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Impact of animal saliva on the performance of rapid antigen tests for detection of SARS-CoV-2 (wildtype and variants B.1.1.7 and B.1.351)

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ABSTRACT

SARS-CoV-2 infects several animal species and SARS-CoV-2 variants of concern (VOCs) may even show (as in humans) enhanced inter- and intra-species transmission rates. We correlated sensitivity data of SARS-CoV-2 rapid antigen tests (RATs) to viral RNA genome equivalents analyzed by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Further, we checked their suitability for testing animals by assessing saliva and VOC effects. Viral loads up to 2 logs (RNA copy number) under the hypothetical SARS-CoV-2 infectivity threshold were detected by most analyzed RATs. However, while saliva from various animal species showed generally no adverse effects on the RATs' analytical sensitivities, the detection of VOCs B.1.1.7 and B.1.351 was in some RATs inferior to non-VOC viruses.

1. Introduction

Seven different coronaviruses are currently known to infect humans and all of them originate from animals (Su et al., 2016). In late 2019 a betacoronavirus of unknown origin, designated SARS-CoV-2 was identified and caused a worldwide pandemic. Apart from humans, SARS-CoV-2 can infect farmed animals, hamsters, minks, ferrets, raccoons, cats and dogs (Abdel-Moneim and Abdelwhab, 2020). Infections of lions, tigers, pumas, snow leopards, cynomolgus macaques, rhesus macaques, treeshrew, gorilla and others were also frequently reported (OIE, 2021). Clinical signs in animals are usually mild, but infections can also be fatal (de Morais et al., 2020; Ferasin et al., 2021). Several SARS-CoV-2 variants of concern (VOCs) are circulating worldwide and may even be more transmissible to and pathogenic for domestic animals than the original strain (Ferasin et al., 2021). There is also a possibility that such infected animals can more easily spill the virus back to humans. To date, rapid antigen tests (RATs) receive much attention as they provide on-site results without the need for elaborate instrumentation and/or expertise (Igloi et al., 2020). RATs are therefore part of most national testing strategies for humans worldwide. Hence, the question arose whether such assays would also be suitable as point-of-care diagnostics for SARS-CoV-2 in animals and, if so, whether the currently circulating VOCs are detected by them just as well. A broad analytical sensitivity study of 122 RATs for use in humans has shown recently that the majority of the assays are detecting SARS-CoV-2 viruses equivalent to about 10^5 genome copies (Scheiblauer et al., 2021). Another study on 5 commercial assays proved their suitability for detecting VOCs (B.1.1.7 and B.1.351) in principle but also revealed differences in analytical sensitivities for the variants (Jungnick et al., 2021). In the aforementioned test, VOCs were better detected than the original SARS-CoV-2 strains. VOCs are primarily defined by differences in spike protein, even though mutations in other viral proteins also exist. Therefore, it is not surprising that variable recognition by RATs, most of which use the nucleoprotein as a target, is observed between VOCs and common SARS-CoV-2 strains. In the described study here, we used saliva samples spiked with cell culture grown virus to show that RATs are also suitable tool for detecting animals shedding SARS-CoV-2. However, as it turned out limits of detection for VOCs can also be substantially lower, calling for detailed assay validations prior to their use.

2. Methods

2.1. Viruses and cells

Vero E6 cells (ATCC CCL-81) were grown and maintained in Eagle's minimal essential medium (EMEM; Lonza) with 8% foetal bovine serum

