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Validation of Pseudo Virus Neutralization Assay (PVNT) for Domestic Cattle SARS-COV-2 Natural Infection

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ABSTRACT

The emergence of the novel coronavirus, SARS-CoV-2, and the subsequent COVID-19 pandemic have posed unprecedented challenges for both global public health for humans and animals alike. With the sudden onset of the pandemic, the need for accurate and accessible diagnostic assays for humans and animals are necessary to track and reduce the spread of the virus. Domestic cattle serve as a potential threatening reservoir due to their ability to contract the virus, proximity to humans, and potential exposure to the virus via white-tailed deer populations. This study focuses on assessing the accuracy of the pseudo virus neutralizing (PVNT) assay in detecting SARS-CoV-2 antibodies in naturally infected cattle populations. Following the collection of both pandemic and pre-pandemic samples, the study evaluated the performance of PVNT assays, with a specific emphasis on the Delta and Omicron variants. The results revealed a complete lack of sensitivity, specificity, with low to 0% positive predictive values, and negative predictive values for both Delta and Omicron PVNT assays. Further investigation into the underlying causes of these inaccuracies was conducted, including exploring potential cross-reactivity with bovine coronavirus (BCoV). However, experiments did not find evidence to support BCoV antibodies as contributors to the observed inaccuracies. Additionally, metadata suggests that maternal antibodies and geographical location did not significantly impact PVNT results. These findings led to proposals of possible strategies to optimize serum dilutions as well as investigate lentiviral vector scaffolding as a means on non-specific binding. In conclusion, this study highlights challenges in PVNT testing for SARS-CoV-2 antibodies in cattle, emphasizing the need to address sample composition and assay methodology for improved diagnostics, crucial for controlling viral spread and minimizing zoonotic transmission risks.

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Introduction

The emergence of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) causing the Coronavirus Disease 2019 (COVID-19) pandemic has presented the world with unparalleled challenges for global health systems regarding treating patients and reducing spread. The virus has since spread rapidly, prompting the need for extensive research and public health interventions. The silent spread of COVID-19 via non-symptomatic and symptomatic carriers, wildlife reservoirs, and domestic animal infection has pushed for a need to develop effective diagnostic tools to track and reduce prevalence of the virus.

The Pseudo-virus Neutralization Test (PVNT) has emerged as a promising method for detecting SARS-CoV-2 specific antibodies from serum of multiple species. Antibody presence indicates current or previous infection with the virus in animals, hence identifying potentially dangerous reservoirs where the virus can circulate and mutate. Specifically, humans have frequent interactions with domestic cattle, a species that can be infected by SARS-CoV-2 and therefore should be monitored for natural infection. This study aimed to address the gap of surveillance in domestic cattle throughout the Northeast United States whilst also testing the viability of both the Delta and Omicron PVNT assay. The investigation reveals the challenges presented through PVNT testing, and the direction needed for future efforts to enhance the diagnostic capability of the assay. Ultimately, these findings contribute to the ongoing effort to enhance more accessible and inexpensive diagnostic assays, such as PVNT, to improve the ability to track and control the spread of COVID-19 throughout cattle populations.

Review of the Literature

SARS-CoV-2 Virus and COVID-19 Disease

Coronavirus disease 2019, otherwise known as COVID-19, is a respiratory and intestinal viral infection caused by the SARS-CoV-2 virus. The virus was first detected in December 2019 in Wuhan, China. On March 11, 2020, the World Health Organization declared COVID-19 a pandemic after there were more than 118,000 cases and 4,291 deaths across 114 countries. As of June 2023, there have been over 691 million documented cases and over 6.8 million documented deaths[1]. Throughout the pandemic, there were numerous efforts to reduce disease spread through distancing policies along with other behavioral changes resulting in less human-to-human contact. These actions, although necessary and effective, resulted in severe socio-economic consequences worldwide due to the global scale of disease spread[2] . After the implementation of the vaccine rollout in December 2020, the fatality and health complications of the virus have drastically decreased allowing the world to slowly recover from the global pandemic. Since the initial vaccine for the ancestral strain, there have been additional vaccines created and distributed to reduce complications of other variants that have emerged and continue to develop.

The etiological agent of COVID-19, the SARS-CoV-2 virus, is a single-stranded, positive sense RNA virus possessing a genome of about 30 kilobases. The virus is transmitted through respiratory droplets and aerosols. It should be noted that both symptomatic and asymptomatic individuals can transmit the virus. It is most likely to be transmitted when infected and noninfected individuals are within six feet of each other, however in poorly ventilated areas the virus may linger in the air allowing for transmission outside of these parameters. SARS-CoV-2 is a part of the coronavirus family which is classified by the crown-like appearance of the envelope

glycoproteins as well as chemical and replicatory features[3]. The virus enters the host cell through binding to the receptor angiotensin-converting enzyme 2 (ACE2). The coronavirus virion is made of structural proteins including the nucleocapsid, membrane, envelope, and spike proteins. Both the attachment and fusion of the virus to the host cell are mediated by the spike glycoprotein. Once the virus has engaged the receptor, multiple conformational changes take place allowing the viral genome to reach the cellular cytoplasm. During this process, the ACE2 engagement causes the exposure of the S2' cleavage site which is ultimately cleaved by transmembrane serine protease 2 (TMPRSS2) at the cell surface, or cathepsin L in the endosome[4].

