Libyan Journal of Medical and Applied Sciences LJMAS
Online ISSN: 3006-1113
Volume 3, Issue 4, 2025, Page No: 94-101
Website: https://ljmas.com/index.php/journal/index

Utilizing Specific Genes to Detect Brucella spp. and Investigating Immunological Alterations in Suspected Brucellosis Patients from Babylon province

Kazem Mehdi Kazem [1], Israa Khudhair Obayes [2*]
[1]Department of Medical Microbiology, Hammurabi College of Medicine, University of Babylon, Hilla 51002, Iraq
[2] Department of Diseases and Forensic Medicine, Hammurabi College of Medicine, University of Babylon, Hilla 51002, Iraq
[*]*Corresponding author:* ham750.israa.khudhair@uobabylon.edu.iq

Received: October 07, 2025 Accepted: December 28, 2025 Published: December 31, 2025

Cite this article as: K, M, Kazem., I, K, Obayes., A, A, Hamza. (2025). Utilizing Specific Genes to Detect Brucella spp. and Investigating Immunological Alterations in Suspected Brucellosis Patients from Babylon province. Libyan Journal of Medical and Applied Sciences (LJMAS). 2025;3(4):94-101.

Abstract:
Brucellosis continued to be a primary cause of illness in people and animals globally.
Objective: Genetic investigation for the identification of Brucella spp. and examination of immune system responses in probable Brucellosis infections in Babylon city.
Materials and methods: The research was carried out from March 2024 to August 2024, a span of six months. In all, 150 male and female patients between the ages of 15 and 55 attended private clinics in the province of Babylon. The Rose Bengal test came up positive for these individuals. Each patient had blood drawn, and 20 samples from healthy people who served as the control group were also taken. Following PCR detection of Brucella spp., a number of immunological markers linked to brucellosis were assessed.
Results: The Rose Bengal test identified Brucella spp. in 53 (46.2%) of 150 blood samples from individuals with brucellosis. Outcomes of research on the identification of Brucella bacteria with a particular Bcsp31 gene primer derived from blood samples. The primer combination used in this study amplified a 223 bp fragment from Brucella, with 22 out of 53 blood samples testing positive for Rose Bengal. Comparison of mean Perforin and granzyme B protein levels between case and control groups. The research revealed substantial elevations in Perforin (19.52±3.88) and granzyme B (74.31±13.88) levels in individuals with Brucellosis relative to the control group (6.39±6.33) ($P \geq 0.05$). The research indicated that individuals with Brucellosis had elevated IL-12 levels (287.54±12.18 ng/ml) relative to the control group (188.08±2.29 ng/ml), demonstrating a statistically significant difference ($P<0.05$).
Conclusion: To fully comprehend the immunological response to Brucella, a thorough knowledge of the functions of perforin and granzyme was required. B.

Keywords: *Brucella*, Immunological Markers, Brucellosis, PCR, Gene.

استخدام جينات محددة للكشف عن بكتيريا البروسيلا ودراسة التغيرات المناعية لدى مرضى مشتبه بإصابتهم بداء البروسيلات في محافظة بابل

كاظم مهدي كاظم[1], إسراء خضير عبيس[2*]
[1]قسم الأحياء المجهرية، كلية طب حمورابي، جامعة بابل، العراق
[2] قسم الأمراض والطب الشرعي، كلية حمورابي للطب، جامعة بابل، الحلة 51002، العراق

الخلاصة
الخلاصة: لا يزال داء البروسيلات مصدرًا رئيسيًا للأمراض لدى البشر والحيوانات في جميع أنحاء العالم.

