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ARTÍCULO DE INVESTIGACIÓN

SARS-CoV-2 in dogs: gamma variant and its transmission from humans in Ciudad de Buenos Aires, Argentina

SARS-CoV-2 en perros: variante gamma y su transmisión desde humanos en la Ciudad de Buenos Aires, Argentina

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ABSTRACT

This article explores SARS-CoV-2 infection in dogs that have had close contact with humans diagnosed with COVID in Argentina. Affirmative molecular identification of the virus was achieved through RT-PCR analysis of oropharyngeal and rectal swabs from the dog as well as a nasal swab from the guardian. Positive results for the dog were confirmed through a LAMP/CRISPR assay and real time PCR. SARS-CoV-2 genomic RNA was successfully obtained from these samples, and the analysis of a partial sequence of the spike gene (ranging from amino acid 428 to 750) allowed us to classify both genomic sequences as Gamma variants, with a single amino acid variation in position 726. We evaluated the specific humoral immune response of the dog by an indirect ELISA using the Argentinian kit COVID AR IgG ELISA test adapted for domestic dogs, and pseudo-neutralization conducted with SARS-CoV-2 pseudotyped particles. We also evaluated the specific cellular immune response of the dog through a lymphoproliferation assay, using purified spike protein as specific stimulus. This work provides evidence of the human-to-dog transmission of SARS-CoV-2 in Argentina, underscoring the One Health concept and reinforcing surveillance methods in pets.

Keywords: (SARS-CoV-2), (Gamma variant), (dog), (human-to-dog transmission), (immune response)

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RESUMEN

Este trabajo explora la infección por SARS-CoV-2 en perros que han tenido contacto estrecho con personas diagnosticadas de COVID en Argentina. La identificación molecular del virus fue realizada por RT-PCR a partir de hisopado orofaríngeo y rectal (perro) o nasal (humano). En el caso del perro, el resultado positivo fue confirmado por LAMP/CRISPR y por PCR en tiempo real. A partir del ARN recuperado de las muestras se realizó un análisis parcial de la secuencia de la proteína de la espícula (del aminoácido 428 al 750) que permitió clasificar a ambos genomas virales como variante Gamma, con una única variación a nivel del residuo 726. Se evaluó la respuesta de anticuerpos del perro por ELISA indirecto a través de la prueba argentina COVID AR IgG ELISA adaptada para perros, y también a través de la prueba de pseudoneutralización con pseudotipos de SARS-CoV-2. Además, se evaluó la respuesta celular del perro mediante la prueba de linfoproliferación, con proteína de la espícula como estímulo. A partir de las evidencias encontradas, indicativas de la transmisión humano-perro del SARS-CoV-2 en Argentina, se destaca la importancia del enfoque de Una Salud y de la incorporación de medidas de vigilancia epidemiológica en mascotas en el abordaje de esta infección.

Palabras clave: (SARS-CoV-2), (variante Gamma), (perro), (transmisión humano-perro), (respuesta immune)

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first detected in Wuhan in December 2019. On January 31, 2020, the World Health Organization declared the coronavirus outbreak a public health emergency of international concern. To date, there have been more than 750 million confirmed human cases worldwide, including about 6,9 million deaths (WHO https://covid19.who.int/, accessed on 08-30-2023).

After the beginning of the SARS-CoV-2 pandemic, human-to-animal transmission has been described in different species, including domestic dogs and cats[19]. Experimental studies and natural cases have demonstrated the susceptibility to SARS-CoV-2 infection of diverse wild and domestic animal species, including cats and dogs. Dogs are occasionally susceptible to SARS-CoV-2, developing mild to moderate[10] or no clinical signs[28].

The angiotensin-converting enzyme 2 (ACE2) receptor that SARS-CoV-2 binds to, is expressed in humans and many other animal species[29]. For this reason, although human-to-human transmission is the main way of virus spreading, there is an increasing concern regarding its behavior as reverse zoonosis, particularly from domestic animals, and the potential role that infected animals could play in perpetuating the spread of the disease[24], and posing a potential Public Health concern in the context of One Health[23].

The Gamma variant (Pango Lineage P.1) of SARS-CoV-2 was first identified in Manaos, Brazil, in December 2020[9]. Since then, it has been associated with a large number of human cases mainly in 2021 and 2022[1]. Regarding pet infections, the Gamma variant has only been reported in one immunosuppressed cat[6].

