Abstract
Following the coronavirus disease 2019 (COVID-19) epidemic peak in Ariano Irpino, Campania region (Italy), we tested cattle for the presence of Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) on a cattle farm at which, prior to the investigation, 13 of the 20 farmworkers showed COVID-19-like symptoms, and one of them died. Twenty-four cows were sampled to detect SARS-CoV-2. All nasal and rectal swabs and milk samples were negative for SARS-CoV-2 RNA. Of the 24 collected serum samples, 11 were positive for SARS-CoV-2 nucleocapsid protein, 14 were positive for SARS-CoV-2 spike protein, and 13 were positive for SARS-CoV-2-neutralising antibodies; all samples were negative for Bovine Coronavirus (BCoV), another betacoronavirus. To our knowledge, this is the first report of natural serological evidence of SARS-CoV-2 infection in cattle. We hypothesise that this may be a case of reverse zoonosis. However, the role of cattle in SARS-CoV-2 infection and transmission seems to be negligible.
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INTRODUCTION

The world is currently fighting an extremely small organism, SARS-CoV-2, the causative virus of the disease that has caused a global pandemic known as COVID-19. SARS-CoV-2, a member of the family Coronaviridae, is a single-stranded enveloped RNA virus. Coronaviruses (CoVs), such as SARS-CoV-2, can emerge from wildlife hosts and infect humans and domestic and/or livestock animals (Dhama et al., 2020; Shi et al., 2020; Hu et al., 2021). Thus, they can cause epidemic or pandemic outbreaks, with low, medium, or high morbidity and mortality. Sequence analyses suggest that SARS-CoV-2 could have originated from bat CoVs, highlighting interspecies transmission of CoVs (Boni et al., 2020; Guo et al., 2020; Guo et al., 2021), although the intermediate host is currently unknown.

Studying the zoonotic aspects of SARS-CoV-2 might aid in the development of a strategy for virus detection and the control of viral dissemination. To date, natural infection with SARS-CoV-2 has been reported in cats, dogs, mink, otter, pet ferrets, lions, tigers, pumas, snow leopards, gorillas, white-tailed deer, fishing cat, Binturong, South American coati, spotted hyena, Eurasian lynx, Canada lynx, hippopotamus and hamster (COVID-19 - OIE - World Organisation for Animal Health; Jo et al., 2021; Decaro et al., 2020a; Delahay et al., 2021; Fernollar et al., 2021; Gortázar et al., 2021; Palmer et al., 2021; Clayton et al., 2022).

However, it has not been detected in farm animals, such as buffaloes, goats, sheep, horses, rabbits, hens, pigs, or cows, despite contact with their SARS-CoV-2-positive human breeders for at least 2 weeks (Cerino et al., 2021). The unknown zoonotic potential of this virus is a cause of concern for pet owners and farmers.

In April 2021, we investigated cattle on a farm, at which 13/20 farmworkers previously had COVID-19-associated disease, and one of them had died, for the presence of SARS-CoV-2 and BCoV. The farm is located in Ariano Irpino, the first city in the Campania region (Southern Italy) to be locked down and declared a red zone in March 2020.

This study is the first to describe natural SARS-CoV-2 infection in cattle. We hypothesise that this may represent a case of reverse zoonosis, that is human-to-animal transmission, which may lead to new reservoirs for the virus as well as the development of new viral variants that are potentially dangerous to humans and/or animals.

MATERIALS AND METHODS

2.1 Samples

The sampling was carried out by Local Official Veterinary Service during Official Eradication Control Plane as specified in the Regulation (Eu) 2016/429 of the European Parliament and of the Council of 9 March 2016 on transmissible animal diseases and amending and repealing certain acts in the area of animal health (‘Animal Health Law’). The permission was obtained from the owners of farm animals for collection of their nasal and rectal swab specimens. All methods were performed in accordance with the relevant guidelines and regulations.
The farm included 150 animals, of which 24 lactating cows were sampled (Table 1). The geographical location of the herd was mapped using a geographic information system (GIS) (Figure 1). The herd had never been vaccinated against BCoV.

All samples were collected by local veterinary authorities. Nasal and rectal swab specimens were collected, frozen, and stored at -80°C until processing. Blood was drawn from the jugular vein using sterile evacuated tubes without EDTA anticoagulant. Upon arrival at the laboratory, the samples were centrifuged, and aliquots of the serum and milk were frozen at -80°C until testing.