الهدف: التحليل الجيني للكشف عن أنواع البروسيلا ودراسة الاستجابات المناعية في حالات العدوى المحتملة بداء البروسيلات في محافظة بابل. المواد والأساليب: أُجريت الدراسة على مدى ستة أشهر، من مارس 2024 إلى أغسطس 2024. زار 150 مريضًا، من كلا الجنسين، تتراوح أعمارهم بين 15 و55 عامًا، عيادات خاصة في محافظة بابل. كانت نتائج اختبار روز بنغال إيجابية لدى هؤلاء المرضى. جُمعت عينات دم من كل مريض، بالإضافة إلى 20 عينة من أفراد أصحاء كمجموعة ضابطة. بعد الكشف عن أنواع البروسيلا... باستخدام تفاعل البوليميراز المتسلسل ((PCR، تم تقييم العديد من المؤشرات المناعية المرتبطة بداء البروسيلات.
النتائج: كشف اختبار روز بنغال عن وجود بكتيريا البروسيلا في 53 عينة (46.2%) من أصل 150 عينة دم مأخوذة من مرضى داء البروسيلات.
نتائج الدراسة: التعرف على بكتيريا البروسيلا باستخدام بادئ جيني محدد لجين Bcsp31 من عينات الدم. وقد ضخمت تركيبة البادئات المستخدمة في هذه الدراسة قطعة (223 زوجًا قاعديًا) من بكتيريا البروسيلا (22 من أصل 53 عينة دم كانت نتائج اختبار روز بنغال فيها إيجابية). متوسط الفروق في بروتينات البرفورين والجرانزيم ب بين مجموعتي الدراسة (الحالات والضوابط). ووجدت الدراسة زيادات معنوية في مستويات البرفورين (19.52±3.88) والجرانزيم ب (74.31±13.88) لدى مرضى داء البروسيلات مقارنةً بمجموعة الضوابط (6.39±6.33) (.(P≥0.05أظهرت الدراسة أن مرضى داء البروسيلات لديهم مستويات أعلى من إنترلوكين-12 (287.54±12.18 نانوغرام/مل) مقارنةً بالمجموعة الضابطة (188.08±2.29 نانوغرام/مل)، مع وجود فرق دال إحصائيًا (.(P<0.05)
الخلاصة: لفهم الاستجابة المناعية لبكتيريا البروسيلا فهمًا كاملًا، من الضروري فهم آلية عمل البيرفورين والجرانزيم ب فهمًا دقيقًا، نظرًا للدور المهم الذي يلعبه هذان البروتينان في هذه العملية.

الكلمات المفتاحية: البروسيلا، المؤشرات المناعية، داء البروسيلات، تفاعل البوليميراز المتسلسل، الجينات.

Introduction

Brucellosis, the contagion of which is attributed to the "*Brucella*" genus poses a great threat to human health, and livestock, especially cattle, suffer huge losses through abortion and infertility (1). In humans' brucellosis can present itself as an acute illness, sub-acute, or chronic depending on the degree of exposure to the bacteria, or the animal, or ingestion of raw milk or meat (2). There are six classical *Brucella* species, each with a distinct host preference: *B. abortus* is mainly a disease of cattle; *B. meitatensis* affects sheep and goats, *B. suis* infects pigs; *B. canis* affects dogs; *B. ovis* affects only sheep and goats; and *B. neotomae* is identified in the desert wood rat (3). There was a discovery of a new species of Brucella later in the 2000s. The marine animals were the sources of the bacteria B. ceti and B. pinnipedialis. Species such as Brucella, which may infect mammals like voles and seals, and B. microti, a disease found in humans, and B. *Strains* inopinata originating from a human breast implant infection have also been recognized to be members of the *Brucella* genus (4). Moreover, new strains of Brucella have recently been identified in wild rodents in North Queensland, Australia and from still birth cases in non-human primates though they have not been still categorised under the *Brucella* genus (3). By reason of the low infective dose and the ability to be spread in the droplet nuclei, *Brucella* species have been deemed as having bioterrorism potential (5).

However, despite the widespread use of novel molecular diagnostic technologies, culture isolation remains the gold standard for brucellosis confirmation. Characterization of *Brucella* and particularly its biotyping can best be achieved using biochemical, serological and molecular techniques (6). Typical biochemical characters are CO_2, H_2S test, dye tolerant test, and enzyme test urease, oxidase, catalase and so forth. Also, the use of monospecific A and M antiserum in agglutination helps in the determination of isolate characteristics (7). The Serum Tube Agglutination Test (STAT), Rose Bengal Plate Test (RBPT), 2-mercaptoethanol (2ME) test, Complement Fixation Test (CFT), and enzyme-linked immunosorbent assay (ELISA) are among the frequently used tests for diagnosing human brucellosis. However, these tests are not competent in certain aspects; for example, there are background antibodies prevalent in the endemic areas and they cannot distinguish between acute and previous infections (8). Many PCR-based tests have been designed depending on genus specificity or a high level of homology of the *Brucella* species including, the 43kDa outer membrane protein-olfactory marker protein (protein-omp) gene, *BCSP 31*, *IS 6501/711*, and *16S rRNA* gene (9).