The aim of this paper is to describe a case of human-to-dog transmission of the SARS-CoV-2 Gamma variant in terms of its molecular and serological diagnosis.

MATERIAL AND METHODS

Animals, Clinical Evaluation and Sample Collection

On April 27th, 2021 (day 0), three crossbred female dogs exhibiting no clinical signs but living with their infected guardian, “Tina”, “Reina” and “Mora” (aged 4, 11 and 16 years, respectively) were sampled by taking oropharyngeal and rectal swabs. The woman responsible for the dogs had begun to experience mild respiratory symptoms and loss of smell and taste on April 25th and tested positive for SARS-CoV-2 at a Buenos Aires city hospital through a nasal swab PCR, on the 26th of the same month. Heparinized blood samples from all 3 animals were taken at days 15, 30 and

45; a further sample from "Tina" was taken at day 200 (Figure 1). All samples were collected with the guardian's consent.

Detection of SARS-CoV-2 and SARS-CoV-2 variant determination

The oropharyngeal swab from the three dogs and the nasopharyngeal swab from the guardian were processed with the Quick-DNA/RNA Viral Kits (Zymo Research). cDNA was synthesized with a random RT-PCR kit (EasyScript First-Strand cDNA Synthesis Super Mix, Transgene Biotech) according to the manufacturer's instructions. cDNA was utilized for a first screening of the canine samples with a pan-coronavirus nested PCR (nPCR) that targets a conserved RNA-directed RNA polymerase (RdRp) region of approximately 440 bp[8].

Extracted RNA from samples was also processed using a LAMP/CRISPR assay (CASPR Biotech) and real time PCR (GeneFinder™ COVID-19 Plus RealAmp Kit). For sequencing purposes, the RNA was used in a one-step RT-PCR reaction (EasyScript® One-Step RT-PCR SuperMix) with specific SARS-CoV2 primers (https://www.nc.cdc.gov/eid/article/26/10/20-1800 article). At this point, RNA from the guardian was included in the RT-PCR assay as well. The sequence of this region, which spans from amino acid 428 to 750 of the S gene, allows discrimination of the different SARS-CoV2 variants, between VOC (variants of concern) and VOI (variants of interest). The fragment was cloned into pGEM-T Easy (Promega), and the selected clones were sequenced with T7 and SP6 primers by the Sanger protocol.

SARS-CoV-2 serology

Anti-SARS-CoV-2 antibodies were evaluated by an indirect ELISA using the Argentinian kit COVID AR IgG ELISA test (Laboratorio Lemos S.R.L.), which evaluates IgG antibodies specific to spike protein and its receptor-binding domain (RBD) in human serum or plasma[17]. The assay was adapted for domestic dogs, by using an anti-canine IgG (Goat anti-canine IgG (H+L) HRP, Invitrogen A18763), at a 1:20000 dilution, as conjugated.

Sera samples were used at a 1:100 dilution. Optical density (OD) was measured at 450/630 nm and Positivity Index (PI) was calculated as a percentage with the formula $OD_{sample}/OD_{positive\ control}$ x 100. The cut-off value used was the average + 3 standard deviations from a panel of sera samples of 20 healthy dogs obtained pre-pandemic, during 2018.

Pseudoneutralization assays (pNA) were conducted with SARS-CoV-2 pseudotyped particles (CoV2pp-GFP), generated in Sean Whelan laboratory[7] as previously described[13]. CoV2pp-GFP consisted in pseudotyped VSVs expressing three variants of the SARS-CoV-2 spike, alfa (B.1.1.7; GenBank accession OU117158.1), beta (B.1.351; GenBank accession MZ212516.1), and gamma (P.1; GISAID EPI_ISL_804823). Results were expressed as the titer showing 80 % infection inhibition.

Lymphoproliferation assays

Lymphoproliferation assays were performed on day 200 in U-shaped 96-well plates (BD Biosciences, USA) as described before[20], using 1 µg/mL concanavalin A (ConA; Sigma-Aldrich Co) or 0.3 µg/mL purified spike protein as stimuli. Cells cultured with purified NG protein (irrelevant protein) were included as negative control; cells incubated without stimulus (RPMI alone, non-stimulated wells) were considered as representative of basal proliferation.

After four days of culture, 0.5 µCi of methyl-[^{3}H]-thymidine (New England Nuclear Radiochemicals, USA) was added to each well. Sixteen hours later, cells were harvested onto Whatman GF/A paper and the incorporated radioactivity (disintegrations per minute, dpm) was measured by liquid scintillation counting (beta counter 1214 Rackbeta, Vallac; Pharmacia, Finland). Experiments were run in triplicate and the stimulation index (SI) was calculated as mean dpm of stimulated cells/mean dpm of non-stimulated cells.