2.2 Nucleic acid extraction

Nucleic acid extraction was performed in biosafety level 3 (BSL-3) laboratories. Aliquots (400 μL) of the milk samples collected from each cow were subjected to extraction and purification using the QIAsymphony DSP Virus/Pathogen Midi Kit (Qiagen, Hilden, Germany) and processed using the QIAsymphony automated system (Qiagen) according to the manufacturer’s instructions, eluted in 60 μL, and stored at -80°C until use.

Nucleic acid was extracted from nasal swabs as follows: 200 μL aliquots of Universal Viral Transport Medium (UTM) (Copan) were used for nucleic acid extraction and purification with the KingFisher Flex system (Thermo Fisher Scientific) using the MVP_2Wash_200_Flex program according to the manufacturer’s instructions. The extracted RNA was eluted in a final volume of 50 μL and stored at –80°C until use.

SARS-CoV-2 RT-qPCR was performed in BSL-2 labs using the TaqPath COVID-19 CE-IVD RT-PCR Kit (Thermo Fisher Scientific), which simultaneously amplifies three viral targets, the ORF1ab gene (FAM), N protein (VIC), and S protein (ABY); MS2 phage (JUN) is detected as the internal positive control. The amplification was carried out in a final volume of 25 μL, which included 5 μL of template, TaqPath 1-Step Multiplex Master Mix (4×), and COVID-19 Real-time PCR assay Multiplex, which contains probes and specific primer sets for different SARS-CoV-2 and internal control genomic regions. Each experiment also included TaqPath COVID-19 IVT RNA as a positive control and a negative control. The thermal cycling conditions consisted of an initial Uracil-DNA glycosylase (UNG) incubation step at 25°C for 2 min, reverse transcription at 53°C for 10 min, and an initial denaturation and enzyme activation step at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 3 sec, and annealing/extension at 60°C for 30 sec. RT-qPCR was performed on a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA).

2.3 SARS-CoV-2 immunoassays

All serum samples were first screened for qualitative detection of anti-SARS-CoV-2 antibodies using the Elecsys® Anti-SARS-CoV-2 test, an electrochemiluminescence immunoassay (Roche Diagnostics, Basel, Switzerland) using a Cobas e 411 instrument. The assay uses a recombinant protein representing the nucleocapsid (N) antigen in a double-antigen sandwich assay format, which favours the detection of high-affinity antibodies against SARS-CoV-2. All serum samples were then quantified using the Roche Elecsys® Anti-SARS-CoV-2 S test for the SARS-CoV-2 spike receptor binding domain. This test enables the determination of both the presence and level of antibodies against SARS-CoV-2 in serum. Both immunoassays were performed according to the manufacturer’s instructions. The efficiency of these kits was determined using standardised controls for each experiment. The results were reported as values of the cut-off index (COI, signal sample/cut-off). All samples were considered negative for anti-SARS-CoV-2 NP antibodies, with a COI value <1.0. As reported above, anti-SARS-CoV-2 NP antibodies in the sera were quantified using the Elecsys® Anti-SARS-CoV-2 S test. The total antibody content in the sample was expressed as U/mL, traceable to the Roche Diagnostics internal standard cut-off for anti-SARS-CoV-2 S, which was 0.8 U/mL.

Both tests were developed for human testing, but the double-antigen method is species independent, as was previously reported (Natale et al., 2021).

2.4 Microneutralisation test (MTN) for SARS-CoV-2 and BCoV

MTN for SARS-CoV-2
The MTN for SARS-CoV-2 was performed in BSL-3 laboratories. All samples were first heat inactivated by incubation at 56°C for 30 min, and then 2-fold dilutions were prepared in Dulbecco’s modified Eagle’s medium (DMEM). The MTN was performed using a modification of a previously described protocol (Gaudreault et al., 2020). Briefly, serum samples were initially diluted 1:10, and then 50 μL per well of each serum sample, in duplicate, was subjected to 2-fold serial dilutions (1:20–1:320) in 50 μL of culture medium. Then, 50 μL of a 100 TCID$_{50}$ of hCoV-19/Italy/CAM-INMI-32803-66/2020 (EPI ISL 493333) was added to each well containing the serially diluted serum and incubated for 1 h at 37°C. Finally, 100 μL of Vero E6 cell suspension, adjusted with maintenance medium to $10^5$ cells/mL, was added to each well and incubated at 37°C and 5% CO$_2$. After 4–5 days of incubation, the cells were fixed and stained with a solution of 0.1% crystal violet in 5% paraformaldehyde (PFA) for 30 min to detect cytopathic effects (CPE).