While perforin is in charge of immune system cell death, granzyme B belongs to the protease family. They are involved in the process of CTL's and NK cells to kill target cells, including virus or bacterial-infected or tumor cells (10). Perforin is a protein that acts as a pore in CTL and NK cells granules. Of these, when these immune cells come across an infected or abnormal cell, they release Perforin that forms a hole in the membrane of the target cell (11). Thus, with the formation of pores the other cytotoxic molecules like the granzyme B, are also allowed to enter the target cell. Granzyme B is a serine protease that has been identified to be present in the granules of CTLs and NK cells. When perforin forms gaps on the outer wall of the target cell, granzyme B gets into the cell through that gap (10). Within the cell, granzyme B engages apoptotic processes that bring about the orderly dismantling of the infected or malignant cell hence contributing to the extermination of the pathogen or the cancerous cell without overproduction of inflammation (12).

Perforin and granzyme B play a role in the immune system's defense against intracellular pathogens including the Brucella organism which causes brucellosis (13). Macs can be infected with *Brucella* and Cytotoxic T cells and NK cells can recognize these infected macrophages and kill them using perforin and granzyme B thus controlling the spread of *Brucella* (14). In brucellosis, cytokines such IFN-γ are generated, leading to macrophage activation and enhancing their capacity to eradicate Brucella. Consequently, if the sources of infection are not well managed, local CTLs and NK cells may be stimulated to eliminate infected cells via the perforin-granzyme pathway. It is used in the destruction of infected cells that harbor *Brucella* during intracellular infections such as brucellosis, hence aiding the immune system (15). The increase in the concentrations of perforin and granzyme B may be related to an active cytotoxic response during the infection by *Brucella*, particularly in chronic or severe cases (16). Several studies indicate that Brucella interferes with immune responses such as the perforin-granzyme pathway, which may help the bacteria to establish a chronic infection in the host (17).

The study's objectives

Molecular identification of Brucella species and assessment of immunological alterations (IL-12), Perforin and granzyme B levels in individuals in Babylon province who may have brucellosis.

Material and method

Patient sample

The research spanned the months of March 2024 through August 2024. The private clinics in Babylon province saw 150 patients, ranging in age from 15 to 55 years old. The results of the rose Bengal test were positive in these people.

Blood sample collection

All patients had their blood tested; this included 150 cases and 20 healthy controls. Every participant had five milliliters of blood drawn from their veins using a vein puncture. The blood was carefully transferred into disposable tubes that contained separating gel. After 30 minutes of waiting for the tubes to clot at room temperature, they were centrifuged at 3000×g for around three minutes. Once collected, the sera were kept at a temperature of -20˚C until analysis.

DNA isolation

Genomic DNA purification kits (Geneaid, USA) were used to extract genomic DNA from each blood sample in accordance with the manufacturer's instructions.

Detection of *Bcsp31* specific gene primer from clinical isolates by RCR technique:

The polymerase Chain Reaction technique we used for amplification of the specific gene of *Brucella* spp. that suspected to be present in blood samples were shown in Table (1). (15)

Table 1: Detection primer sequence with their amplicon size Base pair (bp), and their condition

Specific gene primer	**Primer sequence (5′-3′)**	**Size (bp)**	**PCR condition**
Bcsp31	F:5- TGGCTCGGTTGCCAATATCAA-3 R: 5-CGCGCTTGCCTTTCAGGTCTG-3	223	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 57.6ºC decrease 0.5ºC per cycle, 30 sec. Step 4: 72ºC, 100.0 sec. Step 5: Repeat steps 2-4 14 more times Step 6: 95ºC, 30 sec. Step 7: 50.6ºC, 30 sec. Step 8: 72ºC, 100.0 sec. Step 9: Repeat steps 6-8 19 more times Step 10: 72ºC, 5 min. Step 11: 4ºC, forever

Quantification of Granzyme B and Perforin
This research followed the instructions provided by the manufacturer (Sunlong, China) to quantitatively determine the protein content in blood samples from adult patients using the human Perforin and granzyme B enzyme-linked immunosorbent assay kits.

