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Contents

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The electrocardiographic changes generated by centrally applied arachidonic acid in rats

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ABSTRACT

Arachidonic acid (AA) and its metabolites have multifunctional regulatory effects on the central nervous system. Our previous reports disclosed that centrally injected AA organized the cardiovascular system in normal or hypotensive conditions by regulating the central and peripheral mechanism. In the light of the knowledge of the potential cardiovascular effects of AA, the current study aimed to investigate the effects of intracerebroventricular (ICV) injected AA on the electrocardiography (ECG) of the anesthetized rats. The adult Sprague Dawley rats were anesthetized with ketamine and xylazine mixture (50 mg/kg and 20 mg/kg; i.m., respectively). Under the anesthesia, the guide cannula was inserted into the left lateral ventricle of the rats. The ECG traces obtained from the lead II were written by placing electrodes on the limbs of the rats. Centrally injected AA (150 µg; ICV) statistically significantly ($p<0.05$) caused to the lengthening of the ECG waves and intervals, resulting in a decrease in the heart rate of the rats without changing the ECG waveforms, the amplitude, and also the isoelectric line. The obtained results clearly show that centrally injection of AA caused the deceleration in the heart electrical activity. The deceleration in the electrical activity of the heart caused to show bradycardia in the rats by extending the duration of the ECG waves and intervals.

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Introduction

Arachidonic acid (AA), a membrane phospholipid, is abundant in central nervous system and involved in multifunction tasks (Rapoport, 2008). AA, itself, and its many biologically active cyclooxygenase (COX) and lipoxygenase (LOX) products play a crucial role in homeostasis, including synaptic signaling, neuronal firing, neurotransmitter release, nociception, neuronal gene expression, cerebral blood flow, the sleep–awake cycle, appetite (Bosetti, 2007). They are also involved in the central modulation of ion channels and the activity of many enzymes, including protein kinase A, protein kinase C, and NADPH oxidase (Katsuki and Okuda, 1995). It was reported that the hyperventilation effect with central AA injection could obtain with both central LOX (Guvenc-Bayram et al., 2020) and COX pathways (Erkan et al., 2016; 2017). Moreover, neuroendocrine effects of AA and its metabolites central injection have also been reported (Yalcin and Savci, 2004; 2007; Aydin and Yalcin, 2008; Yalcin et al., 2005a).

The central AA and its pathways are especially very active in cardiovascular modulation. Our previous report clearly showed that centrally administrated AA could produce a pressor effect by activating central COX-thromboxane A2 (TXA2) -prostaglandin (PG) D, -PGE and -PGF2α signaling pathways in normal and stimulated conditions (Erkan et al., 2016; Aydin and Yalcin, 2008; Yalcin, 2011; Yalcin and Aydin, 2009;

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2011; Altinbas et al., 2014). The centrally injected AA also generated bradycardia in normotensive animals (Erkan et al., 2016; Aydin and Yalcin, 2008; Yalcin, 2011; Altinbas et al., 2014) but tachycardia in hypotensive animals (Yalcin and Aydin, 2009; 2011). Recently we reported that intravenous (i.v.) administered AA caused bradycardia along with delay in heart electrical activity according to electrocardiography (ECG) data (Kasikci and Yalcin, 2022).

According to the previous reports, the centrally applied AA is functional in the cardiovascular system for blood pressure and heart rate, but the role of centrally applied AA on ECG reflecting the electrical activity of the heart is unknown. In the light of previous reports, the aim of the current study is to examine the role of ICV injected AA on the ECG waves as the central effect.

Materials and Methods

Ten Sprague–Dawley rats were used in the study with approving The Animal Care and Use Committee of Bursa Uludag University (2020-03/05). The animals were anesthetized by using ketamine/xylazine (50 mg/kg/20 mg/kg; i.m.) mixture. The rats were kept under anesthesia throughout the experiment. Under the anesthesia, the rats were placed in a stereotaxic frame to insert the guide cannula for ICV injection. For this reason, a burr hole was drilled through the skull 1.5 mm lateral to the midline and 1.0 mm posterior to the bregma according to the coordinates, which were taken from the atlas of Paxinos and Watson (2005). The guide cannula made of 22-gauge steel hypodermic tubing was directed through the hole towards the lateral ventricle. The cannula was lowered 4.2 mm below the surface of the skull and fixed to the skull by using acrylic cement. For the ICV injection, a hand-made injection cannula was used. The injection cannula was connected to a polyethylene tubing, which was filled with saline or saline containing the desired dose of the drug of interest in a 10 µl microsyringe. For the ICV treatment, the injection cannula was inserted through the guide cannula and 5 µl volume of saline or the drug solution was infused slowly within 60 s.

The animals were divided into two groups which included 5 rats in each group, as the control and the experimental groups. The animals in the control group and experimental group were treated with saline (5 µL; ICV) and AA (150 µg; ICV), respectively. After the treatments, the ECG of the rats was recorded for 60 min. AA purchased from Sigma-Aldrich Co. (Deisenhofen, Germany) was freshly disolved in saline on the day of the experiment. The dose of AA was chosen from the previous study (Yalcin, 2011).

The leads II ECG of the anesthetized rats was recorded by inserting the ECG electrodes the limbs of the rats. The ECG traces were analyzed in MP36 system having AcqKnowledge software (BIOPAC Systems Inc.). The P and the T waves duration, the QRS complex duration, and the P-R, the Q-T, and the R-R intervals duration were used as ECG parameters in the present study. The heart rate (HR) of the rats was calculated by using the R-R intervals duration formula and expressed as beats per minute (bpm).

Sigma Stat 3.5 software (CA, USA) was used for the statistical analysis of data. For Statistical analysis, repeated-measures analysis of variance (ANOVA; two-way) and the post-ANOVA test of Bonferroni were preferred. The data given as mean ± standard error of the mean (SEM) in the graphs were considered significant at $p<0.05$.

Results

The basal levels of the ECG waves and intervals duration, and the basal HR of the anesthetized rats for both treatments were shown in Table 1 and Figure 1 as “0” min data, respectively. ICV injection of AA statistically significant ($p<0.05$) caused to increase in the duration of the P wave, the T wave, the QRS complex, the P-R interval, the Q-T interval, and the R-R interval compared to saline treatment (Table 1). Also, ICV injection of AA produced the bradycardia by decreasing the HR of the anesthetized rats (Figure 1) compared to the control animals.

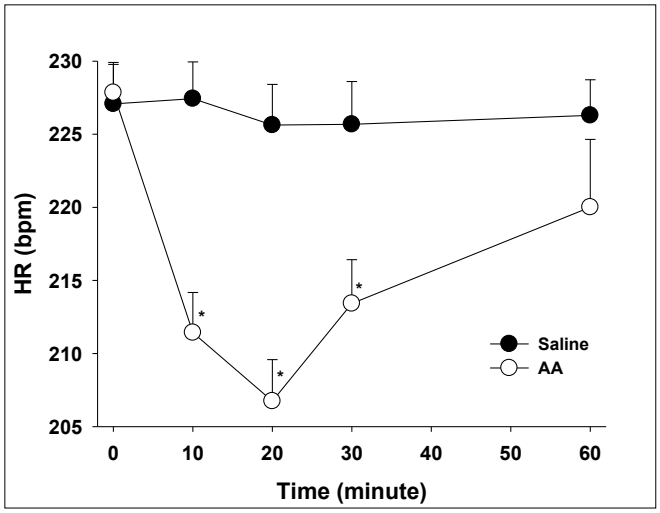

Figure 1. Effect of centrally injected AA on HR in the anesthetized rats. Saline (5 µl; ICV, n=5) or AA (150 µg; ICV, n=5) was injected to the rats. Before and 60 mins after injections, ECG was monitored for the next 60 min. HR measurements obtained from the ECG. Statistical analysis was performed using two-way RM-ANOVA with a post hoc Bonferroni test. *$p<0.05$, significantly different from the value of the saline-treated group.

Table 1. Effect of centrally injected AA on ECG waves and intervals duration in the anesthetized rats.

The duration (Second)		Time (Minute)				
		0	10	20	30	60
P	Saline	$0.0252 \pm 2.0x10^{-4}$	$0.0248 \pm 3.2x10^{-4}$	$0.0248 \pm 4.0x10^{-4}$	$0.0250 \pm 2.0x10^{-4}$	$0.0248 \pm 4.0x10^{-4}$
	AA	$0.0252 \pm 1.7x10^{-4}$	$0.0261 \pm 4.0x10^{-4}$ *	$0.0269 \pm 4.7x10^{-4}$ *	$0.0258 \pm 5.0x10^{-4}$ *	$0.0254 \pm 3.2x10^{-4}$ *
T	Saline	$0.0520 \pm 3.1x10^{-4}$	$0.0510 \pm 3.8x10^{-4}$	$0.0514 \pm 3.4x10^{-4}$	$0.0518 \pm 3.6x10^{-4}$	$0.0508 \pm 3.2x10^{-4}$
	AA	$0.0520 \pm 2.8x10^{-4}$	$0.0618 \pm 2.4x10^{-4}$ *	$0.0686 \pm 3.1x10^{-4}$ *	$0.0604 \pm 2.9x10^{-4}$ *	$0.0568 \pm 2.4x10^{-4}$ *
QRS	Saline	$0.0220 \pm 1.3x10^{-4}$	$0.0219 \pm 4.0x10^{-4}$	$0.0219 \pm 4.0x10^{-4}$	$0.0222 \pm 4.9x10^{-4}$	$0.0218 \pm 4.4x10^{-4}$
	AA	$0.0220 \pm 1.5x10^{-4}$	$0.0239 \pm 6.8x10^{-4}$ *	$0.0244 \pm 4.5x10^{-4}$ *	$0.0236 \pm 4.5x10^{-4}$ *	$0.0234 \pm 4.3x10^{-4}$ *
P-R	Saline	$0.0516 \pm 4.0x10^{-4}$	$0.0515 \pm 4.9x10^{-4}$	$0.0513 \pm 4.0x10^{-4}$	$0.0514 \pm 4.5x10^{-4}$	$0.0516 \pm 4.1x10^{-4}$
	AA	$0.0516 \pm 3.6x10^{-4}$	$0.0526 \pm 4.1x10^{-4}$ *	$0.0529 \pm 4.3x10^{-4}$ *	$0.0520 \pm 5.5x10^{-4}$ *	$0.0524 \pm 3.9x10^{-4}$ *
Q-T	Saline	$0.0700 \pm 4.2x10^{-4}$	$0.0690 \pm 3.3x10^{-4}$	$0.0700 \pm 4.2x10^{-4}$	$0.0690 \pm 4.0x10^{-4}$	$0.0710 \pm 3.5x10^{-4}$
	AA	$0.0700 \pm 2.4x10^{-4}$	$0.0810 \pm 2.4x10^{-4}$ *	$0.0850 \pm 2.9x10^{-4}$ *	$0.0790 \pm 2.7x10^{-4}$ *	$0.0750 \pm 2.6x10^{-4}$ *
R-R	Saline	$0.2900 \pm 1.2x10^{-3}$	$0.2980 \pm 1.4\ x10^{-3}$	$0.2960 \pm 1.3\ x10^{-3}$	$0.2980 \pm 1.4\ x10^{-3}$	$0.2960 \pm 1.5\ x10^{-3}$
	AA	$0.2900 \pm 1.0x10^{-3}$	$0.3120 \pm 0.9\ x10^{-3}$ *	$0.3300 \pm 0.9x10^{-3}$ *	$0.3260 \pm 1.2x10^{-3}$ *	$0.3180 \pm 1.4x10^{-3}$ *

Saline (5 µl; ICV, n=5) or AA (150 µg; ICV, n=5) was injected to the rats. Before and 60 mins after injections, ECG was monitored. The duration of the P wave, the T wave, the QRS complex, the P-R interval, the Q-T interval, and the R-R interval measurements were obtained from the ECG. Statistical analysis was performed using two-way RM-ANOVA with a post hoc Bonferroni test. *p<0.05, significantly different from the value of the saline-treated group.

It was observed that the delay in the duration of the ECG traces and resulting the bradycardia, which was produced by ICV injection of AA, started just after the injection and lasted 60 mins (Table 1, Figure 1). The most potent effects in the ECG traces and the HR were observed 20 min after the AA injection (Table 1, Figure 1). Although ICV injected AA caused to lengthen the rate of the electrical activity of the heart, it did not alter the ECG waveforms, amplitude, and isoelectric line.

Discussion

The current findings demonstrated that ICV administered AA let to bradycardia by prolonging the duration of the ECG waves and intervals without changing the ECG waveforms, amplitude as well as the isoelectric line.

ECG is a simple technic but presents important knowledge about the myocardium's functional and structural characteristics by reflecting the heart's electrical activity. Thus, the ECG gives information about the heart's work with the progression of the action potential produced in the sinoatrial node, which is a natural pacemaker along the atria and ventricles. As a result, an ECG recording shows the P wave during atrial depolarization, the QRS complex during ventricular depolarization, and the T wave during ventricular repolarization (Hall, 2011; Wagner et al., 2009). The current findings have shown that ICV AA administration increases the duration of ECG waves and intervals, resulting in bradycardia. The heart has sympathetic and vagal nerves effects to provide heart rate homeostasis (Zhang and Anderson, 2014). The current findings showing ICV injected AA-induced bradycardia with are consistent with previous reports (Erkan et al., 2016; Aydin and Yalcin, 2008; Yalcin, 2011; Altinbas et al., 2014). The bradycardia and delay in ECG waves observed after the ICV applied AA may be due to the baroreflex response developed as a result of the increase in blood pressure in response to the application of central AA. Because central AA injection causes an increase in plasma catecholamine, vasopressin, and angiotensin levels (Aydin and Yalcin, 2008), which cause an increase in blood pressure and peripheral resistance in normotensive animals (Erkan et al., 2016; Aydin and Yalcin, 2008; Yalcin, 2011; Altinbas et al., 2014), and may mediate the activation of the baroreflex mechanism as a homeostatic mechanism. Moreover, we recently reported that IV injected AA caused bradycardia with delay in ECG waves and intervals in similar way to the current findings (Kasikci and Yalcin, 2022). This effect of IV administered AA may also have exerted a central effect by crossing the blood-brain barrier. Because it is well known that AA can easily cross the blood-brain barrier bi-directly (Pifferi et al., 2021). In addition, it was reported that centrally applied TXA2 mimetic stimulated cardiac vagal afferent fibers to elicit reflex changes in HR resulting the bradycardia (Wacker et al., 2002). This report confirms the bradycardia response with the delay in ECG waves

obtained in the current study. Because centrally administered AA may cause bradycardia by slowing down the electrical activity of the heart by stimulating the afferent fibers of the vagal nerve, similar to the effect of TXA2.

AA, a polyunsaturated phospholipid of the cell membrane, is abundant in the central nervous system (Rapoport, 2008). AA itself is involved in many physiological adjustments, particularly in central cardiovascular regulation (Rapoport, 2008; Bosetti, 2007). Previously we reported that ICV applied AA causes to increase in blood pressure by increasing plasma adrenaline, noradrenaline, and vasopressin levels, and renin activity in normotensive (Erkan et al., 2016; Aydin and Yalcin, 2008; Yalcin, 2011; Altinbas et al., 2014) and hemorrhaged hypotensive rats (Yalcin and Aydin, 2009; 2011). Again, we showed that centrally injected TXA2, one of the AA metabolites, can increase blood pressure in normal conditions and reverse hypotension in hemorrhagic shock conditions by activating brain TXA2 receptors (Yalcin and Savci, 2004; Yalcin et al., 2005a; 2005b; 2006). The activation of peripheral catecholaminergic, vasopressinergic, and renin–angiotensin systems mediates these cardiovascular responses to TXA2 (Yalcin and Savci, 2004). Additionally, our previous report demonstrated that centrally administered melittin, as a phospholipase A2 activator, affects the cardiovascular system and increases blood pressure in both normal (Yalcin et al., 2006; Yalcin and Erturk, 2007) and hypotensive conditions (Yalcin and Savci, 2007). The activation of central TXA2 (Yalcin et al., 2006) or cholinergic nicotinic receptors (Yalcin and Erturk, 2007) is partially involved in these effects of melittin, and the increase in plasma catecholamine, vasopressin, and renin activity mediates the cardiovascular responses to melittin in both conditions (Yalcin and Savci, 2007). Moreover, peripherally injected melittin also causes a pressor effect by activating the central cyclooxygenase (COX) pathway and cholinergic nicotinic receptors (Yalcin et al., 2009). This is because while pretreatment with central indomethacin, a nonselective COX inhibitor, completely blocked the cardiovascular effects evoked by intraperitoneally injected melittin, pretreatment with mecamylamine, a nicotinic receptor antagonist, did so only partially (Yalcin et al., 2009). Central PGD, PGE and PGF2α, AA metabolites (Erkan et al., 2017), and the central lipoxygenase pathway (Guvenc-Bayram et al., 2020) are also involved in the AA produced pressor effect. Centrally administered AA causes an increase in blood pressure in normotensive animals but to decrease in heart rate as similar to the current findings (Erkan et al., 2016; Aydin and Yalcin, 2008; Yalcin, 2011; Altinbas et al., 2014). These studies collectively suggest that the central AA cascade plays a very important role in the central regulation of the cardiovascular system. Consistent with the results of the current study, AA, which plays a role in central cardiovascular regulation, also may direct the work of the heart by affecting the electrical activity of the heart.

Conclusion

In summary, the present findings suggest that ICV administration of AA generates bradycardia by prolonging the rate of the electrical activity of the heart. The similar increase in the duration of the ECG waveforms and intervals might mean that centrally injected AA activates the nervous influence on the heart. The nervous effect on the heart may have occurred directly over the entire heart or through the sinoatrial node. It is possible that the neural effect may have been secondary to the baroreflex response. The fact that the amplitude of the ECG waves and the isoelectric line were not affected strengthens this possibility.

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Exemplifying 'pathobiome' concept through case study: Co-infection with *Vibrio harveyi*, *Photobacterium damsela* and *Cryptocaryon irritans* in Salema (*Sarpa salpa*)

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ABSTRACT

A pathobiome approach has been revealed as a set of organisms (i.e. prokaryotic, eukaryotic, and virus associations) in the aquatic environment that interact with the host (fish) to cause disease. The approach of the one pathogen–one disease is not wholly satisfactory to comment on the impairment of health status and disease process in fish. To exemplify the pathobiome concept, we present a fish disease in which more than one pathogen and possible synergistic interaction of inadequate water quality. In this case, the heavy mortalities were observed in Salema (*Sarpa salpa*) from an exhibition aquaria. Bacterial identification with matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF MS) showed the pathogen bacteria species of Vibrio and Photobacterium. In the parasitic examination, the invasion of marine ciliata, *Cryptocaryon irritans* was observed in the diseased fish. Moreover, poor water quality was considered as contributing factor to disease emergence in this case. The co-existence of pathogen Vibrio species (*Vibrio harveyi*) and *Photobacterium damsela* with its subspecies (subsp. *damsela* and subsp. *piscicida*) as well as marine ciliata, *C. irritans* for a description of a disease picture in a marine fish species are reported for the first time, providing an example of a pathobiome paradigm.

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Introduction

In the aquaculture systems, aquatic organisms, including fish and aquatic microbiome, interact closely with each other, leading to the complexity and dynamism of the marine environment (Tsang et al., 2021). Microbial communities associated with water and fish (host) have been studied for decades. Fish living contiguous to a potpourri of microbes continually interact with the surrounding environment. A single microliter of marine water can contain several microbial communities; thus, marine water has been expressed as a "microbial soup" in terms of a wide variety of microbiome diversity (Bass et al., 2019). Recently, the concept of 'pathobiome' has been very much in the foreground. Basically, the term 'pathobiome' refers to the host-associated pathogens and impaired health status (Stentiford et al., 2017; Bass et al., 2019). Vayssier-Taussat et al. (2014) have put more emphasis on environmental factors in describing the pathobiome concept. The interactions of the various microorganisms are

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remarkable within the context of pathobiome. Increasing scientific facts about the pathobiome phenomenon, integrating the pathogen communities, the aquatic organism, and the water quality in a new understanding of the cause of the disease condition. The one pathogen–one disease approach is insufficient to express how the disease develops particularly in the aquatic environment; therefore, the approach in the aquatic environment has moved away from one pathogen-one disease to the direction of the pathobiome concept.