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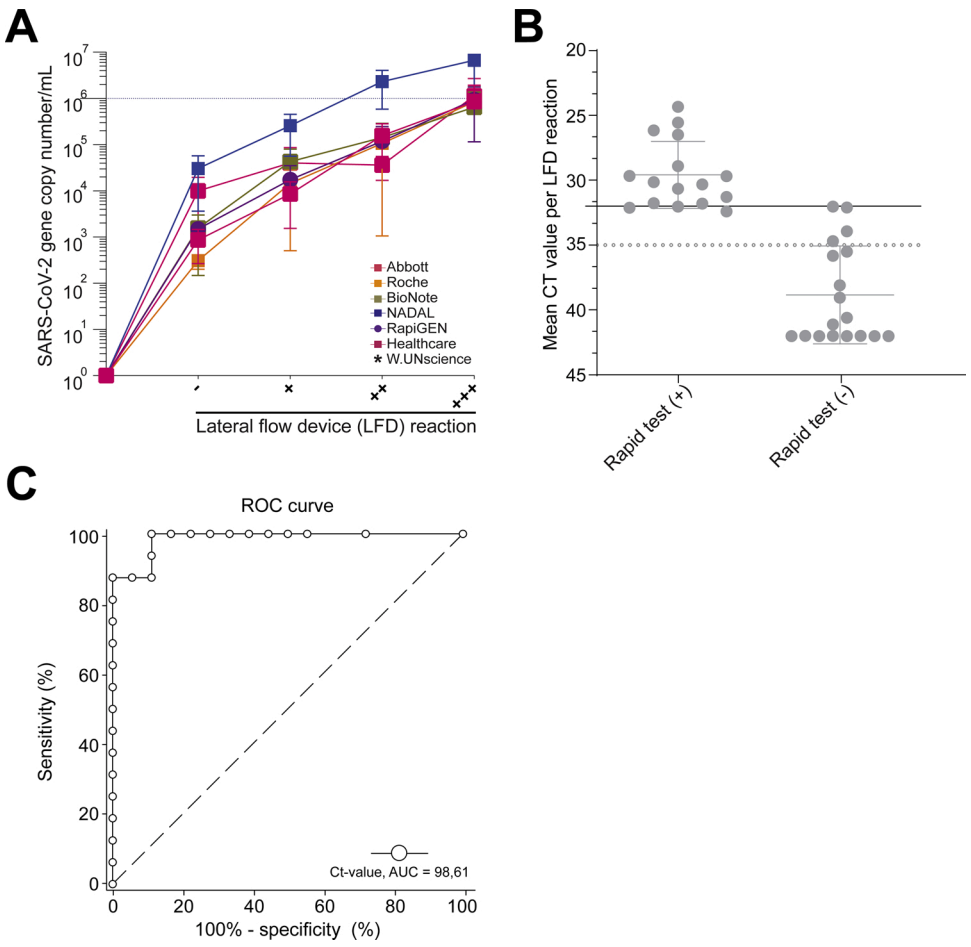

Fig. 1. Limits of detection (LoD) of SARS-CoV-2 rapid antigen assays. (A) Comparison of the rapid antigen test results with the corresponding viral RNA genome copy numbers (per mL) determined by RT-qPCR. * Antigen kits (WuHan UNscience) did not meet the requirements (weak positive at 3.12 E + 06 per mL), as the LoD was significantly different from the other tests (*$p < 0.05$; Bonferroni's multiple comparisons). The dashed line refers to the hypothetical infectivity threshold (10^6/mL) (12). (B) Interactive dot analysis for an exemplary antigen assay (BioNote) results compared to RT-qPCR Ct values. The solid and dashed lines refer to the estimated Ct cut-off value 32.25 and the arbitrary limit of RT-qPCR Ct LoD, respectively. (C) ROC curve for the analysis of sensitivity and specificity of the leading rapid antigen tests (AUC 98.6; $P < 0.001$). No significance was observed between assays (p-value ≤ 0.05). Three to four experiments were evaluated. One-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test was used to analyze the data. Standard deviations (SD±) are represented by error bars.

(PAA), and antibiotics (Sigma) at 37 °C and 5% CO2 as previously described (de Wilde et al., 2013). Infection of Vero E6 cells with SARS-CoV-2 (strain 2019_nCoV Muc-IMB-1, GISAID accession number EPI_ISL_406862) and VOCs was performed as previously described (Case et al., 2020). Virus stocks (passage 3) were stored at −80 °C and viral titres depicted as $TCID_{50}$/mL were calculated before use. All work with live SARS-CoV-2 and VOCs was performed in a biosafety-level 3 (BSL-3) laboratory at Friedrich-Loeffler-Institut, Germany. To perform downstream analysis under BSL-2 conditions, viruses were heat-inactivated at 95 °C for 20 min.

