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Protocols

Approaching the complexity of Crimean-Congo hemorrhagic fever virus serology: A study in swine

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ABSTRACT

Crimean-Congo hemorrhagic fever virus (CCHFV) is a tick-borne zoonotic orthonairovirus of public health concern and widespread geographic distribution. Several animal species are known to seroconvert after infection with CCHFV without showing clinical symptoms. The commercial availability of a multi-species ELISA has led to an increase in recent serosurveillance studies as well as in the range of species reported to be exposed to CCHFV in the field, including wild boar (*Sus scrofa*). However, development and validation of confirmatory serological tests for swine based on different CCHFV antigens or test principles are hampered by the lack of defined control sera from infected and non-infected animals. For the detection of anti-CCHFV antibodies in swine, we established a swine-specific in-house ELISA using a panel of swine sera from CCHFV-free regions and regions with reported CCHFV circulation. We initially screened more than 700 serum samples from wild boar and domestic pigs and observed a correlation of ≃67% between the commercial and the in-house test. From these sera, we selected a panel of 60 samples that were further analyzed in a newly established indirect immunofluorescence assay (iIFA) and virus neutralization test. ELISA-non-reactive samples tested negative. Interestingly, only a subset of samples reactive in both ELISA and iIFA displayed CCHFV-neutralizing antibodies. The observed partial discrepancy between the tests may be explained by different test sensitivities, antibody cross-reactivities or suggests that the immune response to CCHFV in swine is not necessarily associated with eliciting neutralizing antibodies. Overall, this study highlights that meaningful CCHFV serology in swine, and possibly other species, should involve the performance of multiple tests and careful interpretation of the results.

1. Introduction

Crimean-Congo hemorrhagic fever virus (CCHFV) belongs to the genus *Orthonairovirus* and is one of the most significant tick-borne zoonotic viruses of public health concern (Hawman and Feldmann, 2018). The geographical distribution of CCHFV is widespread and closely linked to the range of its principal vector and reservoir, namely ticks from the genus *Hyalomma* spp. (Gargili et al., 2017). Areas where the virus has been shown to circulate include regions in Africa, the middle East, southern Asia as well as countries in Eastern and Southern Europe such as Spain, where autochthonous human cases of CCHF have been reported since 2013 (Febrer-Sendra et al., 2023; Hoogstraal, 1979; Lorenzo Juanes et al., 2023; Magyar et al., 2021; Messina et al., 2015; Negredo et al., 2017). In addition, global warming is largely discussed to contribute to the spread of this tick species to new areas, e.g. France or Germany (Chitimia-Dobler et al., 2019; Kuehnert et al., 2021; Vial et al., 2016). Ticks of the genus *Hyalomma* play a major role in maintaining the virus in nature (Hoogstraal, 1979). They can be infected while feeding on viraemic animals or through co-feeding on a host without detectable viremia (Gordon et al., 1993). The virus can persist in the ticks for their

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whole lifespan by transstadial transmission and is vertically transmitted to offspring (Hoogstraal, 1979). Transmission to humans occurs through the bite of infected ticks (Logan et al., 1989; Shepherd et al., 1989b) or by direct contact with infected animal blood or tissues, e.g. during slaughter (Gümüş et al., 2022; Hoogstraal, 1979). Moreover, clinically diseased humans can pass the virus to close contact persons (e.g. medical personnel and caretakers). Humans infected with CCHFV can develop various clinical pictures ranging from asymptomatic or mild infection up to severe hemorrhagic fever, with a case fatality rate reaching up to 30% among hospitalized patients (Ergonul, 2012; Watts et al., 1988). Given its pathogenicity in humans and the overall lack of approved treatments or vaccines, CCHFV has been prioritized by the WHO on the Research and Development (R&D) Blueprint list (WHO, 2019). Moreover, the European Food Safety Authority (EFSA) has listed CCHFV as a priority pathogen for which sustainable surveillance strategies need to be implemented (Berezowski et al., 2023).

A wide variety of animal species have been reported to be susceptible to CCHFV infection, ranging from livestock such as cattle and sheep to various wildlife species like hedgehogs, hares or ostriches and transient viremia has been described in many of these species (Swanepoel et al., 1998). However, unlike humans, most infected animals have been reported to remain asymptomatic upon infection and only seroconvert (Ergonul and Whitehouse, 2007; Shepherd et al., 1989a; Spengler et al., 2016). This seroconversion observed in different wild and domestic animals provides the basis for serosurveillance studies as a valuable tool for indirect monitoring of virus presence in countries where the virus is endemic as well as in countries at risk for CCHFV emergence (Schuster et al., 2016b).

Simple test methods such as indirect ELISA are preferable to more elaborate tests such as the virus neutralization test (VNT), which requires handling of live virus under highest biosafety level conditions. The recent availability of a commercial multi-species ELISA (ID Screen® CCHF Double Antigen Multi-species ELISA from IDvet, hereafter ID Screen®) for the detection of anti-CCHFV nucleoprotein (N) antibodies in serum or plasma from different animal species (Sas et al., 2018), has enabled laboratories around the world to screen serum samples from a variety of animal species for the presence of anti-CCHFV antibodies without requiring access to high containment laboratories. As a result, an increasing number of serosurveillance studies have recently been conducted in various regions of the world, including new geographical areas and species such as wild boar, for which serological evidence of CCHFV circulation and/or exposure had not previously been reported (Balinandi et al., 2021; Baz-Flores et al., 2024; Cuadrado-Matías et al., 2022; Espunyes et al., 2021; Fanelli et al., 2022; Grech-Angelini et al., 2020; Matthews et al., 2023; Satrovic et al., 2022). Despite the high specificity (100%) and sensitivity (98.9%) of the commercial multi-species assay, additional serological testing is highly recommended to confirm single-test results (WOAH, 2023) before assuming circulation and/or exposure to a specific pathogen. However, particularly in the case of species like swine that have not been associated with CCHFV maintenance before, development and validation of new serological tests can be hampered by the lack of defined positive and negative serum samples. Moreover, to date, there is no systematic study for swine sera comparing the diagnostic performance of the ID Screen® ELISA with other serological tests based on different CCHFV antigens or testing principles.

Therefore, the aim of the present study was to develop additional in-house serological tests based on different target antigens and test principles for the detection of anti-CCHFV antibodies in swine and to compare their diagnostic performance to the commercial test. We established a swine-specific in-house indirect ELISA based on CCHFV N. To account for the lack of defined seropositive samples from swine, we used a panel of swine sera from regions that are currently free of CCHFV circulation and regions with endemic CCHFV circulation and applied different approaches and mathematical models to calculate cut-off values for the differentiation between reactive and non-reactive serum samples. Moreover, an indirect immunofluorescence assay (iIFA) using CCHFV-infected cells and a VNT were developed as additional, confirmatory tools. Overall, we established an in-house workflow using multiple complementary assays and emphasize the need for a careful result interpretation.