Finding the IL-12 concentration
This ELISA kit was developed using the Sandwich ELISA principle. Each kit included with micro-ELISA plates that were pre-coated with an antibody that was specific to Human IL-12. By combining samples (or standards) with the corresponding antibody and then adding them to the wells of a micro-ELISA plate, a positive result may be produced.

Ethical Authorization
Informed permission was obtained from each patient prior to their participation in the research.

Statistical examination
Statistical analysis was conducted with SPSS version 26. Continuous variables were expressed as means ± standard deviation (SD). The student's t-test was used to compare the means of two groups.

Results
The results of the Rose Bengal test were positive in 53 (46.2%) out of 150 blood samples taken from individuals exhibiting brucellosis symptoms, as indicated in Table (2), it was identified as *Brucella* spp.

Table 2: Rose Bengal Tests *Brucella* spp. positive

No. of blood samples	No. of rose Bengal test ***Brucella*** positive %
150	53(46.2%)

The study findings examined the potential for recognizing Brucella bacteria with a unique Bcsp31 gene primer derived from blood samples. Of the 53 blood samples tested with the positive Rose Bengal assays, only 22 (54.3%) of the isolates were identified as Brucella positive. The primer pair used in this investigation successfully amplified a 223 bp fragment from Brucella spp.

Figure 1: Agarose gel electrophoresis (1.5%) of RCR amplified of *Bcsp31* gene (223 bp) of *Brucella* spp. for (55) min at (70) volt L: ladder (DNA marker), (1, 2, 3, 4, 5, 6, 7, 8, to 22) Amplify of *Bcsp31* gene was positive clinical isolates of *Brucella spp.*

The mean differences between Perforin and granzyme B proteins levels according to study groups (case and control groups) are shown in Table (3). The results indicated a significant elevation in Perforin levels (19.52±3.88) (P≥0.05) in patients with Brucellosis infection relative to the control group (6.95±1.96), as well as a notable increase in granzyme B (74.31±13.88) (P≥0.05) in the same patient cohort compared to the control group (6.39±6.33).

Table 3: Mean differences in the levels of Perforin and granzyme B proteins among study groups, including individuals with Brucellosis infection and the control group.

Parameter/ protein	Patients with brucellosis infection	Control group	p-value
	Mean ± SD	**Mean ± SD**	
	No. 22	**No. 20**	
Perforin	19.52±3.88	6.95±1.96	**P≥0.05**
granzyme B	74.31±13.88	6.39±6.33	

* p value ≤ 0.05 was significant

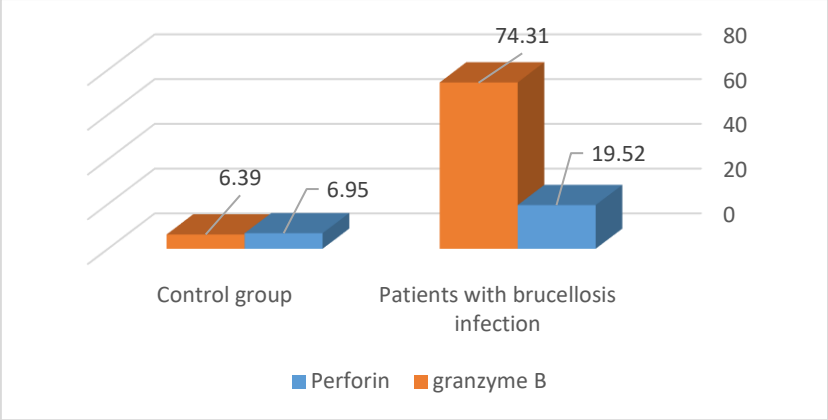

Figure 2: average variations in the levels of the proteins Perforin and granzyme B throughout the research groups, including those with Brucellosis infection and those without.