Throughout the intracellular life cycle, SARS-CoV-2 genomic RNA is replicated creating full-length copies that are then incorporated into viral particles. SARS-CoV-2 as well as other coronaviruses have a large RNA genome flanked with 5'and 3' untranslated regions. The 5' end possesses two open reading frames (ORFa, ORFb) that encode 15-16 non-structural proteins. The majority of these proteins contain the replication and transcription complex used for maintaining the coronavirus genome. The 3' end possesses the ORFs that code the structural and accessory proteins. The accessory protein function is not well conserved between species but is thought to modulate host response and influence pathogenicity. The structural proteins translocate to the endoplasmic reticulum (ER) membrane where they travel through the ER to the Golgi intermediate compartment (ERGIC). Interactions with nucleocapsid-encapsidated genomic RNA lead to the budding of secretory vesicular compartments. The virions are then secreted from the infected cell through exocytosis[5].

In humans, COVID-19 has the potential to cause severe acute respiratory syndrome which has swept the world causing illness, persisting symptoms, and in some cases death. Most

patients will experience fever, cough, difficulty breathing, loss of taste or smell, and fatigue. In about 20% of cases, the infection will spread to the lower respiratory tract causing pneumonia. In about 5% of cases the infection becomes acute respiratory distress syndrome or ARDS[6]. The majority of infections are not severe; however, considerable amounts of the population had to be hospitalized during the height of the pandemic. The cause of death was ultimately the cytokine storm caused by the overreaction of the immune system which led to tissue injury and multiorgan failure[7]. This included complications involving the kidney, liver, heart, and lungs. The severity of the infection was closely related to the age of the patient, causing younger and older individuals to be at the highest risk. Additionally, comorbidities such as diabetes, obesity, and chronic pulmonary and cardiovascular diseases contributed to complications following viral infection.

The Immune Response Following SARS-CoV-2 Infection

The immune response to the virus is mediated by two separate systems, the innate immune system and the adaptive immune system. These two parts of the immune response heavily influence the severity of the infection as well as allow for the development of novel diagnostic methods. Innate immunity is the nonspecific system that serves as the body's first line of defense against a wide variety of pathogens. SARS-CoV-2, like many viruses, activates the innate immune system through Toll-Like Receptor (TLR) signaling. TLR signaling through both the MyD88 and TRIF pathways activates inflammatory and antiviral cytokine production. RIG-I-like receptors (RLRs), play an important role as RNA pattern recognition receptors which can recognize SARS-CoV-2 replication intermediates. The RLRs play a significant role in the production of interferons, proteins that inhibit viral replication[8]. During a COVID-19 infection,

the upregulation of type I interferon is a hallmark of severe symptoms due to its role in signaling inflammatory cytokines. Other receptors, such as the nucleotide oligomerization domain (NOD)-like receptors (NLRs), can detect SARS-CoV-2 and induce the production of type I interferons and proinflammatory cytokines. Additionally, pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) associated with SARS-CoV-2 can be recognized causing cytokine release[9]. cGAS-STING signaling also contributes to proinflammatory signaling after infection. Activating multiple proinflammatory signaling pathways leads to cytokines that lead to inflamed blood vessels within the body. This can be particularly harmful to people who suffer from diabetes and hypertension[10]. The increase in inflammatory cytokines is linked to programmed cell death such as pyroptosis, apoptosis, and necroptosis, all of which can damage respiratory tissue during abrupt events like infection. Additional issues caused by the cytokine storm are respiratory bursts causing superoxide free radicals and hydrogen peroxide and blood clots that can lead to thromboembolism, which is an obstruction of a blood vessel. The innate response to SARS-CoV-2 infection can both be helpful and dangerous in terms of the patient well well-being.

The adaptive immune system is the second subset of the immune system that consists of specialized cells that are manufactured to eliminate one specific pathogen. Adaptive immunity cells can be selected for a single specific antigen that then can target a specific pathogen. This takes place if the innate immune system is unable to eliminate the pathogen, normally four to seven days post-infection. It also can develop memory, meaning the subsequent infections can be quickly recognized and targeted by adaptive immune cells[11]. CD4+ T cells, CD8+ T cells, and antibody-producing B cells are the hallmarks of a viral adaptive immune response. CD4+ T cells are also known as T helper cells and after detecting the virus through MHC II antigen

presentation, they release cytokines that can assist in B cell activation, CD8+ T cells activation, and increase MHC I and MHC II antigen presentation[12]. CD8+ T cells, or cytotoxic T cells, have the capability to kill virally infected cells through recognition of MHC I complexes. It should be noted that higher levels of CD8+ T cells have been associated with better survival and overall disease outcome in the context of SARS-CoV-2 infection[11]. B cells are another major part of the adaptive immune response. Most B lymphocytes differentiate into plasma cells and are then able to secrete proteins called antibodies that are specific for binding to cells infected with SARS-CoV-2. These antibodies, specifically IgM, IgG, and IgA, are able to neutralize the virus by binding to spike protein components ACE2. The neutralizing effects of antibodies are the backbone of diagnostic tools as the presence of antibodies proves prior infection[13]. Overall, the adaptive immune system can help ultimately reduce viral load to help recovery while also serving as a tool for immune surveillance.

The SARS-CoV-2 virus's efficient infection and persistence in the human population has allowed it to evolve, creating viral variants. The zoonotic nature of the virus has also created pressure for it to mutate as interspecies spreading can give rise to variants. The original, or ancestral strain, evolved into various mutants. These include the Alpha stain (UK origin), Beta and Gamma strain (South African and Brazilian origin), Delta (Indian origin), and Omicron (Botswana and South African origin). These variants may alter infectivity and pathogenicity. However, the main differentiating feature between variants is the sequence and structure of the spike glycoprotein. Antibodies produced after exposure to spike from one variant are less effective at neutralizing spike from other variants. Due to this immune escape, variants have also created a need for updated vaccines to protect against disease[14]. While antibody cross-reactivity between ancestral, Alpha, Beta, Gamma, and Delta variants are high, antibodies specific to these

variants are significantly impaired in the ability to neutralize Omicron viruses[15]. Due to low cross-reactivity, diagnostic tools that rely on antibody detection must be modified to detect exposure to the novel variants[16]. Tracking these variants and creating these vaccines and diagnostics is necessary as the virus continues to evolve and persist in both animal and human populations.

