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Differential Expression of TNF-α and IFN-γ in Acute and Chronic Caprine Theileriosis: A Quantitative RT-qPCR Study

Shrooq Sami Turki[1] , Mansour Jadaan Ali[2]
[1,2] Department of Parasitology, College of Veterinary Medicine, University of Al-Qadisiyah, Iraq, Diwaniyah province

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Correspondence
Shrooq Sami Turki
vet.post24.39@qu.edu.iq
Mansour Jadaan Ali
mansoor.ali@qu.edu.iq

Abstract : Background: Caprine theileriosis is a tick-borne hemoprotozoan disease that induces variable immune responses depending on infection stage. Pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) play critical roles in host defense and disease progression. This study aimed to quantify the relative expression levels of TNF-α and IFN-γ in goats with acute theileriosis compared with chronic cases using quantitative reverse transcription PCR (RT-qPCR). Total RNA was extracted from EDTA blood samples, reverse transcribed to cDNA, and analyzed by SYBR Green-based RT-qPCR. Expression levels were normalized to GAPDH as a housekeeping gene. Relative quantification was calculated using the comparative ΔΔCt method. Statistical comparison between groups was performed using independent t-test. TNF-α expression was significantly upregulated in acute theileriosis (1.098 ± 0.069-fold) compared with chronic cases (0.704 ± 0.052-fold) ($P < 0.01$). IFN-γ expression was also significantly elevated in acute infection (1.772 ± 0.261-fold) relative to chronic cases (1.097 ± 0.147-fold) ($P < 0.05$). Amplification efficiencies were consistent with successful normalization using GAPDH. These findings indicate stronger pro-inflammatory immune activation during acute infection. Acute caprine theileriosis is associated with significant upregulation of TNF-α and IFN-γ, suggesting an intensified Th1-mediated immune response compared with chronic infection.

Keywords: Caprine theileriosis, Cytokine expression, IFN-γ, RT-qPCR, TNF-α

Introduction One of the most virulent protozoa of small ruminants is Theileria lestoquardi, the causative agent of caprine theileriosis, and is endemic in many regions of the world. This organism is spread via various ixodid ticks and due to the fever, anemia, lymphadenopathy, and respiratory distress the organism causes, the clinical effects are devastating. Most recent molecular studies have shown that T. lestoquardi is perpetually circulating in small ruminants in the Middle East and South Asia with significant economic and veterinary impacts (1,2). Characterization studies at the molecular level have shown that there is considerable genetic similarity among isolates within a particular region, suggesting there is a continual (transboundary) flow of transmission (3).

Theileriosis is a disease that is the result of a myriad of complex factors including a host's immune response (e.g. the role of pro-inflammatory) of the cytokines). In a given host, an acute infection will result in an activated macrophage and T-cell response, and an increase in TNF-α and IFN-γ production, both of which are significant stimulators of a Th1 type immune response. While the increase in the cytokines is essential for control of the infection, there is the potential for excessive production to result in a

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significant degree of inflammatory pathology. Several previous studies, both molecular and immunologic, have shown that cytokine profiling lends considerable insight into the understanding of the host-parasite relationship in infected small ruminants with Theileria spp. (4,5).

During the duration of an infection, one of the most common and reliable ways to measure the changes in gene expression is comparing the changes in expression of some of the cytokine transcripts using RT-qPCR, which is also one of the most common methods used for gene expression analysis. Using the comparative Ct (ΔΔCt) method, and combined with some custom-made algorithms, the author is able to accurately normalize and quantify the changes in expression of the cytokine transcripts. This method provides valuable insight into the progression of the disease on the molecular scale (6). Recent studies in epidemiology and molecular biology have attempted to assess the cause of the clinical differences of acute and chronic infections with the aid of phylogenetic analysis and immune response studies. (7). Within the body of literature, the specific patterns of cytokine expression in goats infected with acute and chronic theileriosis have been shown to be wildly variable. Therefore, analyzing the expression of certain genes like TNF-α and IFN-γ (which are indicative of certain disease states) is important for clarifying the immunological components that determine the severity and chronicity of an infection in goats.

