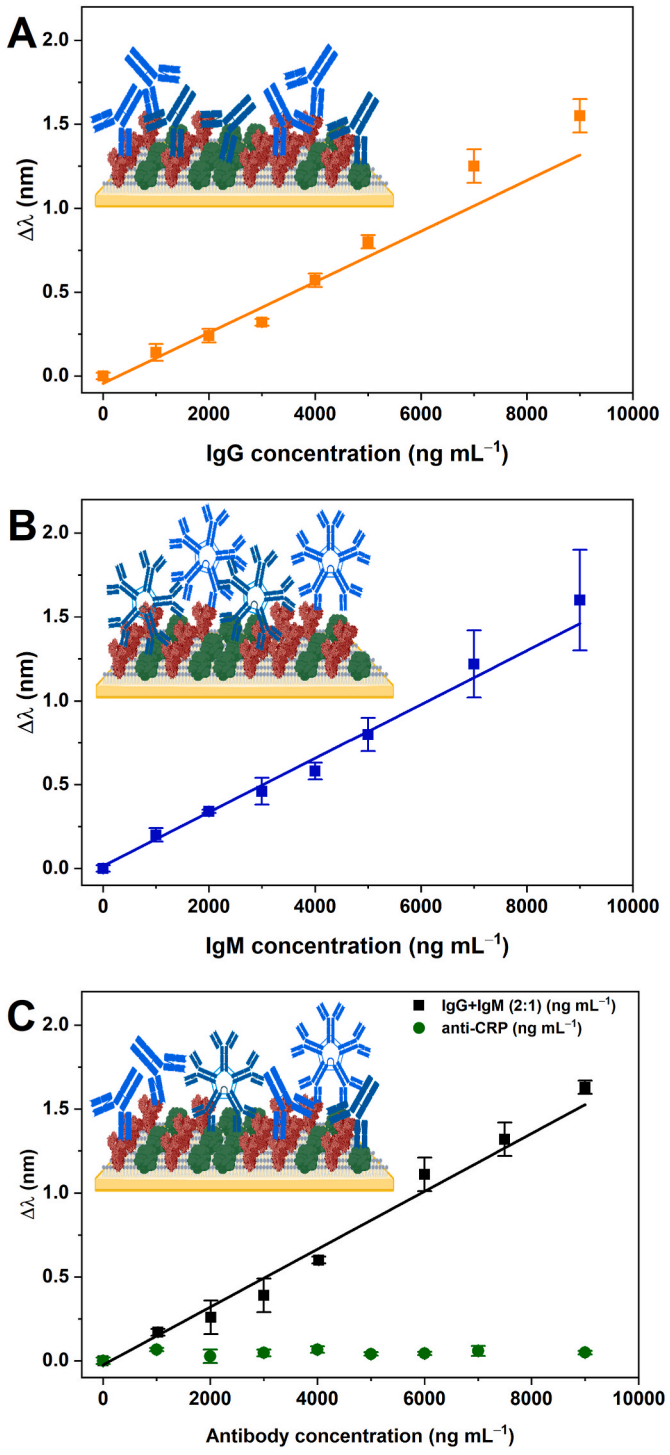

Fig. 2. A) Calibration curve in the standard buffer for the IgG, and **B)** for the IgM. **C)** Total antibody calibration curve in the standard buffer for IgG + IgM (2:1). Control antibody (anti-CRP IgG) at the same concentration ranges is also shown. Each signal corresponds to the mean ± SD of duplicate measurements.

