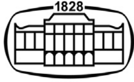

AKADÉMIAI KIADÓ

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

70 (2022) 1, 24–29

DOI:
10.1556/004.2022.00005

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Received: 18 November 2021 • Accepted: 2 February 2022
Published online: 2 March 2022

RESEARCH ARTICLE

ABSTRACT

Stray dogs are inevitably exposed to more infections than owned dogs living indoor. However, no studies have investigated whether the immune system gene expression differs between owned dogs kept in better care conditions and stray dogs living outside. To investigate this, blood samples were taken from 90 dogs (45 owned and 45 stray dogs) that were checked and confirmed as healthy. By using qPCR, the amples were analyzed for the expression of the perforin, granzyme A and granzyme B genes, which are associated with the activation of apoptotic pathways in the immune system. We found that the perforin and granzyme A gene expression levels were higher in stray dogs although the differences were not statistically significant. On the other hand, a 2.81 times higher and a notable difference ($P < 0.001$) was found in the expression level of granzyme B gene in stray dogs. These results indicate that the immune system in stray dogs might be more prepared for diseases than that of the owned dogs and the granzyme B gene plays a more dominant role in the immune response than granzyme A and perforin.

KEYWORDS

canine, gene expression, granzyme, owned dog, perforin, stray dog

INTRODUCTION

Dogs and humans have lived and evolved together for thousands of years since dogs became domesticated. Today, many dogs are owned by humans in many, whereas stray dogs live on the street or in a shelter. Naturally, stray dogs are inevitably exposed to more viral and bacterial agents because they are less likely to be vaccinated or treated, and consume lower quality food. This suggests a potential difference between the immune systems of owned and stray dogs. However, this subject has not been studied before.

The immune response in dogs, just like in many other mammals, especially against viral agents, occurs with two types of cytotoxic cells: cytotoxic T lymphocytes (CTL) and natural killer (NK) cells (Afonina et al., 2010; Lieberman, 2010; Grudzien et al., 2021; Kisseberth and Lee, 2021). These cells mostly use the perforin/granzyme pathway. In this pathway, proteins produced by the perforin gene (PRF1) form pores on the plasma membrane of the target cell after being released onto infected cells by defence cells (Hoves et al., 2009). By deforming the target cell, these pores allow the passage of granzyme proteins into it.

These granzymes have various effects. First, granzyme A (GZMA) induces programmed cell death by disrupting the mitochondria electron transport mechanism (Bots and Medema, 2006; Lieberman, 2010). Second, by targeting nuclear lamin and histone H1, it impairs chromatin integrity and nuclear membrane stabilization to induce apoptosis (Bots and Medema, 2006). GZMA is also sensitive to caspase and granzyme B (GZMB) protease inhibitors (Lieberman, 2010).

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The most studied granzyme B, is produced by other defence cells, such as basophils, mast cells, in addition to CTL and NK cells (Boivin et al., 2009; Afonina et al., 2010). It mostly depends on caspases to cause apoptosis (Bots and Medema, 2006; Boivin et al., 2009). After entering the target cell, GZMB disrupts the mitochondrial outer membrane permeability by cleaving the BH3 Interacting-Domain Death Agonist (BID) protein and activating Caspase 3, Caspase 7 and Caspase 8 *in vitro*, and Caspase 2, Caspase 6, Caspase 7 and Caspase 8 *in vivo* to induce apoptosis (Boivin et al., 2009; Afonina et al., 2010). Apart from the apoptosis pathway, GZMB can also attack viruses by disrupting their replication system and by direct lysis of the viral proteins (Afonina et al., 2010).

The present study aimed to compare owned dogs and stray dogs in terms of activity of the *PRF1*, *GZMA* and *GZMB* genes, which play important roles in the immune response. We wanted to test the hypothesis that the immune system of stray dogs, which live in worse conditions, even if they are healthy, is more stimulated and prepared to respond to pathogen invasion than that of the owned dogs. We expected to show greater expression of the *PRF1*, *GZMA* and *GZMB* genes in stray than in owned dogs.

MATERIALS AND METHODS

Ethics statement

The research protocol of this study was approved by the Ethics Committee of the Erciyes University for the Local Use of Animals in Experiments (No. 19/166).

Animal selection

The study involved 45 owned and 45 stray dogs, of 1–3 years of age, weighing 25–45 kg. None of the sampled dogs was relative. All dogs were mixed breed. Only female dogs were used to eliminate the effect of gender on the results of the study. All samples were collected in Izmir province in Turkey in August of 2020. For owned dogs, blood samples were taken from animals brought to private veterinary clinics for routine check-ups. Extra blood to measure blood parameters was collected with the written permission of the animal owners in accordance with ethical rules. For stray dogs, extra blood was taken during routine veterinary check-ups from dogs in shelters with the written permission of the shelters. Ownership of owned dogs since birth was confirmed from the pedigree documents obtained from the pet owners. Shelter records confirmed that the shelter dogs had been stray since birth.