Material and Methods

Ethical approval

The present study was approved by the Committee for Research Ethics, College of Veterinary Medicine, University of Al-Qadisiyah, Iraq (Approval No. 4914, dated 14/11/2024).

Study Design and Experimental Groups

This study aims to determine the relative expression levels of TNF-α and IFN-γ genes in goats naturally infected with acute and chronic theileriosis. Blood samples were collected from clinically diagnosed goats and divided into two groups according to the clinical examination and the history of the disease: acute theileriosis group and chronic cases group. The infection status has been confirmed in prior studies by molecular detection of Theileria lestoquardi. Blood samples were collected in EDTA tubes and were either processed immediately or stored at minus 80 degrees Celsius degrees until the time of the RNA extraction.

Isolation of RNA

Total RNA was isolated from 200 µL of EDTA anticoagulated whole blood using TRIpure RNA extraction reagent (Elk, China) and as per the protocol provided by the manufacturer. In brief, 600 µL of TRIpure reagent was added to each sample. Each sample was incubated for 5 minutes at room temperature to achieve complete cellular lysis. Subsequently, 300 µL of chloroform was added, and the samples were centrifuged at 13,00 rpm for 10 minutes. The upper aqueous phase was carefully separated and transferred to a new tube. Then 300 µL of isopropanol was added to it. The samples were spun at 13,000 rpm for 10 minutes after they were kept at 4°C for 10 minutes to precipitate RNA. The pelleted RNA was washed with 600 µL of 70% molecular grade ethanol (Sigma, Germany) and was centrifuged again. The pellet was air dried. RNA was then eluted in 50 µL of elution buffer and stored at −80°C until used.

Quantification of RNA

RNA quantification and purity were determined with the help of a Quantus™ Fluorometer (Promega, USA) using the QuantiFluor® RNA System. A 1× TE buffer was prepared from the 20× stock solution and QuantiFluor® RNA dye was prepared as per the manufacturer instructions by a dilution of 1:400. Before analyses, a blank and standard measurements were made to ensure the accuracy of quantification.

cDNA Synthesis

Using an AddScript cDNA Synthesis Kit (AddBio, Korea), we performed the cDNA synthesis. A reverse transcription (RT) reaction mixture, with a total volume of 20 µL, was made with 10 µL of 2× reverse transcriptase master mix, 2 µL of dNTPs, 1 µL of random hexamer primers, 4 µL of total RNA, and 3 µL of nuclease-free water. Reverse transcription was

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performed with the following thermal conditions: 25°C for 10 minutes (for priming), 50°C for 60 minutes (for reverse transcription), and then 80°C for 5 minutes (for enzyme inactivation). The cDNA that was synthesized is stored at −20°C until qPCR analysis is performed.

Quantitative Real-Time PCR (RT-qPCR)
Using AddScript RT-qPCR SYBR Green Master Mix (AddBio, Korea), the analysis of gene expression was performed. Each reaction was prepared with a total volume of of 20 µL, comprising of 10 µL of SYBR Green master mix, 1 µL of a forward primer, 1 µL of a reverse primer, 2 µL of cDNA template, and 6 µL of nuclease-free water. The amplification was done using a Bio-Rad Real-Time PCR system (USA).

For thermal cycling, the following conditions were used: initial denaturation at 95°C for 10 minutes; 40 cycles of denaturation at 95°C for 20 seconds, then an annealing phase at 60°C for 30 seconds (data acquisition step), followed by an elongation/extension phase at 72°C for 30 seconds. A melting curve analysis was performed in order to confirm the specificity of the amplification. The primers used are mentioned in Table 1.