To mimic a more accurate scenario where all antibody types co-circulate in the host blood, we then analysed a mixture of both IgG (anti-N IgG + anti-S IgG) and both IgM (anti-N IgM + anti-S IgM) at a ratio 2:1 IgG-IgM. Previous studies in human samples showed the presence of both immunoglobulins at this ratio after 15–30 days from infection [42–44] and also in cats after 14 days [45]. Fig. 2C shows the total antibody calibration curve obtained in standard buffer (PBS). The LOD was determined at 82.7 ng mL^{-1} (slope = 0.172 nm mL ng^{-1}, R^2 = 0.984), similar to the one obtained for the individual antibody calibrations, indicating that our biosensor surface can efficiently detect with high sensitivity all antibody types present in the sample. Although there are different studies about identifying COVID-19 antibodies in domestic animals, especially cats and dogs [46–49], the concentration levels are not provided. According to previous studies that suggest the antibody levels for SARS-COV-2 in human patients might be in the range of μg mL^{-1} [50], we can assume that the proposed biosensor may provide enough analytical sensitivity for COVID-19 serological testing in domestic animals.

Finally, a specificity assay was performed using a nonrelated antibody (i.e., anti-CRP Ab – an IgG against C-reactive protein) to confirm that the biosensor response is exclusively caused by specific interactions between the viral antigens and their corresponding antibodies. The negative control antibody did not show any signal (green dots in Fig. 2C) compared to specific IgG or IgM, demonstrating the high selectivity and specificity of our serology biosensor methodology.

3.2. *Assessment of domestic animal samples*

To apply the proposed biosensor for direct serological assay, we need to consider the influence of the serum matrix on the biosensor surface and in the antibody recognition. Since our biosensor works in a label-free format, any nonspecific adsorption of molecules on the sensor surface might induce a false positive signal; moreover, the different pH content of salts and other molecules present in the serum could affect the affinity interaction between the antibodies and the viral antigens. Based on our previous studies [14,38,51], we have demonstrated that the addition of different anionic surfactants (Tween 20 and dextran sodium sulfate, DS) to sera dilution buffer (PBS) can effectively reduce the nonspecific adsorptions to the sensor surface as well as can minimize the influence of the matrix components in the recognition interaction, leading to a negligible and reproducible background signal in most of the cases.

To that, we evaluated the biosensor response in the presence of commercial sera diluted 1:10 in PBS containing different concentrations of Tween 20 and DS (Fig. S1 in the SI). Optimal conditions (i.e., lowest signal) were found with a dilution buffer containing 2 % Tween 20 and 2 mg mL^{-1} DS; therefore, this buffer was selected for subsequent experiments. The analytical behaviour of the biosensor was evaluated by measuring total antibody (IgG + IgM) in serum samples (Fig. 3), confirming a good performance with a LOD of 49.6 ng mL^{-1}, nearly two times better than the one calculated in standard buffer conditions (slope = 0.318 nm mL μg, R^2 = 0.967). This sensitivity improvement might be due to the surfactant's effects in the sample, which are known to stabilize the biomolecules, preventing their denaturation or aggregation, thereby enhancing the recognition and interaction efficiency. Furthermore, these surfactants also aid in reducing the assay variability and in increasing the reproducibility. Finally, it is worth mentioning that the high sensitivity achieved by our biosensor technology, in the ng mL^{-1}

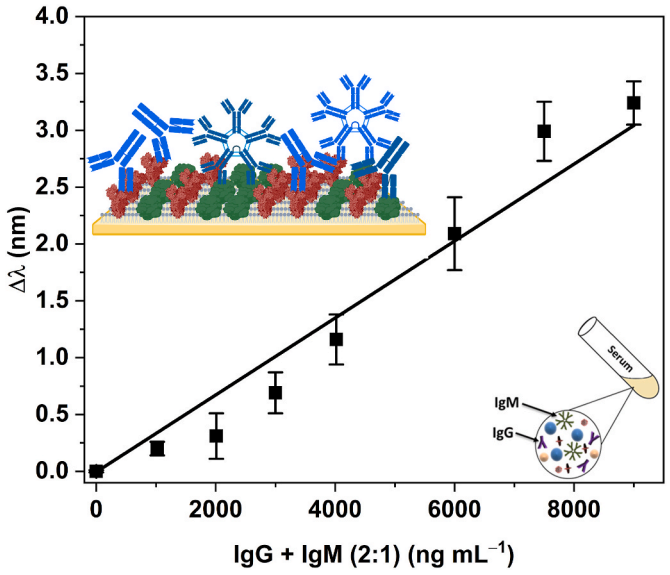

Fig. 3. Total antibody calibration curve (IgG + IgM, 2:1) in standard buffer (SB) with a detergent combination (2 % of Tween 20 and 2 mg mL^{-1} of DS). Each signal corresponds to the mean ± SD of duplicate measurements.