2. Material and methods

2.1. Serum samples

To develop and compare serological assays for the detection of anti-CCHFV antibodies in swine host, 746 serum samples from different geographical regions were used. Of these, 518 serum samples were collected between 2015 and 2021 from extensively reared domestic pigs (n=251) and hunted wild boar (n=267) from South-Western Spain, a European region with endemic circulation of CCHF in wildlife (Baz-Flores et al., 2024; Cuadrado-Matías et al., 2022). This sampling area is characterized by the *dehesa* agroforestry system, where a wide variety of land uses, such as farming, agriculture, and recreational activities (including hunting), are carried out simultaneously and pigs share resources and habitat with sympatric wild boar. Blood samples of wild boar were obtained by puncture of the cavernous sinus (Arenas-Montes et al., 2013) of legally hunted animals during commercial hunting events under Spanish and EU legislation. Additionally, domestic pig sera were taken from animals slaughtered after the final fattening period. These samplings did not involve purposeful killing of animals and, therefore, no ethical approval was deemed necessary. Another 228 domestic pig serum samples were collected from different holdings in Germany within the framework of different animal studies performed at the Friedrich-Loeffler-Institut (FLI). These studies had the specific approval (LALLF 7221.3–2.5–004/10, LALLF M-V/TSD/7221.3–2.1.014/10, LALLF M-V/TSD/7221.3–2.1–017/13. and LALLF M-V/TSD/7221.3–1.1–022/13) from the competent authority of the Federal State of Mecklenburg-Western Pomerania, Germany, on the basis of national (Tierschutzgesetz, Tierschutz-Versuchstier-Verordnung) and European (RL 2010/63/EU) legislation, which also includes the Ethic Committee of Mecklenburg-Western Pomerania. Since Germany is a country that is free of CCHFV circulation, these samples were used as negative control samples. Two serum samples from cattle from a CCHFV-endemic area in Turkey have been described previously (Mertens et al., 2015) and served as positive reference serum for the development of new serological tools in this study. Blood samples were centrifuged at 400 x *g* for 10 min and all obtained sera were stored at −20°C and thawed shortly before analysis.

2.2. Cells and viruses

Human adrenocortical carcinoma (SW13) cells (kindly provided by Ali Mirazimi, National Veterinary Institute, Sweden) were maintained in Leibovitz-15 (L-15) medium supplemented with 5% fetal calf serum (FCS; L-15–5) and incubated at 37°C without CO2. Vero E6 cells (Collection of Cell Lines in Veterinary Medicine, FLI, CCLV-RIE 0929) were cultivated in Minimal Essential Medium (Earl's and Hank's salts 1:1) supplemented with 1% non-essential amino acids, 0.125% sodium hydrogen carbonate, 0.012% sodium pyruvate and 10% FCS. The CCHFV isolates IbAr10200 (Genbank accession number NC005302, MH483988, AY947891) and Kosova Hoti (Genbank accession number DQ133507, EU037902, EU044832) were propagated in SW13 cells. All work with live virus was performed in the biosafety level 4 (BSL4) laboratory at FLI. Viral titers were determined using plaque assay and expressed as plaque forming units (pfu) per milliliter.

2.3. Virus plaque assay

The virus plaque assay was performed in 6-well plates with SW13 confluent monolayers. Ten-fold serial dilutions of the virus stock were prepared and incubated on cells for 1 h at 37°C without CO_2 (250 µl/

well). The inoculum was then replaced with 3 ml of overlay containing 1.2% Avicel® RC-591 (Carboxymethylcellulose sodium; DuPont) mixed 1:1 with L-15 medium supplemented with 4% FCS. Cells were incubated at 37°C for 4 days before fixation with 10% formalin and staining with 0.5% crystal violet dissolved in 10% formalin.

2.4. ID Screen® CCHF Double Antigen Multi-species ELISA (IDvet)

The ID Screen® CCHF Double Antigen Multi-species ELISA (IDvet, Grables, France) was performed according to manufacturer's instructions with slight modifications. Briefly, serum samples were tested in duplicate on each plate precoated with the nucleoprotein (N) of CCHFV [IbAr10200]. According to the manufacturer, a test run is valid if the optical density measured at 450 nm ($OD_{450\,nm}$) of the positive control (OD_{PC}) is greater than 0.35 and the ratio of the OD_{PC} to the $OD_{450\,nm}$ of the negative control (OD_{NC}) is greater than 3. Interpretation of tested samples is based on the ratio of the sample $OD_{450\,nm}$ (OD_S) to OD_{PC} expressed as percentage: (OD_S / OD_{PC}) x 100 (hereafter S/P (%)). A sample was classified as reactive if S/P (%) was over 30%. Serum samples with S/P (%) below or equal to 30% were considered as non-reactive.

2.5. In-house indirect ELISA for swine based on CCHFV N

The N of CCHFV [Kosova Hoti] used as antigen in the in-house indirect ELISA was recombinantly expressed in *E. coli* BL21 (DE3) and purified with buffers containing 8 M Urea as described previously (Schuster et al., 2016a). For coating, recombinant CCHFV N [Kosova Hoti] was diluted (200 ng/well; 0.01 M phosphate-buffered saline (PBS, pH 7.4) with 0.5% bovine serum albumin (BSA; Roth, Germany) and incubated overnight at 4°C (100 μl/well). Empty mock wells coated only with PBS and 0.5% BSA served as control wells to evaluate unspecific binding of the sera. Plates were washed once with 250 μl washing buffer (PBS with 0.1% Tween20, Sigma-Aldrich; PBST) and blocked with blocking buffer (IDvet, France) for 1 h at 37°C followed by three washes. Each porcine serum sample was diluted 1:40 in IDvet Dilution Buffer No.11 and added in duplicate to both the control and antigen containing wells (100 μl/well). After incubation for 1 h at 37°C, plates were washed three times with PBST before goat-anti-porcine IgG HRP conjugate (Dianova) was added in a dilution of 1:10,000 in IDvet Dilution Buffer No. 3 and incubated for another 1 h at 37°C. After three washes with PBST, 3,3',5,5'-Tetramethylbenzidine (TMB) peroxidase substrate (Bio-Rad, Munich) was added to the wells for color development in the dark. The reaction was stopped after 10 min at room temperature with equal amounts of 1 M sulfuric acid. Absorbance was measured at 450 nm. Sera from German pigs served as negative control. Cattle sera from a CCHFV endemic region described in Mertens et al. (2015) were used (1:80 dilution) and served as positive control. For these controls, goat anti-bovine IgG (H+L) Horseradish Peroxidase (HRP) (Southern Biotech) diluted 1:5000 was used. For all samples, the corrected $OD_{450\,nm}$ value was calculated as (mean $OD_{450\,nm}$ value with antigen) – (mean $OD_{450\,nm}$ value without antigen).