Table 1: Primer Sequences Used for RT-qPCR Amplification

Target Gene	Primer Name	Sequence (5′ → 3′)	Amplicon Type	Purpose
GAPDH	Goat-GAPDH-F	GGCAAGTTCCATGGCACAGT	Housekeeping gene	Normalization
	Goat-GAPDH-R	ACGTACTCAGCACCAGCATCAC		
TN	Goat-TNF-α-F	GCCTCAGT	ne gene	gene
	Goat-TNF-α-R	AGAAGGGGATGAGGAGGGTC		
IFN-γ	Goat-IFN-γ-F	TAGCTAAGGGTGGGCCTCTTTTCTCA	Cytokine gene	Target gene
	Goat-IFN-γ-R	TGCAGGCAGGAGAACCATTACATTGA		

All primers were commercially procured. Their specificity was confirmed by melting curve analysis. Using the comparative Ct (ΔΔCt) method [Schmittgen and Livak (2008)], relative gene expression was calculated. For normalisation, GAPDH was used as the internal reference gene. The relative fold change in gene expression was calculated using the formula:
$2^{-\Delta\Delta Ct}$
where ΔCt = Ct (target gene) − Ct (GAPDH), and ΔΔCt = ΔCt (acute group) − ΔCt (chronic group).

Statistical analysis
The statistical analysis was done by using GraphPad Prism software (version 8.4.3). Data were expressed as mean ± standard error (SE). The independent samples t-test was used to compare gene expression levels between the acute and chronic groups. Significance level was considered at $P < 0.05$.

Results

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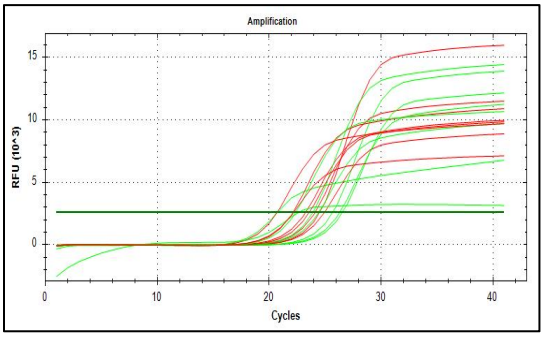
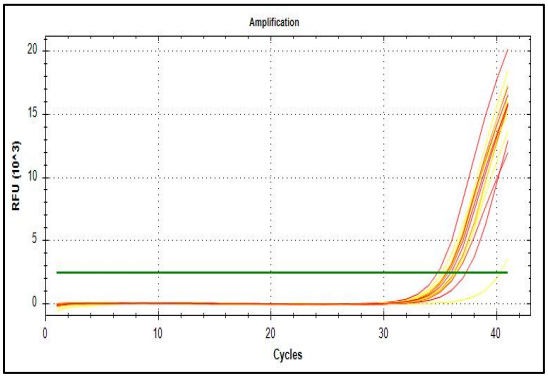
Validation of Amplification of Housekeeping Gene (GAPDH)

To confirm the reliability of normalization GAPDH's internal reference gene amplification efficiency was first assessed. All analyzed samples from both chronic and acute theileriosis cases produced an amplification curve and an exponential phase as well as well-defined threshold cycle (Ct) values. There were no signs of abnormal amplification or primer-dimer pair formation in melting curve analysis suggesting high reaction specificity. The amplification profiles were in fact consistent suggesting minimal variation GAPDH was confirmed as a good reference gene for expression analysis in this study and demonstrated the reliability for normalization in the comparative ΔΔCt analysis (Figure 1).





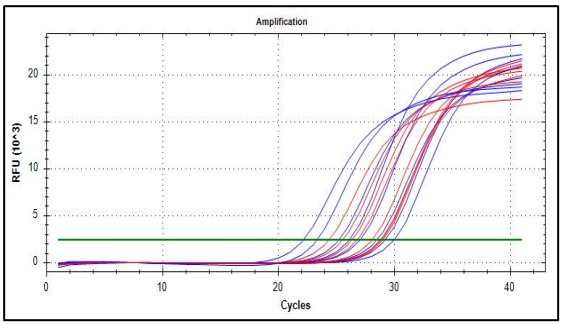


Figure 1: Representative RT-qPCR amplification curves of GAPDH, TNF-α, and IFN-γ genes in chronic (green/yellow/blue) and acute theileriosis (red) goats.

Relative Expression of TNF-α Gene

Goats with acute theileriosis expressed TNF-α significantly higher than the chronic cases. Quantification showed the expression level in the acute infection group as 1.098 ± 0.069 relative fold change, with the chronic group recording 0.704 ± 0.052 which is considerably lower. The groups showed a statistically significant difference ($P < 0.01$, independent t-test).