range, comfortably allows a 10-fold dilution factor of serum, which is indeed much lower than the one usually needed in other techniques such as ELISA or seroneutralization assays, commonly between 100-to10,000-fold [47,52,53].

The suitability of this serum dilution was finally evaluated in animal sera, since there are no such studies applied to these types of samples and the specific composition of the dilution buffer should be optimized. We tested the validity of our multi-target sensing methodology for the accurate discrimination of COVID-19-positive and negative animal samples with this buffer composition. We biofunctionalized three different sensor chips with (i) only the N protein (50 μg mL^{-1}), (ii) only the S protein (50 μg mL^{-1}), and (iii) the N + S protein (1:1, 50 μg mL^{-1}). Fig. 4 shows representative detection signals obtained for the three biofunctionalized sensor surfaces with a hamster-negative sample and hamster-positive sample. In agreement with our previous work [14], it can be clearly observed that the positive sample signal obtained for the multi-target sensor (N + S) corresponds to the sum of the individual

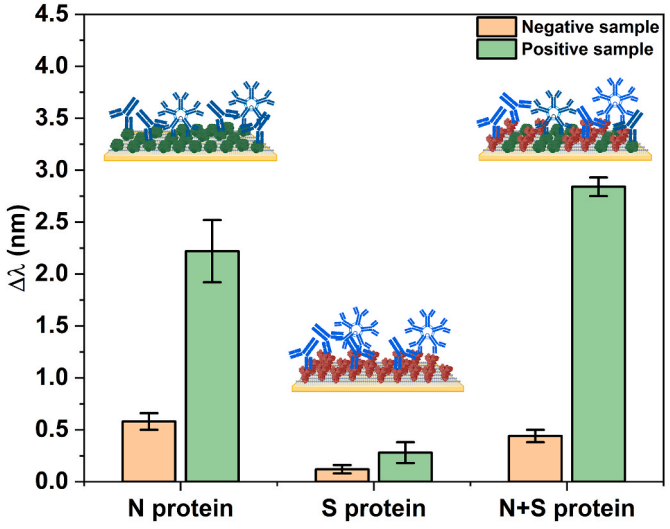

Fig. 4. Biosensor response of a negative and a positive hamster sample diluted 10 % and tested in three different biofunctionalized surfaces (N, S, and N + S protein). Sensor response represents the mean ± SD of two measurements.

surfaces (N and S separately). On the other hand, the background signal of the negative sample signal is relatively lower in the case of the multi-target surface compared to the individual ones, significantly increasing the difference between positive and negative results and, therefore, enhancing the ultimate sensitivity and specificity of the biosensor.

3.3. Validation of the SPR-based COVID serology for domestic animals

A complete clinical validation of the serology biosensor was carried out by analysing 75 serum samples from three different domestic animals with reported susceptibility to SARS-CoV-2 infection (hamsters, dogs, and cats) [54,55]. The sample cohort included 37 COVID-19-positive specimens collected in veterinary healthcare and research institutions during the pandemic and 38 COVID-19-negative specimens. All samples were analysed for active COVID-19 diagnosis through conventional and standard techniques like ELISA, seroneutralization, and Luminex technology (see Table S1 in SI for details).