2.6. Cut-off determination for in-house indirect ELISA

Initially, cut-offs for indirect ELISA were determined using the upper prediction limit obtained from test results from negative serum samples, as defined by the following equation: mean $OD_{450\,nm}$ + 3x standard deviation (Table 1, cut-off method #1). As the German pig serum samples were considered negative, a total of n=228 samples from Germany were used for regional cut-off calculation. In contrast, the calculation of the regional cut-off for Spain was based only on those Spanish porcine serum samples that tested non-reactive in ID Screen® ELISA (n=334). For indirect ELISA, samples with $OD_{450\,nm}$ above the cut-off calculated with this standard method (Table 1, #1: mean $OD_{450\,nm}$ + 3x standard deviation) were classified as reactive. For the serum samples from Spain, additional alternative methods for cut-off determination were applied to compare their impact on the overall analysis and conclusions. All additional methods used in this study are summarized in Table 1. Cut-offs were computed using three standard formulas (Fi, i = 1–3). The coefficients 'f' in formulas #2 and #3 were found to be 3.848 and 2.197 (Table 1), respectively, based on the research by Frey et al. (1998).

2.7. Bayesian latent class model and ROC curve analysis

With the assumption that both components in an indirect ELISA (i.e. antibodies and antigen) were conditionally dependent, and in the absence of known true samples statuses, a Bayesian latent class model (BLCM) based on the approach described by (Branscum et al. (2005) was fitted.

Eventually, to determine the optimal cut-off for classifying positive and negative results, ROC curve analysis was performed. Due to the lack of defined control sera, "true positive" and "true negative" samples had to be defined. Considering the recommendations by WOAH for serological studies (i.e. multiple tests based on different test principles and/or antigens; WOAH, 2023) and the possible public health implications in case of a first report of serological evidence for the circulation of-CCHFV in new areas, we classified only those Spanish serum samples that tested

Table 1
Overview of the different methods used for cut-off calculation for indirect in-house ELISA for Germany and Spain. Swine serum samples that tested non-reactive in IDScreen® ELISA (n=228 for Germany, n=334 for Spain) were analyzed in in-house indirect ELISA. Corrected optical densities ($OD_{450\,nm}$) of the respective samples per country were used to calculate the mean $OD_{450\,nm}$ for Germany (A) or Spain (B). Different methods (#1 A/1B - #4) and formula as outlined were utilized to calculate regional cut-offs. An additional cut-off was calculated (#5) using sample ODs from Spanish swine sera that tested positive in all four serological assays performed, i.e. IDScreen® ELISA, in-house ELISA, iIFA and SNT, and were thus classified as "true positive". f (Standard deviation multipliers, f = t1 +(1/n)) = Standard deviation multipliers are derived from the critical values for a one-tailed t-distribution. α=significance level.

Cut-off method #	Formula	Country	Sample number	Mean $OD_{450\,nm}$	Standard deviation (SD)	Cut-off value	Comment
#1 A	MEAN $OD_{450\,nm}$ + 3*SD of ID Screen® non-reactive	Germany	n=228	-0.011	0.067	0.191	
#1B	MEAN $OD_{450\,nm}$ + 3*SD of ID Screen® non-reactive	Spain	n=334	0.023	0.088	0.288	
#2	MEAN $OD_{450\,nm}$ + f x*SD (with f = 3.848)	Spain	n=334	0.023	0.088	0.361	Confidence level (1-α) for t computation: 99.9%
#3	MEAN $OD_{450\,nm}$ + f x*SD (with f = 2.197)	Spain	n=334	0.023	0.088	0.216	Confidence level (1-α) for t computation: 97.5%
#4	Bayesian latent class analysis	Spain	n=334	0.023	0.088	0.304	
#5	ROC analysis	Spain	n= 19 "true positive" n= 327 "true negative"	0.023	0.088	0.294	

reactive in the four serological assays performed in this study as “true positive” (n=19). In contrast and given the high sensitivity of the commercial test (98%), 327 sera from Spain that tested non-reactive in both ELISAs were defined as “true negative” samples. Therefore, we employed MedCalc software (MedCalc Statistical Software version 19.6, MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2020). The highest combination of Youden’s index (Youden, 1950) in model output was considered to be the optimal cut-off (Table 1, #5).

2.8. Indirect immunofluorescence assay (iIFA)

To test for serum reactivity against whole CCHFV antigens, indirect immunofluorescence assay (iIFA) was performed on Vero E6 cells infected with CCHFV [IbAr10200]. Therefore, cells were seeded in chamber slides and infected with CCHFV at a multiplicity of infection (MOI) of 0.5. At 2 days post infection (p.i.), cells were fixed with 4% paraformaldehyde (PFA). Porcine serum samples were diluted in 1:50 in 0.35% BSA in PBS supplemented with $MgCl_2$ and $CaCl_2$ (PBS^{++}) and added to both CCHFV-infected and mock-infected cells. Cattle serum (TR3) was used as positive control (dilution 1:100 in 0.35% BSA in PBS^{++}). The diluted serum samples were incubated for 1 h at 4°C and washed two times with cold PBS^{++} before incubation with secondary antibodies (goat anti-swine Alexa Fluor 594 or anti-bovine Cy3, both Dianova, 1:500 in PBS^{++}) for 45 min at 4°C. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The fluorescence was observed with the Eclipse Ti-S inverted microscope system. Images were processed with NIS-Element (Nikon) software. Fluorescence was compared between mock- and CCHFV-infected cells and evaluated for CCHFV antigen-specific staining.

2.9. Virus neutralization test (VNT)

For VNT, SW13 cells were seeded in 96-well plates (1.5 $\times 10^4$ cells/well) with L-15–5 medium. The next day, medium was changed to L-15 medium supplemented with 2% FCS and antibiotics (Penicillin-Streptomycin (5.000 U/ml), Thermo Fisher Scientific). Serum samples were incubated at 56°C for 30 min for complement inactivation. Each serum sample was tested in triplicate and serially diluted in L-15 medium starting from 1:4–1:32 and added to empty wells of a 96-well plate. Serial dilutions of serum samples were then mixed with approximately 150 pfu of CCHFV [Kosova Hoti] per well and incubated for 1 h at 37°C. To assess possible cytotoxic effects of the respective samples, serum control wells were included in which no virus was added. After an incubation at 37°C for 1 h, 100 µl of the serum-virus mix was added to SW13 cells per well followed by another incubation at 37°C for 6 days. At 6 days p.i., wells were observed for signs of cytopathic effect (cpe). Neutralizing activity was assumed for those wells in which cpe was fully neutralized. The neutralization titer of a serum was calculated as geometric mean titer (GMT) of three replicates that exhibited neutralizing activity. Samples with a neutralizing titer above 1:8 were classified as VNT positive

2.10. Descriptive statistical analysis and correlation analysis

In this study, we assessed the association between the outcomes of two ELISA methods, designated as IDScreen® ELISA and in-house indirect ELISA, using the Phi Coefficient. Binary variables were assigned (0 for negative, 1 for positive). The Phi Coefficient (φ) was calculated using SPSS software (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0, IBM Corporation, USA). A significance level of $p < 0.05$ was used to determine the presence of statistically significant associations between the variables. Figures were created with GraphPad Prism (version 9.3.1).

3. Results

3.1. Development of an in-house indirect ELISA for screening of swine serum samples and its comparison with the commercial test

For serosurveillance studies in domestic pigs or wild boar, we aimed to complement the commercial multi-species test with a panel of additional in-house assays and compare their performances. Since defined positive serum samples from domestic pigs or wild boar were unavailable, we used two well-characterized cattle sera from a CCHFV-endemic region described previously (Mertens et al., 2015) as positive controls for assay validation. Domestic pig serum samples collected in Germany, a country that is currently free of CCHFV circulation, served as negative controls. Those sera were included in each run and used for the validation of the different assays.