Bacteria belonging to the *Vibrio* genus have been known to be abundant and the most diverse group in marine environments. The *Vibrio* species are critical in marine aquaculture because several species such as *Vibrio harveyi*, *V. anguillarum*, *V. alginolyticus* and *V. parahaemolyticus* can cause serious diseases in fish and shrimp, resulting in increased mortality rates (Mougin et al., 2020). *V. scophthalmi* and *V. ichthyoenteri* are other opportunistic pathogens in the marine environment, leading to disease or death in fish when the environmental conditions are impaired.

Pathogenic ciliate, *Cryptocaryon irritans* is an obligate parasite, causing serious diseases known as cryptocaryonosis or the marine white spot disease in commercial marine farms and ornamental fish culture systems (Vendramin et al., 2016; Munisea et al., 2020; Cascarano et al., 2021). Some characteristics of this parasite are the ability to infect the fish with low host specificity and high virulence levels (Li et al., 2022). The place of fish ciliata and associated bacterial microbiome in the pathobiome concept has been well documented by Jahangiri et al. (2021); hence, symbiosis with *V. harveyi* was evidenced for *C. irritans* by Qiao et al. (2017).

Identification of pathogens, particularly diverse types of bacteria are more substantial in pathobiome concept. The developed techniques in identifying various bacteria species have benefits to pathobiome study. Advanced tools such as matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF MS) enable easier, correct, and rapid discrimination of bacteria species. MALDI-TOF MS has been considered suitable for species identification of the pathogens found in the aquatic environments, particularly for *Vibrio* spp. (Jansson et al., 2020; Mougin et al., 2020). MALDI-TOF MS analysis based on the mass distribution of bacterial proteins has paved the way for the identification of the diverse bacteria in the present study.

In this case study, the multi-species causal pathogens and possible interaction with the water quality in the development of the disease in salema have been studied in the pathobiome concept.

Materials and Methods

Ethical approval: Fish were handled by the international animal ethical rules. Fish examined in this study were moribund.

Case definition: The disease outbreak in salema (*S. salpa*) occurred in an exhibition aquarium of Ankara, Turkey. Clinical signs of the diseased salema (N=30) were skin lesions with ulcerations, scale loss, and extensive hemorrhaging on the body surface (Figure 1). Among the behavioural signs of the outbreak the erratic swimming before death was indicated as an important sign by the aquarium managers. The mortality reached 100 % in several days. Other various fish species such as sharks and rays were living in the aquarium. These species had no disease signs. Water quality parameters in the aquarium were water temperature: 23.5°C; the salinity: 26-27 ppt; the dissolved oxygen concentration: 7.5 mg/L; un-ionized ammonia 0.1 mg/L. Fish were fed *ad libitium* with commercial feed containing approximately 35% raw protein. The model of aquarium made the routine aquarium husbandry, water or fish sampling difficult, resulting in problems in monitoring of fish and water quality.

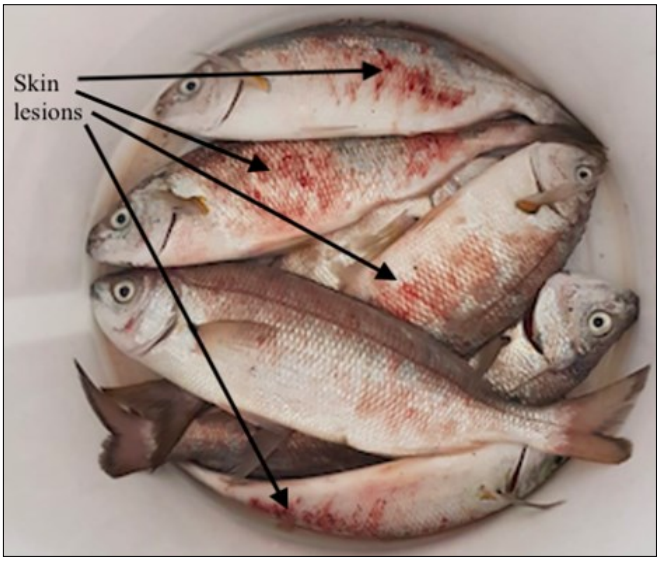

Figure 1. Salema (*S. salpa)* with lesions on the skin

Fish samples: Moribund salema (*S. salpa*) with disease symptoms were obtained from the exhibition aquarium. Fish weight was around 80 to 100 grams.

Isolation of bacteria from diseased *S. salpa:* The collected fish samples were processed in the Fish Health Laboratory, Department of Fisheries&Aquaculture, Ankara University, by following the standard microbiological methods. The samples for bacterial isolation were taken from the kidney and liver of 10 moribund salema and streaked onto Marine Agar 2216 (Difco). The plates were incubated at 22°C

Agar 2216 (Difco). The plates were incubated at 22°C for 48-72 h. The subculture from the fresh bacterial culture was done to obtain a single colony for the MALDI- TOF MS analysis.

Identification of bacteria using MALDI-TOF MS profiling: Bacteria species were identified through Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) by following the workflow for the MALDI Biotyper system (Bruker Daltonics, Bremen, Germany) and previous work (Dieckmann et al., 2010; Sogawa et al., 2011). Briefly, the "ethanol formic acid extraction" protocol was applied for the protein isolation of bacteria. The mass spectrum of bacteria grown on the marine agar, was performed by Microflex LT and analyzed using the MALDI Biotyper software package (version 3.0). Mass spectrum profile (MSP) was compared with the strains in the Bruker reference library (Version 9.0.0.0).

In evaluating the results, log-score values were classified by the MALDI Biotyper. A log score between 2.300 and 3.000 indicated a highly probable species identification. A log-score between 2.000 and 2.299 indicated secure genus identification, and probable species identification. A log-score between 1.700 and 1.999 indicated a probable identification at the genus level only. A log-score below 1.699 indicated no reliable identification.

Parasite examination: All moribund salema were examined under a microscope for the ectoparasites. The gill filaments of moribund salema invaded by *C. irritans* trophonts. To confirm the parasite was *C. irritans* the stages of tomont and theront were also observed through the propagation in seawater-adapted guppies. Briefly, guppies were acclimated to salt water gradually in three days. First day guppies were directly transferred to the water at 10 ppt salinity then, salinity increased to 18 ppt (2nd day) and 25-26 ppt (3rd day). The prevalence (%) and the mean intensity of the parasite in salema were determined as explained by Bush et al. (1997).

Results

MALDI-TOF MS results for culturable bacteria: MALDI -TOF MS results for culturable bacteria from the internal organs of diseased salema showed the log scores in the range of 2.300 and 3.000, corresponding to 'highly probable species identification' (Table 1). The log scores below 2.300 were excluded from the results. MALDI-TOF MS identified the profile for *Photobacterium damsela* with scores 2.25 to 2.009 in kidney samples of salema. The identification index of the MALDI BioTyper software log scores values for the liver sample was 2.24 for *V. harveyi*.

Mass spectrum of culturable bacteria species from the kidney and liver of diseased salema showed the *P. damsela subs*. piscicida and subs. *damselae* as well as *V. harveyi* based on the Bruker database (Figure 2).

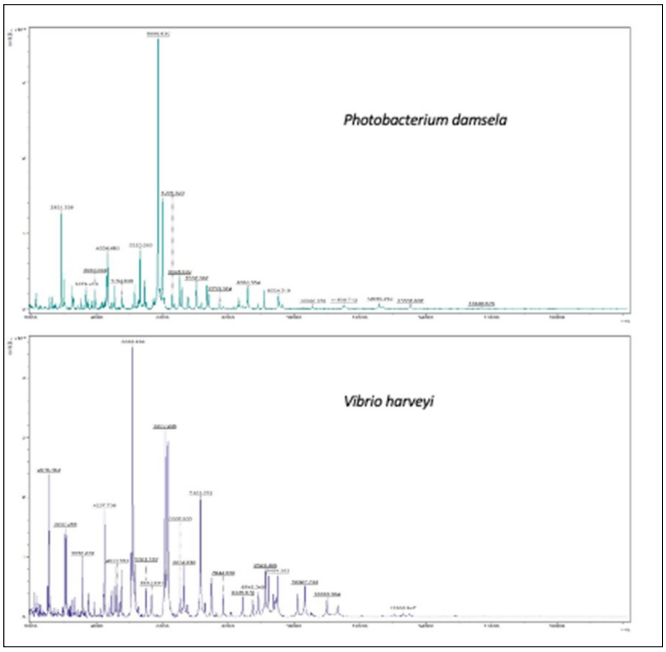

Figure 2. Representative mass spectra for *Photobacterium* and *Vibrio* species.

Table 1. MALDI-TOF MS profile based identification of bacteria species in infected salema, *S. salpa*

Sampling organ	Identification with MALDI-TOF	Log score value*	NCBI Identifier	Identifier isolate by Bruker database
Kidney	*Photobacterium damselae* subs. *piscicida*	2.25	txid38294	DSM 22834T DSM
	Photobacterium damselae subs. *damselae*	2.009	txid85581	DSM 7482T HAM
Liver	*Vibrio harveyi*	2.248	txid669	DSM 19623T DSM

*A log score bigger than 2.3: highly probable identification at the species level; between 2.000 and 2.299: secure genus identification, probable species identification; between 1.700 and 1.999: a probable identification at the genus level; below 1.699 no significant similarity with Bruker reference library 9.0.0.0.

Basic quantitative results for parasite, *C. irritans*: Of the quantitative parameters of parasite, *C. irritans* in the gills of salema, the prevalence was found to be 100% and mean intensity 57.73±1.35. Tomonts passaged from the sea water adapted guppies were about in 450 µm in size (Figure 3).

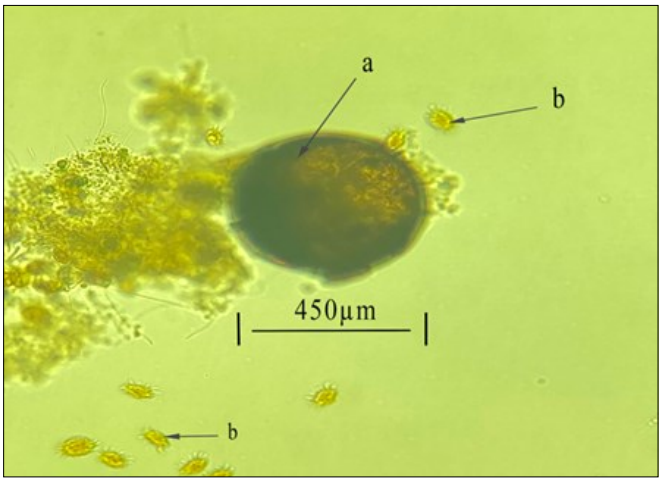

Figure 3. *Cryptocaryon irritans* tomont (a) and theront (b)

Discussion

The heterogenous pathogens involved in a disease case in salema in this study were evaluated in the frame of pathobiome concept. In this study, pathogens found on the moribund salema, *S. sarpa* were both bacteria and parasite; several Vibrionacea family members and marine ciliata, *C. irritans*. The evidence for the inclusion of various bacteria; Vibrio and Photobacterium in one disease picture as provided here help us to understand the factors in a disease occurence. It is clear that the one-pathogen-one disease is not realistic in aquatic environment as exemplified in this study.

The culturable bacteria profiling with MALDI-TOF MS showed *V. harveyi* and *P. damsela* in internal organs of diseased salema. MALDI-TOF MS, which is mainly based on the profiling of bacterial ribosomal proteins is very efficient in distinguishing Vibrio species (Cheng et al., 2015; Osthoff et al., 2017; Mougin et al., 2020). There are difficulties in identifying Vibrios at the species level, even with some molecular techniques used (Moussa et al., 2021). The advantage of MALDI-TOF MS was better utilized in the identification of Vibrio species in this study. MALDI-TOF MS as an advanced tool in bacterial identification has improved our success in the determination of the various bacteria involved in the disease of the salema, contributing to pathobiome approach.

Vibriosis is one of the most common diseases in marine organisms (Ina Salwany et al., 2019). Vibrio spp. is prevailing in seawater aquaria, constituting 60% of the total heterotrophic bacteria (Nagasawa & Cruz-Lacierda, 2004); Various Vibrio species in different fish species have been reported previously (Fabbro et al., 2012; Turgay et al., 2018; Mohamad et al., 2019). *Photobacterium damsela* has been recorded in cultivated marine fish such as meagre, yellowtail, sea bass, and sea bream (Rivas et al., 2013). It should be noted that consists of two subspecies: *P. damselae* subsp. *damselae* and *P. damselae* subsp. *piscicida*. *Photobacterium damselae* subsp. *damselae* has been known as an opportunistic pathogen for fish whilst *P. damselae* subsp. *piscicida* causes pseudotuberculosis in numerous marine fishes (Ina-Salwany et al., 2019). Both subspecies of *P. damsela* were identified in infected salema in this study.

Although the pathobiome approach is relatively new to fish pathology, previous studies have reported the occurrence of diseases due to mixed infections which were referred to as “co-infection” (Kotob et al., 2016). The examples from previous studies on more than one bacterial species were involved in the disease process, such as co-infection with the synergistic interaction of *Aeromonas veronii* and *V. cholera* in koi carp (Han et al., 2021), the concurrent presence of *V. harveyi* and *V. alginolyticus* in hybrid groupers, *Epinephelus polyphekadion* × *E. fuscoguttatus* (Mohamad et al., 2019) and the mix infection with *V. anguillarum* and *Lactococcus garvieae* in rainbow trout (Tanrıkul & Gultepe, 2011).

The parasite, *C. irritans*, at the trophont stage was observed in the all moribund salema, showing the prevalence of 100% in the case presented here. Parasitic invasions that coincide with bacterial infections have been documented in aquatic animals (Kotob et al., 2016; Wise et al., 2021). Pathogen bacteria *V. harveyi* was described as an endosymbiont of the marine Ciliate parasite, *C. irritans*, representing an important example of the relationship between the bacterial microbiome and parasitic ciliates (Qiao et al., 2017). In our study, the co-occurrence of *C. irritans* and *V. harveyi* in salema may be related to this symbiosis. Ciliata parasite and its associated bacteria may render one pathogen-one disease approach pointless; however, it potentially contributes to the pathobiome concept's convenience.

Although the water quality parameters have not been regularly followed in our case unsuitable abiotic factors such as the un-ionized ammonia (0.1 mg/L) in this case is above the acceptable level (<0.05 mg/L) in the water. The increased susceptibility to various pathogens due to adverse water quality in the process of disease development has been documented by Svobodova et al. (2017). For example, the occurrence of *Edwardsiella tarda* has been linked to higher levels

ammonia in water (Karasu Benli & Yavuzcan Yildiz, 2004). In another example, the synergistic effects of *A. hydrophila* and *V. parahaemolyticus* in striped mullet, *Mugil cephalus* have been attributed to adverse water quality including higher levels of nitrogenous products (El-Son et al., 2021). Suboptimal water conditions can facilitate the reproduction of ciliata in warm-water aquaculture systems (Jahangiri et al., 2021). Thus our findings can contribute to the knowledge that poor water quality closes the disease emergence cycle, which can be one of the elements of the pathobiome in aquatic environment.

Conclusion

The one pathogen–one disease paradigm is moving to the pathobiome concept to characterize disease dynamics. In this concept, the combination of multiple pathogens, host, and environment is working together to disease or health of the organism (Vayssier-Taussat et al., 2014). Transitioning from the classic disease triangle comprising the one-pathogen, host, and water environment to a more realistic pathobiome concept would be more useful in solving the disease crisis in aquaculture. Our study contributes to understanding the pathobiome concept, providing the types of pathogens involved, the fish as a host, and the possible effect of impaired water quality holistically.

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JIVS JOURNAL OF ISTANBUL VETERINARY SCIENCES

Pharmacological and clinical approach to plant based complementary health products in lower urinary system diseases in cats and dogs

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Review Article

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ABSTRACT

Medicinal plants, which are widely used in the treatment of many diseases in folk medicine, are alternative treatment approaches that allow to overcome the limitations of modern treatments such as high treatment costs and difficulty in accessing health services. In addition to their traditional uses, the World Health Organization (WHO) also recommends the use of medicinal plants as alternative applications, especially in countries where have limited access to modern medical facilities. Limitations on the methods used in the treatment of lower urinary tract diseases such as urinary tract infection and urolithiasis in both human and veterinary practice (high treatment cost, low tolerability, development of antibacterial resistance, etc.) have brought the use of natural products of herbal origin within the scope of supportive/complementary treatment approaches. Although the mechanism of action of medicinal plants in the treatment of lower urinary tract diseases is not clearly known, studies have shown that they increase the glomerular filtration rate; and they can be complementary alternatives to conventional treatment due to their anti-lithogenic, antibacterial, antioxidant and anti-inflammatory activities. Patients that referred to with one or more of the symptoms of urinary system diseases such as polyuria, pollakiuria, dysuria, stranguria, anuria, hematuria, urinary incontinence constitute the case group of one of the first three systemic diseases most frequently brought to the clinic. Failure to intervene in the diseases shaped in the lower urinary system in a timely manner causes negative consequences such as the disease becoming chronic, the progression of the disease to the upper urinary system in progressive cases, and a decrease in the quality of life. Complementary products used in addition to medical treatment are sometimes used for prophylactic purposes. In this context, many supplements have been prescribed for different diseases in small animal practice in recent years. In this review, it is aimed to convey current developments about medicinal plants, which are used effectively within the scope of supportive treatment practices in lower urinary system diseases of cats and dogs, to veterinary clinical practice.

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Introduction

Plants and herbal materials (phytopharmaceuticals) are increasingly being used by veterinarians and animal owners to prevent and treat animal diseases (Altınok Yipel and Yipel, 2014). Since the development of chemistry, plants and plant-derived materials have largely been replaced by synthetic and semi-synthetic substances in the treatment of human and animal diseases. However with the emergence of side effects associated with conventional drugs, the use of plants and herbal substances has become increasingly popular at human and animal medicine in recent years (Karaoğlan and Özgen, 2011; Raditic, 2015).

It is estimated that more than 80% of drug substances are derived from natural products or are developed from natural compounds, such as antibiotics, cardiovascular drugs, immunosuppressive

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antibiotics, cardiovascular drugs, immunosuppressive drugs, and anticancer agents (Raditic, 2015). Plant-based therapy has a number of advantages, including low cost, fewer side effects, easy accessibility, environmental friendliness. In addition, they also have disadvantages, such as inaccuracies in plant identification, difficulties in dosing, inadequate written records, insufficient scientific studies, and low hygiene standards (Altınok Yipel and Yipel, 2014). Research on plants examines the chemical composition, the clinical and therapeutic effects, but little is known about their toxicological properties. The use of herbal products outside of physician control has been associated with side effects such as direct toxicity, contamination, and drug interactions, which may induce death (Peixoto et al., 2010). Biological activities of medicinal plants should be evaluated in scientific studies in order to determine the effects and toxicities of these plants (Peixoto et al., 2010).

As a result of their antimicrobial, anti-inflammatory and diuretic effects, medicinal plants play an important role in the treatment and/or prevention of diseases such as lower urinary tract infections (UTIs), recurrent lower urinary tract infections (RUTIs), and urolithiasis. Currently, the World Health Organization (WHO) reports that natural products are increasingly used in developed countries to prevent and treat diseases in humans and animals (Bag et al., 2008; Bernal et al., 2011).

Turkey has the richest flora in the Middle East in terms of species rate and diversity of all the countries in Europe with 2891 endemic plants. The Mediterranean (South) region of Turkey has the highest number of endemic species and varieties, with 497 subspecies and 390 varieties (Kendir and Güvenç, 2010). Despite the fact that Turkey has a very diverse flora, studies concerning the use of medicinal plants in lower urinary tract diseases in cats and dogs are relatively limited. The purpose of this article is to provide information about medicinal plants that are effective in supporting treatment protocols for lower urinary system diseases in cats and dogs.