Initially, we evaluated the samples for each animal species separately (Fig. 5). Hamsters are one of the animal species most susceptible to SARS-CoV-2 infection [56], primarily found in bats and rodents, and it is also a common companion animal, especially in European countries – after cats, dogs, and birds. We measured 42 hamster samples (12 negatives and 30 positives) with the plasmonic biosensor, and the results are summarized in Fig. 5A. An evident difference between positive and negative samples is observed, with the nonparametric Kruskal-Wallis's test confirming a significant difference ($p < 0.0001$) at 0.05 level. The diagnostic sensitivity (SE) and negative predictive value (NPV) were determined to be both at 100 %, and specificity (SP) and positive predictive value (PPV) were calculated to be 81.8 % and 93.8 %, respectively, considering the calculated threshold ($\Delta\lambda = 0.65$ nm). Besides, the high values for positive results compared to the negatives ones confirmed the hamster's susceptibility to SARS-CoV-2 infection. Next, we analysed dog samples, which are also known to be susceptible to SARS-CoV-2 infection, and their study could be of great relevance because of their close contact with humans. For dog samples, we could only obtain and analyse 15 samples (11 negatives and 3 positives). Fig. 5B shows a clear difference between the negative and positive biosensor response, with only one significant outsider and two indeterminates among the negative samples, considering the calculated threshold ($\Delta\lambda = 0.28$ nm). However, due to the limited number of positive samples the statistical analysis is not conclusive. Finally, the most particular findings were observed by the evaluation of cat samples (Fig. 5C). In this case, we measured 19 samples (15 negatives and 4 positives), and unlike hamsters and dogs, here we did not observe a significant difference between positive and negative samples, with many COVID-19 negative samples generating relatively high sensor signals, far from the estimated background. The most plausible hypothesis to explain these results can be found in the recently demonstrated cross-reactivity between SARS-CoV-2 viral antigens and Feline Coronavirus (FCoV) antibodies [57], which is a very widespread virus in domestic cats [16]. In the biomedical study published by Hancock et al. [57], pre-pandemic feline serum samples were submitted to an ELISA screening for SARS-CoV-2 RBD antibodies detection, and about 50 % resulted in positive responses, indicating that cat sera might contain antibodies generated in previous CoV infections that can recognize the SARS-CoV-2 viral antigens. Additionally, some studies have been conducted in the literature about how etiological agents could generate an anti-SARS-CoV-2 RBD cross-reactivity [58–60], providing insights into surveillance and developing new methods for managing cat samples and interpreting the results.

Regardless of setbacks with cat antibodies cross-reactivity, to fully validate and position our biosensor as a tool for serological analysis for different domestic animals, we considered all animal samples together (N = 75, 38 negative, and 37 positive), having a well-distributed cohort with sufficient number of positive and negative samples for a significant