Initially, we established an in-house indirect ELISA as a pre-screening tool for large sample sets, for which more laborious tests like the virus neutralization test (VNT) are impractical. We then tested n=228 pig serum samples from Germany in this in-house ELISA. The $OD_{450\,nm}$ values of the 228 German pig serum samples ranged from −0.30–0.37 (Fig. 1). A regional cut-off value for Germany was calculated as follows: mean $OD_{450\,nm}$+3*SD (Table 1, #1 A), which revealed a cut-off of $OD_{450\,nm} = 0.191$ for the in-house ELISA. Additionally, we tested these German sera (n=228) in the commercial ID Screen® ELISA (Fig. 1). In this commercial test, interpretation of tested samples is based on the ratio of the sample $OD_{450\,nm}$ to the $OD_{450\,nm}$ of the positive control and is expressed as percentage (S/P (%)). Serum samples with S/P (%) below or equal to 30% were classified as negative. Here, 225 of the 228 pig sera were considered negative and only 3 were above the S/P (%) 30% cut-off. Mean S/P (%) values ranged from 7% to 47%. Thus, both assays showed consistently non-reactive results for 223 of 228 domestic pig serum samples from Germany.

Next, we investigated whether a regional cut-off calculated for

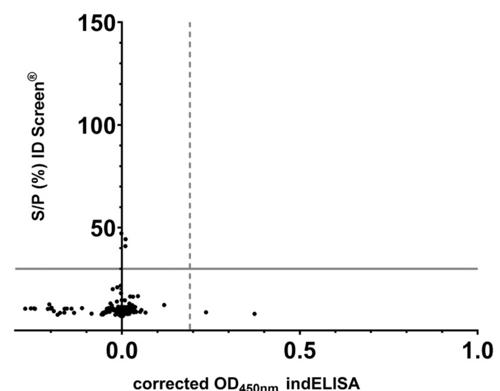

Fig. 1. : Analysis of domestic pig serum samples from Germany in in-house indirect ELISA based on Crimean-Congo hemorrhagic fever virus nucleoprotein (CCHFV N; [Kosova Hoti]) and ID Screen® ELISA. A total of 228 pig serum samples from Germany were selected for analysis in indirect (x-axis) and ID Screen® ELISA (y-axis). For indirect ELISA, serum samples were tested in duplicate on CCHFV N coated wells (dilution 1:40). Additionally, each sample was tested in duplicate in wells without antigen using the same dilution. Corrected optical density ($OD_{450\,nm}$) values are displayed and are calculated as mean $OD_{450\,nm}$ (duplicate wells with antigen) – mean $OD_{450\,nm}$ (duplicate wells without antigen). Vertical dashed line indicates cut-off for Germany at 0.191 calculated as follows: mean (*corrected* $OD_{450\,nm}$ *of all samples of one region*) + (3 x standard deviation). For ID Screen® ELISA, samples were analyzed in duplicate following manufacturer’s protocol. According to the manufacturer, a test run is considered valid if the $OD_{450\,nm}$ of the positive control (OD_{PC}) is greater than 0.35 and the ratio of the OD_{PC} to the $OD_{450\,nm}$ of the negative control (OD_{NC}) is greater than 3. Interpretation of tested samples are based on the ratio of the sample $OD_{450\,nm}$ (OD_S) to OD_{PC} expressed as percentage (OD_S / OD_{PC}) x 100 = S/P (%). Horizontal solid line indicates the cut-off at 30%.

Spanish swine sera would differ significantly from the cut-off calculated for the German sera. Hence, we analyzed a total of 518 sera from Spain including samples from wild boar and domestic pigs in the commercial ID Screen® ELISA to identify non-reactive samples (Supplementary Figure 1). The mean S/P (%) values of the sera ranged from 1% to 304%. The analysis revealed that n=334 out of 518 serum samples scored below the test-specific cut-off of 30% and were classified as non-reactive for further analysis. These sera were subsequently analyzed in the indirect in-house ELISA to calculate a regional cut-off. The cut-off for Spain was calculated as $OD_{450\ nm} = 0.288$ (Table 1, #1B). Mean $OD_{450\ nm}$ values of the ID Screen® non-reactive samples from Spain ranged from −0.18–1.09, with only seven of 334 sera that were above the Spanish cut-off in indirect ELISA (Fig. 2A). Eventually, all serum samples from Spain (n=518) were tested by indirect ELISA (Fig. 2B). Thereof, a total of 119 samples were reactive in indirect in-house ELISA using the Spanish cut-off ($OD_{450\ nm} = 0.288$). When comparing the results between indirect and ID Screen® ELISA, a total of 112 sera were considered reactive and 327 were non-reactive in both ELISAs (Fig. 2B). Taken together, 439 of 518 swine serum samples showed consistent results in both ELISAs. Lastly, we assessed the association between the outcomes of both ELISAs. The Phi Coefficient (φ) was ≃67% indicating a strong correlation of the two tests. The comparison of the positivity rates revealed a rate of 35.5% for the ID Screen® ELISA (184 of 518 samples above the cut-off) and 23% for the in-house ELISA (119 of 518 above the cut-off).

3.2. Confirmation of selected serum samples (n=60) with additional assays

To confirm ELISA-reactive serum samples and thus the presence of anti-CCHFV antibodies in Spanish serum samples, we tested 60 selected sera by iIFA and VNT. Only a limited number of selected samples was tested, as the VNT must be carried out with live CCHFV under BSL4conditions. Therefore, we primarily focused on a randomly selected subset of serum samples from Spain that were reactive in both ELISAs performed in this study (44 of the 112 double-reactive sera). From these, 43 of 44 samples were also positive by iIFA (representative images displayed in Fig. 3). Only about half of the selected ELISA double-reactive serum samples (20/44; 45%) showed CCHFV-neutralizing antibodies by VNT: seven samples were considered weakly neutralizing with a titer of 1:11, thereof one sample that was not reactive in iIFA, while 13 samples showed a neutralizing titer above 1:16 (Fig. 4A). The highest neutralizing titer observed in this study was 1:27. Interestingly, 22 of the 44 ELISA double-reactive sera tested negative by VNT (Fig. 4A). Another two field samples could not be analyzed in VNT due to bacterial contamination.