The range for expression in the acute group (0.8179-1.444) was much higher than the range in the chronic group (0.4569-0.9461). In the acute group, the standard deviation was 0.1971 while the chronic group was at 0.1970. This indicates the presence of a moderate amount of variability in the acute infection group with respect to the cytokine response. Therefore, the data reaffirms the very high level of TNF-α expression during the acute phase of Theileriosis and indicates a high level of pro-inflammatory immune response (Figure 2).

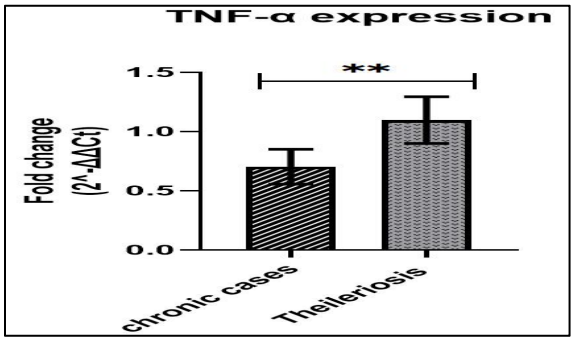


Figure 2: Relative expression of TNF-α in goats showing significant upregulation in theileriosis

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(1.09 ± 0.06) compared with chronic cases (0.70 ± 0.05) (P < 0.01, t-test).

Relative Expression of the IFN-γ Gene

The expression of the IFN-γ gene was found to be significantly higher in the acute infection group when compared to the chronic infection group. The mean fold change of the acute group was 1.772 ±0.2606 while the chronic group was 1.097 ±0.1465 and had a much lower level of expression. It was found that there was a highly significant difference between the chronic group and the acute group (0.05 P, independent t-test).

The chronic group had a smaller expression range than the acute group (0.9727-2.990) by a difference of 2.990 - 1.717 = 1.273. This difference along with the standard deviations (0.7370 for the acute group and 0.4143 for the chronic group) indicates that the immune response in the acute group has a higher degree of variability. Overall, the expression of IFN-γ was much higher in acute Theileriosis than in the chronic form indicating the presence of a Th1 immune response (Figure 3).

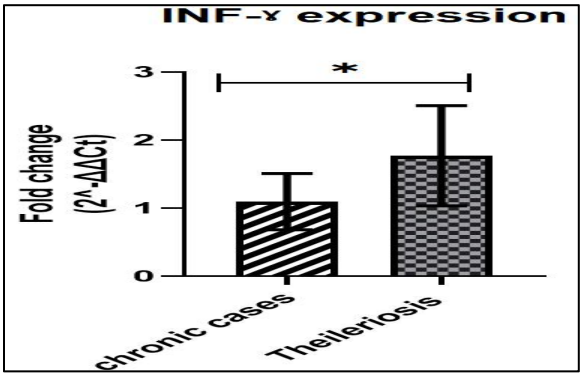

Figure 3: Relative expression of IFN-γ in goats showing significant upregulation in theileriosis (1.77 ± 0.26) compared with chronic cases (1.10 ± 0.15) (P < 0.05, t-test).

Cytokine Expression in Acute and Chronic Theileriosis

The comparison of both Cytokines, TNF-α and IFN-γ, showed both positive regulation in acute infection in contrast to chronic cases, and IFN-γ showed a greater fold change than TNF-α, suggesting greater interferon response immune activation in acute infection. The strong positive fold change of both Cytokines confirms acute theileriosis and chronic infection, and in contrast, chronic infection theileriosis has a less active inflammatory process and therefore less pronounced signal of Cytokine expression (Figure 4).

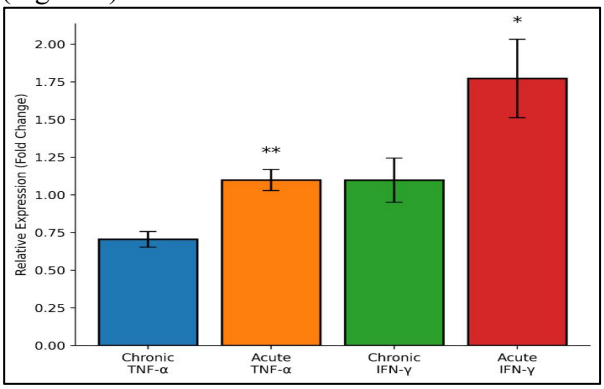

Figure 4. Comparative relative expression of TNF-α and IFN-γ in chronic and acute theileriosis goats. Both cytokines were significantly upregulated in acute infection (**P < 0.01 for TNF-α; *P < 0.05 for IFN-γ; t-test).