RESULTS

The three dogs living with a SARS-CoV-2 positive guardian were clinically examined by a veterinarian and found to be asymptomatic. Only "Tina" tested positive by RT-PCR (pancorona and SARS-CoV-2 specific); oropharyngeal and rectal swabs taken from the other two cohabitant pets yielded negative results. Positive results for "Tina" were

confirmed by LAMP/CRISPR assay and real time PCR. Timeline results are summarized in Figure 1.

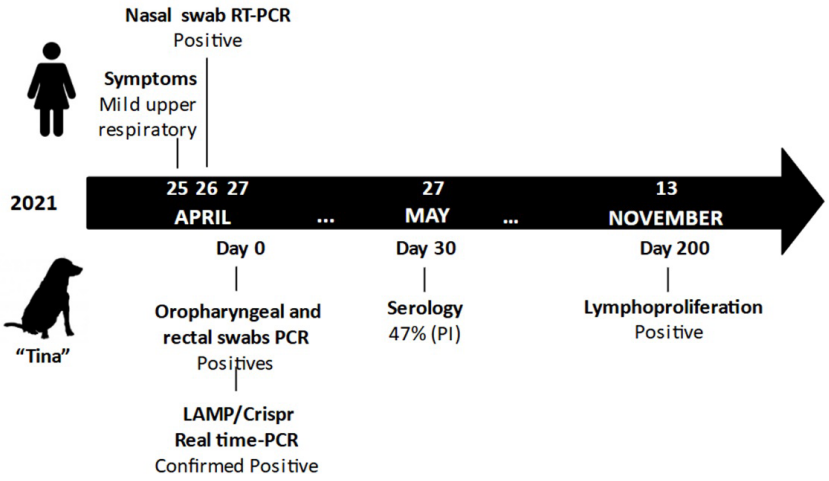

Figure 1. Timeline of the diagnostic tests for SARS-CoV-2 of the positive dog "Tina" (below the timeline arrow) and its guardian (above the timeline arrow). Serology results are expressed as Positivity Index (PI) evaluated by ELISA.

SARS-CoV-2 genomic RNA was successfully obtained from the nasal swab of the guardian as well as from the oropharyngeal and rectal swabs of dog "Tina". Both genomic sequences were identified as Gamma variants (Figure 2). Interestingly, the canine sequence showed an amino acid substitution in position 726 of the S gene (I726V), which is a conservative change. Whole genome sequencing of both sequences was not attempted due to the high real time Ct value.

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hCoV-19/Argenti AWNSNNLDSKVGGNYNYLYRLFRKSNLKPFERDISTEIYQAGSTPCNGVKGFNCYFPLQS
MB/SARS-CoV-2/U AWNSNNLDSKVGGNYNYLYRLFRKSNLKPFERDISTEIYQAGSTPCNGVKGFNCYFPLQS
Tina/SARS-CoV-2 AWNSNNLDSKVGGNYNYLYRLFRKSNLKPFERDISTEIYQAGSTPCNGVKGFNCYFPLQS
                ************************************************************

hCoV-19/Argenti YGFQPTYGVGYQPYRVVVLSFELLHAPATVCGPKKSTNLVKNKCVNFNFNGLTGTGVLTE
MB/SARS-CoV-2/U YGFQPTYGVGYQPYRVVVLSFELLHAPATVCGPKKSTNLVKNKCVNFNFNGLTGTGVLTE
Tina/SARS-CoV-2 YGFQPTYGVGYQPYRVVVLSFELLHAPATVCGPKKSTNLVKNKCVNFNFNGLTGTGVLTE
                ************************************************************

hCoV-19/Argenti SNKKFLPFQQFGRDIADTTDAVRDPQTLEILDITPCSFGGVSVITPGTNTSNQVAVLYQG
MB/SARS-CoV-2/U SNKKFLPFQQFGRDIADTTDAVRDPQTLEILDITPCSFGGVSVITPGTNTSNQVAVLYQG
Tina/SARS-CoV-2 SNKKFLPFQQFGRDIADTTDAVRDPQTLEILDITPCSFGGVSVITPGTNTSNQVAVLYQG
                ************************************************************

