sciendo

Acta Veterinaria-Beograd 2023, 73 (3), 346-354
UDK: 636.8.09:616-097
636.8:575.1
DOI: 10.2478/acve-2023-0026

Research article

THE IMMUNOLOGICAL ADVANTAGE OF OWNED CATS OVER STRAY CATS: A COMPARATIVE STUDY OF PERFORIN AND GRANZYMES GENE EXPRESSIONS

Mehmet Cevat TEMIZKAN[1]*, Gonca SONMEZ[2], Secil SEVINC TEMIZKAN[3], Elmas ULUTAS[4], Muhammed Hudai CULHA[2]

[1]Department of Genetics, Faculty of Veterinary Medicine, Yozgat Bozok University, Yozgat, Turkey;
[2]Department of Genetics, Faculty of Veterinary Medicine, Selcuk University, Konya, Turkey;
[3]Department of Virology, Faculty of Veterinary Medicine, Yozgat Bozok University, Yozgat, Turkey;
[4]Department of Physiology, Faculty of Veterinary Medicine, Yozgat Bozok University, Yozgat, Turkey.

(Received 29 March, Accepted 02 August 2023)

Some cats live at home with their owners while others live unattended on the streets or in shelters. One might expect that the owned cats would be better prepared for diseases with vaccinations, and good care and feeding conditions whereas the stray cats would be prepared for diseases by constant exposure to microbial factors. However, no study has investigated which group has the stronger immune response against diseases. Cytotoxic T cells and NK cells are known to initiate an immune response that causes apoptosis of the affected cells when stimulated by various factors. This immune response occurs due to an influx of perforin and granzyme proteins into the affected cell. Accordingly, this study compared owned and stray cats in terms of perforin and granzymes gene expression. Blood samples were collected from 30 owned and 30 stray cats, whose health conditions were checked. The samples were analyzed by qPCR for perforin, and granzyme A and granzyme B gene expression. All genes were expressed at a higher level in owned cats, although only the granzyme A gene showed a significant difference ($p<0.05$). This indicates that this gene plays a more active and significant role in cats than perforin and granzyme B, and that owned cats have a stronger immune response to diseases than stray cats.

Keywords: felis catus, gene expression, granzyme, owned cat, perforin, stray cat

INTRODUCTION

Microbiological infections in pet animals reduce animal welfare and create an economic burden for animal owners and shelters. In the fight against these infections, immunology studies are important to minimize the costs of vaccination and treatment. Cats are one of the most adopted animal species among pet animals. While some cats live at home with their owners, others live on the streets or in shelters. Owned cats

*Corresponding author: e-mail: m.cevat.temizkan@yobu.edu.tr

generally are vaccinated, and have good care and feeding conditions whereas stray cats are inevitably more likely to be exposed to infections. Nevertheless, no study has investigated whether good care and feeding conditions or exposure to diseases create an immune advantage for cats.

NK cells and Cytotoxic T Lymphocytes (CTL) play an active role in innate immunity [1-3]. Both cells primarily use proteins called perforin and granzyme in the immune response. In this pathway, the protein synthesized by the perforin gene (PRF1) creates pores on the surface of the infected cells that allow granzyme proteins to enter [4]. Granzymes cause the death of the infected cell by activating apoptotic pathways and degrading both cell and viral DNA inside the cell. Two of the most important of these granzymes are granzyme A (GZMA) and granzyme B (GZMB) [5-7].

GZMA, which is the most abundant granzyme in cytotoxic granules that enter the cell, differs from many other granzymes by initiating a caspase-independent apoptosis mechanism. GZMA accelerates programmed cell death by disrupting chromatin integrity and nuclear membrane stabilization, which impairs electron transport mechanisms in mitochondria, oxidative cell repair, and various complexes [3,8,9]. Although some cells may try to resist caspases and GZMB to avoid apoptosis, caspase and GZMB protease inhibitors are sensitive to GZMA [3]. GZMB, on the other hand, simulates caspase-dependent apoptosis [1,8]. GZMB can kill cells by activating the apoptosis pathway and without the need for apoptosis by impairing virus replication systems [1].

This study compares owned and stray cats in terms of PRF1, GZMA, and GZMB genes, which play important roles in the immune response, as outlined above. This study tests the following hypothesis: because of greater exposure to microbiological agents, the immune systems of stray cats are better prepared against diseases than those of owned cats.