Discussion

The current study shows that there is an upregulation of TNF-α and IFN-γ in goats suffering from acute theileriosis in comparison to chronic cases. This illustrates that there is an increased activation of pro-inflammatory immune pathways in acute phases of theileriosis infection. Other research has shown that in acute theileriosis infection there is an increased production of cytokines and that Th1-type immune responses focus on the control of the parasite, but also contribute to the increasing inflammatory response and the subsequent damage to the tissues (8). The increased levels of cytokines in acute infection may be a result of increased activation of macrophages and T-lymphocytes to the infection caused by the parasite.

The increase of TNF-α in acute cases is consistent with previously established articles that state the importance of TNF-α in inflammatory signaling and cellular immunity in response to tick-borne hemoprotozoan infections (9). TNF-α is also understood to drive the phagocytic response and the production of nitric oxide, however, if TNF-α is produced in high

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amounts, it can result in a host of issues including fever, anemia, and inflammation similar to what is seen in Theileriosis infection. Regions where T. lestoquardi is endemic experience an increased response to inflammation in comparison to areas where the species is less virulent (10). The increased amounts of TNF-α is a result of the host's response to the high level of parasitism and is an active infection, therefore TNF-α is justified.

Inflammatory Marker (IFN-γ) also exhibited significantly greater expression during acute infection periods, as compared to chronic infection periods. IFN-γ is an important cytokine in mediated immunity and is important for eliminating intracellular parasites. Prior studies have shown IFN-γ mediated pathways are important for limiting Theileria replication and therefore the activation of macrophages (11), which in acute infections is an indication of strong Th1 immune response. In contrast to acute infections, chronic infections tend to show less IFN-γ expression which provides an immunological explanation of a balance of immune control/adaptation phenomenon, whereby the host immune system permits the intracellular parasites to replicate without any significant clinical manifestations of the infection (12). Despite the presence of parasitemia, chronic carriers have an increasingly lower inflammatory response which explains the decreased cytokine response observed in the study. This is especially true for IFN-γ, as is expected for dominant type 1 cytokine responses.

The observed pattern of the relative (fold) change of IFN-γ > TNF-α during acute infection is more than likely true. The observations are in line with the findings of previous molecular and epidemiological studies which have documented dominant IFN-γ responses in the first stages of an infection (13). In cases of strong dominance of an adaptive immune response, the cytokine and inflammatory responses may become divergent. In previously conducted molecular prevalence studies, it has been shown that immune gene expression (and its variations in a season) may be a result of inflamed adaptive immune responses, which ultimately become the overwhelming factor in the infections (14).

Conclusion

Overall, the current data corroborates the hypothesis that acute caprine theileriosis is linked to considerable Th1-mediated cytokine response, while chronic infection is associated with more balanced regulation of immune response. Hence, chronic theileriosis infection may be accompanied by relatively stable levels of pro-inflammatory cytokines. Thus, measuring transcription levels of TNF-α and IFN-γ may be clinically relevant to developing an infection staging and disease progression model among goats with natural infections of T. lestoquardi.

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Conflict of interest

The authors declare no conflicts of interest.

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Authors' Contributions

All authors participated in the study.

Data availability

Data are available when requested by the corresponding author.

Reference

1. Ashraf S, Hikal WM, Almahallawi R, Muqaddas H, Iqbal F. Molecular prevalence, associated risk factors and phylogenetic evaluation of Theileria lestoquardi in the blood samples of small ruminants. PLoS One. 2024;19(7):e0306697. https://doi.org/10.1371/journal.pone.0306697
2. Celik BA, Kara M, Ayan A, Selcuk MA, Celik OY. Molecular prevalence and phylogenetic analysis of Theileria ovis and Theileria lestoquardi in small ruminants in Siirt Province, Türkiye. Vet Med Sci. 2025;11(5):e70522. https://doi.org/10.1002/vms3.70522
3. Riaz M, Chang SC, Tasawar Z, Sajid M, Nasreen N, Amaro-Estrada I, et al.

