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ON THE COVER

Global article map of Mycobacterium *avium subsp. paratuberculosis* (see page 48).

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Salvia verticillata Improved Cognitive Deficits in a Chronic Cerebral Hypoperfusion Rat Model

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ABSTRACT

CCH, resulting from multiple cerebrovascular diseases, has been considered the primary cause of cognitive impairment in recent years. In this process, oxidative stress plays a critical role and damages hippocampal neurons. Research has shown that *Salvia verticillata* has a significant antioxidant and free radical-scavenging activity due to its polyphenolic compounds. Therefore, the present study aimed to evaluate the effect of *Salvia verticillata* on a rat model of chronic cerebral hypoperfusion. A total of 24 rats were subjected to Salvia verticillata or vehicle orally from one week before 2VO surgery for 14 days. Cerebral hypoperfusion was induced by the bilateral occlusion of the common carotid arteries (2VO, n = 12 and sham, n = 12). The cognition of rats was evaluated 1 week after surgery in the MWM. In the MWM test, 2VO rats showed longer escape latency time and swimming distance and spent a shorter time in the target quadrant ($p < 0.05$). Moreover, we observed that *Salvia verticillata* treatment significantly reduced escape latency time, shortened the swimming distance, and increased target quadrant time ($p > 0.05$). Our results indicated that Salvia verticillata treatment significantly improved cognitive deficits in cerebral ischemic rats, probably by reducing oxidative stress damage.

Keywords

Salvia verticillata, Rat, Hypoperfusion, Dementia

Number of Figures: 4
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Abbreviations

CCH: Chronic Cerebral Hypoperfusion
VD: Vascular Dementia
CBF: Cerebral Blood Flow
BCAO: Bilateral Common Carotid Arteries Occlusion
MWM: Morris Water Maze
2VO: Two-Vessel Occlusion
ROS: Reactive Oxygen Species
ANOVA: Analysis of variance

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Introduction

VD is the second most common form of dementia characterized by progressive mental decline and generally caused by hypoxia-ischemia or hemorrhage brain lesions [1-4]. It has been proposed that cerebrovascular diseases eventually reduce CBF. CCH is the outcome of CBF regulation and is identified as a prominent risk factor contributing to degenerative processes leading to dementia [1, 5-7]. The cerebral blood vessels deliver oxygen and nutrients, which are essential for cellular and neuronal metabolism, to the brain. The anaerobic metabolic capacity of neurons is limited, and adequate CBF is crucial for neuronal function and survival [8, 9]. CCH damages neurons in brain areas, especially the CA1 region of the hippocampus, leading to oxidative stress and inflammation. Studies showed that the mammalian hippocampus is highly involved in spatial learning and episodic memory, and is very sensitive to ischemia and hypoxia [10, 11].

Permanent BCAO in rats significantly reduces cerebral blood flow (hypoperfusion). It is one of the most commonly used CCH animal models for studying neuronal degeneration and memory disturbance, resembling those found in human subjects with vascular dementia [5, 12]. The BCAO surgery is relatively easy to perform and the ligation of both common carotid arteries with sutures takes approximately 10 minutes. Consequently, the use of CCH rat models is beneficial as a preclinical approach for investigating complex questions directly in human research [1, 7, 13].

Numerous studies demonstrated that free radicals play a pivotal role in CCH by causing oxidative damage, brain energy insufficiency, and cell apoptosis [7, 8, 14, 15]. ROS are metabolites produced during oxidative stress and cellular metabolism. Oxidative stress is an imbalance between ROS production and removal due to the uncontrolled production of ROS, decreased antioxidant defenses, or a combination of both [7, 8, 16]. The important role of oxidative stress in the pathogenesis of some neurological disorders, such as epilepsy and depression, has been demonstrated [17]. Augmented ROS values lead to oxidative damage (pathologic effects) to biomolecules, including nucleic acid, proteins, lipids, carbohydrates, or any other essential molecules [7, 8, 18, 19]. Protective mechanisms that neutralize the ROS and maintain free radicals in the physiologic range include an array of systemic enzymes and non-enzyme antioxidant defenses [20]. It has been established that reducing ROS, such as superoxide anion radical, hydroxyl radical, and hydrogen peroxide, by antioxidant therapy can moderate the symptoms of cerebral hypoperfusion and its related disease. Oxygen free radicals and resulting lipid peroxidation are critical to cerebrovascular dysfunction in a variety of conditions that result in CCH. Therefore, antioxidant therapy may be useful for managing cerebrovascular disorders, such as VD [7, 8]. Many antioxidants are reported to reduce ROS-mediated reactions and protect neurons from ischemia-reperfusion-induced neural loss in the animal models of cerebral ischemia [21].

Nowadays, many herbal or chemical medications are available for treating various neurological disorders. *Salvia L.* is the major genera of the family Lamiaceae, which includes large species distributed throughout the world. The main distribution regions of these species are Asia (Iran, Turkey, and Afghanistan), Europe, America, and Africa [22]. Members of this genus have been of extensive research interest due to their diverse medicinal properties [22-24]. It has been reported that many *Salvia* plants are used for treating various diseases, including bronchitis, cancer, hepatitis, other hepatic diseases, cardiovascular diseases, Alzheimer's disease, as well as mental and nervous conditions [24]. In addition, several studies have demonstrated that the *Salvia* genus is a valuable source of powerful antioxidants [25-27]. Phytochemical analysis of different *Salvia* species indicated that these plants contain diterpenoids, sesquiterpenoids, flavonoid glycosides, anthocyanins, and polyphenols [28, 29]. The species Salvia verticillata has shown high antioxidant activity in vitro. This plant is considered an antioxidant and acetylcholinesterase inhibitor [25-27]. *Salvia verticillata* contains a variety of diterpenoids, essential oils, and polyphenols, that may have the potential for being used in cognitive deficits [26]. Some researchers reported *Salvia verticillata* as a natural source of free radical scavengers [27, 30].

Therefore, this study was designed to investigate the effects of the alcoholic extract of *Salvia verticillata* on CCH in rats induced by permanent ligation of the common carotid arteries. We analyzed the effect of *Salvia verticillata* on learning and memory deficits using MWM.

Result

The mean latency to reach the underwater platform (time of escape latency) is shown in Figure 1. In all groups, the escape latencies decreased gradually during the 7 days of training in the MWM test. The saline-treated 2VO group consistently took longer latency to find the platform position than the drug-treated 2VO and sham-operated groups ($p < 0.05$). Two-way ANOVA revealed significant differences between groups. It showed that administration of *Salvia verticillata* decreased the escape latency of 2VO rats ($p < 0.05$).