In addition, we analyzed n=4 samples that were non-reactive in both ELISAs (Fig. 4B) and a few sera with discordant results between the two tests: four samples reactive in the in-house but non-reactive in ID Screen® ELISA (Fig. 4C), and eight non-reactive in the in-house but reactive in ID Screen® ELISA (Fig. 4D), eventually adding up to a total of n=60 serum samples that were tested in iIFA and VNT. The four ELISA non-reactive samples were confirmed negative in VNT with a titer < 1:8 (Fig. 4B). In iIFA, three of these four samples tested negative (Fig. 4B). Regarding the samples with discordant ELISA results, three of four samples reactive in the in-house ELISA but non-reactive in ID Screen® ELISA were also found to be reactive in iIFA (Fig. 4C). The fourth sample, which was non-reactive in iIFA (Fig. 4C), displayed an $OD_{450\ nm}$ of 0.294 and thus ranged just above the regional cut-off for Spain (0.288). From the 8 samples that were non-reactive in the in-house ELISA but reactive in ID Screen® ELISA, the majority of samples (five out of eight) tested iIFA negative (Fig. 4D). Overall, results from iIFA for these samples were more consistent with the results from the in-house ELISA (Supplementary Table 1). However, sample size was too small for further conclusions. Lastly, independent of their reactivity in iIFA, none of the swine serum samples with discordant ELISA results did show

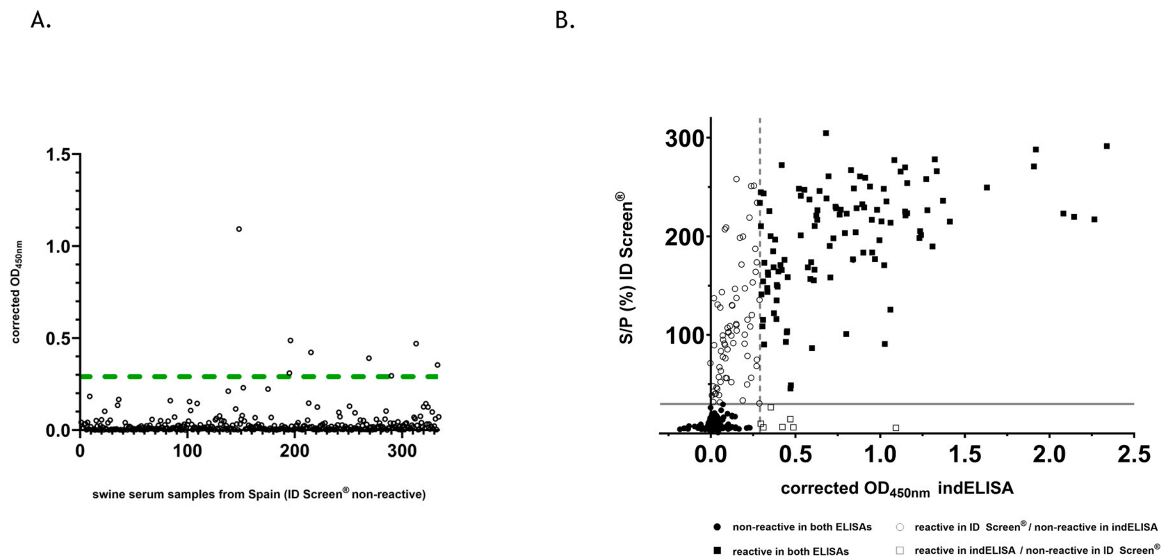

Fig. 2. : Analysis of serum samples from domestic pigs and wild boar from Spain in in-house indirect ELISA based on Crimean-Congo hemorrhagic fever virus nucleoprotein (CCHFV N; [Kosova Hoti]). (A) A subset of 334 swine sera from Spain that previously tested negative in ID Screen® ELISA were selected for determination of a regional cut-off for Spain. Serum samples were tested in duplicate on CCHFV N coated wells (dilution 1:40). Additionally, each sample was tested in duplicate in wells without antigen using the same dilution. Corrected optical density ($OD_{450\ nm}$) values are displayed and are calculated as mean $OD_{450\ nm}$ (duplicate wells with antigen) – mean $OD_{450\ nm}$ (duplicate wells without antigen). Horizontal dashed line indicates the cut-off for Spain at 0.288, calculated as follows: mean (*corrected $OD_{450\ nm}$ of all samples*) + (3 x standard deviation). (B) Using this newly established cut-off, the complete set of 518 swine serum samples from Spain were analyzed in the indirect ELISA (indELISA). Samples were analyzed in duplicates for reactivity against CCHFV N and non-coated mock wells as described above and are shown in a scatter plot to compare reactivity per sample in both the indELISA and the ID Screen® ELISA. The x- axis displays the corrected optical densities ($OD_{450\ nm}$) in indirect ELISA and the y-axis displays the ratio of the sample $OD_{450\ nm}$ (OD_S) to OD_{PC} expressed as percentage: (OD_S / OD_{PC}) x 100 (hereafter S/P (%)) for the ID Screen® ELISA. The indirect ELISA cut-off ($OD_{450\ nm} = 0.288$) is depicted with a vertical dashed line and the ID Screen® ELISA cut-off (S/P % over 30) is depicted with a horizontal solid line. Black dots represent samples that were non-reactive, while black squares represent samples that were reactive in both ELISAs. The white symbols represent samples that were either reactive in the ID Screen® and non-reactive in the indELISA (white dots), or vice versa (white squares).

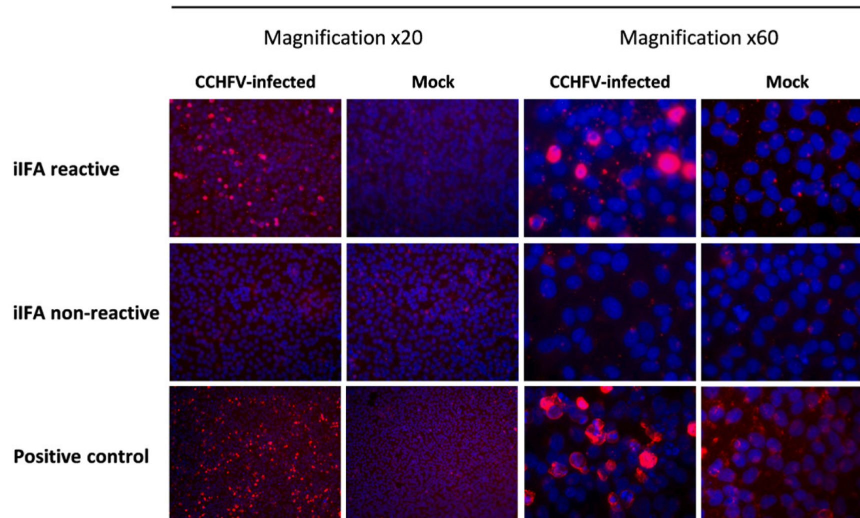

Fig. 3. : Immunofluorescence analysis of selected swine serum samples from Spain. Porcine sera (diluted 1:50) were incubated on fixed Vero E6 cells at 2 days post infection with Crimean-Congo hemorrhagic fever virus (CCHFV; [IbAr 10200]). Mock-infected cells served as negative control. Cattle serum (TR3) was used as positive control (dilution 1:100). Porcine antibodies were stained with anti-pig Alexa Fluor 568 secondary antibodies. Antibodies from cattle were visualized using anti-bovine Cy3-conjugated antibodies. Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Magnification x20 and x60.

neutralizing activity in VNT (Fig. 4C and D).