An overview of lower urinary system infections in veterinary medicine

Veterinary practitioners often treat simple lower urinary tract disorders (LUTDs) such as bacterial urinary tract infections (UTIs) using conventional methods with good results. Most simple infections will resolve after two weeks of oral antibiotic treatment, however persistent or recurrent UTIs caused by resistant bacteria may be difficult to treat. These infections are caused by a breach in the host's defense mechanisms, which allows virulent microbes to adhere, multiply, and remain in the urinary tract for a prolonged period of time. An estimated 14% of all dogs in their lifetime are affected by bacterial urinary tract infections, which are more prevalent in female dogs. It is more common in cats over 10 years old, and the incidence increases with age. It is usually ineffective to repeat courses of antibiotics in these patients to maintain long-term urinary bladder sterility, and it is therefore necessary to develop an antibiotic-free treatment strategy to prevent urinary bladder infection. Dogs and cats are susceptible to the same bacteria that cause UTIs; *E. coli* accounts for more than half of all positive urine cultures (Litster et al., 2011; Thompson et al., 2011). Based on a literature review, evidence was developed for the usefulness of non-antimicrobial therapies of urinary tract infections in veterinary medicine using canine/feline cells in vitro, human cells *in vivo*, and *in vitro* cell lines (Raditic, 2015). In this study, its aimed to evaluate the plants for low urinary tract disorders in cats and dogs and to give information about the use of herbal complementary products to veterinary practitioners.

Cranberry (*Vaccinium macrocarpon*)

In vitro and *in vivo* studies have demonstrated the effectiveness of cranberry products in treating/ preventing urinary tract infections and kidney stones (Kingwatanakul, 1996; Terris, 2001; McHarg, 2003). Initially, it was believed that cranberry juice inhibited bacteria growth through acidification of the urine. However, recent studies have begun to focus on the effects of cranberry juice on the inhibition of bacterial adhesion. An important step in the development of urinary tract infections is the adhesion of bacteria to mucosal surfaces (Reid, 1987). A fimbriae in the bacterial cell wall facilitates the attachment of adhesins produced by bacteria to specific receptors on host uroepithelial cells (Beachey, 1981). Cranberry inhibits the adhesion of uropathogens of type I and P fimbriae (e.g., uropathogenic *E. coli*) to the uroepithelium. In addition, it prevents the spread of infections by preventing colonization. Various components in cranberry can also alter the adhesion ability of P-derived uropathogenic bacteria. As well, cranberry can affect bacterial adhesion by changing the cell surface properties of bacteria and causing a positive shift in the distribution of zeta potentials (electric potentials across the interface of solids and liquids). Cranberry juice alters the conformation of P-fimbriae *E. coli* surface macromolecules, particularly reducing their length and density. The cranberry is also believed to reduce fimbrial activity at the genetic level. It has been demonstrated in a study that proanthocyanidins extracted from cranberries inhibit the adhesion of *E. coli* with P-fimbriae to uroepithelial

cell surfaces, suggesting that proanthocyanidins might contribute to the prevention of urinary tract infections (Howell, 1998). Additionally, cranberry juice cocktail has been shown to provide anti-adherence activity against gram-negative rods, including *Klebsiella,* E*nterobacter*, *Pseudomonas* and *Proteus* species (Schmidt, 1988).

The anti-adherence activity of a cranberry product was examined both *in vitro* and *ex vivo* using mannose -resistant hemagglutination urinalysis in six Beagles. In an in vitro assay, collected urine samples were tested for the ability of P-fimbriated *E. coli* to agglutinate human red blood cells after a daily administration of a cranberry product tablet for three weeks. Hemagglutination activity was observed in control urine. Anti-adhesive activity appeared 3 hours after administration of the cranberry tablet, peaked on day 7, and remained high until day 21. Consequently, the researchers hypothesized that the metabolites found in the urine of 6 healthy dogs were sufficient to reduce the adhesion of Pf E coli and may be useful in preventing E coli-associated UTIs in dogs. It has been reported that a clinical trial on affected dogs indicated the need for further study of this extract (Howell et al., 2010).

Parsley (*Petroselinum crispum*)

Parsley is a potent diuretic that works by inhibiting the activity of Na+/K+/ATPases both in the renal cortex and medulla. This inhibition reduces apical cellular N+ reabsorption, decreases K+ secretion, increases intercellular K+ concentration, and consequently passively inhibits passive K+ across tight junctions. Parsley inhibits the Na+ and K+ pumps, thereby decreasing Na+ and K+ reabsorption, which results in osmotic fluid passage into the lumen and diuresis (Kreydiyyeh, 2002). In an experiment with rats, it was found that after ingestion of parsley seed extract, a greater amount of urine volume was eliminated in 24 hours compared to the control group that drank only water. This provides evidence for the diuretic effect of parsley seed extract (Wynn and Fougère, 2007).

Valerian (*Valeriana officinalis*): Valerian (valerian extract) has soothing and calming properties which are beneficial to cats suffering from FLUTD and feline idiopathic cystitis. Despite the fact that there is little research on pets, one study found that Valerian reduced aggression in cats (Wynn and Fougère, 2007). Another study concluded that approximately 50% of cats responded positively to Valerian (Bol et al., 2017). For maximum therapeutic efficacy, valerian may need to be administered for a period of time ranging from a few days to a few weeks in pets (Wheatley 2005; Wynn and Fougère, 2007). It has been reported that some pets respond to Valerian with hyperactivity, the opposite of the intended sedative and calming effects (Kidd, 2000; Wulff-Tilford and Tilford, 1999; Wynn and Marsden, 2003).

Corn silk (*Zea mays*)

This plant is believed to have diuretic and stone-reducing properties. It has been used to treat cystitis, urethritis, nocturnal enuresis, prostatitis, and especially acute or chronic inflammation of the urinary system (Wren, 1988). A high potassium concentration (2,7%) is thought to be responsible for the diuretic effect observed in animals (Bradley, 1992). Maizenic acid has also been suggested to be active ingredient; it acts as a heart tonic and stimulates diuretic activity (Willard, 1991).

Buchu (*Agathosma betulina*)

It has been reported that it can be used to treat acute cystitis, urethritis, nephritis, prostatitis and inflammation of the urinary bladder (Wren, 1988). The essential oils of the bukko bush are thought to be responsible for the antiseptic properties of the plant in the urinary tract. Monoterpene disophenol is the primary component of essential oils believed to have antibacterial properties. In contrast, Didry et al. (1982) found no significant antibacterial activity of buchu oil. There was very minor activity observed against *E coli*, *Saccharomyces cerevisiae*, and Staphylococcus aureus, suggesting little potential for use as an antimicrobial agent (Lis-Balchin, 2001).

Couch grass (*Agropyron repens*)

Diuretic properties are claimed to be associated with this plant. It has been used for cystitis, urethritis, prostatitis, benign prostatic hypertrophy, renal calculus, lituria, and specifically cystitis with urinary tract irritation or inflammation (Wren, 1988). It contains the ingredient mannitol, an osmotic diuretic that can produce a mild diuretic effect when taken in small amounts. A study conducted in rats with calcium oxalate urolithiasis showed that it had no effect on the main risk factors for the development of this disease (Grases, 1995). Triticum 3% to 8%, which is a polysaccharide related to inulin, and 8% to 10% mucilage are both present in this plant. The antibiotic properties of agropyrene and its oxidation products have been documented by Leung and Foster (1996).

Lavender (*Lavandula officinalis*)

Aqueous extracts of lavender were compared with acetazolamide in order to determine their diuretic properties. A significant decrease in urinary osmolarity and moderate sodium excretion was observed during the peak of the diuretic response when compared to acetazolamide, a synthetic diuretic. With the stability of aldosterone and the absence of correlation with

plasma sodium concentrations, together with the observed clearance of free water, it appears that the increase in diuresis is due to tubular activity (Elhajili, 2001).

Dandelion (*Taraxacum officinalis*)

Dandelion extracts have been shown to induce diuresis in rats and mice after oral administration (Rácz -Kotilla, 1974). Extracts from plants have been found to produce more diuretic effects than root extracts; this effect is comparable to that of furosemide. The administration of leaf or root extracts or purified fractions to mice did not significantly increase urine volume or sodium excretion (Hook, 1993). As well, oral and intravenous administration of dandelion root ethanolic extract did not produce diuretic effects in laboratory animals (Tita, 1993).

Juniper (*Juniperus communis*)

Juniper is estimated to increase urine volume without causing electrolyte losses, such as potassium (Blumenthal, 2000). As a diuretic, juniper berries contain the essential oil terpinen-4-ol and hydrophilic compounds that are reported to increase glomerular filtration rate (Foster, 1999). It has also been stated that terpenin-4-ol may cause irritation to the kidneys, but a later review by the same author made no such claim and concluded that there was no danger associated with the oil (Wynn and Fougère, 2007; Tisserand and Young, 2013).

Bearberry (*Arctostaphylos uva ursi*)

Several properties of uva ursi have been described as being diuretic, antiseptic, and astringent. Cystitis, urethritis, dysuria, pyelitis, lituria, and especially acute catarrhal cystitis with dysuria and highly acidic urine have traditionally been treated with this herb (British Herbal Pharmacopoeia, 1983; Wren, 1988). Hydroquinone derivatives, particularly arbutin, are responsible for the antiseptic and diuretic properties of uva ursi. The active compound in arbutin, hydroquinone, is absorbed intact from the gastrointestinal tract and hydrolyzed during renal elimination to produce astringent and anti-septic effects on urinary mucous membranes (Matsuda, 1992).

It should be noted that arbutin is not effective unless the urine is alkaline. The presence of urinary acidifiers prevents arbutin from converting into an active hydroquinone, which reduces the effectiveness of uva ursi (De Smet, 1993). In a pharmacological evaluation, the aqueous extract caused increased urine flow in rats when tested for diuretic activity (Beaux, 1999).

Tribulus (*Tribulus terrestris*)

The ethanolic extract of *Tribulus* berries showed significant dose-dependent protection against uroliths induced in rats. It prevented the deposition of calculogenic material around a glass bead, as well as preventing leukocytosis and serum urea elevation. Additionally, *T. terrestris* has been reported to increase the contractility of the ileum of guinea pigs in addition to its diuretic properties. Based on its diuretic and contractile effects, *T.terrestris* may be able to dissolve urinary stones (Al Ali, 2003).

Kava (*Piper methysticum*)

Kava rhizomes are traditionally used to treat cystitis, urethritis, infections and inflammations of the genitourinary tract, and rheumatism (British Herbal Medicine Association, 2003).

Gravelroot (*Eupatorium purpureum*)

According to the literature, this plant is anti-lytic, diuretic, anti-inflammatory, and antirheumatic. The herb has traditionally been used to treat urinary stones, cystitis, dysuria, urethritis, prostatitis, rheumatism, gout, and especially renal or vesicular stones (British Herbal Pharmacopoeia, 1983; Wren, 1988, Habtemariam, 2001).

Hydrangea (*Hydrangea arborescens*)

It is reported to have diuretic and antilytic properties. The herb has traditionally been used to treat cystitis, urethritis, urinary stones, prostatitis, and enlarged prostate glands, especially pebbles and cystitis stones (British Herbal Pharmacopoeia, 1983; Wren, 1988).

Crataeva (*Crataeva nurvala*)

This herb significantly inhibited the formation of urinary bladder stones in an experimental model using rats, In comparison with the controls, the bladders of the treated animals showed less edema, ulceration, and cellular infiltration (Deshpande, 1982).

It has been reported that the extract administered orally to rats significantly reduces stone formation (Prabhakar, 1997). The extract from the trunk bark of *Crataeva nurvala* has been reported to improve smooth muscle (intestines and ureters) and skeletal muscle tone in guinea pigs, dogs, and humans in vitro (Das, 1974). In dogs treated with Crataeva for 40 days, there was a significant increase in bladder tone (Deshpande, 1982).

Saw palmetto (*Serenoa repens*)

This plant is reported to possess diuretic, urinary antiseptic, endocrinological, and anabolic properties. A traditional use of this herb is in the treatment of chronic or subacute cystitis, catarrh of the genitourinary tract, atrophia of the testicles, sex hormone disorders, and in particular enlargement of the prostate (British Herbal Pharmacopoeia, 1983; Wren, 1988).

Horsetail (*Equisetum arvense*)

The herb has traditionally been used to treat gravel ulcers of the urinary tract and kidney ailments in general. Irrigation therapy can be used to treat bacterial and inflammatory diseases of the lower urinary tract and kidney stones (American Botanical Council, 1998). The haemostyptic effect of the herb may be due to the presence of silicic acid or flavonoids.

Overview of studies on the effect of plants on lower urinary tract diseases

In traditional medicine, medicinal plants and herbal formulas have been used to treat lower urinary tract diseases in many cultures, but little research has been conducted on lower urinary tract diseases in dogs or cats. According to an in vivo study, twelve cats fed herbal formula showed improvements in symptoms related to struvite in the lower urinary tract. Three traditional herbal remedies are commonly recommended for use in cats suffering from lower urinary tract disease (LUTD), including two formulas, San Ren Tang (SRT) and Wei Ling Tang (WLT), and a single herb, Alisma (A). These three herbal preparations are hypothesized to increase urine volume and decrease urinary saturation for CaOx and struvite. According to the results of study conducted by Daniels et al. (2011) SRT, WLT, or A were not found to increase urine volume in cats or decrease urinary saturation for CaOx or struvite; however, no adverse effects were detected during the study; therefore, it was concluded that these herbal products are safe for cats (Daniels et al., 2011).

The traditional treatment for urolithiasis in veterinary medicine has been to modify the diet in order to affect volume, pH, and solute concentration. As part of the herbal treatment, CrystaClair was administered twice a day and abdominal radiographs were taken to assess urolith size and number on presentation and periodically until the uroliths dissolving or the treatment was discontinued. There were no adverse effects reported, and the overall duration of treatment and end point were determined as the resolution of uroliths or the discontinuation of herbal medicine. There was dissolution reported in 56.5% of patients (58% of dogs and 54% of cats) after taking CrystaClair over a period of 4 to 60 weeks. Despite the fact that the researchers reported this case series was not ideal due to the unknown uroliths, they noted this was similar to clinical practice where the composition of the uroliths is often unknown. This study is difficult to interpret due to problems with its design, definition of known priors (surgery and crystalluria), and the use of diet therapy (Wen and Johnston, 2012; Raditic, 2015).

Fourteen Kampo extracts (10 mg/mL) were tested in vitro to determine whether they inhibited the formation of CaOx stones in Madin-Darby canine kidney cells by inhibiting crystal aggregation and adhesion to the renal tubular epithelium. An ethylene glycol (EG) rat model was used to study the inhibitory effects of the extracts on stone formation. The herbs Sanshishi (Gardeniae Fructus) and Takusha (Alismatis Rhizoma) inhibit CaOx monohydrate crystal aggregation (84.5% and 64.2%, respectively) and crystal adhesion to Madin-Darby canine kidney cells (88.2% and 54.6%, respectively). The researchers concluded that Gorin-san, a Kampo formula containing these two herbs, could be used as a prophylactic against CaOx urolithiasis due to its significantly stronger inhibitory effects (Nishihata et al., 2013; Raditic, 2015).

Conclusion

A drug development process is a lengthy, costly, and potentially risky process. Approximately 5,000 to 10,000 molecules are studied before a drug is approved and released to the market. Drug development costs increase as molecules studied become more complex and the requirements for obtaining sales licenses for drugs become more rigorous (Ernst and Young, 2006). It is estimated that millions of people are still unable to access affordable and high quality essential medicines around the world. In this regard, traditional treatment options based on natural compounds may offer new possibilities. Facilitating access to healthcare and overcoming limitations of modern treatments, such as bacterial resistance or high treatment costs, is essential.

The use of herbal medicines in veterinary medicine is becoming more popular day by day. Extensive research data are available on the effect of herbs from the perspective of their therapeutic effects or active ingredients. Plants as antilithogenic, antibacterial, antioxidant and anti-inflammatory agents have been shown to be effective in the treatment of lower urinary tract diseases in cats and dogs. Furthermore, it is seen that information on the therapeutic efficacy of medicinal plants is obtained based on traditional methods rather than scientific research results. As a consequence, scientific validation of the therapeutic effects of medicinal plants, evaluation of toxicity profiles, in vitro studies to determine whether they are cytotoxic and genotoxic, and evaluation of possible side effects that may arise as a result of drug-drug interactions are critical in accepting such drugs as alternative methods in clinical practice.

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Dietary fiber and animal health: A mini review

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ABSTRACT

This mini review describes dietary fibers, their source and compositions. It explores the importance of fiber in the animal diet, health benefit and how fiber contributes to the production of healthy animals in post antibiotics era. The review also discusses fiber fermentation, role in nutrient digestion, enzyme production and how the gut microbiota responds to a selection of fibers. And the components of fiber that increases microbiota which are commensal to the mucus and epithelium of gut. Lastly, recommendations are made on how dietary fiber could be used to achieve maximum advantages in terms of nutrient utilization, performance, and gut health in both monogastric and ruminant animals.

Keywords: dietary fiber, animal nutrition, gut bacteria, gut health

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Introduction

Dietary fiber and their compositions

The ban and regulation of using antibiotics as a growth promoter and to minimize the burden of sustainable feedstuffs for animals, several co-products and feed additives (probiotics, prebiotics, enzymes, phytogenic products, etc.) are considered (Jha et al., 2020). Among them, dietary fiber has received a considerable amount of attention in recent years as fiber components demonstrating beneficial effects in both monogastric (mainly swine and poultry) and ruminant animals. Most commonly fibers are added to the diet, however, fibers having prebiotic function can be delivered via in ovo technology (Das et al., 2021). Broadly, dietary fiber is categorized into two groups that are soluble and insoluble (figure 1)., Soluble fiber, which includes β-glucans (found in barley and oat), arabinoxylans (in wheat and, rye), and pectin (in fruits, and sugar beet pulp), increases intestinal viscosity and decreases the passage rate which helps in nutrient absorption, promote growth performance, and improve gut health which ultimately improves the animal's overall health (Jha & Mishra, 2021). On the other hand, insoluble dietary fiber is found in oat hulls and sunflower hulls which increases chyme retention time in the upper part of the gastrointestinal (GIT), and consecutively, stimulates gizzard development and improve the digestibility of starch and lipids (Jiménez-Moreno et al., 2019). The soluble fiber is fermented more efficiently than insoluble fiber however soluble and insoluble fiber fermentation vary depending on temperature, water content, buffer as the solvent, and fiber to the solvent ratio (Dhingra et al., 2012). Dietary fiber, by recent definition, includes non-starch polysaccharides (NSP), resistant starch (RS), non-digestible oligosaccharides (NDO), and non-carbohydrate polyphenolic ether lignin (Lunn & Buttriss, 2007). The main polysaccharides of non-starch polysaccharides are cellulose, and a wide

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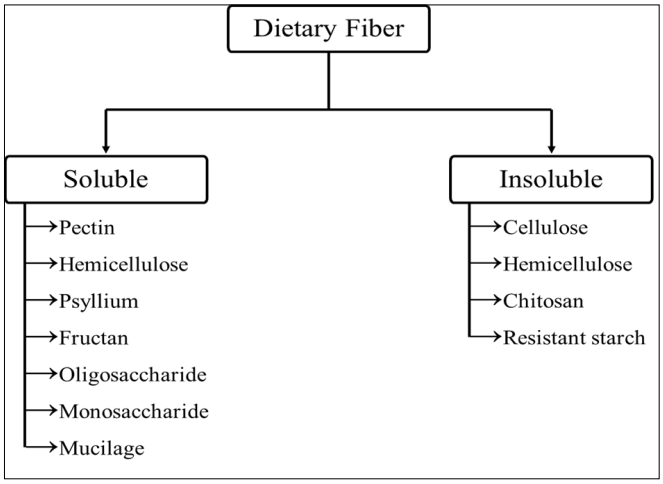

Figure 1. Classification of dietary fiber based on solubility in water . Modified from (Jha and Mishra, 2021).

variety of non-cellulosic polysaccharides (NCP); β-glucan, arabinoxylan, xylans, xyloglucans, and pectic substances to mention the major ones (Knudsen, 2014). The dietary fiber content of the feedstuffs varies widely among cereals generally having a lower concentration in cereals than in legumes and protein-rich crops, and generally, there is a higher concentration of dietary fiber in co-products from cereals and the agro and food industries (Knudsen, 2016). The fiber content of a diet is usually expressed in terms of neutral detergent fiber (NDF) and acid detergent fiber (ADF) (Knudsen, 2016). Acid detergent fiber includes cellulose and lignin as the primary components and concentrations of ADF and lignin are correlated more with digestibility than intake (Van Soest et al., 1991). Neutral detergent fiber includes cellulose, hemicelluloses, and lignin fractions of feeds and is more highly correlated with feed volume and chewing activity than ADF or crude fiber (Jung et al., 1997).