2.2. SARS-CoV-2 rapid antigen assays

Rapid antigen assays were randomly selected from commercially available products. All assays are registered for testing human naso/oropharyngeal swab samples and to detect SARS-CoV-2 N-protein using dual anti-N-antibodies; a dye-labeled antibody in the sample pad and immobilized antibody fixed on a membrane. Upon adding a positive sample to the pad, the N-protein is bound by the labeled antibody and the complex migrates by capillary forces and is finally captured by the immobilized antibody. The test band gets visible by an accumulation of the dye and the control band gets visible by capturing a labeled unspecific antibody. Here, we aimed to evaluate limits of detection of SARS-CoV-2 rapid antigen assays by using serially diluted heat-inactivated wild-type SARS-CoV-2 (Non-VOC; Wuhan-Hu-1 strain; $TCID_{50}$ $10^{3.8}$/mL) and VOCs B.1.1.7 ($TCID_{50}$ $10^{6.75}$/mL) and B.1.351 ($TCID_{50}$ $10^{6.8}$/mL) in cell culture medium, composed of carbonate buffered MEM with Hanks and Earls salts (50:50), pyruvate, non-essential amino acids and without antibiotics. Briefly, 25 µL from each viral dilution were applied to the manufacturer swab, processed as recommended and finally eluted in 300 µL from the supplied lysis buffer. 120 µL from each swab suspension were applied to the lateral flow device and 140 µL were subjected to RNA isolation and subsequent RT-qPCR (Fig. S1A-B). We used these constant volumes in all assays for the comparability of experiments. Results were recorded after 15 min and a 4-grade scaling readout (+++/strong positive; ++/positive; +/weak positive; -/negative) was established to reflect the intensity of the target band, which was furtherly quantified using Image Lab (Image Lab, 6.0.1; BioRad). When results were dubious, the four or six-eye principle was used. Three independent and blinded experiments were performed. To analyze the sensitivity and specificity of an exemplary rapid antigen assay (BioNote), an additional double-fold dilution was performed to the borderline dilution (e.g.10^{-3}) to precisely reflect the borderline sensitivity and corresponding Ct values (Fig. S1C-D). Data were further analyzed by receiver operating analysis (ROC) using Sigmaplot (Systaat Software Inc., Chicago, USA).

2.3. Detection of SARS-CoV-2 RNA by real-time RT-PCR

Viral RNA from each swab suspension was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and subjected to real-time RT-PCR (SuperScript III Reverse Transcriptase, Invitrogen, Germany). An *envelope* (E) gene amplification was performed using CFX real-time PCR systems (Bio-Rad, Germany) and a set of primers and probes as previously described (Corman et al., 2020). Viral genome copy numbers were derived from the standard curves of an *in vitro* RNA transcript harboring the E gene.

Table 1
Detection limits of commercial antigen assays for detecting wild-type SARS-CoV-2.

Antigen Kits (Name/Lot.No./Exp.Date)	Limit of Detection (LOD) (RNA copy numbers/mL)
Standard Q® COVID-19 Ag (SD Biosensor Healthcare PVT.LTD)/C055099/19/10/2022	1,35E + 04
Panbio COVID-19 Ag Rapid Test Device (Abbott Rapid diagnostics)/41ADF058A/04/09/2021	1,68E + 04
SARS-CoV-2 Rapid Antigen Test (Roche Diagnostics)/QCO3020092/01/10/2022	2,43E + 04
Biocredit COVID-19 Ag (RapiGEN INC.)/ H073054SD/29/11/2022	2,99E + 04
NowCheck COVID-19 Ag Test (BioNote, Inc.)/ 1901D023/29/11/2022	6,90E + 04
NADAL® COVID-19 Ag Test (nal von minden)/ 175205/ Nov.20,2022	4,55E + 05
SARS-COV-2 Antigen Rapid Test Kit (WuHan UNscience Biotechnology Co., Ltd)/ 20201030/ 29/04/2022	3,12E + 06