hCoV-19/Argenti VNCTEVPVAIHADQLTPTWRVYSTGSNVFQTRAGCLIGAEYVNNSYECDIPIGAGICASY
MB/SARS-CoV-2/U VNCTEVPVAIHADQLTPTWRVYSTGSNVFQTRAGCLIGAEYVNNSYECDIPIGAGICASY
Tina/SARS-CoV-2 VNCTEVPVAIHADQLTPTWRVYSTGSNVFQTRAGCLIGAEYVNNSYECDIPIGAGICASY
                ************************************************************

hCoV-19/Argenti QTQTNSPRRARSVASQSIIAYTMSLGAENSVAYSNNSIAIPTNFTISVTTEILPVSMTKT
MB/SARS-CoV-2/U QTQTNSPRRARSVASQSIIAYTMSLGAENSVAYSNNSIAIPTNFTISVTTEILPVSMTKT
Tina/SARS-CoV-2 QTQTNSPRRARSVASQSIIAYTMSLGAENSVAYSNNSIAIPTNFTISVTTEVLPVSMTKT
                ************************************************:***********

hCoV-19/Argenti SVDCTMYICG
MB/SARS-CoV-2/U SVDCTMYICG
Tina/SARS-CoV-2 SVDCTMYICG
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Figure 2. Comparison of amino acid sequences for the S gene (spanning from amino acid 428 to 750) obtained from dog "Tina" (Tina/SARS-CoV-2) and its guardian (MB/SARS-CoV-2/U). Sequences were aligned by Blastx (Basic Local Alignment Search Tool, NCBI, NIH). ":" sign under the sequence shows the amino acid substitution.

As for the serological response, only dog "Tina" developed a measurable specific antibody response against SARS-CoV-2 by ELISA, with a peak that could be detected at day 30 (Figure 3). This sample was therefore evaluated by pNA assay, revealing a minimal presence of neutralizing antibodies (pNA assay titer = 4). In this assay, serum samples obtained from dogs confirmed positive or negative by RT-PCR to SARS-CoV-2, showed titers of 139 and 0, respectively.

Dog "Tina" also showed a lymphoproliferative response against the purified spike protein, evaluated on day 200 (Table 1).

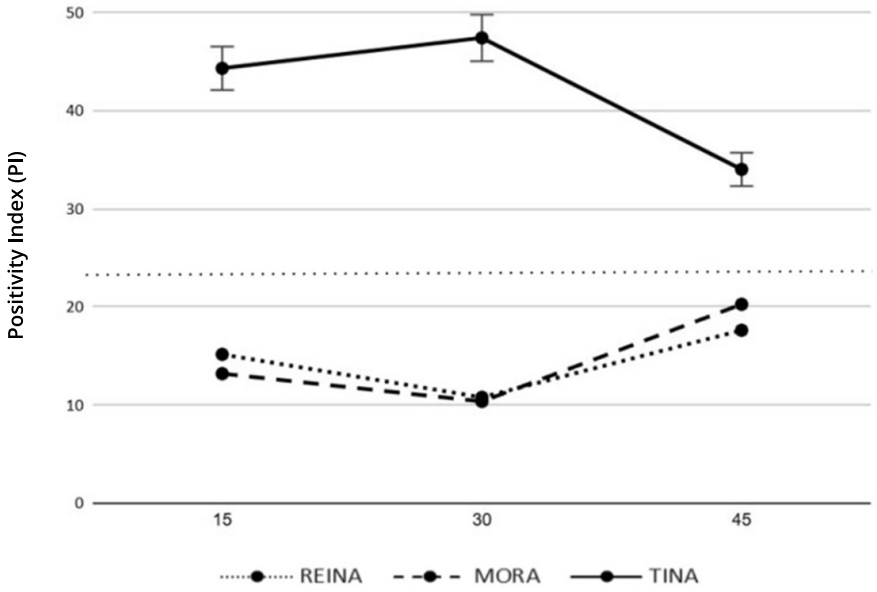

Figure 3. ELISA results are expressed as Positivity Index (PI), calculated as OD sample/OD positive control x 100 and shown as mean ± standard deviation of two independent repetitions. Dotted line shows the cut-off value.

Table 1. Lymphoproliferative response of the dog "Tina" on day 200

Stimulation index (SI) against		
ConA	**Spike**	**Irrelevant protein**
41.63 ± 0.56	2.31 ± 0.89	0.95 ± 0.02

SI are expressed as mean ± S.D. of two independent assays. SI values higher than 10 are considered positive for a non-specific proliferative response. SI values higher than 2 are considered positive for a specific proliferative response.