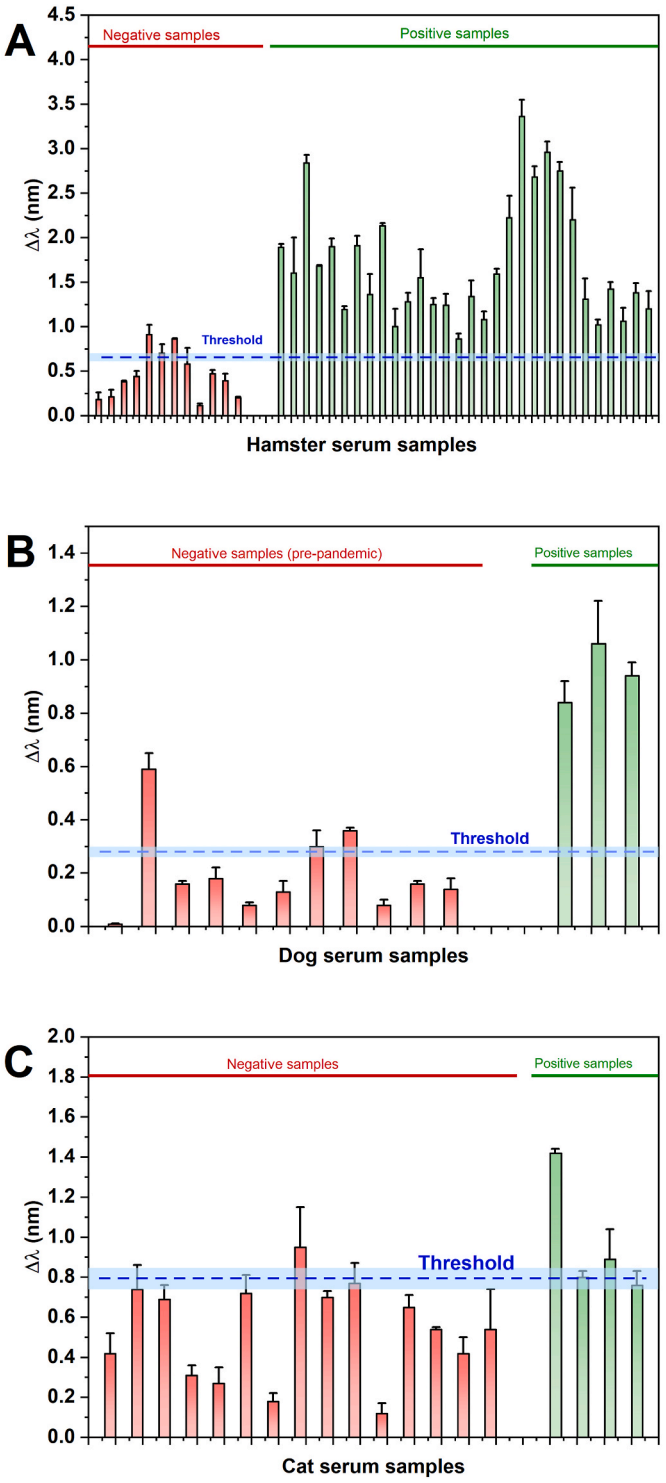

Fig. 5. Summary of the validation of the SPR-based COVID-19 serological assay for domestic animals: **A)** hamsters, **B)** dogs, and **C)** cats. Signals were obtained for negative (red) and positive (green) sera samples (sample diluted 10 %). The threshold (blue shadow) represents the set between positive and negative (indeterminate samples), and it was calculated as described in the experimental section.

statistical analysis. Fig. 6A shows the biosensor performance for all domestic animals evaluated in this study, and the distribution of the sensor signal is depicted in Fig. 6B. In this study, the nonparametric Kruskal-Wallis's test at 0.05 level confirmed that the positive samples are significantly different from the negative samples ($p < 0.0001$), with 10 false-positive samples, 2 indeterminate and 0 false negatives. The analytical and diagnostic performance parameters of our serological biosensor are summarized in Table 1, considering a recalculated threshold that includes all COVID-19 negative samples regardless of the observed FCoV cross-reactivity. Remarkably, the diagnostic SE was determined at 100 %, with 100 % NPV. However, the diagnostic SP was moderate (71.4 %), with a PPV of 78.7 %, mainly due to the presumed cross-reactivity of feline CoVs with the COVID-19 viral antigens, which should be further characterized with a larger number of samples and the corresponding molecular analysis techniques.

In light of the results obtained and considering the lack of decentralized technologies available for animal COVID-19 testing, our SPR biosensor appears as a promising solution for rapid serological analysis of domestic animals, offering a highly sensitive detection in less than 20 min. Moreover, it is integrated into a compact and user-friendly device for facile installation and operation in veterinary healthcare venues or animal research laboratories.

4. Conclusions

In this study, we have implemented a label-free serological biosensor that enables fast one-step identification of total SARS-CoV-2 antibodies in serum samples from European domestic animals (hamsters, dogs, and cats). We have carefully designed and optimized the biosensor assay parameters, including a multi-target sensor chip biofunctionalization and the direct analysis of diluted blood serum with no additional treatment processes. Our biosensor has demonstrated an excellent limit of detection in serum (49.6 ng mL^{-1}) with a short assay turnaround time (20 min sample-to-result). A clinical validation study with 75 samples from different animal species has shown statistically significant discrimination between positive and negative samples, with a diagnostic sensitivity of 100 % and diagnostic specificity of 71.4 %. Interestingly, our experiments reveal a presumed cross-reactivity of common feline coronavirus (FCoV) with COVID-19 viral antigens, as suggested in several biomedical reports recently published. In order to enhance the diagnostic specificity of the biosensor serology, future perspectives would involve the implementation of a multi-channel SPR biosensor [61,62] that enable parallel analysis with different antigens that provide exclusive detection of FcoV and SARS-CoV-2, respectively. Beyond further considerations for enhancing the diagnostic accuracy in cats, this case might contribute to the comprehensive study of animal immunological mechanisms towards coronavirus infections, which may aid in deciphering the pathways for the understanding and surveillance of zoonotic viral transmission, spread, and global incidence.