Zoonotic Nature of SARS-CoV-2

Coronaviruses are a large family of respiratory viruses that cause disease in both humans and animals, specifically circulating camels, bats, and cats. This family of viruses includes the novel coronavirus, SARS-CoV-2 (causative agent of COVID-19), Middle East Respiratory Syndrome (MERS) coronavirus, and Severe Acute Respiratory Syndrome (SARS) coronavirus. The virus's ability to mutate and recombine allows for both sustained infections in populations as well as the ability to cross species barriers. For both MERS and SARS, it has been found that palm civets and camels served as the intermediate host between bats and humans[17].

SARS-CoV-2, like other viruses in its family, is zoonotic. The presence of highly homologous ACE2 receptors in certain animals makes them susceptible to SARS-CoV-2 infections. However, the origin of SARS-CoV-2 is still unclear, but it is believed that it is of bat origin because the virus has a shared identity of 96% with the bat coronavirus RaTG13 strain[18]. It is still not known if the virus was transmitted from bats to humans or if there was an intermediate host that allowed for human infection. The spread of the virus has been detected in 18 animal species from four animal orders (carnivora, artiodactyla, primates, and rodentia). These positive detections have been found in captive, pet, wild, and farmed populations. The number of animals, country of origin, as well as type of animal, is continuously growing. Detection of the virus in wildlife populations is necessary as animals serve as reservoirs for

emerging diseases as well as create pressure for new mutations and variants to potentially spread to the human population[19]. Additionally, there have been suspected transmission of animals to humans through close contact, however, this form of transmission has not significantly contributed to the spread of the disease. One case in Hong Kong reported a hamster-to-human transmission of the virus due to a pet shop-related outbreak[20]. It should also be noted that there is no data to show that humans are infected through meat consumption. Moreover, the potential spillover and virus adaptation to livestock populations poses a great risk to food security through risk to both animal and human populations.

A significant spillover of SARS-CoV-2 has been into the mink population. These outbreaks show great concern as they occurred during the use of biosecurity practices through human-to-mink transmission. The close nature of the animals as well as the ventilation within the farms led to the rapid spread of virus through aerosols amongst the mink[21]. Additionally, the virus was able to efficiently adapt to the mink population through a spike RBD mutation which may have increased the affinity of the virus to the human ACE2 (hACE2) receptor demonstrating the danger of spillover and spillback of viruses. The virus was also shown to spread back to human populations as well as spread to cats and wild mink[22]. This created a wild reservoir for SARS-CoV-2 that would allow for the continuous emergence of novel coronaviruses if it persisted within the wild population. The emergence of these viruses leaves populations, both animal and human, vulnerable to reinfection of new and potentially dangerous coronaviruses. The mink outbreak demonstrates the danger of animal spillover of the virus as well as shows the importance of approaching the COVID-19 pandemic through the One Health philosophy.

An additional significant spillover of SARS-CoV-2 was in the white-tailed deer population as it is believed that deer may serve as a reservoir for the virus. A comprehensive

cross sectional study showed that SARS-CoV-2, specifically variants no longer detected in humans (Alpha, Delta, and Gamma), are currently co-cirulating in the white-tail deer population of New York state[23]. An additional study used evolutionary analysis to show that infections of white-tailed deer across the Washington, DC are from independent spillover events from humans, as well as deer to deer transmission and three cases of deer to human transmission. Overall, this shows multiple introductions and co-circulation of SARS-CoV-2 in the white-tailed deer population[24]. Deer serve as a particularly interesting reservoir for the virus due to the frequency of potential interactions with other species, specifically the cattle population[25]. These interactions lead to possibly spillover and spillback amongst the populations, making cattle a point of interest for SARS-CoV-2 surveillance.

Natural Infection of SARS-CoV-2 in Cattle

The present study investigates the natural infections of SARS-CoV-2 in the cattle population as well as potential antibody cross-reactivity between SARS-CoV-2 and Bovine Coronaviruses. Bovine Coronaviruses (BCoVs) cause respiratory and enteric infections in both domestic cattle and wild ruminant populations. BCoV is an infection of the upper and lower respiratory tract as well as the intestinal tract and can lead to diarrhea and intestinal upset as well as bovine respiratory disease complex or shipping fever for feedlot cattle[26]. Some coronaviruses share antibody cross-reactivity, which is the measure of how similar different antigens appear to the immune system. This means that antibodies created in response to one coronavirus are able to neutralize a different coronavirus. Cross-reactivity is significant as it means the host will be able to protect themselves from multiple viruses from exposure to only one. In terms of diagnostics, cross-reactivity can serve as a barrier to being able to accurately detect the presence of antibodies from a specific infection. For example, there is potential for cross-reactivity between the various

coronaviruses, therefore previous exposure to a coronavirus may impact the ability of one to determine the presence of past infection of the coronavirus of interest[27]. Determining the presence of cross-reactivity between the virus of interest and a related virus is necessary when developing novel diagnostic tools to track zoonotic spillover.