MATERIALS AND METHODS

The current study research protocol received approval from Erciyes University Ethics Committee for the Local Use of Animals in Experiments (No. 19/164). Owners were fully informed of the study and a written informed consent was obtained. Written permission letters were obtained from the shelters.

Thirty owned and 30 stray mixed breed female cats, aged 1-3 years, and weighing 3-5 kg were sampled in Izmir Province in Turkey during August 2021. Blood samples from owned and stray cats were collected at private veterinary clinics and animal shelters, respectively. Shelters records were checked to confirm that each animal had always lived as a stray.

Each cat's health condition was determined by placing a drop of blood on a slide, which was then covered with a coverslip while creating a smear. Prior to the analysis, all samples were stored at room temperature. The analysis was conducted at the Veterinary Faculty Laboratories in Yozgat Bozok University. Each sample was stained

with Giemsa for light microscope examination. As well as leukocyte typing in each sample, leukocytes were counted to confirm that the count fell within the reference range [10,11].

For Peripheral Blood Mononuclear Cell (PBMC) extraction, tubes of ethylenediamine tetraacetic acid (EDTA) were used with at least 3 ml of blood. After centrifugation at 4,000 rpm for 15 minutes at 4 °C, the middle layer of the blood samples between the plasma and red blood cells, which includes both platelets and leukocytes, was added to 140 mM NH4Cl (Ammonium Chloride), 10 mM Tris (Hydroxymethyl) Aminomethane, and 10 ml of RBC Lysis Buffer (Red Blood Cell Lysis Buffer). Following incubation in a shaker for 15 min at room temperature, the samples were centrifuged at 4,000 rpm for 10 minutes at 4 °C. Immediately after extracting the supernatant, the PMBC-containing pellet was frozen in liquid nitrogen. The PMBC pellets were packed in dry ice at -80 °C before being sent to the Genetics Department Laboratories at the Veterinary Faculty in Selcuk University, and stored under the same conditions ready for the RNA isolation procedures described below.

To isolate the RNA, the TRI Reagent Kit (Sigma, Chicago, USA) protocol and Trizol method [12] were used with monophasic solutions of phenol and guanidine isothiocyanate. To eliminate potential contamination with gDNA, the recovered total RNA was treated with DNase I (Thermo Scientific, Germany) following the manufacturer's protocol. Total RNA quantity and quality were assessed through 1% agarose gel inspection and spectrophotometric analysis (NanoDrop ND2000). The latter used wavelength ratios ranging from 1.8 to 2.0 for A260/A230 and A260/A280. After equalizing the RNA concentrations to 1 µg/2.5 µl with nuclease-free water (NFW), cDNA was synthesized from the recovered RNA in a MyCycler-Thermal Cycler (BioRad, Hercules, Calif) with an iScript™ cDNA Synthesis Kit (BioRad, USA) according to manufacturer's protocol.

The National Center for Biotechnology Information (NCBI) GenBank provided the mRNA sequence information while the NCBI Primer Blast and primer design programs IDTDNA and Oligo 7 were used to design the primers. None of the primers formed self- nor hetero-dimers [13]. The NCBI BLAST program was used to test the primers' accuracy and which sequences to amplify. The primers were based on sequences containing exon linkages that did not amplify any other feline genes (Table 1). The tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) gene, is the most suitable gene for blood studies in cats [14-16], was chosen as the internal control gene.

For qPCR, a Light Cycler Nano System (Roche Diagnostics, Germany) using iTaq Universal SYBR Green qPCR Kit (BioRad, USA) was used according to the manufacturer's protocol, and prepared with the following reaction mix: 5 µL master mix, 50 pMol forward primer, 50 pMol reverse primer, 2 µL cDNA in a total volume of 10 µL with NFW. The qPCR steps were as follows: 98 ^{o}C for 3 min, 35 cycles of 95 ^{o}C for 15 s, 56–63 ^{o}C for 30 s, 72 ^{o}C for 30 s, and a melting program that ranged from 60 to 95 ^{o}C at a heating rate of 0.1 ^{o}C /10 s. Continuous fluorescence measurement

was conducted to confirm that the amplification was specific. All reactions were carried out in triplicate for each sample. The amplicons obtained in qPCR were confirmed to be the target sequence by observing the band sizes on 1% agarose gel.