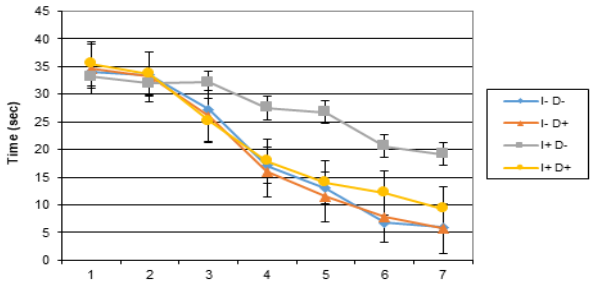

Figure 1.
Effects of *Salvia verticillata* on learning and memory impairment in 2VO rats. Morris water maze tests were performed on the day 7 to 14 post-surgery. Escape latency from the start point to locate the hidden platform.

Figure 2 shows that treatment with *Salvia verticillata* shortened the swimming distance compared to 2VO rats. The results were significantly different between saline-treated 2VO and other groups ($p < 0.05$), while no significant difference was observed between the drug-treated and sham groups ($p > 0.05$). In order to determine whether the animals' swimming ability contributed to swimming distance or platform location latency, swimming speed was also assessed (Figure 3). There was no significant difference in total speed between groups ($p > 0.05$).

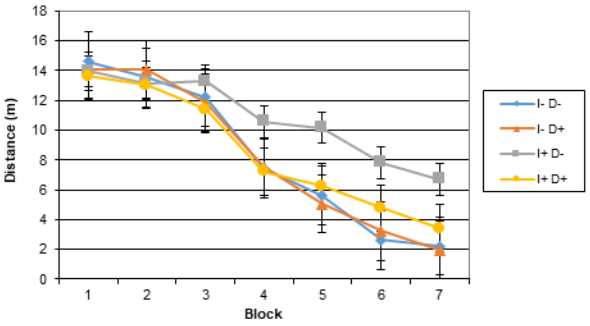

Figure 2.
Swimming distance of each group in the MWM test. Data are expressed as mean ± SD, n=6 for each group. 2VO: permanent bilateral common carotid artery ligation (2-vessel occlusion); HP: hypoperfusion, D: drug; SD: standard deviation.

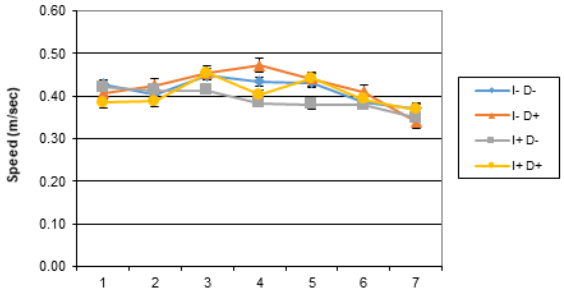

Figure 3.
Average swimming velocity in the MWM test. Data are expressed as mean ± SD, n=6 for each group. 2VO: permanent bilateral common carotid artery ligation (2-vessel occlusion); HP: hypoperfusion, D: drug; SD: standard deviation.

The results of spatial probe trials between groups are presented in Figure 4. It is shown that the 2VO model rats significantly spent less time in the N-E quarter (location of platform), compared to the sham groups ($p < 0.05$). After treatment with *Salvia verticillate*, the rats significantly spent more time in the target quadrant ($p < 0.05$).

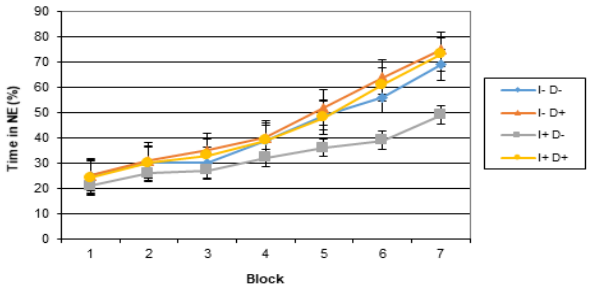

Figure 4.
Percent of time spent in target quadrant in 60 s probe trials (without platform). Data are expressed as mean ± SD, n = 6 for each group. 2VO: permanent bilateral common carotid artery ligation (2-vessel occlusion); HP: hypoperfusion, D: drug; SD: standard deviation.

Discussion

In this study, we demonstrated that administration of *Salvia verticillata* for 14 days markedly improved cognitive function using the MWM in a rat model of the 2VO method. Hypoperfused rats treated with vehicle alone showed significant cognitive deficits in the behavioral test.

In this research, we utilized the 2VO rat model to explore how *Salvia verticillata* affects cognitive impairment induced by decreased cerebral blood flow (hypoperfusion). In behavioral tests, 2VO rats exhibited a significant decline in spatial learning and memory abilities compared to the sham group as evaluated by the MWM test. This finding was consistent with previous studies on 2VO models [31, 32]. The 2VO model is capable of inducing a prolonged decrease in cerebral blood flow, which leads to neuronal damage and a decline in cognitive function [4, 5]. Euler *et al.* observed that the 2VO method resulted in the death of CA1 neurons in the hippocampus region and severe destruction of memory and learning in rats [33]. Therefore, this model is suitable for studying the learning and memory deficits in human dementia with the decline of cerebral circulation and drug effects on the disorder [34, 35].

Many behavioral tests have been designed to in-

vestigate brain lesions. One of the greatest common tests used to evaluate the memory and spatial learning function of cerebral hypoperfused rats is MWM. This test is similar to the non-verbal tests of cognitive function, which are sensitive in diagnosing aging disorders and dementia in the clinical environment [36]. Also, the reduction of non-cognitive functions such as sensorimotor, motor, and visual abilities of demented rats are not related to their performance in the MWM [36]. The probe trial distinguishes between different strategies used by demented rats to find the proper location of the hidden platform [36]. This test is a more accurate and reliable method for measuring memory accuracy [36]. In this type of learning test, considerable evidence has been compiled in support of 2VO-induced impairment [4]. Studies conducted previously have demonstrated that induced cerebral hypoperfusion in rats can adversely affect spatial learning and memory function [4]. In our MWM task, we observed that 2VO rats covered longer swimming distances, displayed longer escape latencies, and spent more time in the target quadrant. These results show that spatial learning and memory impairment were more pronounced in them than those in the sham rats. This finding is consistent with prior findings that indicated cerebral ischemia leads to an increase in the time required to locate the hidden platform and a decrease in the time spent swimming in the target quadrant [4, 37]. This result suggests that Salvia verticillata ameliorates cognitive deficits in 2VO rats.