It has been previously shown that cattle are able to be experimentally infected with SARS-CoV-2. In one study, 6 cattle were inoculated with the virus and put in contact with 3 uninoculated cattle. Only 2 inoculated animals had observable viral replication and none of the uninoculated animals became infected[28]. Another study experimentally inoculated young calves both intratracheally and intravenously. There was the detection of SARS-CoV-2 RNA in one calf that was injected intratracheally and one calf that was injected intravenously. The viral load in these samples was low and infectious virus was unable to be recovered from the samples. These studies suggest that there is a low susceptibility to SARS-CoV-2 due to the low levels of viral replication[29]. However, there has been serological evidence of SARS-CoV-2 in lactating cows in the Campania region of Italy. The research collected 24 samples, all of which were negative for SARS-CoV-2 RNA. However, some samples were positive for the antibodies against the SARS-CoV-2 nucleocapsid and/or spike, and some samples contained SARS-CoV-2-specific neutralizing antibodies. These serological characteristics indicate that there was a natural infection of the virus in the cattle likely from the close human-to-animal contact[30]. As of now, this is the only report of the natural infection of cattle and there is currently little significance in terms of this species' contribution to the spread of the virus. However, there are over 1.5 billion farmed cattle worldwide that sustain close contact with human populations, so the risk of spillover remains. Additionally, there is limited published information regarding SARS-CoV-2 infection dynamics in large animals and determining the potential for transmission and infection

is vital. This information can contribute to the prevention of spread amongst farmed animals and wildlife species, as well as humans. The ability to screen animals for prior virus infection remains critical to ensure early detection of spillover events.

Development of Diagnostic Tools to Detect SARS-CoV-2 Infection in Cattle

The development of diagnostic tools for animal populations has become a vital method of tracking the spread of SARS-CoV-2 throughout animal populations. However, the clinical symptoms of animals are not as widely studied making it difficult to identify active virus infection, furthering the need for diagnostic tools that can detect past exposure. The current gold standard for SARS-CoV-2 serological test that can help determine infection rate, herd immunity, and vaccine efficiency is the conventional virus neutralizing test that requires a live pathogen and must be performed in a biosafety level 3 (BSL 3) laboratory[31]. The virus-neutralizing assay detects antibodies that can block viral entry into host cells, and therefore neutralize the virus. The serum of interest is serially diluted and incubated with both cells and intact virions. Serum from animals previously infected with the virus of interest will likely have neutralizing antibodies against that virus. The amount of virus present following incubation will allow for the determination of previous infection based on the parameters of the assay[32]. Using this assay is incredibly useful as it has become the gold standard for quantifying neutralizing antibody titers and identifying prior infection, however, the use of intact infectious virions demands that the assay is performed in a biosafety level 3 laboratory. This can make the assay both expensive and inaccessible.

An alternative assay has been developed, known as the pseudo-virus neutralization test (PVNT) which uses a manufactured virus that is used to detect the presence of neutralizing antibodies. This assay uses the same methodology as the previous assay, however, instead of

using an intact virion, a pseudo virion is created with the presence of the viral attachment protein. In terms of SARS-CoV-2, the popular packaging systems to create a virus that produces the ACE2 protein are HIV-1 based lentivirus, murine leukemia virus, vesicular stomatitis virus, and others. These packaging systems have viruses that lack certain gene sequences that create virulent viruses, while also being able to be manipulated to express the viral attachment protein of interest. The lack of virulence allows for the virus to be safer to handle and therefore can be used in a Biosafety level 2 laboratory[33]. This aspect makes PVNT both more accessible and less expensive than VNT and therefore an attractive alternative to the gold standard.

However, there are some drawbacks to using PVNT as a pseudovirus may not be able to completely replicate the characteristics of the virus important to virulence and identification from the antibody. The SARS-CoV-2 pseudoviruses only contain the spike protein. This may cause a lack of account for other proteins that can impact the structure of the virus which may impact the ability of the neutralizing antibodies to bind to and neutralize the virus. Additionally, the virus shape of the virus packaging system may impact the ability of the spike protein to be accurately expressed in its natural state. The various shapes and sizes of the virus can impact the distribution, conformation, and density of the virus in the assay and therefore impact the accuracy of the assay[34]. Therefore, results from a PVNT should be compared to the VNT gold standard in order to validate results amidst these shortcomings[33]. Lastly, the use of pseudovirus in other contexts raises caution as genome instability can lead to mutations that allow for the reinstatement of virulence[35]. Overall, the use of PVNT poses an effective alternative to the VNT however possesses drawbacks that can potentially limit the accuracy of the assay.

Other methods for the detection of SARS-CoV-2 antibodies have risen in popularity for animal serological testing such as Indirect Enzyme-Linked Immunosorbent Assays or iELISA. In

this assay, recombinant SARS-CoV-2 spike receptor-binding domain (RBD) is the antigen used for the diagnostic assay to detect serum antibodies against SARS-CoV-2. The recombinant RBD is produced by and purified from bacterial cells, and species-specific detection antibodies are used to create iELISAs capable of detecting virus-specific antibodies from multiple animal species[36].

The SARS-CoV-2 virus has been detrimental to the world and continues to spread causing disease in the form of COVID-19. This virus also continues to mutate, altering its ability to infect and cause disease. The continued screening of animal populations to detect the spread of the virus is necessary as the zoonotic nature of the virus increases its ability to mutate and persist in animal populations. Through the development of diagnostic tools, the ability to effectively detect the virus can help understand the infective nature of the virus as well as limit its spread.

Materials and Methods

Cells and Viruses

Pseudo virus was produced using third-generation human immunodeficiency virus packaging system via the protocol described by Neerukonda *et al*.[37] The transfer plasmid encoded luciferase and ZsGreen (BEI Resources Cat no: NR-52516), the helper plasmid encoded Gag/pol (BEI Resources Cat no: NR-52517), and lastly the spike plasmid encoded with the Delta or Omicron spike were co-transfected in HEK 293T cells (ATCC CRL-1573) that were grown in DMEM with 10% fetal bovine serum (FBS) in 37℃. The subsequent pseudo virus supernatants were collected after 48 hours and subjected to a 0.45 μM low-protein binding filters and stored in -80℃. Bovine Coronavirus strain Mebus (NR-445) was obtained through BEI Resources NIAID, NIH. MBDK cell line (CCL-22) was obtained through American Type Culture Collection (ATCC) and grown in DMEM with 10% FBS at 37℃.