The investigation into IL-12 level determination revealed that, as illustrated in Table (4) and Figure (3), the control group had the lowest average IL-12 level (288.08±2.29ng/ml), while patients with Brucellosis had the highest (287.54±12.18ng/ml). This difference was statistically significant (P<0.05).

Table 4: Determination of IL-12

Parameter	Sample	N	Mean ± S. E	P. value
IL12 (pg/ml)	Patients	22	287.54±12.18	0.0001
	Control	20	188.08±2.29	

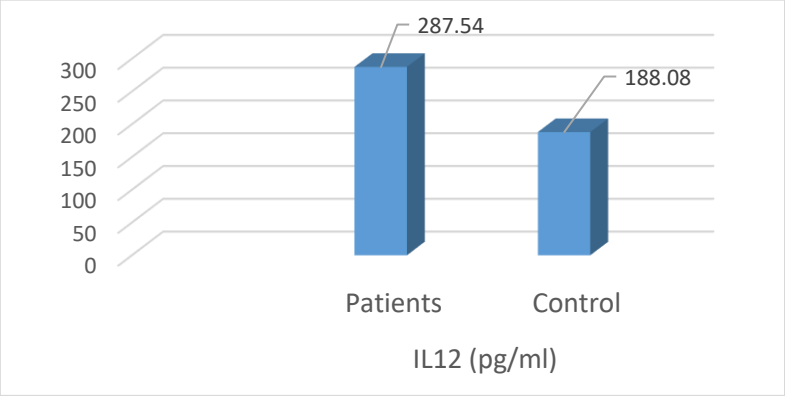

Figure 3: Determination of IL-12

Discussion

Serological testing is crucial for the diagnosis of human brucellosis since the illness may present in a broad range of ways (18). During the process of collecting these samples, the following symptoms were noted: fever, sweating, aches, exhaustion, back and joint pain, arthritis, headache, nausea, vomiting, diarrhea, loss of appetite, weight loss, constipation, abdominal pain, cough, rash, splenomegaly, and hepatomegaly. These samples were collected under the supervision of experienced physicians. Several factors contributed to the low number of *Brucella* DNA extracted from the blood (19). The patient's antibiotic treatment was a major one. Other factors included the patient's immune system's effectiveness in reducing bacterial numbers, the constant flow of blood, and the richness and support of the culture media, which increased the chances of bacterial growth relative to the blood (20). Conventional methods prove unworthy for the diagnosis of infectious diseases brought by fastidious or slow-growing micro-organisms including *Brucella* and therefore, molecular approaches like PCR appear more suitable for this purpose (21). It was investigated in this work, PCR as a diagnostic tool for the identification of *Brucella* species in serums from human beings and calmly looked at the epidemiology of brucellosis (22). It was successful in testing for brucellosis by using specific gene primers and 22 samples from 150 blood samples were positive for brucellosis (23). Similarly, it contrasted the bcsp31 gene target's sensitivity and specificity in PCR tests. PCR assays based on the *bcsp31* gene and its primers were evaluated for their sensitivity and the possibility of detection of *Brucella* DNA in human peripheral blood or serum (24). The present work used serum samples for the PCR test instead of whole blood, since literature suggests that serum contains less inhibitors and facilitates superior DNA extraction compared to whole blood. PCR appears to offer