Furthermore, the average swimming speed of rats during behavioral testing was not different between groups, indicating that swimming motivation and ability were similar between all animals. We concluded that the observed differences in the rats' spatial learning retention were not a result of sensorimotor impairment. Another study demonstrated that the swimming speed of rats did not change in the 2VO models [38].

Another factor discussed after the two-vessel method is the time to conduct behavioral tests after surgery. According to the literature, three phases can be defined for the two-vessel method. Acute phase that starts immediately after obstruction and will last for a maximum of 2-3 days. In this phase, the cerebral blood flow drops significantly and remains at the lowest level, which creates hypoxic-ischemic conditions and starts the electrophysiological activities of the nervous tissue damage [4]. Three days after 2VO surgery, the chronic hypoperfusion phase is started and continues for about 8-12 weeks. This phase closely resembles the conditions of decreased cerebral blood flow in elderly people with mental disorders. In the final phase, cerebral blood flow returns to baseline, and cerebral hypoperfusion and metabolism return to their original state [4].

Research has revealed that the chronic phase of 2VO is significant in the gradual decline of learning ability, but it is essential to consider the damage inflicted during the acute phase as well. With longer times after closing the vessels, cognitive disorders usually become more pronounced [4]. Ohta *et al.* showed that 10 days after vascular ligation, the behavior tests were significantly different between the two groups of 2VO and sham [39]. In another study, 2VO rats had more errors in finding the platform from day seven post-surgery [37]. Moreover, errors in behavioral tests have been reported from 3 days post-surgery in 2VO rats [5]. Therefore, in our study, behavioral tests were started on the 8th day after surgery, and the difference in the rats' learning was determined by test analysis.

There is a consensus that excessive generation of ROS leads to severe damage to cellular lipids, proteins, and DNA. Studies have shown that the brain is highly susceptible to ROS injury due to its dependency on aerobic metabolism, high contents of polyunsaturated lipids in cellular membranes, and low antioxidant defenses. Free radicals can cause degeneration and death of neurons [19]. In the 2VO model, hypoperfusion affects the cerebrum and hippocampus. The involvement of the sensorimotor cortex and hippocampus in memory and learning processes is unquestionable [40].

In the present study, it was found that the learning process in 2VO rats was progressively impaired in the MWM test. This result was confirmed in previous reports. Long-term administration of *Salvia verticillata* ameliorates the memory deficit of 2VO rats. Based on the laboratory studies,

the antioxidant properties of *Salvia verticillata* are significantly higher than other *Salvia* species [41]. The amount of phenolic content and antioxidant properties of the *Salvia verticillata* plant have been measured by different laboratory methods. Tosun et al. showed that the *Salvia verticillata* plant contained the highest amount of phenolic substances in comparison with seven other species of *Salvia* [41]. In a study by Matkowski *et al*., the high antioxidant power of *Salvia verticillata* in comparison with other species of this family has been emphasized [30]. In another research, the ethyl acetate extract of the leaf and stem of this plant had the highest antioxidant activity than other parts. Phenol is very important in scavenging free radicals due to its hydroxyl groups. Consequently, the phenolic content of the plant will probably have a direct relationship with its antioxidant properties [41]. This group allows phenol to remove the hydrogen end more easily to activate free radicals and destroy the antioxidant activation chain. The antioxidant capacity of these extracts is mostly related to their phenolic hydroxyl groups through various ways, such as preventing the formation of free radicals, catalyzing the temporary binding of metal ions, changing the state of peroxides, preventing the continuous accumulation of hydrogen, and scavenging free radicals [41]. As mentioned, the plants of the Lamiaceae family, especially *Salvia verticillata*, have a rich polyphenolic content. In summary, this study demonstrated that *Salvia verticillata* significantly improved cognitive deficits induced by CCH in rats. This effect is likely related to the antioxidant action of the medicine.

Materials and Methods

Animals

The Ferdowsi University of Mashhad's Institutional Animal Use and Care Committee approved this study, which was conducted in Mashhad, Iran.

Twenty-four male white rats aged 12 weeks (180-250 gr) were housed at 22°C ± 2°C room temperature and 60%±5% humidity, with a 12/12 h light/darkness schedule. The rats were provided with ad libitum access to commercial standard laboratory chow and tap water. They were housed in groups of three per cage and were utilized in compliance with regulations governing the examination of experimental animal administration.

The animals were chosen randomly and divided into two groups: 2VO (n = 12) and sham (n = 12). Rats in the 2VO groups underwent bilateral occlusion of carotid arteries through the procedures previously described by Pappas et al [37]. In brief, the rats were administered general anesthesia using isoflurane, following which a midline incision in the cervical region was made to carefully separate the bilateral carotid arteries from the vagus nerve and vein. The arteries were then tightly ligated using a 5-0 type silk suture. The same operation was conducted on the sham group, but without occluding the arteries. The procedure was carried out on a heating blanket, and the animal was kept warm until it regained consciousness. The rats were then randomly divided into four groups: a saline-treated 2VO group (n = 6), a drug-treated 2VO group (n = 6), a drug-treated sham group (n = 6), and a saline-treated sham group (n = 6). All animals were allowed a week of recovery.

Drugs and Administration

The plant *Salvia verticillata* L. was collected from Chalus, Mazandaran province, Iran, and was identified by the Department of Pharmacology, Faculty of Medical Sciences, Shahid Beheshti University, Tehran (6652-THE). The leaves were separated from the stem, dried in laboratory air, and kept in closed containers away from light until use. The dry leaves of the plant were completely powdered and 1000 grams of this powder were soaked in 4000 ml of methanol and placed in the laboratory environment for one night. The extract was evaporated at 40°C and under low pressure to obtain a syrupy extract weighing 264 grams. The methanolic extract was dispersed in 2000 ml of water and extracted with ethanol solvent. The extraction solvent was filtered and evaporated, and 27.5 grams of dry powder was obtained from the alcoholic extract [42]. One gram of *Salvia verticillata* was dissolved in 32 ml of distilled water prior to administration. Seven days before surgery until 7 days after surgery, drug-treated groups (2VO and sham) were treated with prepared *Salvia verticillata* (2 cc/250 gr body weight/d) orally using a stomach tube. Rats in the non-treated groups (2VO and sham) received only normal saline solution orally in a volume similar to *Salvia verticillata* at the same time. All rats were allowed one week to recover from the surgery and then a series of behavior tests were performed for 7 consecutive days.