Samples

A total of 669 serum samples from cattle, goats, sheep and deer were obtained from excess testing material at the Penn State Animal Diagnostic Laboratory. Samples included specimens sent to ADL between September 2019 and September 2023 either for routine testing of healthy animals or for diagnostic testing of diseased animals. Corresponding date of sample collection and ZIP code of collection location were available for 626 samples; of these, the descriptive, approximate or exact age was available for 596. Only cattle samples were evaluated for the results of this study.

Pseudovirus Neutralization Assay (pVNT)

Heat inactivated serum samples from majority cattle, as well as goats, sheep, and deer, were made into a 1:10 dilution in 150 µL using DMEM containing 10% fetal bovine serum (FBS). Serum was heat inactivated by incubating 15 µL of serum samples at 56℃ for 30 minutes.
Both the pseudoviruses for delta and omicron were thawed. Using two 96-well plates, 50uL of the diluted samples were placed in each well, resulting in two plates with the same samples in each well. Each plate also included 4 negative control (cells only) wells and 4 positive control wells (virus only). Both delta and omicron viruses were diluted 1:100. 100 µL of the diluted Delta virus was added onto one plate, and 100u µL of the omicron virus was added to the other. No virus was added to the negative control wells.
The plate was incubated for 1 hour at 37 ℃ with 5% CO2. During incubation, 293T cells were harvested from T75 flasks, counted, and diluted to a concentration of 13000 cells per 20 µL of media (DMEM + 10% FBS). 20 µL of cells were added to all sample and control wells and then the plate was incubated at 37 ℃ with 5% CO2 for 72 hours.

Plate Analysis

Bright-Glo reagent was thawed as plates were removed from incubation and brought to room temperature. 100 µL of Bright-Glo reagent was added to all sample and control wells. The sample and reagent were mixed and incubated at room temperature for at least 2 minutes.

Biotek Neo2 plate reader was used to read the plates. “Luminescence” detection method was used along with “Endpoint/ Kinetic” read type and “Luminescence Fiber” as optic type. Percent inhibition was then calculated, as described below.

Bovine Coronavirus (BCoV)- Virus Neutralization Assay (VNT)

MDBK cells were embedded in a 96 well plate a day before the virus neutralization assay was performed. On the same day as the assay, the serum samples were heat inactivated and a serial dilution was performed by first using 50 µL media (DMEM with 2% serum) to each well. Then an additional 40 µL of the diluent was added to wells A1-A11. 10 µl of the heat inactivated serum was also added to the same wells. A 2-fold serial dilution was performed by transferring 50 µl from row to row for all samples. For the last row, G, 50 µL was removed and discarded.

100$TCID_{50}$ of virus were added all wells except H6-H12, which served as controls for the experiment. The plate was incubated at 37℃ for 1 hour with the lids on, then the spent media from the MDBK plate was removed. The contents from the sample dilution plate were then transferred to the MDBK plate. The plate was incubated at 37℃ for 2-4 days, or until cytopathic effects (CPE) can be observed in the control wells.

Virus Neutralizing AssayPlate Analysis

The CPE was then documented through microscopic observations in the control wells. If the reciprocal of highest dilution of serum showed no CPE, it would be considered as a virus neutralizing titre.

SARS-CoV-2 surrogate virus neutralization test (sVNT)

Samples were invalidated or validated with GenScript SARS-CoV-2 Surrogate Virus Neutralization Test (sVNT) Kit (RUO) and used as described by the manufacturer.

In- House Indirect Enzyme-Linked Immunosorbent Assay

Samples were invalidated or validated with an in- house indirect ELISA as described by Gontu *et al*[36]. The indirect ELISA was developed with a spike protein receptor-binding domain (RBD) antigen and demonstrated to have 100% specificity and sensitivity.

Statistical Analysis

Following performing the PVNT, the chemiluminescent reaction of each plate was measured to determine the relative light units (RLUs). The RLUs were then used to calculate the % inhibition with the following equation: % inhibition = 100-{(RLU of test serum/RLU of virus control)*100}. Following, samples were grouped based on % inhibition and location, and results were demonstrated using app.datawrapper.ed software. Specificity, Sensitivity, Positive

Predictive Value and Negative Predictive Value of the Pseudovirus Neutralization assay were determined via comparison of an in-house previously validated ELISA. The following equations were used to determine various values: Sensitivity= TP/ (TP+FN), Specificity= TN/(TN+FP), Positive Predictive Value= TP/(TP+FP), Negative Predictive Value= TN/(FN+TN). The Fisher's Exact Test was used to determine the two-tailed P value when comparing adult and calf PVNT positives.

Results and Discussion

Initially, 548 pandemic samples and 29 pre pandemic samples were tested using both the Delta PVNT and Omicron PVNT. Partial neutralization of virus by the animal serum was observed in some samples, with maximal percent inhibition 97% against Delta and 84% against Omicron. Using a cutoff of 60% inhibition to indicate positivity, 16.14% of samples tested positive for Delta-specific antibodies and 7.56% for Omicron-specific antibodies using the PVNT assay. These findings were unexpected, and therefore they were further investigated. It was also observed that no samples had a 100% neutralization and so sensitivity and specificity was also explored.