several advantages over conventional methods: it is easy to perform; it is rapid; and it is safe for laboratory staffs because the serum-based PCR-assay will reduce the risk of handling the microorganism in the laboratory (19). Therefore, the use of the *Bcsp31*, based PCR assays described here was a promising method for detection of the *Brucella* genus and also identifying *Brucella* spp in clinical samples (25). An innovative method for identifying the most infectious illness involves the exploration of PCR-based diagnostics for the rapid identification and confirmation of Brucella. Various polymerase chain reaction (PCR) techniques, including conventional, real-time, multiplex, and monoplex PCR, are used. The results validate those of the present study. In contrast, PCR was used by (19) to diagnose brucellosis; they characterized it as highly specific, sensitive, and straightforward, suggesting that it has the potential to become a standard diagnostic tool for the disease (26). Blood and milk, as well as fetal and maternal tissues acquired from infected animals, can be tested for Brucella using the polymerase chain reaction (PCR) method, which has been the subject of numerous studies since its description (27). PCR has an exceptional sensitivity of 98% and a high specificity of 90%. Natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), which are components of the immune system, produced the protein perforin. There were various types, especially the main purpose was to assist these immune cells the eliminating infected and damaged cells including intracellular bacteria as well as viruses, and even cancer cells (28). Perforin function was to penetrate the membranes of target cells (e. g., cells that harbor pathogens). This makes it possible for other cytotoxic molecules like granzyme B to gain entry into the said cell and make it to undergo apoptosis, thus culminating in the elimination of the infected or the abnormal cell (29). One of the most important substances the immune system utilizes to fight intracellular infections, such as Brucella, is perforin. Brucellosis, caused by *Brucella* bacteria was of the intracellular type; that is; *Brucella* parasites live and reproduces within host cells and predominantly in macrophages (30). These intracellular parasites can only be destroyed by cell-mediated cytotoxicity, such as the actions of CTL or NK cells. It could become elevated for the following reasons: *Brucella* bacteria could parasitize host cells and were found essentially within macrophages (16). Subsequently, CTL and NK cells react to the immune system by producing more perforin to target and eliminate infected cells, therefore inhibiting bacterial seeding and proliferation. In response to Brucella infection, cytokines such as IFN-γ are produced, facilitating the activation of CTLs and NK cells. The activated cells produce perforin to neutralize Brucella-infected cells as a defensive mechanism (10). Because of continual and frequent infections of brucellosis, the immune system might be constantly trying to destroy the infected cells thus making perforin to be continually or highly produced in the body as the body tries to control or destroy the diseases (28). High level of perforin in brucellosis was a part of the overall response of the immune system that is usually proactive in the destruction of intracellular organisms. The enhanced levels of perforin enable the immune system to more effectively destroy and eradicate infected cells so as to help contain the spread of the bacteria (31). Perforin is the molecule responsible for lysing infected cells, which is crucial for infection management. Perforin is the molecule responsible for lysing infected cells, which is crucial for infection management. The increase in perforin in brucellosis represents an effort in immune-mediated clearance of infected cells especially where the disease was chronic or severe (32). Perforin and granzyme B have identifiable roles in regulating the intracellular reproduction of Brucella; yet, under certain conditions, Brucella may evade the effects of these immune mediators, leading to persistent infections. This was seen in the increase in the levels of perforin in brucellosis, this was the body's way of targeting cells that have been infected with *Brucella* bacteria (16).

Conclusions

The current study shows that molecular-immunological applications offer a more precise and detailed paradigm on the interpretation of human brucellosis in the endemic regions like Babylon province. PCR with the Bcsp31 gene was found to be an effective confirmatory test in the detection of Brucella spp. in seropositive patients by overcoming a number of shortcomings that are linked to traditional serological tests. In addition to the detection of the pathogen, immunological results showed that cell-mediated immunization in brucellosis patients was highly activated, which was demonstrated by high serum concentrations of perforin, granzyme B, and IL-12. These findings suggest that cytotoxic T lymphocytes and natural killer cells are a key finding in the regulation of Brucella infection by perforin-granzyme-mediated apoptosis of infected host cells, whereas IL-12 may also be involved in maintaining a Th1-biased immune environment necessary to eliminate intracellular pathogens. Together, the results demonstrate that brucellosis is typified by an active cytotoxic and pro-inflammatory immune response, especially in definite cases. These immune interactions do not only help to increase the knowledge on the pathogenesis of the diseases, but also indicate the potential of perforin, granzyme B, and IL-12 as adjuvant immunological proteins in the diagnosis and disease surveillance.

References

1 El-Sayed, A., & Awad, W. (2018). Brucellosis: Evolution and expected comeback. *International Journal of Veterinary Science and Medicine*, 6, S31 - S35.

2 Ran, X., Cheng, J., Wang, M., Chen, X., Wang, H., Ge, Y., Ni, H., Zhang, X., & Wen, X. (2019). Brucellosis seroprevalence in dairy cattle in China during 2008-2018: A systematic review and meta-analysis. *Acta tropica*, 189, 117-123.