Morris Water Maze Test

The learning and working spatial memory ability of rats was assessed using the MWM one week after 2VO surgery. In this model, each rat has to make four sequential performances to find a hidden platform in each trial. The MWM consisted of a circular tank (142 × 80 cm, height × diameter). It was filled with water at approximately 20°C ± 2°C to a height of a bit more than half mixed with innoxious ink. A transparent black metal platform (diameter 10 cm) was located at the center of the northeast quadrant at a constant position (target quadrant) and 1.5 cm below the surface of the water. There were many extra-maze visual cues (e.g., experimenter, window, computer, and rack) on the walls of the testing room to aid navigation. The maze was divided into four quadrants; north (N), south (S), west (W), and east (E). At each trial, animals were carefully placed into the water facing the wall of the tank in one of the four preplanned starting points (south, east, north, and west) that was selected randomly by computer. During the MWM task, the rat was given a time limit of 60 seconds to locate the platform by swimming. In case the rat was unable to find the platform during this time, the examiner gently placed it on the platform, and the escape latency time was recorded as 60 seconds. All rats were permitted to rest on the platform for 15 seconds irrespective of whether they found it or not. Each rat received four trials per day for 7 consecutive days, with a 30-second intertrial interval. Latency, the time required for each rat to find the platform, was recorded. Spatial learning was measured for each rat by averaging the latencies (seconds) across the four trials

per day. After the end of the learning trial on day 7, the platform was removed, and rats were subjected to the probe trial to evaluate the accuracy of the spatial memory. Following the completion of the MWM task, the rats were allowed to swim freely for 60 seconds, and the time spent in the quadrant where the platform was previously located was recorded. The rats' swimming activity and patterns were captured by a video camera, which was linked to a computer for further analysis. For each trial, various parameters, such as the escape latency time, path length, swimming speed, and the time spent in the target quadrant, were measured. The entire experiment, including the recovery period, took 21 days.

Statistical analysis

All data were presented as mean or median ± S.D. The main treatment effect on the escape latency, path length, and swim speed in the MWM was analyzed by repeated measures ANOVA followed by a turkey-Kramer post-hoc test for multiple comparisons between the two groups. Group differences in probe trials were analyzed using one-way ANOVA, followed by Duncan's multiple-range test. One-sample t-test was used to analyze performance in probe trials. $p < 0.05$ was considered statistically significant for all tests.

Funding

No funding was received for conducting this study.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Authors' Contributions

Conceptualization: Amir Afkhami goli, Hossein Kazemi Mehrjerdi; Methodology: [All Authors]; Formal analysis and investigation: [Amir Afkhami Goli]; Writing - original draft preparation: [All Authors]; Writing - review and editing: [Hossein Kazemi Mehrjerdi]; Funding acquisition: [Self-funding]; Supervision: [Hossein Kazemi Mehrjerdi, Amir Afkhami Goli]. All authors checked and approved the final version of the manuscript for publication in the present journal

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Competing Interests

The authors declare no conflict of interest.

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Seroprevalence and Risk Factors for Infection with Bovine Respiratory Syncytial Virus, Bovine Parainfluenza Virus-3, and Bovine Adenovirus-3 in Dairy Cattle Farms of Fars Province, Southern Iran

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ABSTRACT

The seroprevalences of BRSV, BPIV-3, and BAV-3 were studied in the dairy cattle of Fars Province in the south of Iran and their associated risk factors were determined. Serum samples (n = 420) were collected from 36 cattle herds in the northern, central, and southern regions of the study area. Commercial enzyme-linked immunosorbent assay kits were used to detect antibodies against these viruses. The Chi-square test and logistic regression were used to identify potential risk factors. Antibodies were estimated 100% for all the studied viruses at the herd level and 76.43%, 76.90%, and 92.62% at the animal level for BRSV, BPIV-3, and BAV-3, respectively. In logistic regression analysis, age for all the viruses, season for BPIV-3 and BAV-3, and region and farming type for BAV-3 were significantly related to seroprevalence at the animal level. A significant association of dual infections with studied viruses was identified. The present study demonstrated that BRSV, BPIV-3, and BAV-3 are very prevalent in the dairy herds of southern Iran and highlighted the necessity to establish a control program.

Keywords

Seroprevalence, Bovine respiratory syncytial virus, Bovine parainfluenza virus-3, Bovine adenovirus-3

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Abbreviations

BRSV: Bovine respiratory syncytial virus
BPIV-3: Bovine parainfluenza virus-3
BAV-3: Bovine adenovirus-3
BRD: Bovine respiratory disease
CI: Confidence interval
OD: Optical density
PP: Percent positivity

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Introduction

BRD, a global health problem causing severe economic losses due to body weight decline, yield loss, veterinary costs, medical fees, and animal mortality in cattle herds worldwide [1]. They may cause BRD alone or in mixed infections with other viruses or bacterial species, including Mannheimia haemolytica, Mycoplasma bovis, Pasteurella multocida, and Histophilus somni. They are the most well-known organisms that damage the respiratory tract of cattle and create opportunities for bacteria to colonize the lungs. Viruses are usually the first pathogens to intervene, while bacteria act as secondary invaders, aggravating the condition of formerly diseased animals [2].

BRSV is classified in the Pneumovirus genus and Pneumovirinae subfamily within the Paramyxoviridae family [3]. It is enveloped and contains a negative sense, single-stranded RNA genome encoding 11 proteins. Cattle are the natural hosts of BRSV, and a seroprevalence of 30%-70% has been detected. BRSV mostly affects young animals under one year old and calves often show severe clinical symptoms, such as fever, cough, loss of appetite, increased respiratory rate, and nasal discharge. BRSV infection is associated with high morbidity (60%-80%), and mortality can reach 20% in some outbreaks [4]. Previous studies identified environmental and climatic stressors, herd type, size and density, age group, purchasing new animals, and coinfection with bovine viral diarrhea virus as the main risk factors associated with BRSV infection [5-9].

BRSV and BPIV-3 are two closely related viruses often involved in BRD outbreaks [10]. BPIV-3 is a non-segmented, single-stranded, negative-sense, and enveloped RNA virus. It belongs to the Respirovirus genus of the subfamily Paramyxovirinae, family Paramyxoviridae, and spreads primarily by large droplet transmission [11].

Aerosols and fomites contaminated with nasal discharge can transmit BPIV-3 from animal to animal. The morbidity and mortality of BPIV- 3 infections are low, and generally remain subclinical but may present with symptoms, such as reluctancy to eat, cough, discharge from the nose, other respiratory signs, fever, lacrimation, and conjunctivitis [12, 13]. Several studies determined the seroprevalence of BPIV-3 infection in Isfahan (84.4%), Qazvin (95.2%), and Kerman (100%) Provinces of Iran and other countries (85.6% and 43% in Turkey and Mexico, respectively) which indicated broad virus dissemination [5, 6, 14-16].