2.4. Spiking saliva with SARS-CoV-2

To evaluate the use of animal saliva in rapid antigen assays, we collected saliva from different species and spiked it with defined amounts of virus, diluted in a cell culture medium. While human saliva was self-collected, ferrets and bats saliva were obtained by swabbing animals' oral fluid using manufacturer's swabs. The saliva-soaked swabs obtained from ferrets were directly spiked with the diluted virus, whereas bats saliva was eluted from the swabs in PBS and solution was spiked with the virus as described. Saliva from cat and farm animals as sheep, goats, and cattle were obtained by passive drool collection. For collecting substances of swine saliva, a chewing-rope was used as previously described (Gutierrez et al., 2014) and the wet part of the rope was eluted before applying it in antigen assay and RT-qPCR. Of note, collected saliva was processed by centrifugation at 14,000 rpm/30 min and filtration by 0.2 µm filters to remove any debris. However, unprocessed saliva samples were also tested to exclude the inhibitory effect of saliva components such as mucin on RATs performance. Next, SARS-CoV-2 and VOCs were spiked, serially diluted into saliva, and 25 µL were applied on the manufacturer's swab. Further, swabs were eluted in 300 µL buffer and 120 µL were applied on lateral devices. The percent of the target band intensity and corresponding viral gene copy numbers were reexamined as above described (Corman et al., 2020; Kennedy and Oswald, 2011). Importantly, an oropharyngeal swab from SARS-CoV-2-infected hamster was also used as an additional positive control, representing real-world clinical samples.

2.5. Statistical analysis

GraphPad Prism (version 9; GraphPad Software, Inc., CA, USA) was used to analyze data. Normal distribution of data and analysis by Bonferroni's multiple comparisons tests was performed. Statistical analysis was performed using SPSS software (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0, IBM Corporation, Armonk, NY, USA). Significance was considered upon P values ≤0.05.

2.6. Ethics statement

Collected human saliva was collected after required consent and without using any person-related data.

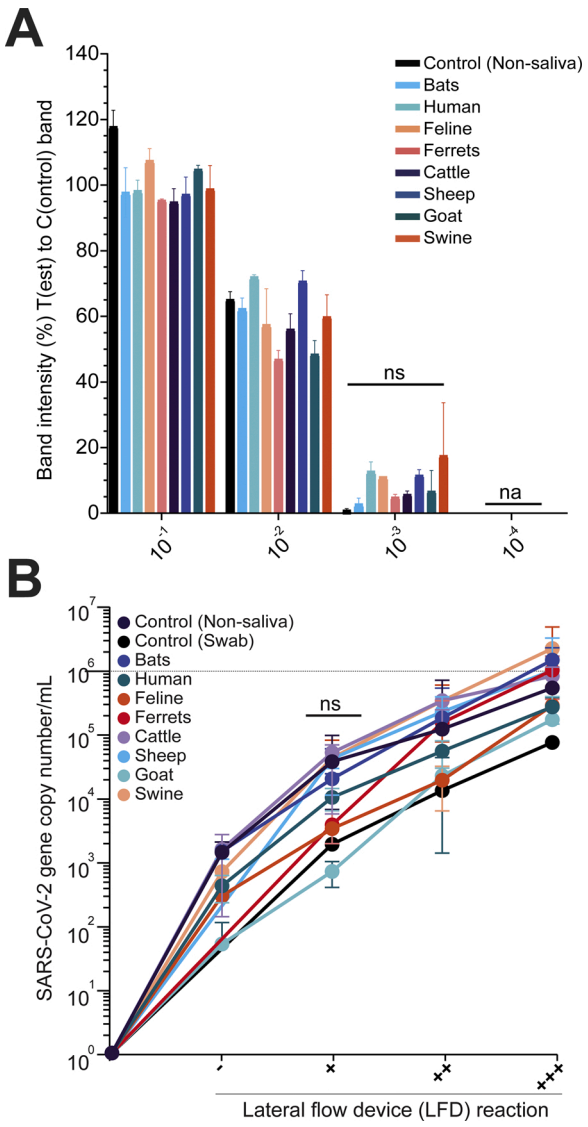

Fig. 2. Saliva from animal species has no adverse effectson the analytical sensitivity of rapid antigen assays. (**A**) SARS-CoV-2 target band intensity (%) in lateral flow device after normalization to the control band. (**B**) SARS-CoV-2 copy numbers from spiked human and animal species saliva with correspondence to the results of lateral flow device using real-time RT-PCR. Controls blue and black respectively represent non-saliva sample and an oropharyngeal swab from SARS-CoV-2-infected hamster (positive clinical sample). Non-applicable data (test band is missing, therefore no calculation was possible) were referred to as 'na'. Three to four average data sets were analyzed. Standard deviations (SD±) are represented by error bars. No significant differences were detected (control vs. human, feline, ferrets, cattle, sheep, swine, and bats; Bonferroni's multiple comparisons test; significance level was set at p-value ≤0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3. Results