3 Dadar, M., Shahali, Y., Fakhri, Y., & Godfroid, J. (2020a). The global epidemiology of Brucella infections in terrestrial wildlife: a meta-analysis. *Transboundary and emerging diseases*.

4 Nejad, R., Krecek, R., Khalaf, O., Hailat, N., & Arenas-Gamboa, Á. (2020). Brucellosis in the Middle East: Current situation and a pathway forward. *PLoS Neglected Tropical Diseases*, 14.

5 Dadar, M., Fakhri, Y., Shahali, Y., & Khaneghah, A. (2020b). Contamination of milk and dairy products by Brucella species: A global systematic review and meta-analysis. *Food research international*, 128, 108775.

6 Ducrotoy, M., Muñoz, P., Conde-Álvarez, R., Blasco, J., & Moriyón, I. (2018). A systematic review of current immunological tests for the diagnosis of cattle brucellosis. *Preventive veterinary medicine*, 151, 57-72.

7 Yagupsky, P., Morata, P., & Colmenero, J. (2019). Laboratory Diagnosis of Human Brucellosis. *Clinical Microbiology Reviews*, 33.

8 Elbehiry, A., Aldubaib, M., Marzouk, E., Abalkhail, A., Almuzaini, A., Rawway, M., Alghamdi, A., Alqarni, A., Aldawsari, M., & Draz, A. (2023). The Development of Diagnostic and Vaccine Strategies for Early Detection and Control of Human Brucellosis, Particularly in Endemic Areas. *Vaccines*, 11.

9 Nyarku, R., Hassim, A., Jonker, A., & Quan, M. (2020). Development of a Genus-Specific Brucella Real-Time PCR Assay Targeting the 16S-23S rDNA Internal Transcribed Spacer from Different Specimen Types. *Veterinary Sciences*, 7.

10 Nicolai, C., & Raulet, D. (2020). Killer cells add fire to fuel immunotherapy. *Science*, 368, 943 – 944.

11 Hay, Z., & Slansky, J. (2022). Granzymes: The Molecular Executors of Immune-Mediated Cytotoxicity. *International Journal of Molecular Sciences*, 23.

12 Zhou, Z., He, H., Wang, K., Shi, X., Wang, Y., Su, Y., Wang, Y., Li, D., Liu, W., Zhang, Y., Shen, L., Han, W., Shen, L., Ding, J., & Shao, F. (2020). Granzyme A from cytotoxic lymphocytes cleaves GSDMB to trigger pyroptosis in target cells. *Science*, 368.

13 Clapp, B., Yang, X., Hoffman, C., & Pascual, D. (2018). Oropharyngeal (OPG) vaccination activates head and neck lymphoid tissues required for immune protection against mucosal Brucella infection. *The Journal of Immunology*.

14 Shringi, B., Choudhary, O., & Kashyap, S. (2019). Cytokines in brucellosis: biological rhythm at the interface of innate and adaptive immunity. *Biological Rhythm Research*, 52, 1031 - 1043.

15 Tang, Y., Ma, C., Sun, H., Yang, S., Yu, F., Li, X., & Wang, L. (2021). Serum Levels of Seven General Cytokines in Acute Brucellosis Before and After Treatment. *Infection and Drug Resistance*, 14, 5501 - 5510.

16 Cuesta, L., & Perez, S. (2021). Perforin and granzymes in neurological infections: From humans to cattle. *Comparative immunology, microbiology and infectious diseases*, 75, 101610.

17 Guo, X., Zeng, H., Li, M., Xiao, Y., Gu, G., Song, Z., Shuai, X., Guo, J., Huang, Q., Zhou, B., Chu, Y., & Jiao, H. (2023). The mechanism of chronic intracellular infection with Brucella spp. *Frontiers in Cellular and Infection Microbiology*, 13.

18 Dal, T., Açıkgöz, Z., Başyiğit, T., Zeybek, H., & Durmaz, R. (2018). [Comparison of two commercial DNA extraction kits and PCR master mixes for the detection of Brucella from blood samples and blood culture bottles]. *Mikrobiyoloji bulteni*, 52 2, 135-146.