Leukocyte count and typing

To determine the dogs' health status, blood smears were created immediately. These samples were stored at room temperature until analysis at Yozgat Bozok University Veterinary Faculty Laboratories. The samples were then stained with Giemsa and examined under a light microscope. Leukocyte counting and typing were performed to determine that the leukocyte count of all dogs was within the reference range (Turgut, 2000; Reece and Swenson, 2004).

Peripheral blood mononuclear cell extraction

For peripheral blood mononuclear cell (PBMC) extraction, at least 7 mL of blood was collected in EDTA containing tubes. The blood samples were centrifuged immediately at 4,000 RPM (1,520 RCF) for 15 min at 4°C. The middle layer, containing the white blood cells and platelets between the plasma and red blood cells, was added to 10 mL of red blood cell lysis buffer, 140 mM ammonium chloride and 10 mM Tris (hydroxymethyl) aminomethane. The samples were then incubated in a shaker for 15 min at room temperature. Finally, each sample was centrifuged at 4000 RPM (1,520 RCF) for 10 min at 4°C. After centrifugation, the supernatant was removed and the pellet, containing the PMBC was immediately frozen in liquid nitrogen. All PMBCs were transported to the Genetics Department Laboratories of the Veterinary Faculty, Selcuk University on dry ice and kept at −80°C until RNA isolation.

RNA extraction and cDNA synthesis

RNA isolation was performed by the TRI Reagent Kit (Sigma, Chicago, USA) protocol and Trizol method (Chomczynski and Sacchi, 1987) using monophasic solutions of phenol and guanidine isothiocyanate. Total RNA was then treated with DNase I (Thermo Scientific, Germany), according to manufacturer's protocol, to eliminate possible gDNA contamination. The quality and quantity of the total RNA were evaluated by inspection after electrophoresis in 1% agarose gel and by spectrophotometric analysis (NanoDrop ND2000) based on wavelength ratios for A260/A230 and A260/A280 between 1.8 and 2.0. The RNA concentrations were equalized to 1 µg/2.5 µL using nuclease free water (NFW), then cDNA synthesis from the RNA was performed in a MyCycler-Thermal Cycler (BioRad, Hercules, Calif) using iScript™ cDNA Synthesis Kit (BioRad, USA) in accordance with the manufacturer's kit protocol.

qPCR analysis

mRNA sequence information was obtained from the GenBank of the National Center for Biotechnology Information (NCBI). PCR primers were designed via the NCBI Primer Blast and primer design programs IDTDNA and Oligo 7. The designed primers did not form a self and/or heterodimers, considering the criteria of Wang and Seed (2006). The accuracy of the primers and sequences to be amplified were checked with the NCBI BLAST program (Table 1). The efficiency of the primers was predicted to range between 1.96 and 2.01 (Table 2). The gene of the TATA-box binding protein (TBP), which is the most suitable gene for blood studies in dogs (Peters et al., 2007; Chimura et al., 2011; Park et al., 2013), was chosen as the internal control.

The qPCRs were performed in a Light Cycler Nano System instrument (Roche Diagnostics, Germany) using the

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Table 1. The sequence of the primers and the annealing temperatures, used in the qPCRs for the mRNA quantifications from the genes of the perforin (PRF1), granzyme A (GZMA) and granzyme B (GZMB) proteins

Gene	Primer Sequence (5′ – 3′)	Amplicon Length	Annealing Temperature	NCBI Accession Number
PRF1 - F	CAGGAGCAGAGAACCTACACG	241 bp	61°C	NM_001197182
PRF1 - R	AGCACTTGGCAATGTAGGAGA			
GZMA – F	TGGTTCCTGGAGATTTCTGTG	213 bp	57°C	XM_038658874
GZMA – R	GTTTTTCCGACTCCTTCTTGG			
GZMB – F	CAAGATACGCAGGTACCCAGA	151 bp	58°C	XM_038673283
GZMB – R	TCCCTGAAAGGAAGACTTGGT			
TBP – F	AGGAAGACAACGTAATGGCTGT	212 bp	59°C	XM_038684469
TBP – R	TTCTCTACGCAGAAGGAAGACC			

The expected size of the amplicons is also shown.