BAV-3 belongs to the Mastadenovirus genus of the family Adenoviridae, which are non-enveloped double-stranded DNA viruses [17]. BAV-3 is considered one of the most important respiratory pathogens in cattle, especially newborn calves. Although BAV-3 infection usually occurs in a subclinical form, clinical symptoms, including fever, dyspnea, as well as nasal and conjunctival discharge have been described in severe infections, especially associated with immunosuppressive factors as crowding or co-infection with other viral or bacterial agents [18]. Data on the serological detection of BAV-3 are rare in Iran and limited to Isfahan and Kerman Provinces which reported 55.6% and 100% seroprevalence [14, 15].

Fars Province ranks first in the south of Iran in terms of cow population with 0.4 million cattle (mostly crossbreeds) and supplies the country with milk and meat. However, there is no official viral respiratory disease control program on dairy cattle farms, and limited knowledge is available on the prevalence of BRSV, BPIV-3, and BAV-3 infection in Fars Province. In order to set up a favored control program, it is important to know the prevalence and potential risk factors of infection. Therefore, the current research aimed to determine the prevalence of antibodies and risk factors of BRSV, BPIV-3, and BAV-3 infections in Fars Province, Iran.

Result

Only apparently healthy cattle were sampled, and finally, 432 blood samples were taken. The lack of kits caused 12 samples to be removed randomly. Finally, 140 specimens from each region (a total of 420 samples) were used in the test, which was considered to be approximately 10% higher than the calculated number. Seroprevalence at the herd level was estimated at 100% for all the studied viruses. Out of 420 animals, 321 (76.43%, 95% CI: 72.17%-80.24%), 323 (76.90%, 95% CI: 72.64%-80.68%), and 389 (92.62%, 95% CI: 89.71%-94.75%) were positive serologically for BRSV, BPIV-3, and BAV-3, respectively. Geographic region, gender, and age significantly affected ($p < 0.05$) BRSV serostatus, and then they were used in logistic regression analysis (Table 1). All the studied risk factors significantly affected the seroprevalence of BPIV-3 at the animal level. The variable "breeding type" was not included in the multivariable logistic regression analysis of BAV-3 due to the lack of statistically significant differences in the univariable model. In logistic regression analysis, age for all the viruses, season for BPIV-3 and BAV-3, and region and farming type for BAV-3 were significantly related to seroprevalence at the animal level (Table 2).

More than half of the sera (66.67%) had antibodies against all three viruses, and 6% of the sera were free of antibodies. The status of co-infection is shown in Table 3. A significant association of co-infections with BRSV and BPIV-3 ($\varphi = 0.494$, $p < 0.001$), BRSV and BAV-3 ($\varphi = 0.337$, $p < 0.001$), and BPIV-3 and

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Table 1.
Univariable analysis of associated variables for Bovine respiratory syncytial virus (BRSV), Bovine parainfluenza virus-3 (BPIV-3), and bovine adenovirus-3 (BAV-3) seropositivity in animal-level.

Viruses	BRSV					BPIV- 3					BAV-3				
Factor	Number		Serop† (%)	$\chi 2^*$	p-value	Number		Serop† (%)	$\chi 2^*$	p-value	Number		Serop† (%)	$\chi 2^*$	p-value
	T	P				T	P				T	P			
Season				1.07	0.30				16.42	0.001				4.21	0.04
warm	210	165	78.60			210	179	85.2			210	200	95.2		
Cold	210	156	74.30			210	144	68.6			210	189	90.0		
Geographic region				29.50	0.001				9.06	0.011				23.89	0.001
Central	140	85	60.7			140	98	70.0			140	118	84.3		
Northern	140	115	82.1			140	119	85.0			140	132	94.3		
Southern	140	121	86.4			140	106	75.7			140	139	99.3		
Gender				31.67	0.001				17.03	0.001				7.45	0.006
Male	38	15	39.5			38	19	50.0			38	31	81.6		
Female	382	306	80.1			382	304	79.6			382	358	93.7		
Age				118.25	0.000				33.14	0.000				44.88	0.000
Calf	80	24	30.0			80	42	52.5			80	60	75.0		
Adult	340	297	87.4			340	281	82.6			340	329	96.8		
Farming type				1.92	0.17				4.38	0.04				9.72	0.002
Industrial	212	156	73.6			212	154	72.6			212	188	88.7		
Traditional	208	165	79.3			208	169	81.3			208	201	96.6		
Breeding type				1.08	0.298				8.69	0.003				2.65	0.103
Artificial	219	189	86.3			219	191	87.2			219	210	95.9		
Mating	113	102	90.3			113	84	74.3			113	112	99.1		

† Seroprevalence *Chi-square T: Tested P: Positive

Table 2.
Logistic regression analysis of associated factors for Bovine respiratory syncytial virus (BRSV), Bovine parainfluenza virus-3 (BPIV-3), and bovine adenovirus-3 (BAV-3) seropositivity in animal-level.

Viruses		BRSV				BPIV-3				BAV-3			
Factor	Class	OR†	95% CI††		p-value	OR	95% CI†		p-value	OR	95% CI†		p-value
			Upper	Lower			Upper	Lower			Upper	Lower	
season	warm					3.15	1.87	5.31	0.000	2.54	1.06	6.07	0.036
	Cold					1				1			
Region	Central	1.014	0.50	2.07	0.21	1				1			
	Northern	0.633	0.31	1.29	0.97	1.65	0.82	3.31	0.164	2.84	1.05	7.66	0.039
	Southern	1				0.63	0.33	1.21	0.169	13.27	1.64	107.48	0.015
Sex	Male	1.01	0.40	2.59	0.98	1				1			
	Female	1				1.72	0.73	4.07	0.219	0.582	0.19	1.82	0.352
Age	Calf	1				1				1			
	Adult	13.44	6.89	26.23	0.000	4.39	2.28	8.43	0.000	6.07	2.43	15.15	0.000
Farming type	Industrial					1				1			
	Tradi-tional					1.61	0.98	2.65	0.062	3.08	1.23	7.72	0.016
Breeding type	Artificial					1							
	Mating					1.86	0.61	5.66	0.273				

† Confidence interval, ††Odds ratio

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Table 3.
The rates of dual infections of Bovine respiratory syncytial virus (BRSV), Bovine parainfluenza virus-3 (BPIV-3), and bovine adenovirus-3 (BAV-3)