The neutralisation endpoint titre was determined as the highest serum dilution at which at least 50% of the wells showed CPE. An MNT titre $\geq$20 was considered positive (Gaudreault et al., 2020).

2.5 MTN for BCoV

MTN was performed by modifying the protocols previously described (Alenius et al., 1991; Tsunemitsu et al., 1991). Advanced RPMI 1640 (1x; Gibco Ref 12633-012) was used in this protocol.

Samples were heat-inactivated by incubation at 56degC for 30 min, and then 50 μL per well of serum samples in duplicates were subjected to 2-fold serial dilutions from 1:20 through 1:320, and from 1:4 through 1:64 in 50 μL culture media.

Each dilution was mixed with an equal volume of a viral suspension containing 100 TCID$_{50}$ of BCoV strain Nebraska, which was kindly provided by Dr. S. Reiche (Friedrich-Loeffler-Institut, Insel Reims, Germany) (Ulrich et al., 2020), and incubated for 1 hour at 37°C. Finally, 100 μL of HRT-18 (a cell line derived from human rectal adenocarcinoma) cell suspension, adjusted with maintenance medium to $10^5$ cells/mL, was added to each well and cultured in 5% CO$_2$ at 37°C. After 6–7 days of incubation, the cells were fixed and stained with a 0.1% crystal violet solution in 5% PFA for 30 min to detect cytopathic effects. The neutralisation endpoint titre was determined as the endpoint serum dilution that inhibited BCoV-induced CPE in at least two out of three parallel wells. The complete inhibition of virus propagation in an individual well was accepted as a positive result (Tuncer & Yeşilbağ, 2015).

2.6 Data analysis

The percentage (with 95% CI) of the results was calculated. Based on age, cows were divided into three age groups: 1–5 years, 6–10 years, and >10 years. Fisher’s exact test was used to compare the age and pregnancy status of the cows with neutralising antibodies. The correlation between antibodies analysed and age was evaluated via Pearson correlation analysis using GraphPad Software InStat 3, (San Diego, CA). P values less than 0.05 were considered statistically significant.

3. RESULTS

To evaluate viral spread, viremia, and seroconversion, nasal and rectal swabs and serum and milk samples were analysed. Milk samples were collected from 21 out of 24 cows due to the presence of two primiparous cows (6 and 10) and a dry cow (19) (Table 1).

All nasal and rectal swabs and milk samples tested negative for SARS-CoV-2 RNA. The results of the analyses performed on the serum samples are reported in Table 1. All tested samples were BCoV-negative (MTN $\geq$ 4). Eight of the 24 samples (33%, 95% CI 14–52) were negative for SARS-CoV-2. Of the 24 serum samples, 11 (46%, 95% CI 26–66) were positive for SARS-CoV-2 nucleocapsid protein (range: 1.38–7.40), 14 (58%, 95% CI 38–78) were positive for SARS-CoV-2 spike protein (range: 1.60–249.00), and 13 (54%, 95% CI 34–74) were positive for SARS-COV-2-neutralising antibodies, with titres ranging from 1:20 to 1:160 (Table 1). Interestingly, six of the samples were from pregnant cows (46%, 95% CI 19–73) (5, 12, 17, 19, 20, and 23) (Table 1). Of the 13 animals with neutralising antibodies, 2 of 13 were aged 1–5 years (15%, 95% CI 5–49), 6 of 13 were aged 6–10 years (46%, 95% CI 36–98), and 5 of 13 were aged >10 years (38%, 95% CI
53–113) (Table 2). Of the six pregnant animals with neutralising antibodies, one was aged 1–5 years (17%, 95% CI 9–31), four were aged 6–10 years (67%, 95% CI 12–76), and one was aged over 10 years (17%, 95% CI 13–47) (Table 2). The correlation between the presence of SARS-CoV-2-neutralising antibodies and the age of the cows was analysed using Fisher’s exact test, which showed a significant relationship (P < 0.05). Comparison of the 1–5-year-old group to the >10-year-old group yielded a statistically significant two-sided p value (p = 0.041). However, no statistically significant difference was observed when comparing the other groups. In addition, no correlation with pregnancy status was detected.