3.1. Effective detection of high and moderate wild-type SARS-CoV-2 loads by rapid antigen assays

Six assays showed detection limits down to 10^3 – 10^4 viral RNA genome copies pro mL (Fig. 1A), while for one assay virus dilutions equivalent to at least 10^5 viral copies and another 10^6 genome copies were needed to obtain positive results (Fig. 1A). Therefore, the least sensitive RAT was excluded from the study (Fig. 1A). Based on these results, all rapid antigen assays were ranked according to their

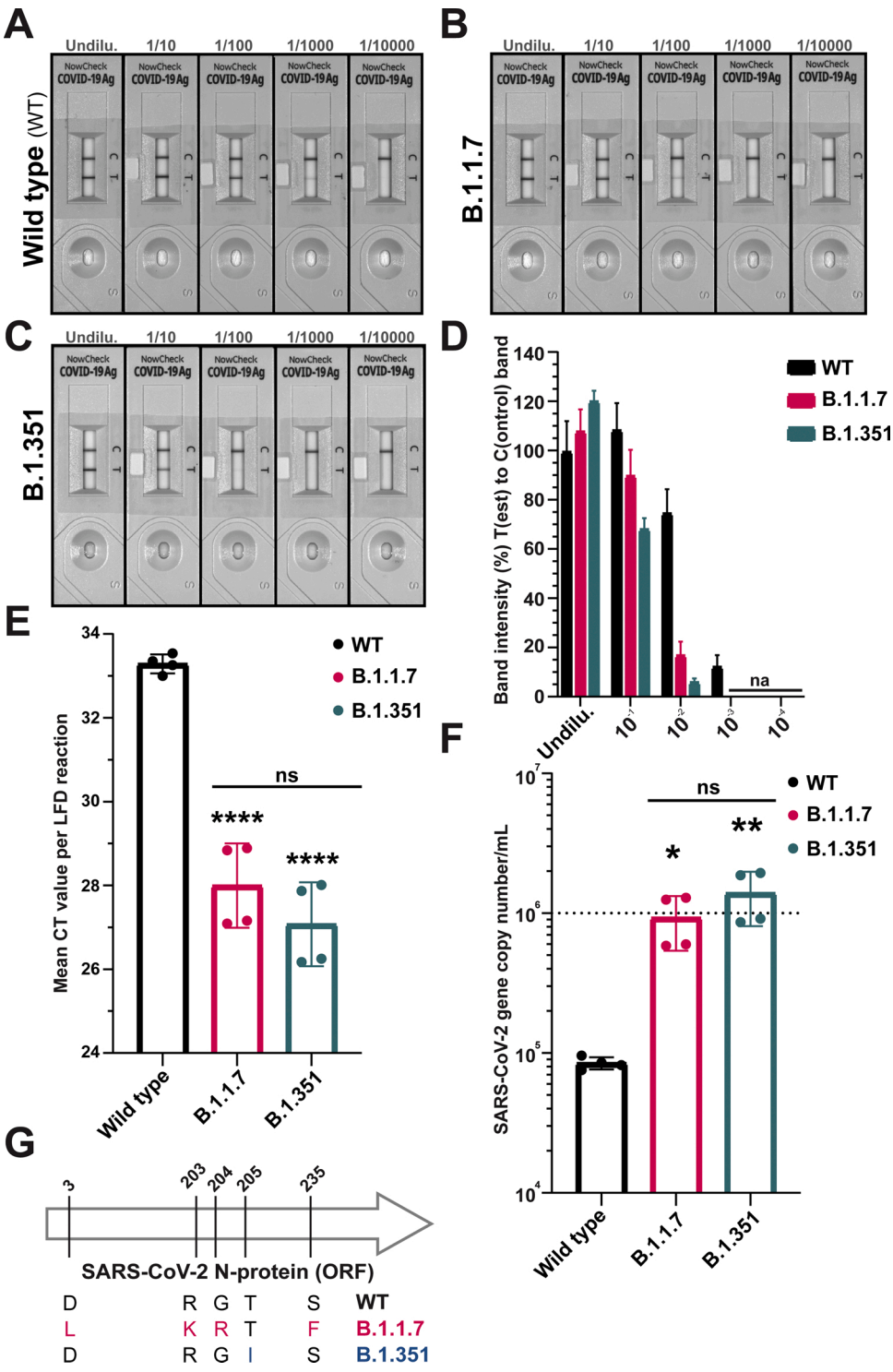

Fig. 3. Partial impairment of analytical sensitivity of antigen assays by B.1.1.7 and B.1.351 VOCs. (**A**), (**B**), and (**C**) Qualitative results of an exemplary antigen assay (BioNote) using serially diluted cell culture supernatants from SARS-CoV-2 and VOC B.1.1.7 and B.1.351 (Fig. S2). (**D**) Quantitative comparison of band intensities (%) of SARS-CoV-2 and VOCs. (**E**) and (**F**) Comparison of borderline detection limit (+) of antigen assay (BioNote/NowCheck) in case of eluted SARS-CoV-2 and VOCs using Ct-values and gene copy numbers (****p < 0.0001; **p < 0.01; *p < 0.05). (**G**) Alignment of N-protein sequences of SARS-CoV-2 and VOCs, highlighting changes in amino acids residues. Three to four average data sets were analyzed. Standard deviations (SD±) are depicted by error bars. The one-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons test was used to analyze data.

sensitivity and detection limits as shown in Table 1. To determine the cut-off value and the overall sensitivity of RATs, an exemplary assay (BioNote) was evaluated. For this experiment, virus dilutions corresponding to high, low and near-to-detection-limit viral loads were used. All samples rated positive by RAT were also positive by RT-qPCR (16/16) and only four samples were considered as false negative (4/18) (Fig. 1B). Therefore, a cut-off value of Ct 32,25 was determined as a limit of detection (LoD) of rapid antigen assay (Fig. 1B). This achieved an overall sensitivity of 93,75 % with a ROC curve area of 98,61 % when compared to RT-qPCR (Fig. 1C).