19 Liu, L., Li, M., Liu, G., He, J., Liu, Y., Chen, X., Tu, Y., Lin, J., Feng, Y., & Xia, X. (2023). A novel, highly sensitive, one-tube nested quantitative real-time PCR for Brucella in human blood samples. *Microbiology Spectrum*, 11.

20 Waringa, N., Waiboci, L., Bebora, L., Kinyanjui, P., Kosgei, P., Kiambi, S., & Osoro, E. (2023). Human brucellosis in Baringo County, Kenya: Evaluating the diagnostic kits used and identifying infecting Brucella species. *PLOS ONE*, 18.

21 Becker, G., & Tuon, F. (2021). Comparative study of IS711 and bcsp31-based polymerase chain reaction (PCR) for the diagnosis of human brucellosis in whole blood and serum samples. Journal of microbiological methods, 106182.

22 Zamanian, M., Jahani, E., & Mahmoudi, H. (2020). Multiplex PCR Assay for the Simultaneous Detection of the Brucella Genus in Human Whole Blood and Serum. *The Open Microbiology Journal*, 14.

23 Abdel-Hamid, N., Beleta, E., Kelany, M., Ismail, R., Shalaby, N., & Khafagi, M. (2021). Validation of real-time polymerase chain reaction versus conventional polymerase chain reaction for diagnosis of brucellosis in cattle sera. Veterinary World, 14, 144 - 154.

24 Saytekin, A., & Ak, S. (2018). Direct diagnosis of Brucella species through multiplex PCR formed by a new method. *Journal of microbiological methods*, 154, 86-94.
25 Boeri, E., Wanke, M., Madariaga, M., Teijeiro, M., Elena, S., & Trangoni, M. (2018). Comparison of four polymerase chain reaction assays for the detection of Brucella spp. in clinical samples from dogs. *Veterinary World*, 11, 201 - 208.
26 Das, A., Kumar, B., Chakravarti, S., Singh, K., , A., & Ekici, G. (2018). Single-tube duplex-PCR for specific detection and differentiation of Brucella abortus S19 vaccine strain from other Brucella spp. *Indian Journal of Animal Research*.
27 Dal, T., Kara, S., Çikman, A., Balkan, Ç., Açıkgoz, Z., Zeybek, H., Uslu, H., & Durmaz, R. (2019). Comparison of multiplex real-time polymerase chain reaction with serological tests and culture for diagnosing human brucellosis. *Journal of infection and public health*, 12 3, 337-342.
28 Hu, H., Tian, M., Li, P., Bao, Y., Guan, X., Lian, Z., Yin, Y., Ding, C., & Yu, S. (2019). Brucella infection regulates peroxiredoxin-5 protein expression to facilitate intracellular survival by reducing the production of nitric oxide and reactive oxygen species. Biochemical and biophysical research communications.
29 Spicer, J., Miller, C., O'Connor, P., Jose, J., Giddens, A., Jaiswal, J., Jamieson, S., Bull, M., Denny, W., Akhlaghi, H., Trapani, J., Hill, G., Chang, K., & Gartlan, K. (2019). Inhibition of the cytolytic protein perforin prevents rejection of transplanted bone marrow stem cells in vivo. *Journal of medicinal chemistry*.
30 Tampio, J., Huttunen, J., Montaser, A., & Huttunen, K. (2020). Targeting of Perforin Inhibitor into the Brain Parenchyma Via a Prodrug Approach Can Decrease Oxidative Stress and Neuroinflammation and Improve Cell Survival. Molecular Neurobiology, 57, 4563 - 4577.
31 Gapud, E., Trejo-Zambrano, M., Gómez-Bañuelos, E., Tiniakou, E., Antiochos, B., Granville, D., Andrade, F., Casciola-Rosen, L., & Rosen, A. (2020). Granzyme B Induces IRF-3 Phosphorylation through a Perforin-Independent Proteolysis-Dependent Signaling Cascade without Inducing Cell Death. The Journal of Immunology, 206, 335 - 344.
32 Temizkan, M., & Sonmez, G. (2022). Are owned dogs or stray dogs more prepared to diseases? A comparative study of immune system gene expression of perforin and granzymes. *Acta veterinaria Hungarica*.