First, to evaluate the Delta PVNT 56 positive samples (>60% inhibition), 50 of those samples that had over a 60% inhibition along with 38 pre pandemic samples were evaluated using a SARS-CoV-2 surrogate virus neutralization test (sVNT). None of the 50 PVNT-positive samples tested positive in the sVNT. Two pre-pandemic samples, one with 52% inhibition by PVNT and one with 33% inhibition by PVNT, tested positive using the sVNT. This data defined the samples that were true positives (TP), false positives (FP), false negatives (FN), and true negative (TN). These findings were used calculate that the delta PVNT had a 0% sensitivity, 39% specificity, 0% positive predictive value and 94% negative predictive value and therefore is entirely inaccurate. The Omicron PVNT had a total of 48 samples with over 60% inhibition. 55 samples over 55% inhibition and 35 pre pandemic samples were tested using the Omicron sVNT. Only one sample tested positive by sVNT, which had 31% inhibition by PVNT. These results were used to calculate that the Omicron PVNT had a 0% sensitivity, 44% specificity, 0% positive predictive value and 94% negative predictive value. In addition to this comparison, an

in-house indirect ELISA was used to test 88 of the positive PVNT samples, only one of which was positive by ELISA. Therefore, both the Delta and Omicron PVNT were inaccurate.

These findings raised questions regarding what aspects of the diagnostic assay was leading to the consistent inaccuracy for both positive and negative results. Initially, potential cross reactivity of the samples with bovine coronavirus (BCoV) was suspected. BCoV is a pneumonteric virus that infects cattle and wild ruminant upper respiratory system, lower respiratory system, and intestinal tract causing respiratory and enteric infections[26]. It is not well characterized how common BCoV infection is in cows, however there is substantial evidence that this disease may have circulated through some of the populations that samples were taken from[38]. Therefore, 8 PVNT positive samples including 3 SARS-CoV-2 RBD hyperimmune sera and 10 PVNT negative samples including 3 pre-pandemic samples, were tested using a BCoV live virus neutralization test. Cytopathic effect consistent with BCoV infection was observed in all wells with sera meaning that the sera was unable to neutralize BCoV, allowing the virus to infect and kill the cells. These observations suggested that no BCoV-specific antibody was present in the sera, and therefore there was no cross reactivity between BCoV and the antibodies that seemingly neutralized SARS-CoV-2 in the PVNT test.

Delta SARS-CoV-2 PVNT % Inhibition

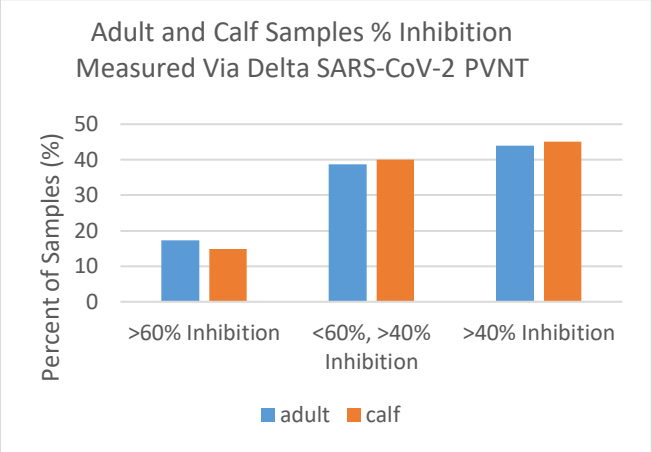

Figure 1. Comparison of Adult and Calf % Inhibitions Measured Via SARS-CoV-2 Delta PVNT. To determine possible reasons for PVNT, metadata, including age of animals was collected. Percent inhibitions were measured, and animals under 6 months of age were classified as calves and animals over 6 months of age were classified as adults.

Omicron SARS-CoV-2 PVNT % Inhibition

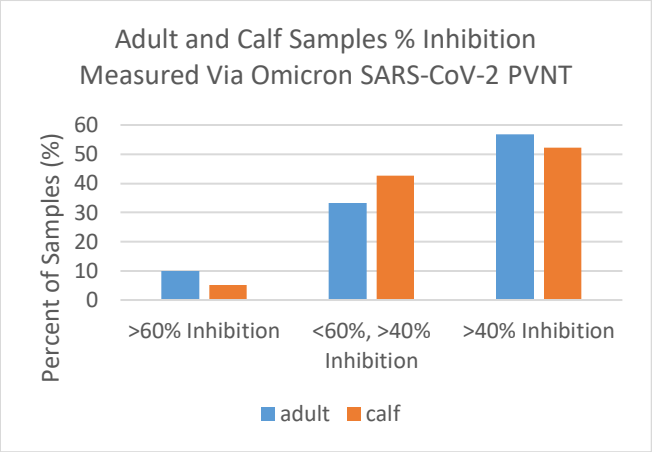

Figure 2. Comparison of Adult and Calf % Inhibitions Measured Via SARS-CoV-2 Omicron PVNT. To determine possible reasons for PVNT, metadata, including age of animals was collected. Percent inhibitions were measured, and animals under 6 months of age were classified as calves and animals over 6 months of age were classified as adults.

Following these observations, metadata from the samples was analyzed to further characterize the inaccurate PVNT readings. It was initially hypothesized that maternal antibodies in the younger animals may be interfering with the PVNT. It is documented that calves maintain their maternal antibodies up to 6 months of age, and therefore animals tested were categorized by either calved (6 months or younger) or adults (older than 6 months) using the metadata from the samples[39]. Percent of positive tests from each category was calculated and statistical analysis was conducted. PVNT assay positivity (defined as >60% inhibition) was not significantly different between adults and calves for either Delta (Fisher's exact two-tailed p value 0.8474) or Omicron (p value 0.4353). Neither Omicron nor Delta PVNT showed a preference for calf serum (Figure 1) (Figure 2). Hence, it is unlikely that maternal antibodies were interfering with the PVNT leading to no further investigation of this hypothesis. It should be noted that there is no data regarding the type of production farm these animals come from and no documentation of whether animals were nursed by their mothers.