Molecular epidemiology and phylogeny of Theileria ovis and Theileria lestoquardi in sheep and goats from Southern Punjab, Pakistan. Vector Borne Zoonotic Dis. 2024;24(10):656-665. https://doi.org/10.1089/vbz.2023.0118

4. Al-Hamidhi S, Weir W, Al-Fahdi A, Johnson EH, Bobade P, Al-Maawali M. A comparative study of single Theileria lestoquardi and mixed infections with Theileria ovis. Parasites Vectors. 2021;14(1):370. https://doi.org/10.1186/s13071-021-04864-6
5. Tanveer M, Farooq M, Amjad M, Asif M, Kashif M, Latif M, et al. Molecular prevalence, associated risk factors and phylogeny of Anaplasma marginale, Theileria ovis and T. lestoquardi in sheep from Pakistan. Comp Immunol Microbiol Infect Dis. 2022;86:101822. https://doi.org/10.1016/j.cimid.2022.101822
6. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nat Protoc. 2008;3(6):1101-1108. https://doi.org/10.1038/nprot.2008.73
7. Schnittger L, Ganzinelli S, Bhoora R, Omondi D, Nijhof AM, Florin-Christensen M. The Piroplasmida Babesia, Cytauxzoon, and Theileria in farm and companion animals: Species compilation, molecular phylogeny, and evolutionary insights. Parasitol Res. 2022;121(5):1207-1245. https://doi.org/10.1007/s00436-022-07424-8
8. Al-Hamidhi S, Weir W, Al-Fahdi A, Johnson EH, Bobade P, Al-Maawali M. A comparative study of single Theileria lestoquardi and mixed infections with Theileria ovis. Parasites Vectors. 2021;14(1):370. https://doi.org/10.1186/s13071-021-04864-6
9. Adil Khan, Muhammed AA, Nasreen N, Iqbal F, Cossio-Bayugar R, Ali Sha SS, et al. Tick-borne haemoparasitic diseases in small ruminants in Pakistan: Current knowledge and future perspectives. Saudi J Biol Sci. 2022;29(4):2014-2025. https://doi.org/10.1016/j.sjbs.2021.12.046
10. Al-Hosary AA, ElSify A, Salama AA, Nayel M, Elkhtam A, Elmajdoub LO, et al. Phylogenetic study of Theileria ovis and Theileria lestoquardi in sheep from Egypt: Molecular evidence and genetic characterization. Vet World. 2021;14(3):634-639. https://doi.org/10.14202/vetworld.2021.634-639
11. Schnittger L, Ganzinelli S, Bhoora R, Omondi D, Nijhof AM, Florin-Christensen M. The Piroplasmida Babesia, Cytauxzoon, and Theileria in farm and companion animals: Species compilation, molecular phylogeny, and evolutionary insights. Parasitol Res. 2022;121(5):1207-1245. https://doi.org/10.1007/s00436-022-07424-8
12. Shahedi A, et al. Molecular identification of Theileria spp. in ruminants and ticks from southern littoral of Caspian Sea, Iran. Trop Anim Health Prod. 2022;54(3):157. https://doi.org/10.1007/s11250-022-03156-0
13. Nangru A, Sharma A, Kumar R, Jindal N, Moudgil P. Identification, molecular characterization and risk factors of Theileria infection among sheep: A first comprehensive report from North India. Anim Biotechnol. 2023;34(8):3658-3670.
14. Tanveer M, Farooq M, Amjad M, Asif M, Kashif M, Latif M, et al. Molecular prevalence, associated risk factors and phylogeny of Anaplasma marginale, Theileria ovis and T. lestoquardi in sheep from Pakistan. Comp Immunol Microbiol Infect Dis. 2022;86:101822. https://doi.org/10.1016/j.cimid.2022.101822