Furthermore, evaluating the analysed antibodies and age of the cows via Pearson correlation analysis showed a significant relationship (P < 0.05) between anti-S and neutralising antibodies against SARS-CoV-2 with age (Table 2) and interestingly, Pearson correlation analysis showed a strongly significant correlation (P < 0.01) between anti-S and anti-N antibodies (Table 3).

None of the cows with neutralising antibodies displayed fever, diarrhoea, and/or respiratory signs at sampling.

### 4. DISCUSSION

The role of cattle in SARS-CoV-2 transmission remains unclear. Although cattle are potentially sensitive to SARS-CoV-2, this study aimed to determine the susceptibility of cows to active SARS-CoV-2 infection at a farm where there was a COVID-19 outbreak among the farmworkers. SARS-CoV-2 and BCoV are both betacoronaviruses, and BCoV is very similar to human coronavirus (HCoV) OC43 (Betacoronavirus 1) (Wensman & Stokstad, 2020). Thus, after the samples tested positive for SARS-CoV-2, they were also analysed for BCoV.

In addition to seroneutralisation, the other methods used for the diagnosis of SARS-CoV-2 include PCR (to amplify and quantify nucleic acids) and new ELISA tests (using serum from infected or vaccinated animals), which allow the detection of antibodies in a wide range of animal species or the discovery of reservoirs or intermediate hosts (Wernike et al., 2021). In our study, we introduced chemiluminescence, a methodology mainly used for qualitative and quantitative evaluation of the antibody response in humans to infection with the wild strain of SARS-CoV-2 and to the vaccine antigen, specifically anti-S (quantitative). Using this test, our results showed that 15 of 24 cows were positive for SARS-CoV-2, which was the only betacoronavirus detected by serological tests.

The limited data on cattle, including our results, indicate that cattle show low susceptibility to SARS-CoV-2 and probably do not function as reservoirs. However, we suppose that in areas with large cattle populations and a high prevalence of SARS-CoV-2 infection in humans, close contact between livestock and farmworkers may cause reverse zoonotic infections in cattle, as has already been described for highly sensitive animal species, such as minks, cats, and dogs (Fenollar et al., 2021; Decaro et al., 2021a; Clayton et al., 2022).

Interestingly, in our study, Pearson correlation analysis showed a strongly significant relationship (P < 0.01) between anti-S and anti-N antibodies, and a significant correlation (P < 0.05) between anti-S and neutralising antibodies against SARS-CoV-2 with age. Particularly, of the 13 cows with neutralising antibodies to SARS-CoV-2, only two were young animals (aged 1–5 years), whereas 11 were adults (aged 6–10 years); this significant difference is similar to reports of SARS-CoV-2 infections in humans. In humans, children seem to be less susceptible to SARS-CoV-2 than adults. This could be a result of several factors, such as the decline of immune protection due to aging, modulation of angiotensin converting enzyme 2 (ACE2) receptor expression, and previous human-CoV infections (Felsenstein et al., 2020; Wu et al., 2020; Dioguardi et al., 2021; Zhang et al., 2021).

Of the 13 cows with neutralising antibodies against SARS-CoV-2, six were pregnant. Further studies are required to evaluate this interesting result. To date, there is very little data on COVID-19 during pregnancy and vertical transmission in animals. For example, adult white-tailed deer are highly susceptible to SARS-CoV-2 infection and can transmit the virus vertically (Cool et al., 2022). Thus, there are concerns about the risk of neonatal infections in the postpartum phases (Salma, 2021).

The data from the current study indicate that cattle do not seem to be vehicles for the transmission of SARS-
CoV-2. Indeed, after experimental infection of a group of calves with SARS-CoV-2 by intranasal inoculation, no intraspecies transmission of the virus to uninoculated cattle that were in contact with inoculated cattle was detected at 24 hours post infection (Ulrich et al., 2020). Therefore, based on the results of our study, we conclude that there is no indication that cattle play a role in the SARS-CoV-2 human pandemic.