BRSV and BPIV-3		BRSV and BAV-3		BPIV-3 and BAV-3	
BRSV(+)&BPIV-3(+)	67.62%	BRSV(+)&BAV-3(+)	74.52%	BPIV-3(+)&BAV-3(+)	75.48%
BRSV(+)&BPIV-3(-)	8.81%	BRSV(+)&BAV-3(-)	1.90%	BPIV-3(+)&BAV-3(-)	1.43%
BRSV(-)&BPIV-3(+)	9.29%	BRSV(-)&BAV-3(+)	18.10%	BPIV-3(-)&BAV-3(+)	17.14%
BRSV(-)&BPIV-3(-)	14.29%	BRSV(-)&BAV-3(-)	5.48%	BPIV-3(-)&BAV-3(-)	5.95%
Phi value	0.494	Phi value	0.337	Phi value	0.385
p-value	0.001	p-value	0.001	p-value	0.001

BAV-3 ($\varphi = 0.385$, $p < 0.001$) has been identified in cattle.

Discussion

Antibodies against BRSV, BPIV-3, and BAV-3 were found in all herds in this study. Vaccination against BRSV, BPIV-3, and BAV-3 was not practiced in the herds of Fars Province. Therefore, the presence of antibodies indicates exposure to these viruses. Limited knowledge is available on the herd-level prevalence of BRSV, BPIV-3, and BAV-3 infections in Iran. BRSV, BPIV-3, and BAV-3 were found in all the herds representing a seroprevalence of 100% at the herd level in Fars Province, and these rates are similar to the findings in Kerman Province, Iran [15]. The results from the Aegean Region in Turkey, São Paulo State in Brazil, and Northern Italy also demonstrated that antibodies to BRSV were detected in 100% of studied dairy herds [7, 8, 20]. The high herd prevalence of BRSV was also reported in dual-purpose cattle herds in some Latin American countries (91.3% and 93.2% in Ecuador and Mexico, respectively) [21, 22]. Poor biosecurity measures, such as failure to quarantine newly purchased animals, inability to diagnose subclinical BRSV cases, and the lack of vaccination programs against respiratory diseases may play a role in the high seroprevalence rate of BRSV at the herd level [7]. In endemic areas, observing biosecurity can protect herds from invading viral infections and reduce the morbidity rate [20].

The prevalence of BRSV (76.43%) in our study was considered high at the animal level, compared to the other report (51.1%) from the central region of Iran [14]. High individual seroprevalence of BRSV in the present study is also consistent with 80.48% and 79.5% of individual seroprevalences observed in Brazil and Ecuador, respectively [7, 22]. The animal-level seroprevalence of BRSV was reported 69.1% in Italy and 52.2% in Mexico [8, 21]. Some explanations for these variabilities are the differences in the number of samples, time of sample collection, route of antibody detection, housing and management, and inadequate knowledge of the disease [20]. In this study, a Chi-square analysis of the variables showed that region, gender, and age significantly affected the prevalence of antibodies to BRSV. The probability of BRSV infection in adults increased significantly ($p < 0.05$) by a factor of 13.44 compared to calves. The high risk of BRSV associated with age is in agreement with other reports. This association was explained by the longer exposure to the pathogen, decreased maternal antibodies, and reinfection with BRSV throughout life for older animals [20, 22]. A study in eastern and southeastern Poland confirmed the presence of BRSV infections in young cattle under 12 months of age in 60% of the dairy and beef herds examined, which was similar to other parts of Poland and Europe [23].

The reports from various countries have shown a great variation in BPIV-3 seroprevalence. In Iran, the seroprevalence of BPIV-3 was reported 100% at the herd level in Kerman and Qazvin Provinces, and 84.4%, 90%, and 95.5% at the animal level in Isfahan, Khorasan Razavi, and Qazvin Provinces, respectively [14-16, 24]. Others reported

a lower prevalence of antibodies against BPIV-3 in Saudi Arabia (67.6%), Turkey (56.2%), western Kenya (20.1%), and Grenada (13.4%) [25-28]. Despite a high seroprevalence, BPIV-3 has been identified less frequently in livestock farms. This is probably due to the lack of clinical cases, the similarity of symptoms to other respiratory diseases, and the lack of diagnostic kits. It has been hypothesized that small ruminants, particularly goats with high BPIV- 3 prevalence, may act as reservoirs or vectors in the transmission of BRDC to cattle [25]. The most common respiratory virus in our study was BAV-3, with a seroprevalence of 92.62%, which was in agreement with the findings in north-western Turkey, which reported a seroprevalence of 92.3% for BAV-3 [5]. Other reports from Iran showed that although the herd level seroprevalence of BAV-3 in Fars Province (100%) is similar to that in Kerman Province, the animal level prevalence of the virus is higher than that reported in Isfahan province (55.6%)[14, 15]. The prevalence of BAV-3 was 61.9% in serum samples from calves showing respiratory disorders symptoms [24]. Although preliminary Chi-square tests showed associations ($p < 0.05$) between the presence of antibodies to BPIV-3 and all the studied factors, season and age were significant in logistic regression analysis. Older animals had 4.39- and 6.07-fold greater odds of seropositivity for BPIV-3 and BAV-3 than calves, respectively. This higher seropositivity probably results from the fact that older animals were exposed to the active substance longer than younger animals [6, 27]. The prevalence of BPIV-3 and BAV-3 was higher in the warm seasons than cold seasons in the present study. Immunosuppressive stress is induced by various factors, such as dehydration, and high temperature may contribute to a difference between seroprevalence in seasons. A higher seroprevalence of BAV-3 in the southern region than in the northern region of the study area was manifested by risk factors analysis, which may be due to a higher temperature in the southern region compared to the northern region. Regarding the type of husbandry, the static analysis confirmed that cattle in traditional farms were 3.08 times more likely to be seropositive for BAV-3 than industrial farms. The likely causes for this difference have been ascribed to variations in herd hygiene, diet, and management system. Poor diet, early weaning, dehydration, low or high temperatures, inadequate rest, and transportation can trigger immunosuppressive stress [29]. A lower prevalence of BAV-3 in industrial farms can be due to many factors, for instance, the control of environmental factors, the establishment of biosecurity measures, and good management practices [14].

A significant association of dual infection with BRSV, BPIV-3, and BAV-3 was shown in the current study. The frequencies of mixed infection in the present study were higher than those in Isfahan Province. They reported 3.7% triple virus infection and 10% and 18.9% dual infections of BRSV plus BPIV-3 and BPIV-3 plus BAV-3, respectively [14].