Map of SARS-CoV-2 Delta Antibody Detection With PVNT

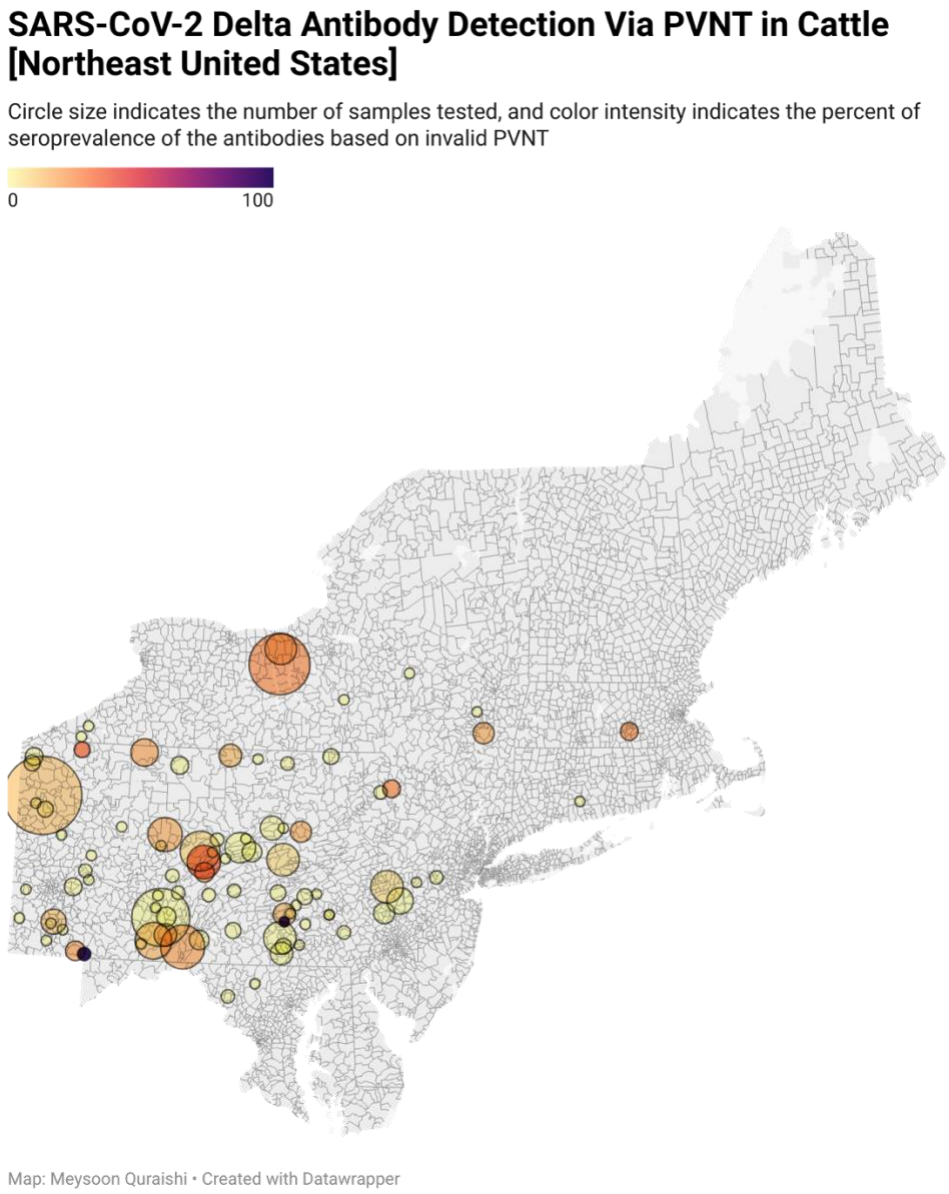

Figure 3. Map of Seroprevalence of SARS-CoV-2 Delta Antibodies via PVNT in Cattle Across the Northeast United States. Samples were grouped by the zip code correlating to where retrieved from and the mean of the % inhibition of all samples from that zip code is represented via the color intensity. Map was created using Datawrapper program.