Table 1. Primer sequences and informations

Gene	Primer sequence	Amplicon length	Annealing temperature	NCBI GeneBank Number
PRF1 – F PRF1 – R	TGGTGGAATGTCGCTTCTACAG GGTGCCGTAGTTGGAGATGAG	146	61ºC	NM_001101660
GZMA– F GZMA – R	AACGTCCCAGGTCATTCTTG TGGTTCCTGGTTTCACATCA	218	56ºC	XM_045060721
GZMB – F GZMB – R	CTCTGGTGGGAGCTAAAAGAGA ATCAGGAACCCACCACACTTAC	232	62.6ºC	XM_006932823
YWHAZ – F YWHAZ – R	GATGGCTCGAGAATACAGAGAGA CAACCTCAGCCAAGTAACGATAG	167	56ºC	XM_006943327

The relative gene expression fold changes of the studied genes between groups were calculated using the criteria of Livak and Schmittengen [17] with the $2^{-\Delta\Delta Ct}$ method. As mentioned earlier, the internal control gene was YWHAZ while the control group was the stray cats group. The significance of the differences in gene expression between the two groups was tested with independent T-Tests with $p<0.05$ representing statistical significance [18].

RESULTS

Our results show that in owned cats PRF1 was expressed 2.20 times more, GZMA 2.67 times more, and GZMB 1.68 times more. However, while the difference in gene expression between owned and stray cats was statistically significant for the GZMA gene ($p < 0.05$), it was not for the PRF1 and GZMB genes (Figure 1; Table 2).

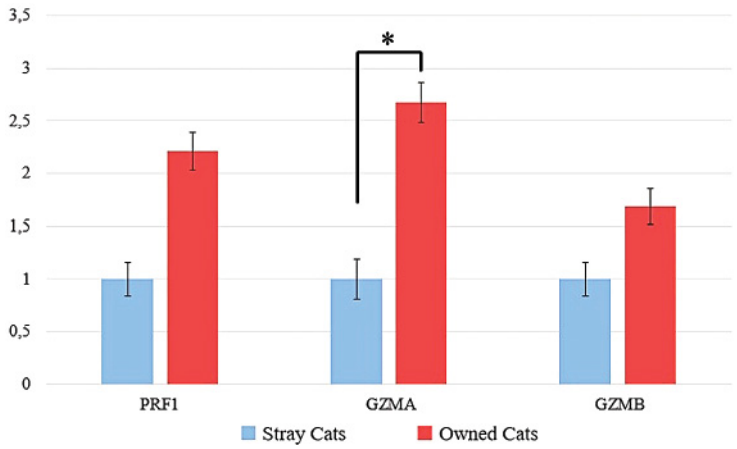


Figure 1. Relative Gene Expression Differences; *: $p<0.05$

Table 2. Relative Gene Expression Differences

Gene	Group	$2^{-\Delta\Delta Ct}$	Standard Error	p value
PRF1	Owned cats	2.2077	0.158986628	0.1592
	Stray cats	1	0.178989151	
GZMA	Owned cats	2.6754	0.192615590	0.0276*
	Stray cats	1	0.187892422	
GZMB	Owned cats	1.6879	0.155766898	0.3131
	Stray cats	1	0.174038369	

*: $p<0.05$

DISCUSSION

Studies of the feline PRF1 gene have shown that PRF1 expression is increased in vaccinated cats [19,20]. Feline Immunodeficiency Virus (FIV), one of the most common diseases in both owned and stray cats worldwide [21], was the subject of a study by Simoes et al. [22] which showed that in vitro stimulation of pathogen-free cats and cats previously infected with FIV increases PRF1 gene expression in pathogen-free cats. However, they found no difference in PRF1 gene expression of NK cells in cats treated with Listeria monocytogenes compared to untreated cats. Similarly, PRF1 gene expression did not change after NK cells were stimulated with Interleukin-15 (IL-15) [23]. In a case report, PRF1 proteins were found to be concentrated in tumoral regions [24]. This indicates that PRF1 expression is greater in regions induced by the tumoral factor. Previous studies indicate that PRF1 gene expression increases after vaccination, infection, or in tumoral conditions, but not with parasitic infections. The cats used in our study were found to have no infections, parasites, or tumoral conditions while the owned cats had definitely been vaccinated. Although the difference was not statistically significant, PRF1 gene expression was higher in owned, vaccinated cats than stray cats. Thus, our results are at least consistent with those of previous studies.