3.3. *Cut-off determination by ROC analysis*

Besides expanding the panel of serological tools available for the detection of anti-CCHFV antibodies in swine, we aimed to test different mathematical methods described for the determination of threshold for the indirect in-house ELISA and assess their impact on result interpretation (Lardeux et al., 2016). Using the corrected $OD_{450\ nm}$ values from the Spanish swine serum samples that were non-reactive in ID Screen® ELISA (n=334), we calculated cut-offs using different algorithms (Table 1: cut-off #2, #3, and #4). In ascending order, these cut-offs ranged between $OD_{450\ nm}$ = 0.216 (#3), 0.304 (#4, Bayesian latent class analysis), and 0.361 (#2) and resulted in n=137, n=114, or n=100 in-house ELISA-reactive samples, respectively. In comparison, using the initially calculated cut-off of $OD_{450\ nm}$ = 0.288 (Table 1, #1B), n=119 samples were considered reactive in the in-house ELISA.

Moreover, we defined selected serum samples from Spain that tested reactive in all four assays (i.e. ELISAs, VNT and iIFA) as “true positive” (n=19) and used their $OD_{450\ nm}$ for cut-off calculation by ROC analysis (Supplementary Table 2 and Supplementary Figure 2). In addition, given the high sensitivity of the ID Screen® ELISA (98%), we considered 327 samples from Spain that tested non-reactive in both ELISAs as “true negative” (n=327). The seven samples that gave discordant results between the two ELISAs (Fig. 2A) were excluded from the ROC analysis. Using “true positive” and “true negative” samples, the resulting cut-off (Table 1, #5) was $OD_{450\ nm}$ = 0.294, and thus very similar to the one initially calculated (Table 1, #1B; $OD_{450\ nm}$ = 0.288). In total, only ten serum samples ranged in between these two different thresholds in indirect ELISA. From those, nine samples were reactive in ID Screen®. Due to limited sample volume, only two of them could be tested in iIFA and of these again only one in VNT. However, both samples were interpreted as reactive in iIFA and VNT with a titer of 1:11.

4. Discussion

Recent serological studies from Spain using a commercial multi-species ID Screen® ELISA have indicated a role of wild boar as possible sentinels for CCHFV circulation (Baz-Flores et al., 2024; Cuadrado-Matías et al., 2022). Despite the reported high sensitivity and specificity of the commercial multi-species ELISA (Sas et al., 2018), each serological test has its own limitations and should thus be combined with other assays, e.g. targeting different antigens or using different test principles (WOAH, 2023). Therefore, we first developed a swine-specific indirect ELISA as an additional screening tool for large sample sets. The overall principles of both the ID Screen® and the in-house ELISA are compared in Fig. 5. Despite many advantages of the commercial test, the in-house ELISA compensates for a critical limitation of the ID Screen® ELISA in terms of specificity, as each well of the commercial test is pre-coated with the CCHFV N antigen. It is therefore not feasible to test a serum sample for non-specific binding to the well. To reduce false positive results, each serum sample in the indirect in-house ELISA is tested in duplicate for reactivity against CCHFV N as well as against mock wells. Corrected $OD_{450\ nm}$ values are then calculated by subtracting the mean $OD_{450\ nm}$ of a sample in mock wells from the mean $OD_{450\ nm}$ in antigen-coated wells, which has also been practiced by others (Mertens et al., 2016; Schuster et al., 2016b; Waritani et al., 2017). Moreover, differences in the initial serum sample dilution (1:2.7 in the ID Screen® vs. 1:40 in the in-house ELISA) as well as in the binding of the respective conjugates (IgG and IgM antibodies are bound by the commercial species-unspecific conjugate while only IgG antibodies are detected in the in-house test) may result in a higher sensitivity of the commercial ELISA. Nonetheless, we observed at least three samples to be reactive in indirect ELISA and iIFA that were non-reactive in the ID Screen® test, which further supports the recommendation to increase the strength and accuracy of a serological study by combining different serological tests for thorough interpretation.

Because all viral antigens are present in their native confirmation in virus-infected cells, some laboratories consider iIFA as the most sensitive serological method for detecting IgM or IgG antibodies, e.g. in human sera taken during early phase of infection (Emmerich et al., 2021; Emmerich et al., 2018). However, iIFA may be more prone to misinterpretation as the analysis solely relies on the investigator’s expertise to differentiate between specific staining and unspecific background fluorescence. In addition, iIFA lack specificity as staining by cross-reactive antibodies elicited after exposure to closely related CCHF-like viruses cannot be distinguished from CCHFV-specific staining. While sequence similarities between CCHFV and closely related orthonairoviruses have been reported over decades (Guilherme et al., 1996; Hartlaub et al., 2021; Papa et al., 2022; Walker et al., 2015), serological