In summary, this study demonstrated that BRSV, BPIV-3, and BAV-3 are very common in dairy cattle farms in the study area. Although the high seroprevalence found is not synonymous with disease, it represents a worrying epidemiological scenario as it is potentially important in the bovine respiratory disease complex. Therefore, a comprehensive epidemiological study on bovine respiratory viruses and other related bacterial species, including Mycoplasma spp, Mannheimia/Pasteurella, and Haemophilus/Histophilus, in Fars Province is proposed. Some preventive measures, such as quarantine, mass vaccination, and biosecurity, alongside raising farmer awareness of known risk factors, can help establish a control program on dairy farms.

Materials and Methods

Study location

This cross-sectional study with random cluster sampling was designed in Fars Province, southern Iran. This Province is located between latitude 27O3' to 31O40' N and longitude 50O36' to 55O35' E in an area of about 133000 km2 with a mean annual rainfall of about 230 mm in the south of Iran and contains 29 counties. Fars Province is classified into three regions based on topographic features. The northern region surrounds an area of the north, northwest, and west of the Province with mild cold winters. Central region is characterized by a relatively temperate climate with rainfall in winter and a hot and dry climate in summer. Finally, the southern region, which extends from south to southeast area, is defined by very warm summers (Statistical Yearbook of Fars Province 2019). The counties of Fars Province were assigned into northern, central, and southern regions based on

their geographical locations. Three industrial and three traditional dairy farms were randomly selected in each of these regions. Sampling was performed two times; one in the warm season (June-July) and another in the cold season (November-December) 2017.

Sample size

A total of 36 dairy farms were selected. Selected farms were visited, the purposes and details of the research project were described to the farm owners at the start of the study, and verbal consent was obtained. The target population was cattle herds, and the sampling unit was cattle. The formula below was used to calculate the sample size [19].

n=((1.96)^2 ×EP ×(1-EP))/d^2

Where n is the sample size, d represents precision of 0.05 at a 95% confidence level, and EP refers to an abbreviation for the expected prevalence, which was assumed to be 50% because the data on the seroprevalence of studied viruses are scarce in the south of Iran. Blood samples were taken from 12 cows on each farm, including six adult females, three adult males, and three calves under six months of age. Adult females were substituted if there were insufficient young or male animals. Generally, no vaccine was used against BPIV-3, BRSV, and BAV-3 in the study location.

Samples and antibodies evaluation

The sterile vacuum tubes without anticoagulant (VACUETTE®, Greiner Bio-One GmbH, Kremsmünster, Austria) were used for blood sample collection from the jugular vein. The tubes were labeled and immediately transported to the laboratory in a chilled state. Sera were collected after 10 min centrifugation at 3000 rpm and stored in a microtube at -20°C until analysis.

Antibodies against BRSV, BPIV-3, and BAV-3 were screened using an ELISA kit developed commercially by Bio-X Diagnostics (Rochefort, Belgium). According to the kit

instructions, a dilution buffer was prepared, and the samples were diluted in a dilution plate. The kit's reference sera were also diluted in a tube. The samples and controls were poured into the wells of antigen-coated microtiteration plate and incubated at 21°C for 1 h. The plate was rinsed with washing solution, and after three rinses, the diluted conjugate solution was added to each well. The plate was washed again after another incubation, and the reaction was made visible by chromogen combination for 10 min. Next, 1 M phosphoric acid stopped the reaction in the last step, and the ODs were recorded at 450 nm. The following formula was used to calculate percent positivity:

PP=(ODcorr of sample)/(ODcorr of positive control)×100

ODcorr is an abbreviation for corrected optical density, which is equal to ODtest of antigen or positive control minus ODcontrol.

The sample was considered positive for BRSV, BPIV-3, and BAV-3 if PP was greater than 20%, 20%, and 10%, respectively. Herds were considered positive for herd prevalence calculation when at least two antibody-positive samples were detected.

Statistical analysis

All statistical analyses were performed using the SPSS software version 22. Descriptive data analysis was carried out to calculate the animal and herd level seroprevalences. Associations between outcomes (BRSV, BPIV-3, and BAV-3 serostatus) and geographic region, cattle specifications, and farm features at the animal level were investigated by the Chi-square test. Logistic regression was used to find the effects of potential risk factors on the seroprevalence outcomes. The strength of the association between outcome and variables was assessed using odds ratios and a 95% confidence interval. Phi and Cramer's V measures were used for the correlation of the coexistence of antibodies to BRSV, BPIV-3, and BAV-3.

Authors' Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Majid Hashemi, Mehran Bakhshesh and Mohsen Manavian. The first draft of the manuscript was written by Majid Hashemi and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Competing Interests

The authors declare no conflict of interest.

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A Comparison of Bacteriological Culture, Serology, and Quantitative PCR for Detecting Brucellosis in Ewes with a History of Abortion

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ABSTRACT

The zoonotic disease brucellosis is a serious public health and livestock industry concern. In the present study, we used bacteriological culture, RBT, and qPCR to determine the prevalence of brucellosis in the serum and milk samples of sheep with a history of abortion. Serum and milk samples were obtained from 100 sheep aged 3-5 years. In order to determine the prevalence of brucellosis, a modified RBT was performed on serum samples, Brucella was isolated from milk by bacteriological culture, and qPCR was applied to detect bacterial DNA in milk. The prevalence of brucellosis using modified RBT, bacteriological culture, and qPCR was 32%, 42%, and 44%, respectively. By considering qPCR as the standard, modified RBT showed a sensitivity of 95%, a specificity of 100%, an accuracy of 98%, a PV+ of 100%, and a PV- of 97%. The sensitivity, specificity, accuracy, PV+, and PV- for bacteriological culture were 77%, 100%, 90%, 100%, and 85%, respectively. The agreement between qPCR and modified RBT was 0.959 (95% CI: 0.896-1), between qPCR and bacteriological culture was 0.792 (95% CI: 0.667-0.897), and between modified RBT and bacteriological culture was 0.831 (95% CI: 0.709-0.38). Based on the results, bacterial isolation from sheep milk is not recommended except in specific cases due to its low sensitivity, as well as its time-consuming and hazardous nature. However, the modified RBT can be used as a routine method because of its cost-effectiveness, higher sensitivity, and higher accuracy compared to bacterial isolation. Moreover, qPCR is recommended as the gold standard test for detecting brucellosis in sheep milk, especially in those with a history of abortion.