PRF1 and GZMB are generally examined together in studies of cats [2,15,16,23,25,26] because GZMB needs PRF1 to operate apoptotic pathways inside the cell [27,28]. In addition, GZMB, which enters the cell without pores, can be effective in the presence of PRF1 [26]. Stimulation of NK cells with IL-15 increases PRF1 and GZMB gene expression [2,15,23], and expression of both genes increases in allergic reactions [25]. Spiri et al. [16], examined immune gene expression in cats vaccinated against Feline Calicivirus Virus (FCV) and cats infected with FCV. GZMB was expressed at a higher level than PRF1 in both groups. Studies of the GZMB gene suggest that it plays a more active role in the immune system than PRF1. Although neither PRF1 nor GZMB gene expression was statistically significant in our study, PRF1 expression was 2.20 times higher and GZMB was 1.68 times higher in owned cats. This is consistent with most previous studies but inconsistent with Spiri et al. [16], who reported that PRF1 gene expression was higher than GZMB in both FCV-vaccinated and FCV-treated cats. The

reason for the difference in our study could be that the owned cats were sampled a long time after vaccination while the stray cats were healthy at the time of sampling.

In cells that are resistant to GZMB, GZMA affects caspases and various IL pathways [3,8]. Simoes et al. [22] showed that the application of *Listeria monocytogenes* to cats does not affect GZMA gene expression. Additionally, Simoes et al. [22] found no difference in GZMA gene expression following in vitro stimulation of pathogen-free cats and cats previously infected with FIV. In another study, GZMA gene expression increased significantly in FIV-positive cats. Following continuous stimulation with lipopolysaccharide, GZMA expression was suppressed, although it remained at a higher level than in the control group [29]. In our study, it is noteworthy that GZMA gene expression was 2.67 times higher ($p < 0.05$) in owned than stray cats. These results suggest that owned cats gain a significant immunological advantage over stray cats regarding GZMA gene expression.

PRF1, GZMA, and GZMB genes are highly effective in primary defense [5]. The results of a study on owned and stray dogs reveal that GZMB gene expression is significantly higher in stray dogs whereas there are no significant differences for PRF1 and GZMA gene expression [30]. In another study examining 17 different felid species, nucleotide sequences of PRF1, GZMA and GZMB showed high similarities. All trees derived from coding sequences expressed phylogenetic relationships corresponding to the zoological taxonomy of the Felidae, except GZMA [6]. Bubenikova et al. [31] found no significant differences between different cat breeds for PRF1, GZMA, and GZMB genes. Li et al. [7] reported significant differences in NK cells across 12 different species, including pet animals like cats and dogs. More specifically, GZMB genes are expressed more than any other genes in NK cells in dogs, so this gene can act as a marker. Similarly, GZMA genes are expressed more than any other genes in NK cells in cats, so can also act as a marker. Our study also clearly demonstrated the importance of the GZMA gene for cats. Taken together, our study and previous studies [6,7,30,31] indicate that there are important immune system differences between pet cats and dog species, although not between breeds and related species. While stray dogs gain an immunological advantage in the fight against diseases [30], the opposite is true for cats: owned cats gain an immunological advantage compared to stray cats.

Our findings do not imply that PRF1 and GZMB gene expression is unimportant. Rather, it suggests that GZMA expression is more important than the other two genes in cats. Therefore, immune system research on cat species will provide more meaningful results by focusing on the GZMA gene and its protein. Evaluated together with other studies, granzymes are more important for immunity than perforin. That is, owned cats, which have been vaccinated, and have better care and feeding conditions, have a stronger immune response than stray cats.

Acknowledgements
This work was supported by the Yozgat Bozok University Scientific Research Projects Unit, (Grant Number: 6602b-SMYO/19-336).

Authors' contributions

MCT and SST conducted the field study and processed blood samples for analysis. Health check was applied by EU. RNA isolation, cDNA synthesis, qPCR and statistical analysis were carried out by GS and MHC. MCT controlled the final results and wrote the manuscript draft. GS, SST, EU and MHC revised the manuscript draft. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Statement of Informed Consent

The owner understood procedure and agrees that results related to investigation or treatment of their companion animals, could be published in Scientific Journal Acta Veterinaria-Beograd.