Dietary fiber in poultry nutrition

The addition of fiber in the poultry diet is in trend to utilize non-conventional feedstuffs and contribute as an alternative to the antibiotic in the post-antibiotic era (Lillehoj et al., 2018). The antibiotic is a synthetic, microbial agent which is used widely in poultry to control and treat disease and infection. The researcher found that the inclusion of fiber in the poultry diet help to improve growth performance and overall health (Ali et al., 2021; Mehdi et al., 2018). The co-products of conventional feedstuffs namely wheat bran, sunflower meal, fuzzy cottonseed, oat hulls, soybean hulls, and pea hulls are rich in fiber, and adding these fibers to the poultry diet improves weight gain and feed efficiency (Jha & Mishra, 2021). Considering the laying bird feeding of alfalfa during molting may reduce the stress of egg production (Landers et al., 2005).

Dietary fiber has a great impact on the length and weight of the gastrointestinal tract which helps in the proper function of the gizzard, ultimately improves gut motility, and reduces the risk of gut pathogens and gut diseases like salmonellosis, coccidiosis, and so on (Jha & Mishra, 2021). It may influence epithelial morphology which depends on the type of dietary fiber and its level of inclusion, age of the bird, and site of the intestinal tract. Dietary fiber added to the diet increases the absorption of the mucosal surface of the small intestine by increasing villi height, and mucus secretion (Mtei et al., 2019). Fibers in diets also improve the diversity of the microbiota and stimulate intestinal activities whereas many soluble fibers function as prebiotics which promotes the beneficial bacteria in the gut and induce the production of short-chain fatty acid (SCFA) (Liu et al., 2021). While insoluble fiber shows the preventive method in cannibalism and feather pecking (Mens et al., 2020).

Poultry has both innate and acquired immune systems, the dietary fiber can be used as a cost-effective nutrient to modulate the poultry immune system (Kheravii et al., 2018). With its high fiber content, alfa-alfa has been shown to have a very long transit time in the gastrointestinal tract of chickens (Jiang et al., 2012). Similarly, the inclusion of polysavone (Alfalfa extract) in the diet also increases serum Anti-Newcastle disease virus (Dong et al., 2007). The arabinoxylan from wheat bran increases the goblet cells which produce a protein barrier factor that protect intestinal epithelial cells (Jha & Mishra, 2021).

Dietary fiber in swine nutrition

Globally, a diverse range of fiber-rich feed ingredients is added to swine diets. They include wheat bran, wheat middling, oat husk, maize bran, rye bran, sugar beet pulp and fiber, corn cobs and bran, distillers grains, rapeseed, soya bean hulls, kiwi fruit, and chicory (Jarrett & Ashworth, 2018). Dietary fiber, usually defined as the indigestible portion of food derived from plants, forms a key component of many swine diets. Although not fully digested, dietary fiber can affect different physiological processes, both directly (e.g. by gut filling) and indirectly by the production of short chain fatty acid and physiologically active co-products through fermentation in the colon (Jarrett & Ashworth, 2018).

Although these diets do not always maximize swine performance but provide effective and economical use of locally grown feedstuffs and hence contribute to sustainable production. High fiber diets fed from the beginning of the estrous cycle could have a positive impact during this maturation process (Jiang et al.,

2012). It is believed that this is because the high fiber-rich diets create endocrine profiles and ovarian follicular fluid content that enhance oocyte quality (Revelli et al., 2009). Such oocytes are then more likely to form viable embryos which, potentially through epigenetic mechanisms, are expected to survive throughout gestation and as piglets (Ashworth et al., 2009). A high fiber diet is most advantageous when fed before mating (Gianaroli et al., 2012). The most likely reasoning is that the high fiber diet affects the very early stages of development of the oocyte and embryo, more than factors such as ovulation and fertilization rates.

The inclusion of dietary fibers in the swine diet is sometimes limited because of their anti-nutritive properties (Jarrett & Ashworth, 2018). These include a reduction in the digestibility of dietary energy and protein which may lead to an inadequate amino acid, particularly threonine absorption (Jarrett & Ashworth, 2018). Fiber-rich diets can reduce the post-prandial activity of the pig, including the incidence of non-feeding oral and other stereotypic behaviors (Leeuw et al., 2008). It was also found that pigs fed a high lipid, high fiber diet had lower plasma concentrations of β-hydroxybutyrate, leptin, glucose, insulin, and urea (Yde et al., 2011).

A major concern when including dietary fiber for swine is that high dietary fiber content is associated with decreased nutrient utilization and low net energy values (Lindberg, 2014). Negative impacts of dietary fiber on nutrient utilization and net energy value will be determined by the fiber properties and may differ between fiber sources (Lindberg, 2014). Positive impacts are that it stimulates gut health, increases satiety, affects behavior, and overall improves animal well-being (Lindberg, 2014). Dietary fiber plays a major role in reducing ammonia emission by the growth of beneficial gut bacteria which shifts nitrogen excretion from urea in urine to feces (Jha & Berrocoso, 2016).

Dietary fiber in ruminants

Fiber plays an important role in ruminant nutrition. Fiber is essential to maintain animal health and is required to maintain an appropriate rumen function and physiology (Zebeli et al., 2012). In ruminants, fermentation of fiber occurs in the rumen. Thus, the microbial protein that is produced, as fiber is being digested, and is available for digestion to amino acids with subsequent absorption for use by the animal (Firkins et al., 1998).

Fiber is important in the diet of ruminants because it helps to make their stomach active. Fiber added to the diet 'tickles' the ruminant's stomach to get it to stay active and digest food. Fiber stimulates rumination, chewing, and salivation. It also maintains a normal fat test, normal rumen pH, and normal rumen mat (Ishler & Heinrichs, 2016). Simplistically, the fermentation of fiber (cellulose and hemicellulose) results in the production of acetic acid in liver which is used by the ruminant animal for energy and is the primary precursor of fat in the milk (Kung, Jr, 2014). The amount and size of fiber particles(NDF should be 35% of diet and eNDF) in the diets of lactating dairy cows are important to maintaining optimal rumen function (Zebeli et al., 2008).

Lignification of the plant cell wall generally increases with increasing plant maturity and within specific forage species; increased lignification is associated with reduced digestion by interfering the digestion of cell wall polysaccharides by acting as a physical barrier to microbial enzymes (Moore & Jung, 2001). White rot fungi i.e. Phanerochaeote chrysosporium-like fungi play a primary role in fiber digestion (Susmel & Stefanon, 1993). When ruminants are fed more starch and glucose and less fiber then rumen pH starts to fall causing acidosis (Beef Cattle Research Council, 2019). If rumen pH falls below 6.0-6.2, fiber digestion in the rumen begins to decline. As rumen pH decreases, fibrolytic bacteria in the rumen become less active and fiber digestion is decreased. When ruminal pH falls below 5.8-5.9, the rumen is mildly acidic and fiber digestion in the rumen ceases completely. When ruminal pH drops below 5.2 to 5.5, animals can succumb to acidosis (Kung, Jr, 2014).

Conclusion

Dietary fiber has an important role in the complex interaction between the diet, the endogenous enzymes, the mucosa, and the commensal microflora – all of which are considered important in the assimilation of nutrients and a key component for optimal intestinal health. These include enhanced welfare through increased satiety and reduced stereotypic behaviors (like wall licking, bar biting), a reduced environmental footprint, and improved reproductive efficiency. Studies described in this review have shown both positive and negative effects of feeding a diet high in fiber to poultry, pigs, and ruminants.

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Detection of neutralizing antibodies against SARS-CoV-2 in companion animals in Istanbul

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Research Article

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ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in late 2019 and rapidly spread throughout the world. After the susceptibility of various animals to SARS-CoV-2 infection was reported, the prevalence of COVID-19 infection in pet animals and their role in virus transmission became a source of concern. Seroprevalence of SARS-CoV-2 in pet animals has been reported from various parts of the world to date. In this study, 206 serum samples of household dogs and cats were obtained from a diagnostic laboratory in Istanbul. Ninety-nine of the samples were collected during the second wave (November and December 2020) of the pandemic, and 107 were collected between August and September 2021. Neutralizing antibodies against SARS-CoV-2 in companion animals was investigated by virus neutralization test. Among the 99 serum samples which were collected during the second wave (November and December 2020) of the pandemic, while no neutralizing antibodies were found in 61 sera of cats, one of 38 dogs was positive with a titer of 1/256. Similarly, 0/54 cats and 1/53 dogs were tested positive for the neutralizing antibodies among the samples collected between August and September 2021. This study is demonstrating the evidence of SARS-CoV-2 infection in dogs.

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Introduction

SARS-CoV-2 emerged in Wuhan, China in December 2019 (Zhu et al., 2020) and was defined as a pandemic by the World Health Organisation (WHO) in March 2020. During the rapid spread of SARS-CoV-2 to the entire world via person-to-person transmission, the susceptibility of animals and their role in transmission dynamics have arisen as a concern. The first COVID-19 report in animals was from Hong Kong, viral RNA was detected from oral and nasal swabs of a pomeranian dog with a history of a COVID-19 positive owner in February 2020 (Sit et al., 2020). According to the WOAH Situation Report (31.08.2022), 692 SARS-CoV-2 outbreaks in animals have been reported, affecting 25 species, including animals from zoos (Puma, Lion, Tiger, Gorilla, Ottor, Binturong, Fishingcat, and Coatimundi), pets (dogs, cats, ferrets), wild animals (white-tailed deer), and farm animals (Minks) (WOAH, 2022). Generally, infected companion animals had a

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history of a COVID-19 affected owner (Hamer et al., 2020; Segalés et al., 2020; Sit et al., 2020; Neira et al., 2021; Ruiz-Arrondo et al., 2021). The transmission of the virus from human-to-animal occurs as a result of the close contact between infected people and household animals. Recently, evidence of cat-to-human transmission reported that a pet cat infected a veterinary surgeon in Thailand (Sila et al., 2022) and mink-to-human transmission reported from the Netherlands (Munnink et al., 2021) after mink farms were infected with SARS-CoV-2. Transmission of animal-to-human cases indicate the importance of monitoring the SARS-CoV-2 infections in animals.

With the increasing number of reports stating that the SARS-CoV-2 RNA is detected by molecular methods in pet animals from various countries, further studies were conducted in which serological tests were performed to determine the prevalence of the disease in pet animals. Studies from China (Zhang et al., 2020), Croatia (Stevanovic et al., 2021), Italy (Patterson et al., 2020), Netherlands (Zhao et al., 2021), Germany (Michelitsch et al., 2020, 2021), United Kingdom (Smith et al., 2021), France (Laidoudi et al., 2021) Brazil (Calvet et al., 2021) and several countries (Fritz et al., 2020; Barua et al., 2021; Schulz et al., 2021; Yilmaz et al., 2021) have shown that pet animals develop antibodies against SARS-CoV-2.

In this study, the presence of neutralizing antibodies was investigated in serum samples of cats and dogs which were collected in the second wave of the pandemic and during the dominance of variants.

Materials and Methods

Samples: In this study, 206 sera (91 dogs and 115 cats) were tested, which were provided from a veterinary diagnostic laboratory, where the samples were sent from veterinary clinics for hematological tests for other reasons under the cold chain. Samples were provided in two apart periods. The first group consisted of samples from 61 cats and 38 dogs, which were collected in November and December 2020. As the second group, 54 cat sera and 53 dog sera were collected in August and September 2021. Bio-information of animals was not available in both groups.

Test Virus: hCoV-19/Turkey/Pen07/2020 strain (GISAID Access. ID: EPI_ISL_491476) which was isolated in Pendik Veterinary Control Institute from an oropharyngeal swab of a SARS-CoV-2 patient before (not published), were used in neutralization test.

VNT: Serum samples were heat-inactivated at 56°C for 30 min. The tests were performed in Vero cell culture (3×10^5 cell/ml) grown in 96-well microplates. Serial two-fold dilutions of serum were mixed with the same volume of virus suspensions containing 100 TCID50 virus and incubated at 37°C for 1h. The serum and virus suspension inoculated to four wells of Vero cells and incubated at 37°C for 3 days. Neutralizing antibody titers were recorded as the reciprocal of the highest serum dilution that inhibited 50% of the cytopathic effect per well. With a titer of ≥ 16 samples were considered positive (Zhao et al., 2021). The tests were performed in BSL-3 laboratories of Pendik Veterinary Control Institute.

Statistical Analysis: The number of seropositive animals among samples collected between two separate periods were tested for significant using chi-square test and values of $P \leq 0.05$ were considered significant.

Results

The first cohort was including 99 sera samples collected during the second wave (November and December 2020) of the pandemic. Among these, one serum sample from a dog out of 99 pet animals tested positive for neutralizing antibodies with a titer of 1/256. Neutralizing antibodies were not detected from 61 cat sera, while 1/38 dog sera were positive.

The second cohort consisted of 107 sera (54 cats, 53 dogs), collected in August and September 2021, in which one dog sera was positive with a titer of 1/32. None of the 54 cat sera gave positive results in VNT.

No significant difference detected between the two periods of pandemic in number of seropositive animals ($P>0.05$).

Discussion

SARS-CoV-2 emerged from China and spread to the whole world rapidly. During the spread of the disease via person-to-person transmission, studies have been carried out on the susceptibility of animals to SARS-CoV-2 due to the possibility of inter-species transmission. Experimental infection studies showed that cats, ferrets, dogs, white-tailed deer, golden hamsters, and fruit bats are susceptible to SARS-CoV-2 (Schlottau et al., 2020; Shi et al., 2020; Sia et al., 2020; Palmer et al., 2021). The experimental study on cattle demonstrated that cattle showed low susceptibility and the transmission didn't occur from the infected cattle to naive ones (Ulrich et al., 2020). Also, natural infections revealed that minks, tigers, otters, lions, and gorillas are susceptible as well (WOAH, 2022).

As a result of the quarantine process applied during the pandemic, people have spent more time with pet animals than pre-pandemic period. Close contact of COVID-19 positive owners with pets have let the transmission of the virus to the animals. To

date, SARS-CoV-2 positive companion animals have been reported from various countries. Although it was a question of curiosity whether pet animals play a role in the transmission of the virus to humans, it is now the general opinion that reverse zoonotic cases from humans to companion animals are common. Since investigating SARS-CoV-2 infections in animals by molecular methods is challenging because of the low chance of sampling in the viral shedding period, serological surveillance studies were conducted to put forward the prevalence of SARS-CoV-2 infections in pet animals.

In this study, we investigated the presence of neutralizing antibodies against SARS-CoV-2 in the sera of 91 dogs and 115 cats. The samples were obtained from a diagnostic laboratory, which were sent from the veterinary clinics for hematological tests during routine veterinary visits of pets in Istanbul. Our results are consistent with a study conducted in the United Kingdom, in which none of the 96 serum samples collected from cats during the first wave were positive for VNT (Smith et al., 2021). Although seroprevalence of cats from COVID-19 positive households were remarkably high, such as 23.5% in France (Fritz et al., 2020), 43.8% in the USA (Hamer et al., 2020) and 20% in Brazil (Calvet et al., 2021), studies investigating the seroprevalence of cats without the information of their owners' COVID-19 status, as in this study, resulted in low seropositivity (0.36-1.36%) in Germany (Michelitsch et al., 2020, 2021), the USA (Barua et al., 2021), Thailand (Udom et al., 2021), and Croatia (Stevanovic et al., 2021). Focusing on Istanbul, the sampling area of this study, Yilmaz et al. (2021) investigated the seroprevalence of domestic cats and found three positive with surrogate virus neutralizing test, out of 34 ELISA-positive sera among 155 samples. The discordance between the results of this study and ours may be due to the different testing methods used between the studies.

In the present study, 91 dog sera were tested with VNT, and 2 (2.2%) samples were positive, with titers of 1/256 and 1/32, respectively. Our results are in line with the study from Italy (Patterson et al., 2020), in which after excluding the known COVID-19 status households, the samples collected from the unknown status of COVID-19 environments showed 2.3% positivity by neutralizing tests in dogs during the early pandemic. Results of studies that conducted in the United Kingdom (Smith et al., 2021), the Netherlands (Zhao et al., 2021), France (Fritz et al., 2020), the USA (Barua et al., 2021), and Thailand (Udom et al., 2021) remained below 2% seropositivity.

One of the aims of this study was also to make a comparative assessment between the original SARS-CoV-2 virus circulating period in the second wave and during the dominance of SARS-CoV-2 variants in the late pandemic, which was expected to exhibit higher positivity. According to studies in Germany and the United Kingdom, the seropositivity was higher in the second wave than in the first wave (Michelitsch et al., 2020, 2021; Smith et al., 2021), which is probably due to the cumulative increase of people with a history of COVID-19, reflecting on animals. Additionally, since it is known that the variants are more transmissible (Davies et al., 2021; Public Health England, 2021), it could have resulted in higher rates of infection in companion animals. But these hypotheses are in contrast with our results, which showed that there was not a significant difference between the two periods in this study ($P>0.05$). This might be due to the limited number of our samples.

In this study, both cats and dogs were subjected to investigation. Experimental infection studies have shown that cats are highly susceptible to SARS-CoV-2 and can shed the virus for a prolonged period; however, dogs have low susceptibility, but develop antibodies (Bosco-Lauth et al., 2020; Shi et al., 2020). Based on this information, some seroprevalence studies in companion animals have focused solely on cats. The studies that investigated both cats and dogs generally showed no significant difference on seropositivity between the two species. Companion cats spend most of their lifetime inside the house. In contrast to cats, dogs take their daily walks outside, may interact with other dogs and people closely in common areas, such as parks, etc. As they are more social, in case of the virus shed by dogs, they may be considered potentially more spreader than cats. Since there is still a lack of knowledge about SARS-CoV-2 and it is a highly mutagenic virus, following the transmission of the virus among pet animals is important, but dogs should not be excluded from the seroprevalence studies.

VNT was the method of our study, which requires at least biosafety level 3 conditions. Despite the infectious virus used in some studies (Michelitsch et al., 2020; Patterson et al., 2020; Zhang et al., 2020; Smith et al., 2021) to determine the seroprevalence, some others used pseudotyped viruses with SARS-CoV-2 spike protein (Fritz et al., 2021; Zhao et al., 2021) and surrogate virus neutralization tests (Barua et al., 2021; Yilmaz et al., 2021), which enabled to investigate the neutralizing activity of sera without the need of high-containment laboratories. ELISA is also one of the widely used method that showed more positivity than neutralization tests for the same samples in several studies (Michelitsch et al., 2020; Zhang et al., 2020; Zhao et al., 2021) which might be because of

non-neutralizing but binding antibodies can be detected with this method (Tan et al., 2020), but sometimes it is because of the low specifity of in-house ELISA methods. For this reason, serum samples that are detected positive by in-house ELISA methods should be confirmed by neutralization tests. The variety of antibody detection methods limits to draw a precise comparison among the studies on seroprevalence. In this study the samples were not tested with other coronaviruses since it has been reported previously that there was no cross-reactivity between SARS-CoV-2 and canine respiratory coronavirus (CRCoV),feline coronavirus (FCoV) and canine enteric coronavirus (CeCoV) (Michelitsch et al., 2020; Patterson et al., 2020; Zhang et al., 2020; Smith et al., 2021; Stevanovic et al., 2021).

Conclusion

This study conducted in Istanbul, the biggest city of Turkey which was affected severely by COVID-19. The results of this study shows the evidence of SARS-CoV-2 infection in dogs. Companion animals are susceptible to SARS-CoV-2 and can develop neutralizing antibodies. COVID-19 positive people should avoid close contact with the animals they live with at home, and they should include the companion animals in-home quarantine procedures. The results of this study reinforce the importance of the One Health approach.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical statement: Pendik Veterinary Control Institute Animal Experiments Local Ethics Committee decided that no ethical approval was required for this study.

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Could polymerase chain reaction be an alternative diagnostic method for dermatophytes?