Map of SARS-CoV-2 Omicron Antibody Detection With PVNT

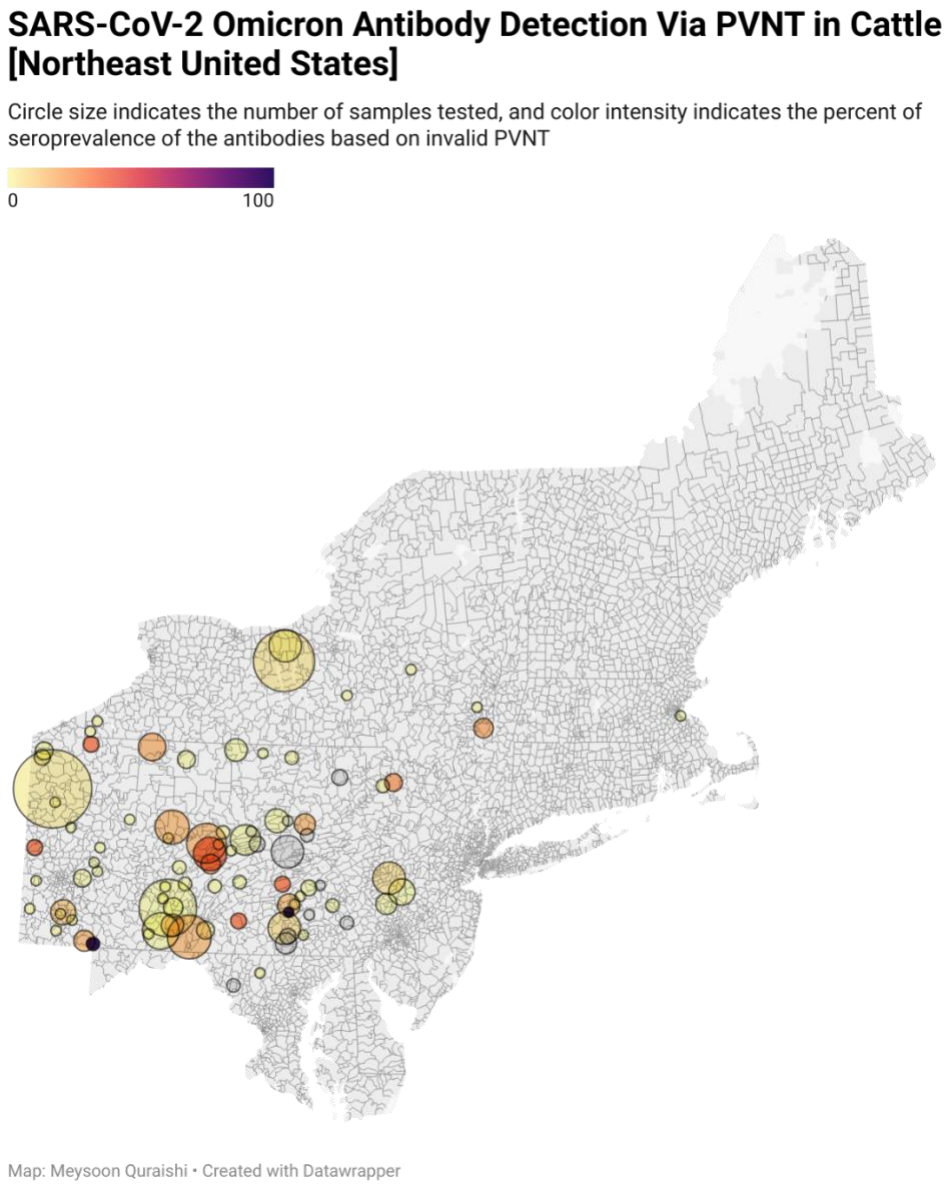

Figure 4. Map of Seroprevalence of SARS-CoV-2 Omicron Antibodies via PVNT in Cattle Across the Northeast United States. Samples were grouped by the zip code correlating to where retrieved from and the mean of the % inhibition of all samples from that zip code is represented via the color intensity. Map was created using Datawrapper program.

Further investigation of metadata was analyzed to determine that location did not impact the sero-prevalence detected by the PVNT, as well as other insights regarding the underlying causes of the inaccuracy was revealed. First, the Delta PVNT results were analyzed, and every sample was paired with the ZIP code from where the sample was taken from. The samples along with their % inhibition were grouped by ZIP code and processed by Datawrapper. The map represented the number of samples via the size of the circle and the intensity of the color represented the seroprevalence measured. The Delta PVNT Northeast map showed no obvious correlation between geographical location and seroprevalence (Figure 3). Another map was generated using the same methodology for the Omicron PVNT results revealing no correlations regarding geographical location (Figure 4). However, the map did reveal that samples that tested positive for the Delta PVNT also showed high % inhibition for the Omicron PVNT. This suggests that these specific samples from these areas are causing inaccuracy.

No further investigation was conducted throughout the study, however these results narrowed down possible explanations for the inaccuracy of the PVNT assay. First, considering that there is similar seroprevalence of the same samples from both Delta and Omicron PVNT there may be a specific aspect of these sample contributing to the inaccurate readings. Considering the PVNT uses a lentiviral vector, there is scaffolding not including the RBD which may be causing binding to antibodies within the serum. Samples taken from the specific farms that had high seroprevalence may have preexisting exposure to different parts of the lentiviral vector and therefore are able to neutralize the PVNT. For example, prior exposure to Bovine Immunodeficiency Virus (BIV), a worldwide lentiviral infection of cattle, may have induced antibodies that rendered a lentiviral vector inappropriate for these samples.

Another possible interference with the PVNT assay may be due to the dilution of the serum used for the assay, as undiluted or low levels of dilution contribute to false positives[40]. Undiluted sera can cause increased background due to non-specific binding caused by other components of the serum. To combat this, a threshold for most accurate dilutions should be calculated by determining % inhibition of serial dilutions of hyperimmune and pre-pandemic serum samples. The data may reveal which dilutions lead to accurate readings therefore determining the appropriate dilutions to be used for testing samples. In all, further investigation is still needed to address the inaccuracy of the PVNT through addressing aspects of the sample as well as optimizing serum dilutions to improve the sensitivity and specificity of delta and omicron SARS-CoV-2 PVNT assay.

Conclusions

Overall, the study has offered valuable insights into the challenges related to detecting SARS-CoV-2 antibodies in naturally infected domesticated cattle using the PVNT assay. Through comprehensive experimentation and data analysis, aspects of sample composition and assay methodology that may have impacted the accuracy or results were revealed. Specifically, potential cross-reactivity with bovine coronaviruses (BCoV) was explored but eliminated as a potential contributing factor. Analysis of metadata suggested no interference from maternal antibodies or correlations between geographical location and positive samples. However, samples from specific farms exhibited high seroprevalence for both the Delta and Omicron PVNT, indicating potential prior exposure to lentiviral vectors or issues with serum dilution affecting assay accuracy. These findings highlight the need for further investigation into sample factors and assay optimization to improve PVNT reliability for detecting SARS-CoV-2 infections in cattle populations. By addressing these challenges, future diagnostic tools can be developed to improve sensitivity and specificity, thereby enabling more accurate and reliable detection of SARS-CoV-2 antibodies in cattle populations. This study contributes to the broader effort of One Health, recognizing the interconnectedness of human, animal, and environmental health, and underscores the importance of interdisciplinary collaboration in addressing emerging infectious diseases. Moving forward, further research is warranted to explore additional factors influencing the accuracy of PVNT. By continuing to advance our understanding of SARS-CoV-2 infection dynamics in animal populations through diagnostics, the ability to effectively control and mitigate the impact of the ongoing pandemic can be strengthened.

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