3.2. Saliva from animal species has no adverse effects on the sensitivity of rapid immunoassays

To test whether saliva could have an impact on the analytical sensitivity of rapid antigen assays, we collected plain saliva from human and animal species and spiked them with SARS-CoV-2. Saliva was collected either by swabbing oral fluids (as in bats and ferrets), passive drool collection (as in human, cat, sheep, goats, and cattle), or by chewing-ropes in swine as previously described (Gutierrez et al., 2014). SARS-CoV-2 non-VOC and VOCs were serially diluted into saliva and

applied on lateral devices. Interestingly, both target and control bands were detected on lateral devices using spiked animal saliva with no significant differences when compared to previous results without using saliva (control) (Fig. 2A). This clearly demonstrates that saliva has no adverse effects on the sensitivity of antigen assays. Further, we examined the corresponding viral genome copies by real-time RT-PCR and found no significant impact on viral copy numbers upon using saliva as the detection limits were also about 10^3 – 10^4 viral copies (Fig. 2B). Together, these results underline that saliva from the animal species studied did not adversely affect the sensitivity of rapid antigen assays and/or real-time RT-PCR.

3.3. Lower performance of rapid antigen tests for SARS-CoV-2 variants of concern B.1.1.7 and B.1.351

To examine the potential effect of SARS-CoV-2 variants of concern on the sensitivity of antigen assays, VOCs B.1.1.7 and B.1.351 were also included in this study. Surprisingly, the tested antigen assays showed a significant reduction by nearly 1 log in LoD when VOCs were used (Fig. 3A–D). Further, we compared the corresponding SARS-CoV-2 Ct-values and gene copy numbers and found a significant reduction by 1-2 logs ($p < 0.0001$, Fig. 3E; $p < 0.001$, 3F). Next, we aligned N-protein from wild-type SARS-CoV-2 and B.1.1.7 and B.1.351 variants and found multiple amino acids substitutions in N-protein (Fig. 3G). Whether these substitutions significantly hampered efficient immunodetection of such variants by RATs remained to be determined and worth future studies.