Overall, our plasmonic biosensor is presented as a unique tool for decentralized, accurate, and efficient animal infection diagnosis and screening. In our technological system, all components are integrated into a compact and user-friendly device suitable for point-of-care operation in different scenarios, from laboratory environments to veterinary clinics or farms. Our findings confirm the value of plasmonic biosensors as a highly sensitive and efficient serology tool for the screening of large populations, and in addition, corroborate the need for targeted developments and validation studies for the application of novel diagnostics in the veterinary field, where different pathogens may co-exist imposing a high risk for cross-reactivity and low-specificity in the diagnosis assays.

CRediT authorship contribution statement

Juliana Fátima Giarola: Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Maria Soler:** Writing – review & editing, Project administration, Methodology, Conceptualization. **M.-Carmen Estevez:** Writing – review & editing, Supervision. **Anna Tarasova:** Investigation. **Sophie Le Poder:** Resources, Funding acquisition. **Marine Wasniewski:** Resources, Funding acquisition. **Nicola Decaro:** Resources, Funding acquisition. **Laura M. Lechuga:**

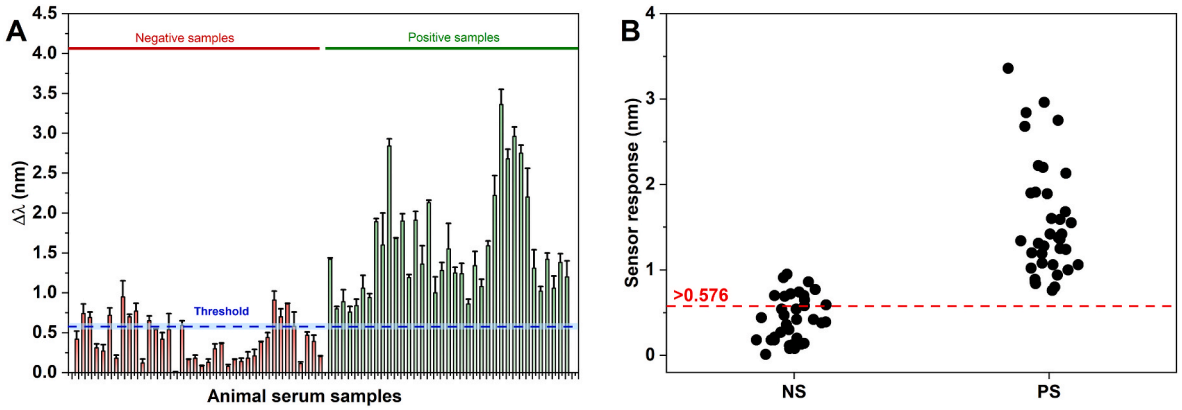

Fig. 6. A) Summary of the validation of all the animal sera samples with the SPR-based serological assay. A positive result was considered for samples above the set threshold (blue dotted line). **B)** Sample signal distribution of 37 COVID-19-positive (PS) and 38 negative (NS) sera samples.

Table 1
Analytical and diagnostic performance of the proposed plasmonic biosensor.

Biosensor performance in sera 10 %	
LOD (ng mL^{-1})	49.6
LOQ (ng mL^{-1})	163.7
Diagnostic validation (all samples)	
SE	100 %
SP	71.4 %
PPV	78.7 %
NPV	100 %

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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