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Review Article

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ABSTRACT

Dermatophytosis are cutaneous mycoses caused by *Microsporum* spp., *Trichophyton* spp. and *Epidermophyton* spp. dermatophytes. Dermatophytosis resembles other skin diseases due to its various clinical manifestations such as multifocal alopecia, circular lesions, scaling, crusting, papular and pustular lesions, follicular obstruction, erythema, hyperpigmentation, miliary dermatitis and dystrophic nail growth, and its diagnosis is based on the use of many different methods. Generally used methods; clinical appearance, microscopic examination, examination with Wood's lamp and mycological culture but these methods have some disadvantages. Microscopic examination requiring expertise, fast and cost-effective method, but in some cases, microbiologists encounter specimens that are microscopically negative but give positive results in mycological culture. Examination with Wood's lamp can only be used for the diagnosis of *M. canis* and its specificity is low. On the other hand mycological cultures require a long time (3-6 weeks) to give definitive results, and their sensitivity may decrease due to common contaminant growth. Considering these reasons, new Polymerase Chain Reaction (PCR) based methods have been developed for the diagnosis of dermatophyte agents. Compared to other molecular methods, the PCR method is easy, fast and applicable for the identification of dermatophyte species that do not show typical morphological features. Although PCR-based diagnostic methods are widely used in humans in the diagnosis of dermatophytosis, its usefulness in dogs and cats has also been confirmed. As a result, the PCR method used in the diagnosis of dermatophytosis; it is emphasized that it can be used in the diagnosis of dermatophytosis due to the ease of obtaining samples, providing faster results compared to mycological culture, and not requiring expertise, and it is emphasized that new and different methods should be used in the diagnosis of diseases. In this study, it was aimed to demonstrate the effectiveness of the PCR method and its applicability in clinical practice, as well as mycological culture, which is frequently used in the diagnosis of dermatophytosis.

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Introduction

Dermatophytosis are cutaneous mycoses caused by *Microsporum* spp., *Trichophyton* spp. and *Epidermophyton* spp. dermatophytes (Cafarchia et al., 2013; Katiraee et al., 2021). Infection is also described as "ringworm" (Tel and Akan, 2008). Dermatophytes are common all over the world and cause infections in various animals (Katiraee et al., 2021). Although there is no difference in terms of age, sex or race in getting the infection, it is generally more common in animals with low immunity (Tel and Akan, 2008). Among the factors affecting the incidence of dermatophytes are humidity and temperature, which vary depending on

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location and season (Cafarchia et al., 2004; Boehm and Mueller, 2019). Dermatophytes, which are classified as geophilic, zoophilic and anthropic according to their primary habitat (Or et al., 1999), also have zoonotic features (Tel and Akan, 2008). While geophilic dermatophytes are associated with the decomposition process of the surrounding keratinous structures such as hair, feathers, hooves and horns; zoophilic and anthropophilic dermatophytes are superficial mycoses that infect the stratum corneum layer, hair, nails and paws on the host (Baldo et al., 2012). About 20 different species have been identified that cause dermatophytosis in cats and dogs, (Tel and Akan, 2008), but among these agents, the most common species that cause dermatophytosis in cats and dogs are *Microsporum canis*, *Microsporum gyps*eum and *Trichophyton mentagrophytes* (Moriello et al., 2017). According to one study, infections caused by *M. canis* account for approximately 80% of dermatophytoses in cats and dogs (Cafarchia et al., 2013), while according to another study, *M. canis* is the cause of more than 90% of dermatophyte infections in cats (Paryuni et al., 2020).

Dermatophytosis resembles other skin diseases due to its various clinical manifestations such as multifocal alopecia, circular lesions, scaling, crusting, papular and pustular lesions, follicular obstruction, erythema, hyperpigmentation, miliary dermatitis and dystrophic nail growth (Tel and Akan, 2008; Kaya et al., 2022), and its diagnosis is based on the use of many different methods (Cafarchia et al., 2013). Although generally used methods of dermatological clinic examination include microscopic examination, examination with Wood's lamp and mycological culture (Verrier et al., 2019), have some disadvantages.

Rapid and accurate diagnosis is very important for the successful treatment of dermatophytes (Gräser et al., 2012). The validity of diagnoses made only in the light of clinical findings is low (Faergmann and Baran, 2003). For this reason, the diagnosis should be supported by laboratory tests.

Microscopic examination requiring expertise, fast and cost-effective method, but in some cases, microbiologists encounter specimens that are negative microscopically but give positive results in mycological culture (Piri et al., 2018). Examination with Wood's lamp can only be used for the diagnosis of *M. canis* and its specificity is low (Larry and Francis, 2015; Marsella R., 2022). On the other hand mycological cultures require a long time (3-6 weeks) to give definitive results, and their sensitivity may decrease due to common contaminant growth (Cafarchia et al., 2013; Verrier et al., 2019). In addition, the identification process on the basis of species becomes even more complicated, since some dermatophytes do not show any characteristic features as a result of the first isolation (Gräser et al., 2012; Piri et al., 2018).

Considering these reasons, new Polymerase Chain Reaction (PCR) based methods have been developed for the diagnosis of dermatophyte agents. Among these methods, pulsed-field gel electrophoresis, random amplified polymorphic DNA analysis (RAPD), PCR amplification using non-transcribed spacer (NTS) primers and internal transcribed spacer (ITS) primers, nested-PCR, PCR-restriction fragment length polymorphism (RFLP) analysis, arbitrary primer PCR and ITS region sequencing, have been used for the identification of dermatophyte species and strains (Liu et al., 2014). Compared to other molecular methods, the PCR method is easy, fast and applicable for the identification of dermatophyte species that do not show typical morphological features (Faggi et al., 2001). Although PCR-based diagnostic methods are widely used in humans in the diagnosis of dermatophytosis (Moriello and Leutenegger, 2018), its usefulness in dogs and cats has also been confirmed (Katiraee et al., 2021). In studies comparing microscopic examination, mycological culture and PCR methods, it has been observed that the PCR method can diagnose samples with positive results with conventional methods (Faggi et al., 2001; Dabrowska et al., 2014; Moriello and Leutenegger, 2018; Piri ve ark., 2018; Verrier et al., 2019). To date, many PCR methods have been developed for the diagnosis of dermatophytes. These PCR methods have advantages and disadvantages compared to the methods used for the diagnosis of dermatophytes.

In this study, it was aimed to demonstrate the effectiveness of the PCR method and its applicability in clinical practice, as well as mycological culture, which is frequently used in the diagnosis of dermatophytosis.

Sensitivity and specificity

In many studies in which samples taken from cats and dogs for the diagnosis of dermatophytosis were examined using microscopic examination, mycological culture and PCR methods, it was seen that PCR methods gave reliable results (Tel and Akan, 2008; Moriello and Leutenegger, 2018; Piri ve ark., 2018; Verrier ve ark., 2019). In the study in which 268 samples (97 dogs, 57 cats) were examined by microscopy and mycological culture and 40 samples were isolated, it was observed that all isolated dermatophyte agents were positive as a result of the detection of 3390 bp bands by PCR method (Tel and Akan, 2008). In a study where qPCR was used to

measure clinical utility, confirm dermatophytosis and evaluate mycological recovery in cats with suspected dermatophytes, qPCR and mycological culture results were consistent in 49 of 52 cats. qPCR was able to correctly identify 47 of 50 cases with infection and 2 of 2 cases without infection. In addition, 2 negative mycological cultures were defined as mycological recovery in the study. qPCR correctly defined 30 patients for *Microsporum* spp. and 39 patients for *Microsporum canis*. in 46 patients diagnosed with mycological recovery by mycological culture (Moriello and Leutenegger, 2018). Using a newly developed PCR method (pan-dermatophyte nested-PCR), in a study conducted on 140 samples taken from cattle, sheep, goats, cats, dogs and horses with clinically suspected dermatophytosis, the positivity rates of the samples were 90% with nested-PCR and %85.7 with microscopy and 75% with mycological culture. For this reason, it was concluded that the newly developed nested-PCR method is a fast, sensitive and specific method for the detection of dermatophytes in suitable clinical samples (Piri et al., 2018). In another developed method (PCR assay based on terminal restriction fragment length polymorphism (TRFLP)), the detection rate of agents was found to be 97.1% with PCR-TRFLP from samples that were found positive by both microscopic examination and culture. The PCR method did not give positive results in any of the samples that were detected negative with the classical methods used in this study (Verrier et al., 2019). While the one-step PCR method showed high accuracy in samples obtained from dogs, the accuracy rate seen in cats was lower. However, it was determined that the samples taken from cats with the nested-PCR method showed higher accuracy. The specificity determined by the nested-PCR method in dogs and cats was 94.1%, 94.4%, and the sensitivity was 100% and 94.9%, respectively. At the same time, the nested-PCR method can distinguish *Microsporum canis* from *Microsporum gypseum*, *Trichophyton interdigitale* and *Trichophyton terrestre*, which is not possible in the single-step PCR method (Cafarchia et al., 2013).

False positivity

It has been thought that false positive results seen in the samples examined using the PCR method may be due to false negative microscopic examination and culture results, the presence of non-viable DNA fragments on the hair cover during treatment, contamination of the skin, fomite carrier or environmental fungal spores (Cafarchia et al., 2013; Moriello and Leutenegger, 2018). Positive PCR results seen in animals, although there are no clinical signs, are thought to be related to early diagnosis (Moriello et al., 2017; Moriello and Leutenegger, 2018). In order to avoid false positive results, it is recommended to repeat the PCR test by cleaning the environment and the animal (bath) before starting any treatment (Moriello and Leutenegger, 2018).

Jacobson et al. (2018a) determine that in samples from 132 cats, PCR detected positive in correlation with all samples found positive by mycological culture. However, 12 samples with negative mycological culture results were evaluated as positive. In this study, mycological culture was accepted as the gold standard, and mycological culture and PCR were repeated for 9 out of 12 samples. Mycological culture was positive in 2 out of 9 samples, and it was thought that the first mycological culture gave false negative results and PCR results gave true positive results. Results for the remaining 7 samples did not change and PCR was associated with a false positive result. However, 5 out of 7 cats had a history of past exposure to dermatophytosis, and positive PCR results for samples from these cats may have revealed the presence of fungal agents that could not be detected by culture.

False negativity

In a study in which 52 samples from cats were analyzed using the qPCR technique, the results of 3 samples were observed to be negative. All of these samples had clinical lesions. By cytology and mycological culture, 1 of these patients was confirmed as M. canis and 2 as *Trichophyton* spp. (Moriello and Leutenegger, 2018). It is thought that the amount of hair and crust in the sample taken from the lesions is important when using the qPCR technique, and false negative results may be caused by the inadequate sampling technique (Moriello et al., 2017; Moriello and Leutenegger, 2018). In addition, it was thought that due to the self-grooming feature of cats, fungal DNAs could be found less on the hair cover and their detection by PCR could be detected at a lower rate compared to dogs (Cafarchia et al., 2013).

Diagnostic expenses and relationship with treatment

Although it is thought that the biggest disadvantage of the PCR method when compared to classical methods is its high cost (Faggi et al., 2001), it has been reported that the cost is now approaching classical methods thorugh the developing technology and developed PCR methods (Piri et al., 2018). In addition, it can provide significant savings, especially as negative results can be detected in a short time and thus unnecessary medication, care and accommodation costs can be reduced (Jacobson et al., 2018b). Because of these savings, the cost difference of the PCR method

compared to mycological culture is reduced.

Despite the culture results can be detected as negative in the samples taken from the treated cats and dogs, the PCR method is not affected by the systematic treatment and thus enables the monitoring of the efficacy of the treatment (Cafarchia et al., 2013; Moriello and Leutenegger, 2018). In a study, 3 samples from treated animals were negative in mycological culture but positive by the nested-PCR method. These results suggested that PCR can be used as a routine method for monitoring treatment (Cafarchia et al., 2013). Although the negative PCR results seen during the treatment are reliable, the positive PCR results do not show that there is no improvement due to the false positive reasons we have listed above (Moriello et al., 2017; Jacobson et al., 2018b; Moriello and Leutenegger, 2018).

Antifungal Resistance

In countries where dermatophytosis needs to be confirmed by culture before starting antifungal treatment, the need to take samples again due to false negative culture results causes both loss of time, financial loss and discomfort for the patient and the patient's relatives (Piri et al., 2018).

Today, the emergence of antifungal resistant clinical isolates may lead to failure in dermatophyte treatment. Especially the treatments created as a result of misdiagnosis contribute to the spread of antifungal resistance (Kaya et al., 2022). It is important to determine the species before starting the treatment, as it will prevent antifungal resistance that may occur with incorrect and incomplete treatment (Katiraee et al., 2021). Although the identification of dermatophytes by culture is difficult and the specificity is low, PCR has been found to give more successful results (Piri et al., 2018).

Conclusion

Comparison with other methods, the advantages of the PCR method are that it gives reliable results, the potential to guide early diagnosis, and its ability to determine the effectiveness of treatment. It can also prevent antifungal resistance through species identification and the use of specific antifungals. The disadvantages are that non-living DNA fragments give false positive results, false negative results are seen due to the inadequacy of the sampling technique, and its cost is still high compared to other methods.

As a result, the PCR method can be used in the diagnosis of dermatophytosis; due to the ease of samples collection, providing faster results compared to mycological culture, and not requiring expertise, and it is emphasized that new and different methods should be used in the diagnosis of diseases because of alternative techniques are improving recently.

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A morphological study on sinus and atrioventricular nodes in Saanen goats

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ABSTRACT

In mammals, the sinus node and atrioventricular node are defined as the heart's specific stimulus and conduction systems. The electrical stimulation that starts in the sinus node continues with the atrioventricular node, and the rhythmic stimulation occurs in this way. There are differences in the sinus and atrioventricular nodes' location, size, and cell structures between species. In this study, which was carried out to determine the morphological structures and locations of the sinus and atrioventricular nodes in Saanen goat hearts, nine hearts from healthy goats were used. After performing the routine tissue follow-up procedure for histological examinations, 5 µm-thick serial sections taken from paraffin-blocked samples were stained with Crossman Trichrome and Periodic Acid Schiff (PAS) and photographed. It was determined that the sinus node was in a subepicardial position near where the cranial vena cava opens up to the right atrium. The sinus node, with an average length of 8-10 mm was crescent-shaped. The atrioventricular node was located in the subendocardium layer in the right half of the interatrial and the interventricular septum, where the coronary sinus opened into the right atrium. An atrioventricular node with an average length of 4.5-5 mm was observed to be roughly oval. Two types of cells were identified in both nodes: cells with small, rounded with empty cytoplasm and rather large nuclei, and cells with thin, elongated structures and darker staining.

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Introduction

The heart has a sinus node, an atrioventricular node, and many conduction pathways, which provide its stimulation and conduction system (Pawlina and Ross, 2018; Wiyarta and Karima, 2022). Electrical impulses are produced in the sinus node (Keith-Flack) (Ambesh and Kapoor, 2017; Lang and Glukhov, 2018; Treuting et al., 2017; Wiyarta and Karima, 2022). The impulse starting from the sinus node spreads along the internodal tracts consisting of the muscle fibers of the atria and modified cardiac muscle fibers and collects in the atrioventricular node (Aschoff-Tawara) (Pawlina and Ross, 2018). They are transmitted to the septum interventricular via the His bundles originating from the atrioventricular node. These bundles pass through the fibrous skeleton of the heart and divide into right and left branches at the interventricular septum (Pawlina and Ross, 2018; Wiyarta and Karima, 2022). The last branches of these bundles, divided into two, are Purkinje fibers (rami subendocardial) in the endocardium layer (Pawlina and Ross, 2018; Wiyarta and Karima, 2022).

While the sinus node is found where the cranial vena cava opens into the right atrium and below the epicardium in ruminantia (Ghazi and Tadjalli, 1996;

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Ghonimi et al., 2015; James, 1965; Nabipur et al., 2000; Türkmenoğlu et al., 2017), horses (Bishop and Cole, 1967) and carnivores (Ghazi et al., 1998; James, 1962), it is located in the more caudal part of the terminal sulcus below the epicardium in guinea pigs (Nabipour, 2004) and rabbits (James, 1967a). In the horse (Bishop and Cole, 1967), dog (James, 1962), goat (Nabipur et al., 2000), and camel (Ghazi and Tadjalli, 1996; Ghonimi et al., 2015), the sinus node is surrounded by a connective tissue capsule. The cells in the node, which has a rich connective tissue matrix, are small, pale, and contain fewer sarcomeres and mitochondria than atrial muscle cells (Dobrzynski et al., 2013). These cells are P and T cells (Duan et al., 2012; Ghazi and Tadjalli, 1996; Ghonimi et al., 2015; Nabipour, 2012; Nabipur, 2002). P cells are called different names such as perinuclear clear zone, pacemaker, pale cell, and nodal cell. They are oval-shaped cells with light-colored cytoplasms and with getting together can be seen as a bunch of grapes (Duan et al., 2012; James, 1965; Mitrofanova et al., 2018). Impulses produced by P cells are believed to be delivered to the myocardium via T cells (James, 1967b). T cell with longitudinal course stains darker than P cell (Merideth and Titus, 1968; Nabipur, 2002). In some studies (Ghonimi et al., 2015; Mitrofanova et al., 2018), different from these cells (P and T cells) Purkinje-like cells are also mentioned. Purkinje-like cells are large cells located at the periphery of the node (Ghonimi et al., 2015; Mitrofanova et al., 2018). Numerous fibroblasts, nerve tissue, and capillaries are seen in the interstitium between P and T cells (James, 1977).

In domestic mammals such as goat (Nabipur, 2002), cattle (James, 1965), sheep (Frink and Merrick, 1974), yak (Duan et al., 2017) and buffalo (Türkmenoğlu et al., 2017), the atrioventricular node is located where at the junction of the interatrial septum and the interventricular septum, on the right side of the interatrial septum, at the end of the septal cusp of the tricuspid valve, anterior to the coronary sinus and under the the subendocardium. The cells in this node are similar to the cells in the sinus node, and only His bundles surrounded by connective tissue are observed in the continuation of the atrioventricular node (Duan et al., 2017; Türkmenoğlu et al., 2017). Purkinje fibers, which are smaller branches of the His bundles, distribute in the muscle layer of the right ventricle and left ventricle (Pawlina and Ross, 2018). Purkinje fibers provide rapid spread of electrical conduction in the right ventricle and left ventricle wall and contraction of both ventricles (Pawlina and Ross, 2018).

The morphological structure of the sinus node and atrioventricular node in many domestic mammals has been studied (Duan et al., 2017; Duan et al., 2012; George et al., 2017; Ghonimi et al., 2015; James, 1964, 1965; Nabipour, 2004, 2012; Nabipur et al., 2000; Türkmenoğlu et al., 2017). In the literature review, no information was found about the location and structure of these nodes in Saanen goat hearts. Therefore, with this study, it is aimed to determine the localization and morphological structure of the nodes mentioned in Saanen goat hearts in detail and to contribute to the anatomy literature.

Materials and Methods

In this study, a total of nine healthy Saanen goat hearts taken from the animal slaughterhouse were used. After the hearts were fixed in 10% formaldehyde solution, a routine tissue follow-up procedure was performed on the samples and embedded in paraffin. Serial sections of 5 µm thickness were taken from the embedded samples with a Leica (RM2125RT Leica, Wetzlar, Germany) microtome. Then these sections were stained with Crossman Trichome and Periodic Acid Schiff (PAS) (Luna, 1968). Histological examinations were performed using the Nikon Eclipse E600W (Nikon, Tokyo, Japan) light microscope, and microscopic photographs were taken with the Nikon DS Camera Head DS-5M imaging system.

Results

The localization and morphological structures of the sinus node and atrioventricular node, located in the heart-specific stimulation and conduction system, were determined. The sinus node was located at the lateral wall of the atrium, on the terminal crest, near the opening of the cranial vena cava to the right atrium, and in the subepicardial (Figure 1A). The mean length of the sinus node, which was observed as a crescent-shape in the subepicardial, was 8-10 mm (Figures 1B, 2A).

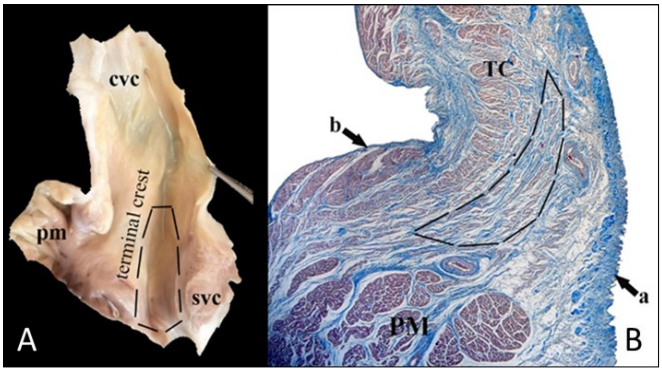

Figure 1. (**A**) Localization of the sinus node. The where cranial vena cava (cvc) opens into the right atrium, pectinate muscle (pm), sinus of vena cava (svc) and sinus node (dashed lines). (**B**) A histological view of the sinus node. Epicardium layer (a), endocardium layer (b), terminal crest (TC), pectinate muscle (PM), and sinus node (area delimited by dashed lines), 2X objective magnification (Crossman trichrome).