4. Discussion

Here most antigen assays were able to find positive results up to the level of Ct-value 32,25 and 10^3-10^4 viral RNA genome copies respectively. However, there were also two assays with lower sensitivities, rendering them less suitable for field applications (Fig. 1A and Table 1). Our data demonstrate that detection limits of rapid tests were up to 2 logs under the hypothetical infectivity threshold (Wölfel et al., 2020), underlining their safety margin for detecting SARS-CoV-2 infectious individuals and/or animals (Fig. 1A). Initial reports on SARS-CoV-2 natural infections in animals as cats, dogs, and lions showed relatively high Ct values >30/35 for positive animals showing clinical signs, suggesting missed infections upon using RATs (Abdel-Moneim and Abdelwhab, 2020; de Morais et al., 2020; Segalés et al., 2020). Nonetheless, as the virus continues to evolve and adapt in hosts, natural infections with lower Ct values <25 were recently reported, for instance in lions, where a RAT was successfully used to detect the infection (Fernández-Bellon et al., 2021).

Hundreds of rapid tests from different vendors are now available on the market (FIND, 2020) and it is not surprising that some assays perform better than others. This, however, strongly underlines the need for a regular and rigorous evaluation of rapid antigen tests before their market approval. A leading-edge assay should be able to detect minimum SARS-CoV-2 and VOC loads equivalent to 10^3-10^4 viral RNA copies (per mL). Overall, rapid antigen tests showed an analytical sensitivity of 93,75 % when compared to RT-qPCR (Fig. 1C).

SARS-CoV-2 rapid tests rely on antigen-antibody reactions that can be inhibited by different physiochemical properties of samples, e.g. viscosity and extreme pH values (Reverberi and Reverberi, 2007). It is well known that animal saliva possesses a variable viscosity and pH range (Reid and Huffman, 1949). Accordingly, whether animal saliva might affect the sensitivity of SARS-CoV-2 antigen assays was an intriguing question that has been answered in this study. Neither saliva composition nor collection methods showed adverse effects on the sensitivity of rapid tests as interpreted qualitatively by lateral flows and quantitatively by relative band intensity (Fig. 2A), and RT-qPCR (Fig. 2B). Hence, antigen-antibody reactions can tolerate pH changes between 6.5–8.5 (Barnes, 1966; Hughes-Jones et al., 1964). Our data align with previous reports that recommended the use of saliva for detecting SARS-CoV-2 by antigen tests or RT-qPCR in humans (Basso et al., 2021; Wyllie et al., 2020).

B.1.1.7 and B.1.351 variants harbor mutations mainly in the S-gene that significantly impact S-gene targeting by RT-qPCR, a phenomenon known as S-gene dropout (Washington et al., 2020). Therefore, it is of utmost importance to detect whether mutations in other genes (e.g. N-gene) have an impact on the performance of these tests, especially commercial antigen assays that target N-protein (Fig. 3G) (Azad, 2021). We observed that analytical sensitivities of RATs were significantly reduced especially at lower viral concentrations (Fig. 3), which suggests a partial interaction between N-protein variants and lateral flow antibodies. The reason for that could be attributed to altered stability or epitopes of N-protein of B.1.1.7 and B.1.351 variants.

5. Conclusion

In summary, saliva of the species tested has no impact on the functionality and performance of SARS-CoV-2 RATs. Therefore, the use of RATs can be recommended as a point-of-care surveillance tool for SARS-CoV-2 infections in these species. However, the tests should be checked beforehand for their suitability to equally detect VOCs B.1.1.7 and B.1.351.

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Author contributions

ITH, MHG, MK conceived and designed the experiments. ITH, SW, MK performed experiments. ITH, SW, BS, MHG, MK analyzed data. ITH, SW, MK contributed reagents/materials/analysis tools. ITH, MK, MHG attained funding. ITH, MHG, MK wrote the paper. All authors reviewed, edited, and approved the final version.

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. Supplementary data

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