Table 2. The predicted efficiency of the qPCR primers to the four genes

Gene	Formula	R^2	Efficieny
PRF1	$y = -3.3013x + 26{,}74$	0.9916	2.01
GZMA	$y = -3.4125x + 21{,}939$	0.9999	1.96
GZMB	$y = -3.4154x + 19{,}781$	0.9993	1.96
TBP	$y = -3.4223x + 23{,}284$	0.9951	1.96

PRF1: perforin; GZMA: granzyme A; GZMB: granzyme B; TBP: TATA-box binding protein.

iTaq Universal SYBR Green qPCR Kit (BioRad, USA) according to the manufacturer's protocol. The reaction mix was prepared as follows: 5 µL master mix, 50 pMol forward primer, 50 pMol reverse primer, 2 µL cDNA complemented with NFW to a total volume of 10 µL. The thermal profile of the reactions was the following: initial denaturation at 98 °C for 3 min, followed by 35 cycles of 95 °C for 15 s, 57–61 °C for 20 s, 72 °C for 30 s, ending with a melting program ranging from 60 to 95 °C with a heating rate of 0.1 °C/10 s and continuous fluorescence measurement to confirm amplification specificity. All reactions were carried out in triplicate for each sample. The specificity of the amplicons, obtained in the qPCR, was also controlled by checking the band size after electrophoresis in 1% agarose gel.

Statistical analysis

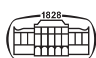
The relative fold changes in the mRNA transcription of the studied genes between the groups were calculated using the criteria of Livak and Schmittengen (2001) with the $2^{-\Delta\Delta Ct}$ method. The *TBP* gene was used as the internal control in the calculation, while the value of the owned dogs (control group) was considered as the base level. Independent *T*-tests were used to test the significance of the differences between the mRNA values of the genes. All these comparisons were done at a significance level of $P < 0.05$ using the data analysis software IBM SPSS 22.

RESULTS AND DISCUSSION

The mRNA level from the *PRF1* gene was 1.53 times higher in the stray dogs than in the owned ones but this difference was not statistically significant ($P < 0.1238$). Similarly, the expression of the *GZMA* gene was measured to be 1.39 times higher in the stray dogs than in the owned ones but this difference was not statistically significant ($P < 0.1618$) either. In contrast, the *GZMB* gene was found to be expressed 2.81 times higher in the stray dogs, and this difference was highly significant statistically ($P < 0.0002$). As it can be seen on Fig. 1 and Table 3, all the three genes, related directly to the immune system, were expressed at a higher rate in the stray dogs than in the owned animals.

GZMA acts through different interleukin pathways in cells resistant to caspases and to GZMB (Bots and Medema, 2006; Lieberman, 2010). Vanherberghen et al. (2012) have found that the *GZMA* gene expression was significantly higher in dogs affected by sino-nasal aspergillosis while *GZMA* expression was higher in dogs with lymphoplasmacytic rhinitis than in healthy dogs but the difference was not notable. In another study, the *GZMA* gene expression has been found higher in individuals with high CXC chemokine receptors, which can be associated with allergy, and lower in individuals with low CXC chemokine receptors (Schulten et al., 2018). In addition, increased *GZMA* gene expression has been found in stimulated NK cells (Gingrich, 2020). Although the *GZMA* gene expression did not differ significantly in our study, it was higher in stray dogs. Hence, our findings are in line with previous studies in terms of *GZMA* gene expression in dogs.

Studies on canine interleukins have shown that interleukin administration increases *PRF1* and *GZMB* gene expression in dogs (Lee et al., 2015; Shin et al., 2015; Grudzien et al., 2021). Chimura et al. (2013) have found that the *PRF1* and *GZMB* gene expression was lower in dogs with mycosis fungoides than in healthy dogs. In another cancer-related study, Inoue et al. (2017) have found that GZMB proteins were considerably more frequent in the tumour tissue of the sick dogs. Canter et al. (2017) have reported increased *GZMB* and *PRF1* gene expression in stimulated canine NK cells. The *PRF1* gene expression has also been found increased in dogs with autoimmune pigmentation disease (Egbeto et al., 2020). Increased *GZMB* gene expression has been observed in tissue rejection in the first two weeks of tissue transplantation in dogs (Ito et al., 1998). Wilke et al. (2012) have reported that *GZMB* gene expression is three times higher in dogs with chronic enteropathy