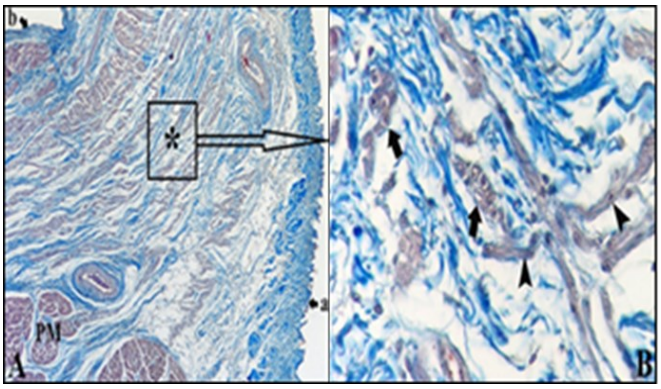

Figure 2. (**A**) A histological view of the sinus node. Epicardium layer (a), endocardium layer (b), pectinate muscle (PM) and sinus node (*), 4X objective magnification, (Crossman trichrome). (**B**) Different cell groups in the sinus node. Cell group I (arrow), cell group II (arrowhead), 40X objective magnification, (Crossman trichrome).

In histological examination, different cell groups were observed in the sinus node as well as structures such as connective tissue and nerve tissue (Figures 3A, 3B). One of these groups (cells group I) was cells with pretty large nuclei, which were small, round and had an empty cytoplasm (Figures 2B, 3A, 3B). These cells, in coming together in some places, were observed in a bunch of grapes shape (Figures 2B, 3B). The other cell group (cell group II) was elongated and darkly stained cells (Figures 2B, 3A, 3B). There was no intercalated disc in these cells. The connective tissue observed in the sinus node was densely located among the cells mentioned above. Arteriola and peripheral nerve fibers were found in the periphery of the node.

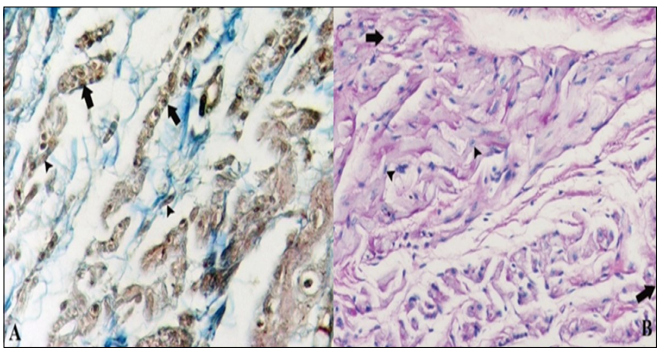

Figure 3. (A, B) Different cell groups in the sinus node, cell group I (arrow), cell group II (arrowhead), A: (Crossman trichrome) 40X objective magnification. B: (PAS) 20X objective magnification.

The atrioventricular node was located in the area extending from where the opening of the coronary sinus to the right atrium in the subendocardium to the junction of the interatrial septum and interventricular septum (Figure 4A). It was determined that this node was oval in shape at the base of the septal cusp of the tricuspid valve and had an average length of 4.5-5 mm (Figure 4B).

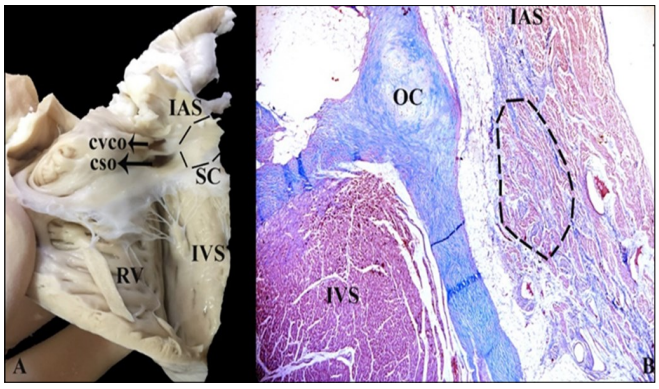

Figure 4. Localization of the atrioventricular node (**A**). Caudal vena cava ostium (cvco), coronary sinus ostium (cso), interatrial septum (IAS), interventricular septum (IVS), septal cusp (tricuspid valve) (SC), right ventricle (RV). Where atrioventricular node is found (dashed lines). Histological view of the atrioventricular node. (**B**) Atrioventricular node (area delimited by dashed lines), os cordis (OC), interatrial septum (IAS), interventricular septum (IVS), 2X objective magnification, (Crossman trichrome).

Cell groups residing in the sinus node were also found in the dense connective tissue layer of the atrioventricular node (Figure 5). Peripheral nerve fibers and a few arterioles were located around this node, as in the sinus node.

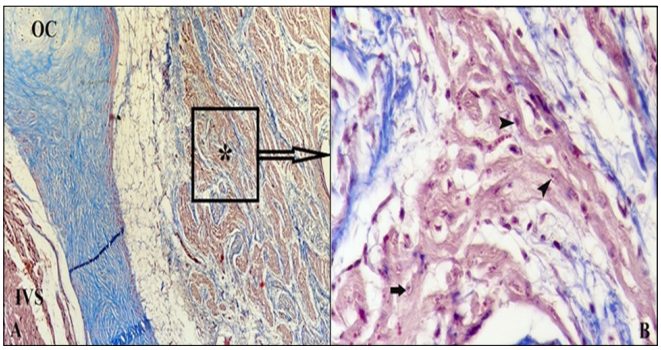

Figure 5. (**A**) A histological view of the atrioventricular node. Atrioventricular node (*), os cordis (OC), interventricular septum (IVS), 4X objective magnification, (Crossman trichrome). (**B**) Different cell groups in the atrioventricular node. arrow: cell group I, arrowhead: cell group II, 40X objective magnification, (Crossman trichrome).

His bundles were observed in the subendocardium layer of the interventricular septum after the atrioventricular node (Figures 6A, 6B). This structure consisted of interconnected light-colored and large cells surrounded by connective tissue.

Discussion

The conduction pathways originate from the sinus node (pacemaker) and atrioventricular node and spread to the heart for the heart's unique warning and conduction system (Pawlina and Ross, 2018). In domestic mammals, there are many studies on the location and macro anatomical structure of these

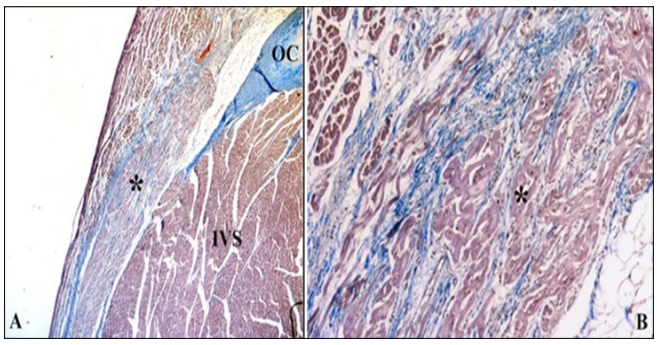

Figure 6. (**A**) Histological view of his bundles. His bundle (*), os cordis (OC), interventricular septum (IVS), 2X objective magnification, (Crossman trichrome). (**B**) Transition from the atrioventricular node to His bundles (*). 10X objective magnification, (Crossman trichrome).

histological appearance (Duan et al., 2017; Ghazi and Tadjalli, 1993; Ghonimi et al., 2015; Nabipour, 2012). In previous studies, it has been stated that the location of the sinus node in cattle (James, 1965), goats (Nabipur et al., 2000), camels (Ghazi and Tadjalli, 1996; Ghonimi et al., 2015), horses (Bishop and Cole, 1967), dogs (James, 1962), cats (Ghazi et al., 1998), water buffalo (Türkmenoğlu et al., 2017), and humans (Perde et al., 2016; Sanchez-Quintana et al., 2005) is at the cranial vena cava opening into the right atrium below the epicardium. This node is located in the caudal part of the terminal sulcus below the epicardium in guinea pigs (Nabipour, 2004) and rabbits (James, 1967a). The localization of sinus node in the Saanen goat was found to be similar to those of the above-mentioned species, except for guinea pigs and rabbits. The length of sinus node, which has different sizes among species, is specified as 2.75 mm in female cats (Ghazi et al., 1998), 2.78 mm in male cats (Ghazi et al., 1998), 10-20 mm in humans (Perde et al., 2016) or 15 mm (Merideth and Titus, 1968), 4 mm in guinea pigs (Opthof et al., 1987), 12.75 mm in goats (Nabipur et al., 2000), 3 mm in ferrets (Truex et al., 1974), 0.5-0.8 mm in rabbit (James, 1967a), 28.25 mm in camel (Ghazi and Tadjalli, 1996), and 18 mm in buffalo (Türkmenoğlu et al., 2017). In the study, the length of the sinus node was observed to be between 8-10 mm on average and was smaller than the lenght reported by Nabipur et al. (2000) in goats.

It has been reported that the surrounding of the sinus node consists of a collagen framework in horses (Bishop and Cole, 1967), dogs (James, 1962), goats (Nabipur et al., 2000), and camels (Ghazi and Tadjalli, 1996; Ghonimi et al., 2015). In this study, connective tissue was seen in the sinus node but did not form a clear border around the node. The shape of the sinus node has been reported to be oblong or spindle -shaped in the dog (James, 1962), elongated or bent oblong in the camel (Ghonimi et al., 2015), and almost triangular in the cat (Ghazi et al., 1998). This node was observed to be crescent-shaped in Saanen goats. It is stated that there is an artery responsible for the arterial nutrition of the same node, and this artery's location varies according to the species. The artery of a sinus node is located in the center of the node in dogs (James, 1962), horses (Bishop and Cole, 1967), camels (Ghazi and Tadjalli, 1996), and humans (James, 1977; Merideth and Titus, 1968; Perde et al., 2016; Sanchez-Quintana et al., 2005). In cattle (James, 1965), buffalo (Türkmenoğlu et al., 2017), sheep (Copenhaver and Truex, 1952), and goats (Nabipur et al., 2000), no artery has been found in the node's center, while arterioles have been determined around the node. This situation was consistent with our findings in Saanen goats.

In the literature (Ghonimi et al., 2015; James et al., 1966; Nabipur et al., 2000; Türkmenoğlu et al., 2017), it has been stated that there are different cells in the structure of both nodes. It has been reported that the cellular structure of the sinus node consists of pale staining cytoplasm and round-oval shaped P (perinuclear clear zone) cells, darker staining, and longitudinally courses T (transitional) cells, and pale staining atrial muscle cells (Ghonimi et al., 2015; James et al., 1966; Nabipur et al., 2000; Türkmenoğlu et al., 2017). The cell group I and II seen in the sinus node of the Saanen goat were similar to P and T cells mentioned in the literature, respectively. The collection of some cells in group I cells in the form of grape bunches was consistent with what Duan et al. (2012) and Nabipour (2012) reported about P cells. Other than these cells, Mitrofanova et al. (2018) have also mentioned the existence of Purkinje-like cells in humans and stated that these cells are considerably larger than P cells. These cells were not found in Saanen goats. It was observed that the modified muscle cells in the sinus node of the studied animals did not have intercalated discs. This was consistent with that reported in humans (James, 1961), cats (Ghazi et al., 1998), dogs (James, 1962), horses (Bishop and Cole, 1967), camels (Ghazi and Tadjalli, 1996), and cattle (James, 1965). While many nerve fibers are found in and around the sinus node in cats (Ghazi et al., 1998), horses (Bishop and Cole, 1967), dogs (James, 1962), and humans (James, 1961), it is observed that nerve fibers around the node in cattle (James, 1965) and camels (Ghazi and Tadjalli, 1996) are similar to our study.

The location of the other node of the heart, the atrioventricular node, differs between species. In goats (Nabipur, 2002), cattle (James, 1965), and sheep (Frink and Merrick, 1974), yak (Duan et al., 2017), buffalo (Türkmenoğlu et al., 2017), and human (Titus et al., 1963), it is stated that the border of an atrioventricular node is at the right side of the interatrial septum, above the septal cusp of the tricuspid valve, anterior of the coronary sinus and located in the subendocardium. Researchers reported that in hedgehogs (Nabipour, 2010) and sheep fetuses (Nabipour, 2007), the atrioventricular node is located in a position similar to the above literature. The only difference is that the node extended to the aorta in these animals. The location of the atrioventricular node in the Saanen goat was consistent with what has been reported in the literature except for hedgehog and sheep fetuses (Duan et al., 2017; Frink and Merrick, 1974; James, 1965; Nabipur, 2002; Titus et al., 1963; Türkmenoğlu et al., 2017). The length of an atrioventricular node is 7.5 mm in humans (Titus et al., 1963), 9 mm in buffalo (Türkmenoğlu et al., 2017), 2 mm in weasels (Truex et al., 1974), 4.23 mm in goats (Nabipur, 2002), 2.2-4.5 mm in the yak (Duan et al., 2017), 0.13 mm in the sheep fetus (Nabipour, 2007), and approximately 2 mm

node varied between 4.5-5 mm. In our study, the cell diversity in the atrioventricular node and the cell configuration in the sinus node were similar and consistent with what was reported by and Duan et al. (2017).

The shape of the atrioventricular node, which varies between species, is ovoid in the cattle (James, 1965), oval in the hedgehog (Nabipour, 2010), roughly oval or fan-shaped in the human (Titus et al., 1963), roughly oval in the yak (Duan et al., 2017), elongated oval-shaped in goats (Nabipur, 2002), and almost spherical-shaped in sheep fetuses (Nabipour, 2007). In the current study, the shape of the atrioventricular node was observed to be roughly oval. While the nerve fiber density in the atrioventricular node is reported to be low in humans (Titus et al., 1963), guinea pigs (Nabipour, 2004), and dogs (James, 1964), it was observed in our study that nerve fiber was quite dense as in cattle (James, 1965), goats (Nabipur, 2002), and yaks (Duan et al., 2017). Duan et al. (2017) reported that intercalated discs were not observed in the yak atrioventricular node, while James (1965) mentioned the presence of ganglia around the node in cattle. The absence of intercalated discs and peripheral nerve fibers around the node was detected in Saanen goats. It is mentioned that the His bundle, seen in the continuation of the atrioventricular node, consists of cells connected by chain style, surrounded by connective tissue (Duan et al., 2017; Machida et al., 2005). The appearance of her bundles in the present study was consistent with the abovementioned literature (Duan et al., 2017; Machida et al., 2005). The transition between His bundles and the node could not be determined.

Conclusion

The localization of the sinus node, one of the nodes of the special conduction system of the heart, was determined to be subepicardial near the opening of the cranial vena cava to the right atrium. The other node, the atrioventricular node, was located subendocardial on the right side of the interatrial septum and interventricular septum, where the coronary sinus opened to the right atrium. The absence of an artery in the center of the oval-shaped atrioventricular node and the crescent-shaped sinus node was noted. The cellular configuration of the sinus node, which varies between 8-10 mm in length, and the atrioventricular node, which varies between 4.5-5 mm in length, was similar and consisted of P, T, and atrial muscle cells. This study, in which the length, position, and cellular structure of the atrioventricular node and sinus node were determined, was carried out for the first time in Saanen goats. We think that the obtained data will contribute to the veterinary anatomy literature.

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Is it possible to change milk secretion of drugs with soy enriched diets in lactating ruminants?

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ABSTRACT

Soy is the most commonly used protein supplement in beef and dairy diets. Soy, which is also used as a common protein source in animal feed, is palatable and has a good amino acid balance and high bioavailability. In vivo and in vitro interaction of flavonoids, including isoflavones such as genistein and daidzein, with several ABC transporters, including breast cancer resistance protein (BCRP/ABCG2), has been demonstrated. BCRP presence in ruminants could affect the efflux of hydrophobic toxins and drugs, including their active secretion to milk and a reduction in the withdrawal time of the drug milk residues. As a result of inhibition of efflux transporters such as BCRP, changes in drug pharmacokinetics and drug transfer into milk have been observed. In this respect, the use of forage supplemented with BCRP inhibitors may be beneficial to control drug accumulation in milk and prevent undesirable contamination of milk. It is aimed to reduce the drug withdrawal periods for dairy animals with the procedure in question. In this review, it is aimed to give information about the importance of soy-enriched diets in the nutrition of ruminants during the lactation period and the effect of transport proteins on the transfer of drugs into milk.

Keywords: BCRP/ABCG2, pharmacokinetics, soy, withdrawal time, ruminant

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Introduction

Milk secretion of drugs and xenotoxins

The main mechanisms that ensure the milk secretion of drugs are; passive diffusion, active transport by ABC (ATP-binding cassette) transport proteins, and ion trapping. Other examples of mechanisms include facilitated diffusion, pinocytosis and exocytosis (Ito and Alcorn, 2003).

Drug excretion into milk varies depending on many factors such as lipophilicity, molecular weight, plasma protein binding rate, and ionization (Agatonovic-Kustrin et al., 2002; Ito and Lee, 2003).

Drug residues that are released due to the accumulation of drugs used for the treatment of diseases or to increase animal production or their metabolites in products such as animal tissues or milk pose risks to food safety and public health (Behnke et al., 2008). Since residue levels in milk are harmful to health, it is necessary to pay attention to drug withdrawal times (Alvarez et al., 2006). Maximum residue limit (MRL) values have been determined for many drugs by the European Union in order to eliminate possible public health problems that residues may cause (EMA, 1999).

Breast cancer resistance protein (BCRP/ABCG2), which is in the family of ABC transport proteins, is one of the main factors in the transfer of drugs into milk, which leads to the presence of undesirable products in milk; causes exposure of suckling pups and consumers

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to xenotoxins (Jonker et al., 2005; van Herwaarden and Schinkel, 2006; Schrickx and Fink-Gremmels, 2008).

BCRP affects the bioavailability of many compounds and causes drug-drug interactions by enabling the excretion of many toxic elements from the cells in the intestine, liver and other organs (Szakacs et al., 2006). BCRP/BCRP1 is located in the apical membrane of epithelial cells of the small and large intestines, the bile canalicular membrane of hepatocytes, the apical membrane of renal proximal tubular epithelial cells, the luminal membrane of endothelial cells of capillaries in the brain, the syncytiophoblasts of the placenta, and the apical of mammary alveolar epithelial cells, which is the main area of milk production (van Herwaarden and Schinkel, 2006).

BCRP is highly expressed in the mammary tissues of cows and sheep and the apical membrane of the alveolar epithelial cells that form the blood-milk barrier during lactation (Pulido et al., 2006). The blood -milk barrier consists of mammary alveolar epithelial cells, with tight junctions with the apical side facing the milk and the basolateral side facing the mammary gland capillaries (Zhao and Lacasse, 2008). BCRP/ ABCG2 provides active transport of various drugs and toxins to milk by crossing the blood-milk barrier (Jonker et al., 2005). BCRP plays an important role in the active transport of some drugs and carcinogenic xenotoxins (cimetidine, nitrofurantoin, topotecan, acyclovir, PhIP) into milk (Jonker et al., 2005; Merino et al., 2005). Lactation period affects BCRP expression, and BCRP in the mammary gland of lactating animals is higher than in non-lactating animals (Jonker et al., 2005). Although BCRP has been shown to have an increased expression in lactating mice, cows and humans, and play an important role in the transfer of substrate drugs into milk, ABC transport proteins similar in structure to BCRP, such as MRP1, MRP2 and P-gp, are not significantly expressed in lactating mice (Jonker et al., 2005). These results support the view that BCRP substrate drugs have a higher rate of excretion into milk (Jonker et al., 2005). In another similar study, immunoblot and Western blot experiments showed high BCRP expression in the mammary glands of sheep and cows. The results obtained by the researchers on the vanadate-sensitive ATPase activity of BCRP-specific substrates and inhibitors in cow mammary gland homogenates confirmed the functional expression of BCRP in cows and sheep (Pulido et al., 2006).

The role of BCRP in the excretion of drugs used in veterinary medicine

BCRP is critical to drug pharmacokinetics and safety in veterinary field. BCRP has been shown to mediate the excretion of some substrate drugs, such as enrofloxacin and nitrofurantoin, into milk in sheep. In addition to numerous studies on potential drug interactions mediated by P-glycoprotein (P-gp) in ruminants, BCRP-mediated interactions in the mammary gland are also gaining importance. In these studies, antiparasitic drugs such as monepantel, triclabendazole, ivermectin, moxidectin and antibiotics such as danofloxacin, nitrofurantoin, and enrofloxacin secreted into milk by BCRP-mediated active transport (Virkel et al., 2018).