DISCUSSION AND CONCLUSIONS

In this study, we identified the natural infection of one dog by a Gamma variant of SARS-CoV-2 in Argentina, two days after its guardian was molecularly confirmed as a COVID-19 case. Viral RNA isolation and sequence analysis revealed that the virus infecting the guardian and the dog were closely related genetically. Although animal-to-human transmission is possible[25], the timeline of appearance of the symptoms and the guardian's contacts history, would suggest that in this case the most likely scenario was the transmission from human to dog.

We evaluated the specific antibody levels of the positive dog. A specific antibody kinetic was detected by ELISA from day 15 to 45, though the level of pseudoneutralization in that serum sample was minimal. It has to be considered that this study was conducted using SARS-CoV-2 pseudoviruses, which could not reflect the virus's real behavior[14] due in part to the fact that pNA also involves alfa and beta SARS-CoV-2 variants. For example, other researchers found that the median neutralizing antibodies titer was significantly higher for P.1 than for B.1.1.7 and 20A.EU1 variants when analyzing sera from 22 infected human patients[12]. Unfortunately, no other samples could be taken to deeply evaluate this humoral response.

Studies in humans have shown that approximately 90 % of the sera collected from COVID-19 patients on the 10th day after infection carries specific antibodies against the spike and N proteins[26]. Those sera can exert a significant neutralizing effect, especially RBD specific, peaking about 4-6 weeks after infection[26]. However, in dogs experimentally infected with SARS-CoV-2, the antibody response against spike protein was reported to be relatively low, peaking by day 14 but plateauing and/or waning by day 42[4]. These authors also evaluated the neutralizing ability of antibodies developed after experimental infection in cats (n=7) and dogs (n=3) and reported a comparatively low level of detection in dogs, peaking at 14 dpi, with titers ranging from 40 to 80 (while these exceed 2560 in cats). This neutralizing response detected in positive dogs was also reported to be low when compared to the magnitude of response detected in human patients[10]. In another study, the frequency of SARS-CoV-2 positivity in dogs as assessed by neutralizing antibodies was eight times lower than the frequency of positivity by RT-PCR[5]. Overall, these studies suggest that the neutralizing antibody response against SARS-CoV-2 is not as prevalent in dogs as it is in humans.

We also evaluated the lymphoproliferative response of the positive dog. Interestingly, we found a weak specific response against spike protein 200 days after confirmation of the case. This result indicates the induction of a long lasting anamnestic cellular response, in agreement with studies performed in humans which demonstrate the presence of elevated levels of T memory cells 6 months[27] or even 15 months after infection[16].

To the best of our knowledge, this report constitutes the first confirmed case of SARS-CoV-2 in a dog published in Argentina, where two other papers reporting natural infection in felines had previously been published[11, 18]. The Gamma virus variant that was identified in this work, both in the dog and in its guardian, was the predominant variant circulating in Argentina at the time this case took place, according to the Argentinian Health Ministry. Comparative analysis of the obtained sequences revealed one amino acid substitution in the canine sequence, although this modification was not associated with predicted variations in the physicochemical characteristics of the protein. Nevertheless, the possibility of other non-silent mutations might not be excluded, as adaptation of coronaviruses to new hosts usually occurs via mutations that alter viral tropism[3] and mutations that allow cross-species transmission[15, 31].

In conclusion, we herein report evidence of the human-to-dog transmission of SARS-CoV-2 in one out of three exposed dogs, two of which remained asymptomatic and negative by ELISA and PCR. While the potential role of domestic dogs in COVID-19 spread has been considered unlikely[22], a recent study has suggested a possible role of dogs as passive mechanical carriers of the SARS-CoV-2 virus among humans[30]. Moreover, in a recent study of viral sequences identified in a dog and its guardian in Colombia, it has been proposed that variants of the virus with mutations in critical spike sites may become more transmissible to animals, increasing the risk of reverse zoonosis[21]. A report published about the Amazonian region[28], suggests the need for further research on the role of dogs in SARS-CoV-2 transmission to other companion and wild animals, as well as

humans, particularly in low- and middle-income countries, where free-roaming and feral dogs are common. Furthermore, a recent serosurvey in Spain detected more SARS-CoV-2 infections in pets than previous molecular research reports[2], emphasizing the need for preventive measures to avoid reverse zoonosis events.

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