REFERENCES

1. Afonina IS, Cullen SP, Martin SJ: Cytotoxic and non-cytotoxic roles of the CTL/NK protease granzyme B. Immunol Rev 2010, 235: 105-116.
2. Fehniger TA, Cai SF, Cao X, Bredemeyer AJ, Presti RM, French AR, Ley TJ: Acquisition of murine NK cell cytotoxicity requires the translation of a pre-existing pool of granzyme B and perforin mRNAs. Immunity 2007, 26: 798-811.
3. Lieberman J: Granzyme A activates another way to die. Immun Rev 2010, 235(1): 93-104.
4. Hoves S, Trapani JA, Voskoboinik I: The battlefield of perforin/granzyme cell death pathways. J Leukoc Biol 2009, 87: 237-243.
5. Aranyos AM, Roff SR, Pu R, Owen JL, Coleman JK, Yamamoto JK: An initial examination of the potential role of T-cell immunity in protection against feline immunodeficiency virus (FIV) infection. Vaccine 2016, 34: 1480-1488.
6. Futas J, Oppelt J, Vychodilova L, Burger P, Horin P: The deadly face of felid killer cells: The cytotoxic proteins and their genes. HLA 2022, 100: 37-51.
7. Li Z, Sun C, Wang F, Wang X, Zhu J, Luo L, Ding X, Zhang Y, Ding P, Wang H, Pu M, Li Y, Wang S, Qin Q, Wei Y, Sun J, Wang X, Luo Y, Chen D, Qiu W: Molecular mechanisms governing circulating immune cell heterogeneity across different species revealed by single-cell sequencing. Clin Transl Med 2022, 12: e689.
8. Bots M, Medema JP: Granzymes at a glance. J Cell Sci 2006, 119: 5011-5014.
9. Pinkoski MJ, Green DR: Granzyme A: the road less traveled. Nat Immunol 2003, 4: 106-108.
10. Reece WO, Swenson MJ: The composition and functions of blood. Dukes' physiology of domestic animals. 12th Edition. Cornell University Press, Ithaca, New York, 2004, 26-52.

11. Turgut K: Veteriniary Clinical Laboratory Diagnosis. 2th Edition. Bahcivanlar Press, Konya, Turkey, 2000, 599-665.
12. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987, 162: 156-159.
13. Wang X, Seed B: High-throughput primer and probe design. In Real-time PCR, Taylor & Francis, 2007, 121-134.
14. Kessler Y, Helfer-Hungerbuehler AK, Cattori V, Meli ML, Zellweger B, Ossent P, Riond B, Reusch CE, Lutz H, Hofmann-Lehmann R: Quantitative TaqMan real-time PCR assays for gene expression normalisation in feline tissues. BMC Mol Biol 2009, 10: 106.
15. Robert-Tissot C, Rüegger VL, Cattori V, Meli ML, Riond B, Gomes-Keller MA, Vögtlin A, Wittig B, Juhls C, Hofmann-Lehmann R, Lutz H: The innate antiviral immune system of the cat: molecular tools for the measurement of its state of activation. Vet Immunol Immunopathol 2001, 143: 269-281, 201116.
16. Spiri AM, Novacco M, Meli ML, Stirn M, Riond B, Fogle JE, Boretti FS, Herbert I, Hosie MJ, Hofmann-Lehmann R: Modified-live feline calicivirus vaccination elicits cellular immunity against a current feline calicivirus field strain in an experimental feline challenge study. Viruses 2021, 13: 1736.
17. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method. Methods 2001, 25: 402-408.
18. SPSS: SPSS for Windows, Release 22. SPSS Inc, 2013.
19. Omori M, Pu R, Tanabe T, Hou W, Coleman JK, Arai M, Yamamoto JK: Cellular immune responses to feline immunodeficiency virus (FIV) induced by dual-subtype FIV vaccine. Vaccine 2004, 23: 386-398.
20. Pu R, Coleman J, Omori M, Arai M, Hohdatsu T, Huang C, Tanabe T, Yamamoto JK: Dual-subtype FIV vaccine protects cats against in vivo swarms of both homologous and heterologous subtype FIV isolates. AIDS 2001, 15: 1225-1237.
21. Battilani M, Kaehler E, Tirolo A, Balboni A, Dondi F: Clinicopathological findings in cats tested for feline immunodeficiency virus (FIV) and feline leukaemia virus (FeLV), Acta Veterinaria 2022, 72: 419-432.
22. Simoes RD, Howard KE, Dean GA: In vivo assessment of natural killer cell responses during chronic feline immunodeficiency virus infection. PLoS One 2012, 7: e37606.
23. Rüegger VL: Induction of resistance to herpesvirus and calicivirus in the domestic cat by stimulation of the innate immune system. Doctoral dissertation, University of Zurich, Zurich, Switzerland, 2001.
24. Neta M, Naigamwalla D, Bienzle D: Perforin expression in feline epitheliotropic cutaneous lymphoma. J Vet Diagn 2008, 20: 831-835.
25. Geraldes L, Morgado J, Almeida A, Todo-Bom A, Santos P, Chieira C, Pais ML: Expression patterns of HLA-DR+ or HLA-DR-on CD4+/CD25++/CD127low regulatory T cells in patients with allergy. J Investig Allergol Clin Immunol 2010, 20: 201-209.
26. Metkar SS, Wang B, Aguilar-Santelises M, Raja SM, Uhlin-Hansen L, Podack E, Trapani JA, Froelich CJ: Cytotoxic cell granule-mediated apoptosis: perforin delivers granzyme B-serglycin complexes into target cells without plasma membrane pore formation. Immunity 2002, 16: 417-428.
27. Browne, KA, Blink, E, Jans, DA, Sutton, VR, Froelich, CJ, Trapani, JA: Bacterial toxins replace perforin in the cytosolic delivery of the pro-apoptotic protease granzyme B: further