The secretion of exogenous compounds into milk by BCRP can lead to many beneficial or undesirable results. A useful example of BCRP-mediated drug transfer into milk is to provide effective treatment with parenteral antibiotic administration in the treatment of mastitis. However, the excretion of parenterally administered fluoroquinolones into milk may cause some adverse drug reactions, such as fluoroquinolone-induced cartilage disorders in suckling puppies. Especially in dairy animals, the transfer of drugs into milk by BCRP may cause undesirable contamination in milk for human consumption (Mealey, 2012). In other studies in humans, mice, rats, cows, and sheep, bile acids, uric acid, enterolactone, enterodiol, ciprofloxacin, danofloxacin, moxidectin, flunixin, 5-hydroxyflunixin, pantoprazole, nifedipine, aflatoxin B1 and heterocyclic amines are transported into milk by BCRP (Garcia-Lino et al., 2019).

Drug excretion characteristics based on some studies in ruminants

Benzimidazoles, avermectins and milbemycins are excreted in ruminant milk, but the mechanisms underlying the excretion process have not been fully elucidated (De Liguoro et al., 1996; Barker and Kappel, 1997; Moreno et al., 2005).

Although most drugs secreted from maternal plasma to milk by passive diffusion, the milk/plasma (M/P) ratio, which is used to determine the equilibrium concentration between breast milk and blood, is affected by the composition of the milk and the physicochemical properties of the drug (Agatonovic-Kustrin et al., 2002; Ito and Lee, 2003).

Drugs with steady-state concentrations such as cimetidine, ranitidine, and nitrofurantoin are excreted in human and rat milk, possibly by active transport mechanism. High M/P ratio has been reported for some drugs such as moxidectin and ivermectin in

domestic animals and these compounds can be actively transported into milk (Imperiale et al., 2004).
In a study conducted in goats and cows, benzylpenicillin can cross the blood-milk barrier by active transport and this transport can be inhibited by probenecid (Schadewinkel-Scherkl et al., 1993). Furthermore, some transporters such as MDR1 and OCTN2, which have a potential role in drug disposition in the mammary gland, increase significantly in lactating cell (Alcorn et al., 2002).

Use of inhibitors to reduce the excretion of substrates of ABC transport proteins into milk

The importance given to the use of inhibitors to increase the oral bioavailability and penetration of drugs into tissues for effective treatment or to reduce the transfer of substrates of ABC transport proteins into milk has been increasing (Merino et al., 2010; Shukla et al., 2011). Many in vivo and in vitro studies have shown that inhibition of ABC transport proteins, such as BCRP, with various drugs may also leads to drug interactions. The bioavailability and therapeutic effect of the substrate drugs increase as a result of the use of BCRP substrate drugs co-administered with BCRP inhibitors. In contrast, the bioavailability and therapeutic effects of the substrate drugs are weakened as a result of the use of BCRP substrates co-administered with BCRP inducers (Kunta and Sinko, 2004).

BCRP inhibitors

BCRP inhibitors have two clinically significant pharmacological effects. The first and the reason for the intensification of research on these drugs is that they can overcome BCRP-mediated multi-drug resistance in tumor cells (Henrich et al., 2007). Second, when BCRP inhibitors are administered with drugs that are BCRP substrates, the BCRP substrate could change the disposition (absorption, distribution, and excretion) of the drug in the body (Breedveld et al., 2004; Kuppens et al., 2007).

Flavonoids are herbal components that have recently attracted attention as BCRP inhibitors and have been extensively researched. There are thought to be two possible mechanisms of BCRP inhibition by flavonoids (Morris and Zhang, 2006). The first is that flavonoids are inhibited by interactions with the nucleotide-binding domain of BCRP. The second mechanism appears to result from interaction with BCRP substrate binding sites; because many flavonoid BCRP substrates can stimulate BCRP ATPase activity as seen in mitoxantrone (Cooray et al., 2004, Pulido et al., 2006).

Soy

There are many studies showing the interaction and inhibition of BCRP with polyphenols (tannins, gossypol, fagopyrin) that are largely found in ruminant feed (Broderick 1995; Cooray et al., 2004; Zhang et al., 2004b). Soybean, which is used as a protein source in ruminant feed, is rich in isoflavonoids containing herbal active ingredients (100-200 mg of isoflavonoids are found in 100 g of soy) (Broderick 1995 and 2003). BCRP is inhibited as a result of the interaction of isoflavonoids, which are secondary metabolites of soy and are found in high amounts in polyphenols, and BCRP in the mammary gland (Pulido et al., 2006; Perez et al., 2009). Studies conducted in line with this information have shown that adding BCRP inhibitors such as isoflavonoids to forage prevents the secretion of toxins into milk and is beneficial in controlling drug residues. It is also expected that this procedure may make it possible to reduce the drug withdrawal time in lactating sheep (Pulido et al., 2006).

Soy Active Ingredients

Flavonoids are polyphenols commonly found in fruits and plants. Flavonoids, which give these products their flavor and color, are also the main component of many herbal products (Zhang et al., 2004a). Flavonoids contain two or more aromatic rings, each carrying at least one aromatic hydroxyl and connected by a carbon bridge.

Flavonoids are divided into different subclasses such as chalcones, flavonols, flavones, procyanidins, flavan-3 ol (catechins), flavanones and isoflavones according to the change of C ring and oxidation state (Morris and Zhang, 2006).

Isoflavonoids are secondary metabolites naturally found in plants of the Leguminosae family, such as soy, red clover, peanuts, chickpeas, and alfalfa (USDA, 2008). Isoflavonoids attract bacteria to plant roots to aid in nodulation and nitrogen fixation (Rolfe, 1988). The most abundant source of isoflavones is soybean containing genistein (approximately 2.3 mg/kg), daidzein and glycitine (USDA, 2008). In addition, they contain trace amounts of formononetin and biochanin A (Burdette and Marcus 2013). Isoflavones exist in plants in two forms: glycoside (genistin or genistein-7-O-β-D-glycoside) and aglycone (genistein) (Barnes, 2010).

Glycosides are found in higher concentrations than aglycones in soybean and other plants (Song et al., 1998). After oral ingestion, isoflavone glycosides are converted to aglycon forms with high biological activity by epithelial and microbial β-glucosidases in the oral cavity and small intestine (Akiyama et al., 1987; Day et al., 1998; Morito et al., 2001; Walle et al., 2005).

Isoflavones such as genistein, daidzein and biochanin A are found in soybeans, and their

precursors together with lignans found in a wide variety of plants (Yao et al., 2004).

BCRP-Inhibitory Effects of Some Soy Active Ingredients

Recent studies have begun to investigate the ability of isoflavones to alter the pharmacokinetics or pharmacodynamics of compounds transported by BCRP. To date, a variety of in vitro and in vivo experimental models have been used to detect isoflavones that inhibit BCRP function and to better understand the potential effects of concomitantly consumed isoflavones (e.g. soy-rich diet) with drugs transported by BCRP (Bircsak and Aleksunes, 2015).

Isoflavones in animal feeds and diets can alter the in vivo pharmacokinetics and pharmacodynamics of many drugs. For this reason, it is necessary to pay attention to the administration of BCRP substrate drugs, especially to animals in the lactation period. On the other hand, there are also therapeutic uses, such as inhibiting the secretion of xenobiotics into milk with this function, leading to the protection of the infants from drug-induced toxic effects (Bircsak and Aleksunes, 2015).

When BCRP inhibitors are administered together with drugs that are BCRP substrates, a change occurs in the in vivo disposition of drugs (absorption, distribution, excretion, and excretion into milk) (Ballent et al., 2012; Mealey, 2012). The bioavailability of drugs used in sheep increased with the inhibition of ABC transport proteins (Dupuy et al., 2003; Merino et al., 2003). Polyphenols (tannins, gossypol, fagopyrin and isoflavones) are present in moderate amounts in ruminant feeds. The main isoflavones genistein and daidzein in soy found in ruminant diets have been shown to act as BCRP inhibitors both in vitro and in vivo (Imai et al., 2004; Merino et al., 2010; Zhang et al., 2004b). Although genistein and daidzein are metabolized to glucuronide and sulfate forms in vivo, they interact with and inhibit BCRP (Alvarez et al., 2011). In a study conducted in ewes, daidzein monoglucurunoids and equol are the main isoflavone conjugates found in plasma and tissues (Urpi Sarda et al., 2008). Various studies have shown that flavonoids such as genistein, daidzein, apigenin and luteolin, which are also found in soybean and cow milk in ruminant feeds, reverse BCRP-mediated drug resistance (Antignac et al., 2003; Zhang and Morris, 2003; Cooray et al., 2004; Imai et al., 2004; Zhang et al., 2004b).

In a study conducted in sheep, the flavonoid quercetin increases the plasma moxidectin bioavailability, which is the result of decreasing the secretion of moxidectin into bile and intestine due to P -glycoprotein inhibition of quercetin (Dupuy et al., 2003). Studies in sheep have revealed that the drug penetration into milk decreases as a result of the use of BCRP substrates such as enrofloxacin, danofloxacin and nitrofurantoin together with BCRP inhibitors such as genistein and daidzein (Pulido et al., 2006; Perez et al. 2009 and 2013).

In a study to understand the effect of genistein on the concentrations of enrofloxacin in milk, enrofloxacin was administered to lactating sheep with genistein and without genistein (Pulido et al., 2006). There was no change in enrofloxacin peak plasma concentration (Cmax) value between sheep administered genistein and sheep not administered (control group). On the other hand, a 1.5-fold decrease was found in the area under the concentration versus time curve (AUC) of enrofloxacin in the milk of animals administered genistein, and genistein reduced the concentrations of enrofloxacin in milk. Researchers have suggested that the drug withdrawal period in milk may be shortened, as genistein reduces enrofloxacin concentrations in milk.

In another similar studies, the effect of isoflavone combinations (genistein + daidzein) on the secretion of BCRP substrates (danofloxacin and nitrofurantoin) into milk in lactating sheep was investigated (Perez et al. 2009 and 2013).

In the nitrofurantoin experiment, there was a significant decrease in milk AUC of nitrofurantoin in sheep given exogenous isoflavones (10 mg/kg genistein + 10 mg/kg daidzein) by oral gavage compared to the standard diet (Perez et al., 2009). In addition, higher plasma nitrofurantoin AUC and Cmax values were found in animals in the control group given only nitrofurantoin compared to sheep exposed to isoflavones (Perez et al., 2009). Since this is not compatible with the BCRP inhibition mechanism, the researchers suggested that the plasma nitrofurantoin concentration may be decreased in sheep given isoflavones due to decreased absorption or increased excretion of nitrofurantoin (Bircsak and Aleksunes, 2015).

In the danofloxacin experiment, the AUC and Cmax of danofloxacin in milk were significantly reduced in the group fed with soy-enriched diet for 15 days compared to lactating sheep fed with feed that do not contain isoflavones (Perez et al., 2013). A similar decrease in milk concentrations of danofloxacin was not observed in sheep given orally the soy active ingredients 10 mg/kg genistein and 10 mg/kg daidzein. Researchers have explained that this is because the form in which the compounds are administered (solid and liquid, aglycone and glycone) or the compounds in the diet can change the effect of other chemicals on the pharmacokinetics. Although the components in

the soy diet were specified in these studies, the amount of genistein, daidzein or other isoflavones in the rations was not reported.

Conclusion

Milk is one of the main sources of nutrients and bioactive components in all mammals and is essential in the early stages of neonatal development. Milk production is a very complex process that develops depending on transport mechanisms. Many studies in recent years have focused on transporters in the mammary gland to reveal this mechanism (Garcia-Lino et al., 2019).

One of the most important transporter superfamilies that ensure the secretion of compounds into milk is the ABC transporter protein family. The expression of these transport proteins vary according to the lactation period of the mammary gland. There is a relationship between BCRP, one of the ABC transporter proteins in the mammary gland of ruminants, and xenobiotic residues in milk, and this is important in shortening the drug withdrawal period as a result of interaction with ABC transport proteins (Garcia-Lino et al., 2019).

As a result of the interactions between BCRP drug substrate and inhibitors, many implications can be made for the pharmacotherapy of dairy animals. It is aimed to contribute to a better understanding of the potential role of BCRP inhibitors in the transfer of drug residues into milk, thereby protecting dairy consumers against possible drug residues and other xenobiotics.

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A study on toxin genes and cytotoxicity levels of *Bacillus cereus* in various ready-to-eat foods and pastry products in Turkey

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ABSTRACT

Bacillus cereus is a spore-forming and toxin-producing gram-positive bacteria widely isolated from soils, meat, milk, and vegetables. It is recognized as one of the pathogenic bacteria that can lead to food poisoning and food spoilage in food service systems due to its ease of contamination of foods and lack of guarantee of elimination by pasteurization and sanitation practices. *B. cereus* causes two types of diseases mainly characterized by diarrhea and vomiting type syndrome with the toxins it produces. Toxins produced by *B. cereus* are mainly heat-stable emetic toxin and three different heat-labile enterotoxins. Foodborne illnesses of the diarrheal type are caused by the single protein toxin; cytotoxin K (CytK), and both tripartite toxins; hemolysin BL (Hbl), and the non-hemolytic enterotoxin (Nhe), whereas the emetic type, is caused by an emetic toxin cereulide. In this study, 225 ready-to-eat foods and pastry products were analyzed for *B. cereus*, its toxin profiles, and cytotoxicity effects. Multiplex PCR is used to identify the presence of the Hbl, CytK, and emetic toxin encoding genes. Component-specific antibody-based ELISA tests were utilized to determine the Hbl-L_2 and NheB components. Cytotoxic activity of the *B. cereus* isolates on Vero cells was also identified. In total, *B. cereus* was detected in 37 out of 225 (16.4%) food samples. From the positive 37 *B. cereus* isolates, the *ces* gene was not identified, whereas 91.9% (34) Nhe, 56.8% (21) Hbl, and 8.1% (3) CytK encoding genes revealed positive results on PCR analysis. PCR results were also compatible with ELISA and Cytotoxicity tests. In a nutshell, 16.4% prevalence of *B. cereus* in foods is insufficient, and the presence or absence of toxin genes may not yield reliable results. It is critical to detect pathogenic *B. cereus* toxin gene profiles as well as toxin production ability at the same time. This study presents for the first time, data from a cell culture cytotoxicity test using specific monoclonal antibody-based sandwich ELISA and multiplex PCR for ready-to-eat foods and pastry products in Turkey

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Introduction

B. cereus is an aerobic or facultative anaerobic, gram-positive endospore-forming soil bacteria that grows at 28-35 °C optimum and is mainly isolated from meat, milk, and vegetable products. Its enterotoxins cause emetic or diarrheal characterized foodborne illness (Schoeni and Lee Wong, 2005; Wijnands et al., 2007; Stenfors Arnesen et al., 2008).

Single protein cytotoxin K (CytK) and three compounds Hemolysin BL (Hbl) and Non-hemolytic enterotoxins (Nhe) cause diarrheal-type foodborne illness. Hbl consists of B, L_1, and L_2 protein complexes, and Nhe consists of NheA, NheB, NheC protein complexes. In addition to these enterotoxins, there are recently published article claiming BceT, enterotoxin FM toxins, enzymes hemolysis II and III, InhA2 protease and phospholipase C enzymes are also associated with the formation of the diarrheal form (Lund and Granum 1996; Lund et al., 2000; Fagerlund et al., 2004; Lindbäck et al., 2010). More importantly, Hbl, Nhe, CytK and emetic toxins of *B. cereus* have been affiliated with food poisoning (Lindbäck et al., 2010).

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Contrasting to the single protein enterotoxin CytK and the three component enterotoxins Hbl/Nhe (Agata et al., 1995), the 17 different isoforms of the cyclic depsipeptide emetic toxin cereulide (Marxen et al., 2015) are responsible for emetic-type foodborne illness. Having said that, the diarrheal type of illness usually has mild symptoms for a longer period of time than the emetic form, such as mild abdominal pain and watery diarrhea. These symptoms start to appear 8 to 16 hours after consuming food and can last for 12 to 24 hours (Gaulin et al., 2002; Logan and Rodrigez-Diaz 2006; Murray et al., 2007). While produced in the small intestine during vegetative cell proliferation (Logan and Rodrigez-Diaz 2006) heat-labile diarrheal enterotoxins have an infectious dosage of 10^4–10^9 cell/g of food consumed. In contrast to the diarrheal form, the emetic form of the foodborne illness manifests its symptoms within a shorter period of 1 to 6 hours after food consumption. The infective dose of nausea and vomiting in the emetic form is 10^5-10^8 cells per gram, and it often goes away within 24 hours (Logan and Rodrigez-Diaz 2006).

Numerous studies have reported the detection of more than 90% Nhe and 43-83% in about 50% frequency of Hbl encoding toxin genes (Hansen and Hendriksen 2001; Yang et. al., 2005; Ehling-Schulz et al., 2006) diarrheal-type foodborne illnesses (Didier et al., 2016). Three components of the Nhe (A, B, C) encoded by the *nheA*, *nheB, nheC* genes; additionally the *hblC*, *hblD* and *hblA* genes encode the three partite toxins Hbl (L_2, L_1 and B) respectively (Granum et al., 1999).

For the highest level of biological activity, Nhe and Hbl require all three complex proteins (Clair et al., 2010; Sastalla et al., 2013; Dietrich et al., 2021), while the maximum toxic activity can be obtained with the optimum molar ratio between the three components A, B, C (10:10:1) and L_1, L_2, B (1:1:1) respectively (Lindbaeck 2004; Stenfors Arnesen et al., 2008; Didier et al., 2016).

Due to its proteolytic, lipolytic, and saccharolytic activities, the common contaminant *B. cereus* can cause food to spoil and become poisonous. It is considered one of the pathogenic bacteria that can endanger human life in food service systems (Ehling-Schulz et al., 2004; Tewari and Abdullah, 2015), as inactivation of *B. cereus* is not insured by pasteurization and sanitation techniques. In 413 epidemics between 2007 and 2014, the European Food Safety Authority (EFSA) reported that *B. cereus* was the source of 6657 cases. It has been noted that these cases most frequently occurred due to mixed-type foods and open buffet meals (EFSA Panel, 2016). The three main causes for *B. cereus*-related food poisoning are currently thought to be Hbl, Nhe, and emetic toxin (Schoeni and Wong, 2005; Stenfors Arnesen et al., 2008).

Foodborne outbreaks linked to *B. cereus* may become more frequent, necessitating quick, accurate test procedures. Currently, ELISA, PCR, rapid strip tests, various cell culture assays and LC-MC technics are used to detect *B. cereus* and its toxins. However, most rapid toxin kits have been used to qualitatively detect Hbl-L_2 or NheA components (Granum et al., 1993; Beecher and Wong, 1994), and the results of these rapid toxin kits are incompatible with molecular-based techniques (Ouoba et al., 2008; Ankolekar et al., 2009).

Dietrich et al. (1999, 2005) obtained monoclonal antibodies for the accurate immunochemical detection of each individual protein of Hbl and Nhe (mAbs). It's important to obtain consistent results when using molecular and immunological techniques. It is also crucial to determine the potency of cytotoxic capability and the detection of enterotoxins. Therefore, the objectives of this study were to investigate the consistency of 36 *B. cereus* foodborne isolates' molecular, immunological, cell culture, and cytotoxic test results.

Materials and Methods

Bacterial strains and isolates: Enterotoxigenic *B. cereus* reference strains; MHI163 (Hbl, Nhe), MHI165 (cereulide), MHI1307 (CytK1) and MHI 241 (Nhe) were obtained from the Milk Hygiene Institute (MHI). All other isolates were obtained from various ready-to-eat (RTE) foods and pastry products.

Microbial analyses: Enumeration of *B. cereus* in foods was carried out through ISO 7932:2004. Reference strains and 36 *B. cereus* isolates were cultured on supplemented with 5% blood sheep Columbia agar (Oxoid, PB 5039 A) and incubated for 24 h at 32 °C. After the first incubation, one colony was selected and inserted into a 5 mL Brain Heart Infusion (BHI) broth (Merck, 10493) for an additional 24 h at 32 °C incubation. DNA of *B. cereus* isolates were extracted from the 24 h incubated BHI cultures.