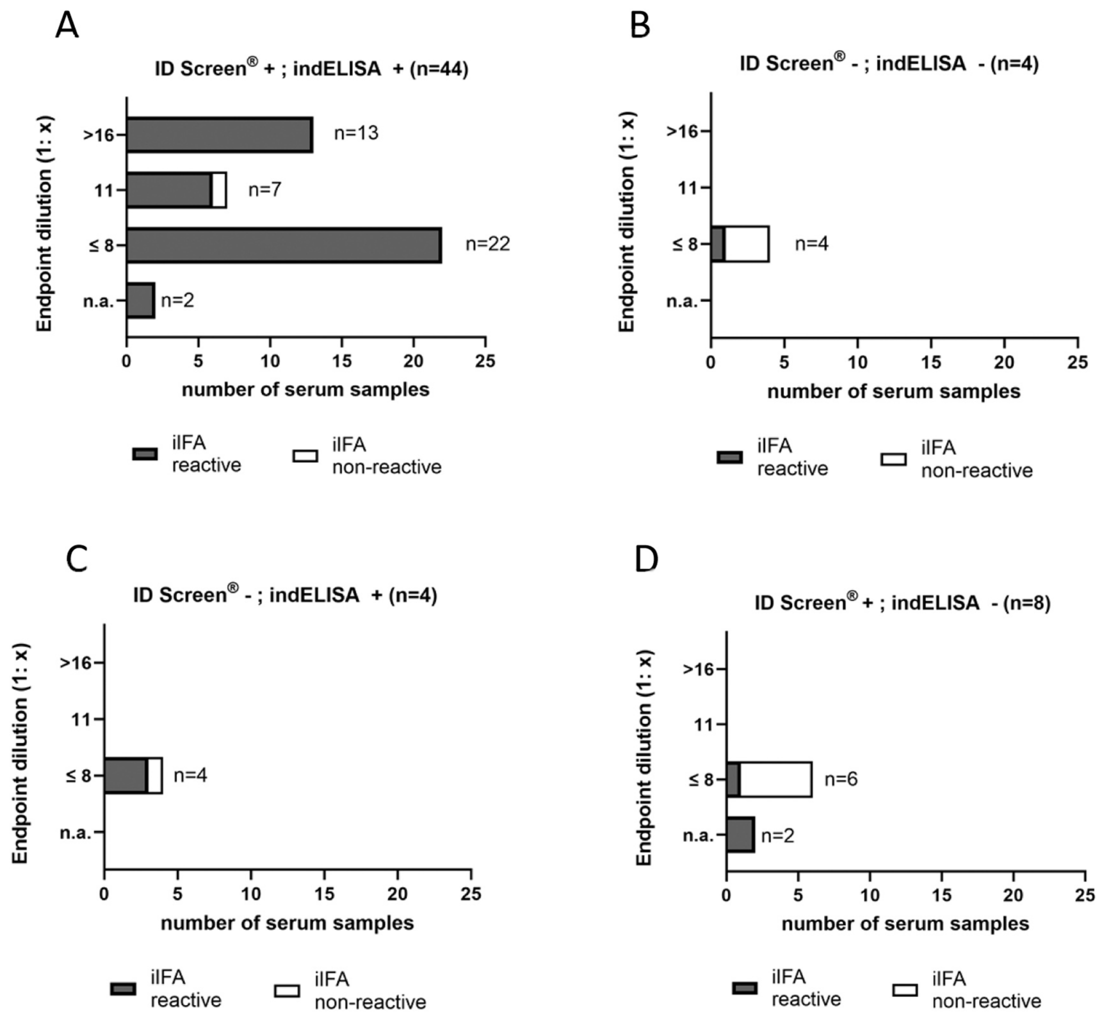

Fig. 4. : Neutralizing capacity and reactivity of selected swine serum samples from Spain (n=60) in indirect immunofluorescence assay (iIFA). Samples that were (A) reactive (n=44) or (B) non-reactive (n=4) in both ELISAs as well as (C and D) ELISA-discordant serum samples (n=4 and n=8, respectively) were tested in virus neutralization test (VNT) and iIFA. For VNT, serum samples were serially diluted and incubated with Crimean-Congo hemorrhagic fever virus (CCHFV; [Kosova Hoti]; dilution 1:8–1:64) for 1 h at 37°C. After that, the serum-virus mixture was added to SW13 cells followed by an incubation of 6 days before cytopathic effect (CPE) was assessed. Neutralization titers were calculated as geometric mean titer of three replicates that exhibited neutralizing activity. Samples with a neutralizing titer above 1:8 were classified as VNT positive. The y-axis depicts endpoint dilutions of samples in VNT. Bars represent the total number of serum samples tested in VNT and iIFA; in white, iIFA non-reactive; in grey, iIFA reactive. A small subset of field samples was not analyzable (n.a.; n=4) in VNT, e.g. due to bacterial contamination.

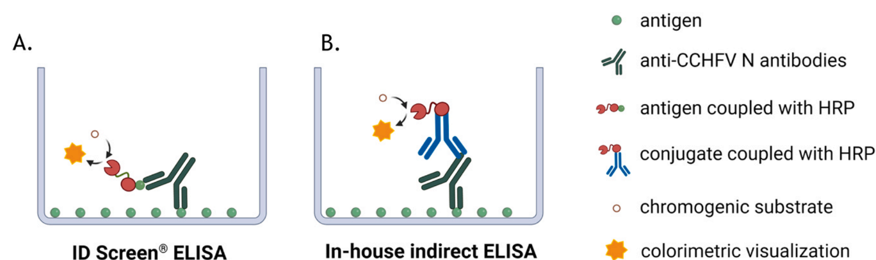

Fig. 5. : Schematic of ELISA formats used in this study for the detection of anti-CCHFV antibodies. For CCHF Double Antigen Multi-species ELISA (ID Screen®) depicted in (A), 96-well plates were pre-coated with the nucleoprotein (N) of Crimean-Congo hemorrhagic fever virus (CCHFV; [IbAr 10200]) and serum samples were added directly. For indirect in-house ELISA (B), 96-well plates are coated with the N of CCHFV [Kosova Hoti] overnight before a one-hour blocking step. Serum samples are diluted in (A) 1:2.7 or (B) 1:40 and added to the antigen-coated wells. In (B), additional mock wells containing only the coating buffer serve as control wells to evaluate nonspecific binding of a sample. For detection of anti-CCHFV N antibodies, different conjugates are used: while the N antigen linked to horseradish peroxidase (HRP) is used in (A) as species-unspecific conjugate, species-specific secondary antibodies are used in (B). A chromogenic substrate is added in both formats and optical density is measured at 450 nm ($OD_{450\ nm}$).

cross-reactivities between CCHFV, CCHF-like viruses and closely related orthonairoviruses have only been poorly investigated. One study analyzed serological cross-reactivities in ovine and bovine sera between different orthonairoviruses including CCHFV, Hazara virus, Dugbe virus and Nairobi sheep disease virus. Interestingly, all CCHFV ELISAs based on the viral nucleoproteins displayed high diagnostic specificities to distinguish between these related orthonairoviruses, whereas significant cross-reactivities were observed in iIFA (Hartlaub et al., 2021).

To increase overall specificity of our study, we performed VNTs. We indeed detected CCHFV-neutralizing antibodies in serum samples from domestic pigs and wild boar that were previously reactive in both ELISAs and iIFA, but only in a subset of samples. In contrast to reports from other animal species (Grech-Angelini et al., 2020; Khamassi Khbou et al., 2021; Müller et al., 2016; Simo Tchetgna et al., 2023), neutralizing activity in serum samples was weak and corresponded more with a historic report in which most human patients developed only low levels of neutralizing antibodies (1:8 – 1:32) (Shepherd et al., 1989c). However, test principles used for detection of neutralizing antibodies differed significantly between the studies and are therefore not directly comparable. It is conceivable that the neutralizing response in swine hosts is generally weak or that the neutralizing titer has dropped below the detection limit over time. In mice experimentally infected with CCHFV for instance, only a marginal neutralizing antibody response has been detected at 28 d.p.i., and no increase in neutralizing capacity was observed during convalescence (Hawman et al., 2019). Moreover, the general kinetics of anti-CCHFV antibody development and persistence in animals after CCHFV exposure are poorly understood. While anti-CCHFV IgG antibodies in sera of convalescent patients may last for up to five years (Charrel et al., 2004) a study in cattle described that IgM and IgG antibody levels in serum increased quickly after CCHFV exposure but decreased again after a short period of time (Zeller et al., 1997). Similarly, experimentally infected small African mammals developed CCHFV-specific antibodies reaching a maximum titer between day 14–21, which was shown to decline around day 28–35 post infection (Shepherd et al., 1989a). Concerning domestic pigs or wild boar, no data on antibody kinetics are available at all and only a few serological field studies based on ELISA testing have been conducted so far. A recent study using the ID Screen® ELISA reported an overall sero-reactivity of 40% (184 of 452 samples) in wild boar from Southern Spain sampled over fifteen years (Cuadrado-Matías et al., 2022). More recently, a high seroprevalence (19.4%; 1026/5291) was also found in a national serosurvey carried out in this species in Spain using the same commercial ELISA (Baz-Flores et al., 2024). In contrast, only five of 156 wild boar serum samples from northeastern Spain and only 1 of 40 wild boar serum samples from Turkey were reported to be reactive in ELISA (Espunyes et al., 2021; Nurettin et al., 2022). In the present study, we thus provide the first evidence for a CCHFV-neutralizing response in extensively reared pigs and wild boar. However, since we only performed serological tests based on CCHFV and single CCHFV antigens, we cannot exclude cross-reactivities to CCHF-like viruses or closely related orthonairoviruses such as Aigai virus (Papa et al., 2022).