support for an endosomolytic mechanism in cytotoxic cell granule-mediated apoptosis. Mol Cell Biol 1999, 19: 8604–8615.

28. Froelich CJ, Orth K, Turbov J, Seth P, Gottlieb R, Babior B, Gottlieb RA, Shah GM, Bleackley RC, Dixit VM., Hanna W: New paradigm for lymphocyte granule-mediated cytotoxicity: target cells bind and internalize granzyme B, but an endosomolytic agent is necessary for cytosolic delivery and subsequent apoptosis. J Biol Chem 1996, 271: 29073-29079.
29. Maingat F, Viappiani S, Zhu Y, Vivithanaporn P, Ellestad KK, Holden J, Silva C, Power C: Regulation of lentivirus neurovirulence by lipopolysaccharide conditioning: suppression of CXCL10 in the brain by IL-10. J Immun 2010, 184: 1566-1574.
30. Temizkan MC, Sonmez G: Are owned dogs or stray dogs more prepared to diseases? A comparative study of immune system gene expression of perforin and granzymes. Acta Vet Hung 2022, 70: 24-29.
31. Bubenikova J, Vychodilova L, Stejskalova K, Futas J, Oppelt J, Cerna P, Plasil M, Horin P: The Population Diversity of Candidate Genes for Resistance/Susceptibility to Coronavirus Infection in Domestic Cats: An Inter-Breed Comparison. Pathogens 2021, 10: 778.

PREDNOSTI IMUNSKOG ODGOVORA VLASNIČKIH MAČAKA U ODNOSU NA ULIČNE MAČKE: UPOREDNA STUDIJA EKSPRESIJE GENA ZA PERFORIN I GRANZIME

Mehmet Cevat TEMIZKAN, Gonca SONMEZ, Secil SEVINC TEMIZKAN, Elmas ULUTAS, Muhammed Hudai CULHA

Neke mačke žive kod kuće sa svojim vlasnicima, dok druge žive bez nadzora na ulici ili u skloništima. Moglo bi da se očekuje da će mačke u vlasništvu biti bolje pripremljene za bolesti, a kao posledice vakcinacije, dobrim uslovima nege i ishrane, dok bi mačke lutalice bile pripremljene za bolesti stalnim izlaganjem mikrobnim faktorima. Međutim, nijedna studija nije istraživala koja grupa ima jači imunski odgovor na bolesti. Poznato je da citotoksične T ćelije i NK ćelije (ćelije prirodne ubice) iniciraju imunski odgovor koji izaziva apoptozu inficiranih, ćelija kada su stimulisane različitim faktorima. Ovaj imunski odgovor nastaje kao posledica ulaska perforina i proteina granzima u inficiranu ćeliju. Shodno tome, ova studija je upoređivala mačke u vlasništvu i mačke lutalice u smislu ekspresije gena za perforin i granzime. Uzeti su uzorci krvi od 30 vlasničkih i 30 mačaka lutalica, čije je zdravstveno stanje prethodno provereno. Uzorci su analizirani qPCR metodom na perforin i ekspresiju gena granzima A i granzima B. Ekspresija svih gena bila je na višem nivou kod vlasničkih mačaka, iako je samo gen granzima A pokazao značajnu razliku ($p < 0,05$). Ovo ukazuje da ovaj gen igra aktivniju i značajniju ulogu kod mačaka od perforina i granzima B, i da mačke u vlasništvu imaju jači imunski odgovor na bolesti od mačaka lutalica.