Conventional multiplex PCR: The Qiagen DNeasy blood and tissue kit was used in this study to extract the DNA of bacterial isolates in accordance with the manufacturer's instructions. The genes of the *B. cereus* toxin were identified using multiplex PCR. The samples were screened for the presence of *ces*, *cytK1*, *nheA*, *nheB*, *nheC* and *hblC* genes. A single primer was used to detect for the nheB and nheC target genes . Table 1 provides an overview of the PCR conditions and primers. Amplified PCR products with 5 µL loading buffer were run by 2% agarose gels in Tris acetate

Table 1. PCR conditions and primers

Target Gene	Primer	Primersequans (5'→3')	Amplification length (pb)	PCR conditions	References
hblC	L2aF	CGA AAA TTA GGT GCG CAA TC	411	94°C, 60 s; 51°C, 60 s; 72°C, 60 s (30 cycles)	(Moravek et al., 2004)
	L2aR	TAA TAT GCC TTG CGC AGT TG			
hblD	L1aF	AGG TCA ACA GGC AAC GAT TC	205	94°C, 60 s; 52°C, 60 s; 72°C, 60 s (30 cycles)	(Moravek et al., 2004)
	L1aR	CGA GAG TCC ACC AAC AAC AG			
hblA	HA-F1	ATT AAT ACA GGG GAT GGGA GAA ACT T	237	94°C, 60 s; 52°C, 60 s; 72°C, 60 s (30 cycles)	(Yang et al., 2005)
	HA-R1	TGA TCC TAA TAC TTC TTC TAG ACG CTT			
nheA	45c1	GAG GGG CAA ACA GAA GTG AA	186	94°C, 60 s; 52°C, 60 s; 72°C, 60 s (30 cycles)	(Moravek et al., 2004)
	45c2	TGC GAA CTT TTG ATG ATT CG			
nheBC	NhEBC1	ACA TTG CGA AAG ATA GCT GGA	300	94°C, 60 s; 48°C, 60 s; 72°C, 60 s (30 cycles)	(Dietrich et al., 2005)
	NhEBC2	TGT TCT GCT GCA AAA GGA TG			
ces	cesF1	GGT GAC ACA TTA TCA TAT AAG GTG	1271	94°C, 60 s; 52°C, 60 s; 72°C, 60 s (30 cycles)	(Ehling-Shulz et al., 2005 a,b)
	cesF2	GTA AGC GAA CCT GTC TGT AAC AAC A			
cytK1	F2 R7	AAC AGA TAT CGG TCA AAA TGC CGT GCA TCT GTT TCA TGA GC	623	94°C, 60 s; 52°C, 60 s; 72°C, 60 s (30 cycles)	(Lund et al., 2000)

EDTA (Sigma-Aldrich, T4038-5x1L) antigen retrieval buffer at 200 volts and 400 ma. for 45 minutes was used in nucleic acid electrophoresis.

Pre-enrichment of *B. cereus* isolates in CGY medium: Pre-enrichment in CGY (casein hydrolysate glucose yeast) broth (Merck, 101868) was used to stimulate enterotoxin production of *B. cereus* isolates. Pure cultures were added into supplemented CGY (18 mL CGY + 2mL 10% glucose) broth in the shaking water bath for 17 h at 32°C. The next day, 200µL of the enriched isolates were transferred into a new 20 mL of CGY broth. Liquid cultures were incubated for another 6 h with shaking at 32 °C. After the final incubation, the enriched culture was centrifuged at 3000 rpm at 4 °C for 20 minutes. At the end of the centrifugation, supernatants were transferred with a sterile filter (0,2 µm) into a new 2 mL tube containing 1mmol/L EDTA. Subsequently, aliquots were stored at -20 °C for use in the ELISA and cytotoxicity assays.

Enzyme immunoassay analyses (EIAs): Sandwich enzyme immunoassays (Sandwich EIAs) were carried out to determine NheB and Hbl-L_2 enterotoxin components. For this reason, specific mAbs; (5 µg/mL) 2B11 and 1E11; Horseradish peroxidase (HRP) 1:2000 against NheB, (10 µg/mL) 1A12 and 8B12 HRP 1:1000 against Hbl-L_2 were used as previously described (Dietrich et al., 1999, 2005). The same test procedures and chemicals were used as before (Jeßberger et al., 2017; Schwenk et al., 2020). The color-forming reaction was stopped after 20 minutes by adding 1 mol of H_2SO_4 (100L per well), and the plates were read at 450 nm in the final step of sandwich EIAs. Titer was defined as the reciprocal titer for the highest absorbance dilutions greater than or equal to 1.0. (Dietrich et al., 1999, 2005).

Cell cultures: African green monkey kidney (Vero) cell lines (Bio Whittaker, 76-108B) were studied for the cytotoxic effect of *B. cereus* isolates.

Cytotoxicity assays: As previously described, Vero cell lines were studied to associate the cytotoxic activity of *B. cereus* culture supernatants (Dietrich et al., 1999). Serial dilutions of the supernatants were added to 1 mL microplates containing 10^4 cells per well. Earle's salts containing 1% fetal bovine serum and 2 mmol L^1 glutamine were added to the growth medium and Eagle Minimum Essential Medium (EMEM). The plates were incubated at 37 °C in 5% CO_2 for 24 h. Finally, the absorbance of the tetrazolium salt WST-1 reagent at 450 nm was measured to determine the mitochondrial activity of viable cells. The 50% inhibitory concentration was calculated using the resultant dose-response curve and expressed as the reciprocal dilution giving 50% decrease in mitochondrial activity.

Results

***Bacillus cereus* results**: *B. cereus* was isolated from 37 contaminated RTE foods and pastry products from caterers, cafés, restaurants, bakeries, delicatessens, supermarkets, and mobile vendors in Istanbul. *B. cereus* was found at a level of $2x10^1$ to $2x10^4$ cfu/g in 26/150 (17.3%) RTE foods. Raw meatballs, minced meat pie and rice pilaf had the highest level of bacteria per gram. The amount of *B. cereus* were also enumerated between $2x10^1$ cfu/g and $7x10^2$ cfu/g in 11/75 (14.7%) pastry products. Results are presented in Table 2.

Table 2: *B. cereus* results

Food group	Total	*B. cereus* positiv (%)	*B. cereus* level (cfu/g)			
			>10-100	>100-1000	>1000-10000	>10000
Ready-to-eat foods	150	26 (17.3%)	10	12	3	1
Pastry products	75	11 (14.7%)	8	3	-	-

Multiplex PCR: For the *hblC*, *hblD*, *hblA*, *nheA*, *nheBC*, *ces*, and *cytK1* genes, agarose gel electrophoresis was used to amplify DNA fragments based on their size. In 26 RTE foods; 24/26 (92.3%) *nhe* (*ABC*), 16/26 (61.5%) *hbl* (*CDA*), 2/26 (7.7%) *cytK1* genes and a total of 11 pastry products; 10/11 (90.9%) *nhe* (*ABC*), 5/11 (45.5%) *hbl* (*CDA*) and 1/11 (9.1%) *cytK1* toxin-encoding genes were detected, whereas the *ces* gene was not determined in any samples. Figure 1(A), Figure 1(B) and Figure 1(C) represent different toxigenic patterns of certain *B. cereus* isolates after PCR assays.

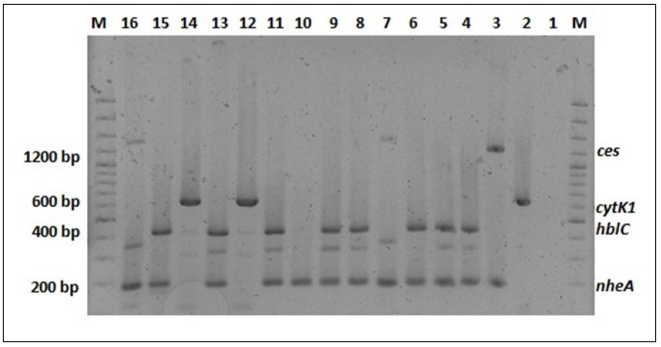

Figure 1(A). Toxigenic patterns of *B. cereus* isolates.
M: marker, 1: negative control, 2: MHI11307, 3: MHI165, 4: MHI163, 5-14: Food samples.

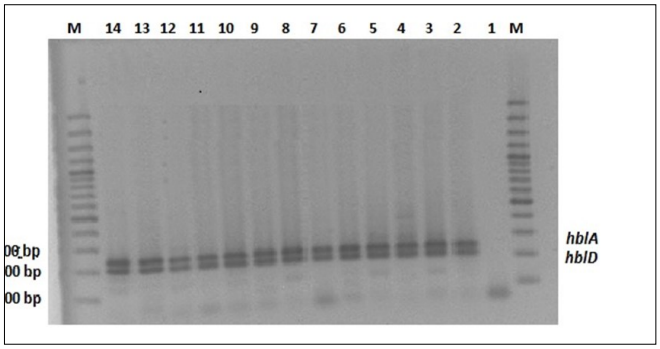

Figure 1(B). Toxigenic patterns of *B. cereus* isolates.
M: marker, 1: negative control, 2: MHI11307, 3: MHI165, 4: MHI163, 5-16: Food samples.

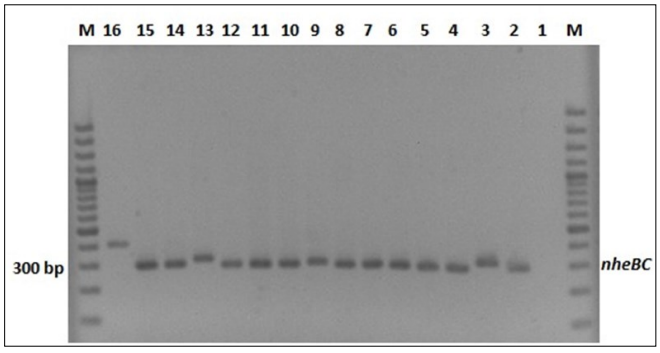

Figure 1(C). Toxigenic patterns of *B. cereus* isolates
M marker, 1 negative control, 2:MHI241, 3-16: Food samples

Enzyme immunoassay: Sandwich EIAs using monoclonal antibodies to detect Hbl-L_2 and NheB protein components were fully consistent with the multiplex PCR results. Nhe was detected in all Nhe positive isolates. Figures 2(A) and 2(B) show NheB and Hbl-L_2 titers .

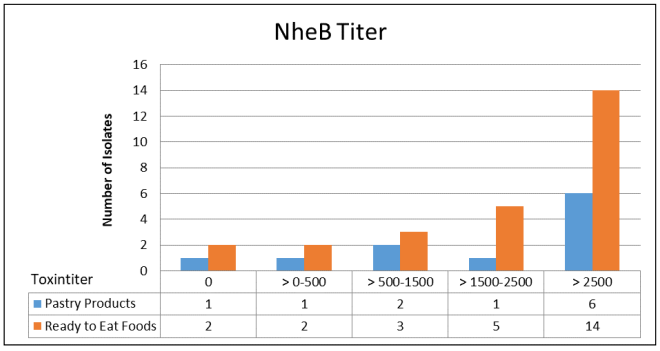

Figure 2(A). NheB titer

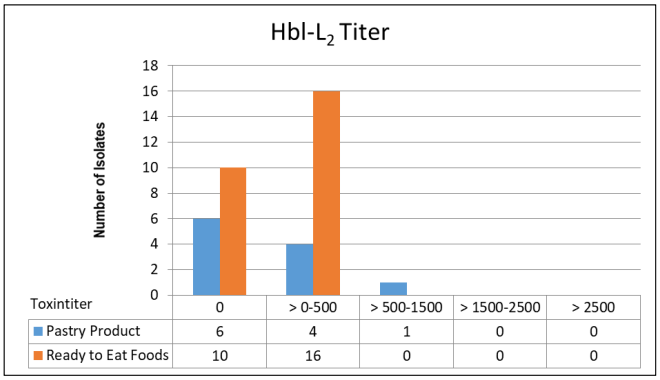

Figure 2 (B). Hbl-L_2 titer

Cytotoxicity assays: Vero cells were used to demonstrate the general cytotoxic activity of the isolates. The in-vitro cytotoxicity test yielded positive results in 24 of 26 RTE food samples and 11 pastry products. Cell culture test results show an agreement between immunoassay and PCR results. Table 3 shows the results of the PCR, EIAs and cytotoxicity tests.

Discussion

Foodborne infections are serious public health issues that affect millions of people worldwide. Because *B. cereus* is frequently found in RTE and reheated foods due to spore formation and its virulent nature. It is extremely difficult to prevent RTE food contamination by *B. cereus* that causes diarrheal or emetic type food poisonings (Agata et al., 1995).

Several studies have reported *B. cereus* levels in RTE foods. In the Netherlands, Van Netten et al. (1990) discovered B. cereus in 6% of 551 rice dishes, 3% of 35

Table 3. Results of PCR, ELISA and cytotoxicity tests

		PCR				ELISA		Cytotoxicity [&]
						NhE	Hbl	
Food Group	No	*nhe*	*hbl*	*cytK*	*ces*	B (+)	L_2 (+)	
Pastry products	11	10	5	1	0	10	5	1.2 -848
Ready-to-eat food	26	24	16	2	0	24	16	0-893

[&] Reciprocal cytotoxicity titre, Zero indicates negative ELISA result, Nhe (nonhaemolytic enterotoxin), Hbl (hemolysin BL)

lasagna dishes, and 1% of 72 RTE dishes (Van Netten et al., 1990). Ehling-Schulz et al. (2004) found *B. cereus* levels ranging from 10-100 cfu/g in 28% of RTE foods. Valero et al. (2007) discovered *B. cereus* in 109(48%) of 229 food samples, including bakery and meat products, milk, chocolate, spices, etc. *B. cereus* had a range of 10^2 to 10^6 cfu/g or ml. In our study, *B. cereus* levels ranged from $2x10^1$-$2x10^4$ cfu/g in 26 of the 150 RTE foods tested. *B. cereus* was isolated between $2x10^1$ cfu/g and $7x10^2$ cfu/g in 11 (14.7%) of the 75 pastry products collected from various production and sales points in Istanbul.

Aksu and Ergün (1996) isolated *B. cereus* from 5 (7.0%) of 71 pastry products, including milk-based desserts, in their study in Istanbul. Çadırcı et al. (2013) also determined *B. cereus* from 7 (7%) out of 100 milk pudding samples. Our result was higher than the rates stated in other studies mentioned above. Bonerba et al. (2010) isolated *B. cereus* from 34 (45%) of 74 patisserie products, which was higher than we found. In our study, eight of the eleven pasty products were milk-based desserts (rice pudding, panna cotta, creme brulee, and so on), with the remaining three being chocolate cake, fiora pandi, spagna, and pistachio dessert.

In the current study, it's found that positive enterotoxin gene PCR results (*hblC*, *hblD*, *hblA*, *nheA*, *nheBC*, *ces*, *cytK1*) confirmed component specific ELISA findings. NheB levels were higher in the isolates than in Hbl-L_2, and cytotoxicity titers ranged from 0 to 893. The majority of the isolates exhibited cytotoxic activity.

The findings from various studies (Hansen and Hendriksen, 2001; In't Veld et al., 2001; Guinebretiere et al., 2002) indicate that almost all *B. cereus* strains contain at least one diarrheal enterotoxins gene. Although the percentage of emetic strains in food isolates varies greatly depending on the food contents, studies have shown very few strains produce the *ces* gene in general, but the level of the percentage could be up to 20.2% (Jung et al., 2017). Our study found no evidence of the ces gene in any food isolates.

The results of PCR showed that 24 (92.3%) out of the 26 Nhe positive RTE food isolates produced complete Nhe consisting of the three components, whereas 16 (61.5%) out of 26 were Hbl-positive isolates for the B, L_1 and L_2 components. 2 (7.7%) out of 26 positive isolates contained the *cytK1* gene. In pastry products, 10 (90.9%) out of 11 isolates were Nhe positive for the 3 components, 45.5% (5) positive for the 3 components of Hbl isolates, 1 (9.1%) and out of 11 positive isolates contained the *cytK1* gene.

Wijnands et al. (2006) reported that 796 isolates and 182 different food samples contain approximately 95% Nhe encoding genes, with a further 66% and 50% determining positive for the genes encoded by Hbl and CytK, respectively. Except for the *cytK1* gene percentage, 50% of our study findings matched those of Wijnands et al. (2006). However, in our current study, we discovered Hbl levels lower than 95% of those found by Hwang and Park (2015) in RTE foods.

Çadırcı et al. (2013) obtained 20 isolates from 7 positive samples of 100 milk-based desserts. 70% (14/20) of isolates did not carry the Hbl encoding genes..In the Hbl encoding genes, 30% (6/20) of isolates contained three enterotoxic Hbl complex coding genes (*hblA*, *hblC* and *hblD)*. Additionally , 40% (8/20) of the isolates had three Nhe complex coding genes (*nheA*, *nheB* and *nheC*). 45% (9/20) contained two encoding genes (*nheA* and *nheB*) and 15% (3/20) had at least one Nhe gene (two *nheA* and one *nheB*) The *cytK1* gene was not detected in any sample.

The findings of our study were consistent with those of Çadırcı et al. (2013). While NheA, NheB and NheC components were found to be positive in 10 (90.9%) of 11 positive samples, 5 of the samples (45.5%) were found to have 3 components of Hbl. The number of samples containing both Nhe and Hbl genes was determined as 5/11 (45.5%). Cerelude was not found in any of the isolates, and only one sample (9.1%) tested positive for CytK1.

In RTE foods and pastry products isolates' cytotoxicity titers ranged from 0 to 893 and 1.2-848, respectively. In this study, the highest cytotoxicity levels were found in potato salad, creme brulee, lamb shank, and lentil meatballs. Two isolates containing *cytK1* (rice pilafs) genes had no cytotoxicity on Vero cells.

Moravek et al. (2006) demonstrated that the cytotoxicity titer of Hbl positive strains was lower than that of Nhe positive strains and that food poisoning strains produced more enterotoxins, particularly Nhe, showing that food poisoning strains produce more enterotoxins, particularly Nhe. Our findings revealed that the strain with the highest cytotoxicity titer was the Nhe positive, Hbl negative. Other strains containing only Nhe, on the other hand, had low cytotoxicity titers. This study's EIAs and cytotoxicity findings suggest that there was a good correlation between the level of NheB and the toxic activity of B. cereus isolates on Vero cells, as Moravek et al. (2006) discovered.

Based on the current information, the presence of only a small or large number of vegetative cells and spores is insufficient for food contaminated with B. cereus to be deemed dangerous to health if consumed. At the same time, the presence of all Hbl, Nhe, CytK encoding genes and cytotoxicity tests should determine the inhibition of the cells (Hep-2, Vero) mitochondrial metabolic activity of B. cereus toxins. Our results show that most of the B. cereus isolates have cytotoxic capability. When the B. cereus strains possess all 3 genes of the Hbl or Nhe enterotoxins at the same time, they have the potential to cause foodborne illness.

Conclusion

In Turkey, most research has focused on *B. cereus* enumeration, with only a few studies on *B. cereus* toxigenic gene profiles and commercial tests based on enterotoxin levels. Although PCR-based methods give fast results in detecting profiles of enterotoxigenic *B. cereus*, they are not a reliable way to predict toxin levels in food isolates (Dietrich et al., 2021). As a result, the presence of *B. cereus* at a 16.4% rate does not guarantee pathogenicity for *B. cereus*. Although potential toxin genes are present, they may not be expressed for the production of the related toxin. As a result, the production of toxins is an important factor in the risk of *B. cereus*-related foodborne illness. However, while much research has been conducted to date, there is little information about the incidence of *B. cereus* food poisoning cases in Turkey in terms of toxin production and genetic characterization. Çadırcı et al. (2013, 2018) used PCR analyses to detect enterotoxigenic structures of *B. cereus* isolated from dairy desserts and ice cream. Yibar et. al. (2017) used enterotoxin production in dairy products (milk and cheese) via using Duopath® Cereus Enterotoxins Test Kit (Merck) Overall, the majority of the studies are concerned with the enumeration of *B. cereus* and some with the detection of toxigenic profiles of *B. cereus* isolates. As a result, our current study contains data for the first time in Turkey containing *B. cereus* enterotoxigenic gene profiles, mAbs-based ELISA, and cytotoxicity assay using Vero cells in RTE foods and pastry products. As a result, future research on the cytotoxic potency of *B. cereus* in various food products is recommended in our country.

Compliance with ethical standards

Conflict of interest

The authors declare that there are no competing interests associated with the manuscript.

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