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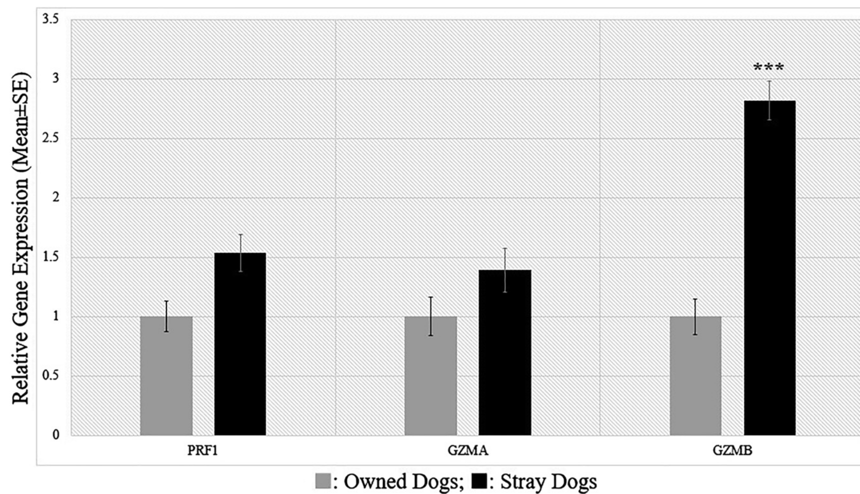


Fig. 1. Relative differences in the expression levels of the genes of the perforin (PRF1), granzyme A (GZMA) and granzyme B (GZMB) proteins between owned and stray dogs. The abundance of the specific mRNA in the peripheral blood mononuclear cells was determined by qPCR. Statistically significant differences and the different confidence intervals are marked. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$

Table 3. Numerical values of the relative gene expression differences

Gene	Group	$2^{-\Delta\Delta Ct}$	Standard Error	*p*-value
PRF1	Owned Dogs	1	0.1555	0.1238
	Stray Dogs	1.5376	0.1304	
GZMA	Owned Dogs	1	0.1823	0.1618
	Stray Dogs	1.3910	0.1607	
GZMB	Owned Dogs	1	0.1652	0.0002***
	Stray Dogs	2.8195	0.1484	

*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.
PRF1: perforin; GZMA: granzyme A; GZMB: granzyme B.

than in healthy dogs. Considering the nature of the diseases in these studies, it can be seen that the results are similar to each other and to our findings. Although the difference was not statistically significant in our study, the *PRF1* gene expression was 1.5 times higher in stray dogs, which does not contradict previous studies. Our statistical findings, especially the higher *GZMB* mRNA level in stray dogs, confirms the important role of GZMB in the immune response.

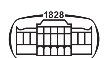
The GZMB and PRF1 proteins play an active role in disease response, especially in the primary defence against viral agents (Chimura et al., 2013; Lee et al., 2015; Shin et al., 2015; Canter et al., 2017; Grudzien et al., 2021). To activate apoptotic pathways in the cell, GZMB and PRF1 must act together (Froelich et al., 1996; Browne et al., 1999). GZMB proteins that enter the cell without pore formation in the plasma membrane are more effective in the presence of PRF1 (Metkar et al., 2002). The significant difference in GZMB expression level in stray dogs in our study suggests that this protein might play a more active role in primary defence than GZMA and PRF1. Indeed, GZMB is more abundant in defence cells than GZMA and PRF1 to activate more apoptotic pathways and act more rapidly in activating the cell's apoptotic mechanisms (Bots and Medema, 2006; Boivin et al., 2009; Afonina et al., 2010). In addition, *GZMB* gene expression is independent of GZMA and PRF1 in some defence cells, such as basophils and mast cells (Tschopp et al., 2006; Afonina et al., 2010). This may explain the higher expression of GZMB. Most importantly, the proteins produced by the *GZMB* gene inhibit viral replication and cause directly the proteolysis of viral proteins (Afonina et al., 2010), suggesting that it is more active that the *GZMA* and *PRF1* genes in the primary defence.

Our study found that *GZMA* and *PRF1* gene expression was higher in stray dogs although did not differ significantly between owned and stray dogs. In contrast, *GZMB* gene expression was much higher in stray dogs. This corroborated our initial hypothesis that stray dogs, which have to live in worse conditions, are more stimulated than owned dogs, even if their immune systems are in the same health condition. Thus, while owned dogs generally live in good conditions, stray dogs are more prepared for the diseases.

In conclusion, the current results indicate that the canine immune response requires prior activation of GZMB because it plays the dominant role but not GZMA or PRF1. The reason for higher GZMB expression in stray dogs is likely to activate more apoptotic mechanisms, be used by more defence cells, and play a direct role in combating viral agents. Although the results show that stray dogs are more prepared for diseases, this should not mean that dogs living on the streets or in shelters are healthier and will live longer. Nonetheless, they may indicate that in the long run, stray dogs can stand more easily the harsh conditions compared to owned dogs.

ACKNOWLEDGEMENTS

This work was supported by the Yozgat Bozok University Scientific Research Projects Unit [Grant Number: 6602B-



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ŞMYO/19-337]. Special thanks are due to Kivanc Karabiyik and Cilem Nur Yildiz for their support in the sample collection.

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