The susceptibility of various animal species to SARS-CoV-2 is of great interest to the international scientific community, and it has been hypothesised that the host range of SARS-CoV-2 may depend on the interaction of the virus spike protein with host cell receptors. ACE2 plays a crucial role in host cell entry of the virus. Based on phylogenetic and expression pattern analyses of ACE2, various mammals may be susceptible to SARS-CoV-2. However, the amino acid sequence of ACE2 is highly conserved in cattle (83% homology) (Lean et al., 2021) and cattle share four of the five hotspot residues with humans, suggesting a good probability of interaction between ACE2 and the spike protein of SARS-CoV-2 (Liu et al., 2020). Other studies confirmed these data (Bentum et al., 2022; Clayton et al., 2022; Lupala et al., 2022). In humans, ACE2 is mainly expressed by the epithelial cells of the lung, intestine, kidney, heart, and blood vessels (Liu et al., 2020). In contrast, ACE2 receptor expression in cattle is only moderate in the lungs but is higher in the liver and the kidneys (Sun et al., 2020).

Despite earlier reports on SARS-CoV-2 replication in respiratory ex vivo organ cultures of cattle (Di Teodoro et al., 2021) and detection of low viral RNA levels after experimental intranasal inoculation of SARS-CoV-2 in cattle (Ulrich et al., 2020), the nasal swabs collected in our study were negative for SARS-CoV-2. A possible explanation may involve the different distribution of ACE2 receptors in cattle. Indeed, a recent investigation established that in cattle (Bos taurus), ACE2 was detected in the bronchiolar epithelium in the lungs, but not in the nasal mucosa epithelium (Lean et al., 2021). According to Lean et al. (2021), we hypothesise that this pattern of distribution may explain the difference in the susceptibility of animals to SARS-CoV-2.

Despite the negative results from swabs, the serological analyses in our study confirmed infection of cattle with SARS-CoV-2 for the first time, although no active viral replication was detected in colostrum-deprived calves that were experimentally infected (Falkenberg et al., 2021). To the best of our knowledge, no cases of natural SARS-CoV-2 infection in cattle have been previously reported. As stated above, the serum samples were negative for BCoV infection. Thus, we ruled out the possibility of cross-reactivity due to other betacoronaviruses.

Finally, to avoid economic losses and threats to animal health, biosecurity measures to control SARS-CoV-2 infections may be useful. Conventional measures, including spray disinfection of each vehicle entering the farm, ultraviolet light, and incoming and outgoing showers for service personnel, as well as emerging technological measures, such as electrostatic air filtration systems and heat treatments at high temperatures for disinfection (Subedi et al., 2021), which have been tested on chicken and pig farms in the USA, may be effective methods.

In conclusion, further studies are needed to validate the chemiluminescence technique used to detect SARS-CoV-2 antibodies in cattle, which appeared to seroconvert in the presence of circulating virus. The advantages of the chemiluminescence techniques described above are their speed, performance, and the fact that they are inexpensive and can be carried out in BSL-2 labs.

To our knowledge, this is the first report of natural SARS-CoV-2 seroconversion in cattle. We hypothesise that this may represent a case of reverse zoonosis. However, the role of cattle in SARS-CoV-2 infection seems to be negligible. Further studies are needed to better define the role of SARS-CoV-2 infection in cattle as well as its potential role in the emergence of novel recombinant coronaviruses.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTION STATEMENT
F.F., U.P., G.I. and G.F. designed the study and wrote the first draft of the manuscript. F.F., C.C. and G.F. edited the manuscript. V.I. and V.T. contributed to the study’s design. F.F., V.I., C.C., S.B., M.L., L.M., G.Fe. and G.F. performed experiments. F.F., U.P., C.C. E.D.C., G.I. and GF. analysed data.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal’s author guidelines page, have been adhered to. No ethical approval was required for sampling of the involved animals.

COMPETING INTERESTS

The authors declare that they have no competing interests.

REFERENCES


FIGURE LEGEND

Figure 1. Map of the cattle collection site in the area of Ariano Irpino.
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Table 1.docx available at https://authorea.com/users/474446/articles/564283-first-description-of-serological-evidence-for-sars-cov-2-in-cattle

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Table 2.docx available at https://authorea.com/users/474446/articles/564283-first-description-of-serological-evidence-for-sars-cov-2-in-cattle

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