The circulation of as of yet unknown orthonairoviruses that may lead to cross-reactivity, as well as the general limitations of serological tests, should be given special consideration when interpreting a serological study and assuming the introduction of CCHFV into new geographical areas, as such an assertion has implications for national and international health security. In Spain, a number of confirmed autochthonous human cases of CCHF have been reported to date (Lorenzo Juanes et al., 2023). Moreover, CCHFV RNA has been detected in *Hyalomma* ticks infesting red deer and wild boar (Estrada-Peña et al., 2012; Sánchez-Seco et al., 2022), making exposure and thus seroconversion of extensively reared pigs or wild boar to CCHFV more likely. Similarly, when seroreactivity is reported in a species not previously associated with CCHFV, such as pigs or wild boars, where a serological gold standard with defined positive control sera is missing, performing multiple serological assays is highly recommended for thorough interpretation (Garnier et al., 2017; Peel et al., 2013).

Several methods are known to determine cut-offs with different requirements. One is to use defined negative samples, which is easy in an environment where no vector is present and molecular detection has not been reported such as Germany. It becomes more difficult in countries at risk where the probability of virus presence or emergence is higher. In this study, we first used porcine sera from Germany as defined negative samples. Additionally, we utilized local swine serum samples from Spain that tested non-reactive in commercial ID Screen® as a proxy for defined negative samples. With these, we adapted and revalidated the cut-off of our in-house ELISA to Spain as the geographic area of interest, as it is recommended by WOAH for serosurveillance studies (WOAH, 2023). Using standard methods for cut-off calculation as well as Bayesian latent class analysis recommended for cut-off determination without gold standard (Lahuerta-Marin et al., 2018; Nielsen et al., 2002; Olsen et al., 2022), we only observed small differences between the calculated cut-offs for Germany and Spain. However, this may be different for intercontinental comparisons, e.g. between African and European breeds.

On the other hand, we defined serum samples that were reactive in all four serological assays in this study as “true positive” and used ROC analysis for assessing a potentially more appropriate cut-off. However, when comparing the impact of the different methods on our results for the Spanish cut-off, we found that the calculated cut-offs were similar irrespective of the mathematical model applied. Nonetheless, this finding is study-specific and may depend on the respective sample sets used, e.g. in terms of origin and number of samples tested. For example, in regions with low seropositivity and depending on the sample size of a study, the differences between the cut-off values calculated in this study may already have an impact on the interpretation of the results.

Taken together, ELISAs based on CCHFV N as antigen seem to be suitable tools for screening a large number of swine samples. However, single test results should always be confirmed by a second method considering the limitations of the individual assays and possible cross-reactivities with CCHF-like viruses or closely related orthonairoviruses. Overall, this is the first study using four different serological assays (summarized in Table 2) to screen porcine field serum samples for the presence of anti-CCHFV antibodies. Our results emphasize that meaningful CCHFV serology should be based on the performance and analysis of multiple serological tests. Furthermore, our results indicate the suitability of wild boar as sentinel animals including the presence of CCHFV-neutralizing antibodies in a subset of samples, albeit at low titers. Future studies are needed to investigate the significance of virus neutralizing antibodies in CCHFV infection in swine species and possible serological cross-reactivities against closely related CCHF-like viruses.

Author Summary

Serosurveillance studies using a commercially available multi-species ELISA have suggested wild boar as sentinels for monitoring silent CCHFV circulation. In this study, we established additional serological tests for comprehensive screening of swine serum samples, namely a swine-specific in-house ELISA, an indirect immunofluorescence assay (iIFA) on CCHFV-infected cells and a virus neutralization test (VNT). We compared the diagnostic performance of these assays based on different CCHFV antigens and test principles using a panel of sera from CCHFV-free regions and regions with CCHFV circulation. Initially, we screened over 700 serum samples from wild boar, extensively and conventionally reared domestic pigs in the in-house and the commercial ELISAs and observed a correlation of ≃67% between the tests. From these, we selected 60 sera for confirmatory testing in iIFA and VNT with a particular focus on reactive samples. Interestingly, we found CCHFV-neutralizing antibodies only in a subset of ELISA- and iIFA-reactive serum samples. Observed discrepancies between the tests may result from different test sensitivities, antibody cross-reactivities or suggests that the immune response to CCHFV in porcine host is not

Table 2
Serological tests used in this study. Commercially available as well as in-house tests were used to characterize pig serum samples for the presence of anti-Crimean-Congo hemorrhagic fever virus (CCHFV) antibodies. Enzyme-linked immunosorbent assay (ELISA); positive control (PC); negative control (NC); optical density ($OD_{450\ nm}$); S/P percentage (S/P (%)).

Assay	Virus	Antigen	Serum dilution	Validation and Interpretation	Controls
ID Screen® CCHF Double Antigen Multi-species ELISA	CCHFV IbAr 10200	Nucleoprotein (N)	1:2.7	$\frac{S/P\ (\%)\ PC}{S/P\ (\%)\ NC} > 3$ OD450nm of PC > 0.350 Sample with S/P(%) ≥ 30% are considered positive	Commercial PC and NC provided by the manufacturer.
Indirect in-house ELISA	CCHFV Kosova Hoti	Nucleoprotein (N)	1:40	$OD_{450\ nm}$ of PC >0.9, $OD_{450\ nm}$ of NC < cut-off, sample % variation <20% Samples with $OD_{450\ nm}$ ≥ cut-off value are considered reactive	PC: cattle sera from CCHFV-endemic region (Mertens et al., 2015), NC cattle and pig sera from non-endemic region (PC and NC validated in SNT and IFA)
Virus neutralization test (VNT)	CCHFV Kosova Hoti	Glycoprotein Gc	1:8–1:64	Virus control wells, serum toxicity wells, virus-neutralizing control wells (PC) Samples with a neutralizing titer above 1:8 were classified as VNT-reactive	PC: cattle serum from CCHFV-endemic region (neutralizing titer of 1:64; Mertens et al., 2015), NC: cattle and pig sera from non-endemic region
Indirect immunofluorescence assay (iIFA)	CCHFV IbAr 10200	Whole virus antigen	1:50	Analysis of mock and infected cells via fluorescence microscopy	PC: rabbit-derived polyclonal hyperimmune serum (antigen used for immunization: CCHFV N [Kosova Hoti])

necessarily associated with eliciting neutralizing antibodies. Overall, this study highlights that meaningful diagnostic CCHFV serology in animal species should include the performance of multiple tests and requires a careful interpretation of the results.

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CRediT authorship contribution statement

Martin Hermann Groschup: Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Ignacio Garcia-Bocanegra:** Writing – review & editing, Resources, Funding acquisition. **Mario Frias:** Writing – review & editing, Investigation. **Saúl Jimenez-Ruiz:** Writing – review & editing, Investigation. **Kerstin Fischer:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Conceptualization. **Balal Sadeghi:** Writing – review & editing, Formal analysis, Data curation. **David Cano-Terriza:** Writing – review & editing, Resources, Investigation, Conceptualization. **Caroline Bost:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation. **Sabrina Castro-Scholten:** Writing – review & editing, Resources, Methodology, Investigation.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2024.114915.

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