Keywords

Brucellosis, Modified Rose Bengal, qPCR, Sheep

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Number of Pages: 11

Abbreviations

RBT: Rose Bengal test
qPCR: quantitative PCR
CI: Confidence Interval
PV+: Positive Predictive Value

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Introduction

The *Brucella* genus is a non-motile, gram-negative, and intracellular *coccobacilli* bacteria that causes the zoonotic disease brucellosis [1-3]. Human Malta fever caused by *Brucella*, with more than 500,000 cases annually, is the most common contagious disease between humans and farmed species worldwide [4]. The genus Brucella, with 12 main species, can cause disease in several animal breeds, leading to economic loss. For example, abortion, stillbirth, and reproductive disorders are common clinical manifestations of brucellosis in sheep [5, 6]. Human infections are mainly caused by B. *melitensis*, B. *abortus,* B. *suis*, and B. *canis*, of which B. *melitensis,* with three biovars, is the most contagious [7-9]. *Brucella* infects humans by direct contact with contaminated tissues (e.g., placenta, fetus, and uterine secretion) or by consuming unpasteurized dairy products [10, 11]. Contrary to the cow milk used in industrial dairy production, ewe milk is routinely used for producing raw milk products, increasing the risk of contracting Malta fever [12]. In addition, B. melitensis, as the major causative agent of *brucellosis* in ewes, displays higher pathogenicity in humans than B. *abortus* due to its 10,000 times lower infectious dose [13]. As mentioned, *brucellosis* significantly impacts the livestock industry and public health. The control strategies are based on prevention and eradication. Sheep infected by *Brucella* are considered reservoirs in herds. In order to lessen the risk of disease and subsequent economic losses, the infected sheep must be identified and removed from the herd by the fastest, most cost-effective, and least hazardous method. Indeed, the detection of *Brucella* is the fundamental step in any control program. To achieve this goal, laboratory diagnosis could be performed in three diverse areas: 1) direct detection of living bacteria using culture media, 2) indirect diagnosis by serological methods, and 3) rapid diagnosis by molecular assays based on PCR [14].

Bacterial isolation is the most accurate method for brucellosis detection. However, the chronic stage of the disease is challenged by several limitations, including a long incubation period and low sensitivity. In addition, appropriate safety precautions need to be implemented for exposed laboratories and workers due to the hazardous nature of the Brucella organism classified as a class III pathogen [15]. Although serological methods are recommended for detecting brucellosis, they can have false positive and/or false negative results. Indeed, they are either too sensitive causing false positives, or too specific causing false negatives [16]. Moreover, the presence of antibodies in a serum sample does not always indicate an active case of brucellosis. Sustained immune responses that form after vaccination are an example of antibody formation in the absence of brucellosis [17]. Furthermore, in serological tests several gram-negative bacteria, especially *Salmonella* group N (O: 30), Escherichia coli O157:H7, Yersinia enterocolitica O:9, and Vibrio cholerae O1, can induce antibodies with cross-reactivity and cause false-positive results for brucellosis [18]. Therefore, employing two serological tests simultaneously to decrease the number of false positive and false negative results is highly recommended. According to the available protocols in Iran, RBT is applied for primary screening. Next, SAT and 2-ME confirm positive RBT samples. Despite the limited and conflicting information about RBT [19, 20], this test has been internationally approved for monitoring brucellosis in small ruminants [21]. Rose Bengal can be used as a rapid test for monitoring, but more specific tests are needed to confirm RBT results. SAT is routinely used for confirmation, and titers above a certain threshold are considered active brucellosis. Moreover, 2-ME, combined with SAT, differentiates between the agglutination of IgG and IgM-specific antibodies [22]. Because of the problems raised by the bacteriological culture and immunological methods, developing new diagnostic examinations for directly detecting Brucella species in milk has been increasingly under investigation. Recently, qPCR, as a well-established method, has been widely used to detect unculturable or slow-growth bacteria in microbial communities. The number of investigations on *Brucella* detection from ewe milk by qPCR is relatively limited. Consequently, evaluation of the efficiency of this method for detecting Brucella in ewe milk is not applicable. However, it seems that a molecular detection method, such as qPCR, which targets the specific region of Brucella with high sensitivity, could be an appropriate approach for the rapid and safe diagnosis of *Brucella* with the lowest rate of false negative and false positive results. We conducted a real-time PCR assay based on designing an alternative pair of primers to detect *Brucella. spp*. The present study aimed to: 1) determine the prevalence of brucellosis in ewes with a history of abortion by bacterial culture and qPCR on milk samples and serological methods (Rose Bengal, Wright, 2-ME) on serum samples, 2) compare the efficiency of three diagnostic methods (molecular, serological, and bacteriological) for identifying the infected ewes, and 3)

Abbreviations-Cont'd

PV-: Negative Predictive Value
SAT: Serum agglutination test
2-ME: 2-mercaptoethanol
LR: Likelihood ratios
MRT: Milk ring test
ELISA: Enzyme-linked
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

detect B*rucella* species circulating in the ewe population by Bruce-ladder multiplex PCR assay.

Result

To ensure the efficiency of DNA extraction from milk, all samples were evaluated for the integrity of GAPDH (housekeeping gene) in sheep. The 467bp GAPDH amplicon detected on a 1% agarose gel (Figure 1) showed an appropriate DNA extraction efficiency.

Comparison of Serological Tests and Culture with qPCR

Out of 100 milk specimens, Brucella spp. were isolated from 34 samples (34%), demonstrating the phenotypic and biochemical characteristics of typical Brucella species, including small and smooth colonies, non-hemolytic small gram-negative coccobacilli, catalase positivity, oxidase positivity, and urease positivity. All the isolates grew well in both aerobic and 8%-10% CO_2 atmospheres at 37°C, 4-8 days after incubation. Cultures that did not show any sign of growth until day eight did not grow until the end of 16 days in either atmosphere. Along with the phenotypic assays, the isolated bacteria were confirmed by PCR using genus-specific primers (Figure 2). Moreover, the species of *Brucella* strains were defined by the Bruce-ladder multiplex-PCR as well. Forty-two serum samples (42%) were diagnosed as positive by all three serological tests. Furthermore, all the positive cultures showed positive results in the serological tests. However, eight samples with positive serological results did not show any growth in culture. Using qPCR, the genomic elements of Brucella spp. were detected in 44 milk samples (44%), 42 of which were serologically positive. All the serological- and culture-positive samples were also positive in qPCR (Tables 1 and 2).

The sensitivity, specificity, PV+, and PV- of serological tests and cultures were calculated based on the qPCR results. The sensitivity, specificity, PV+, and PV- of serological tests compared to qPCR were 95%, 100%, 100%, and 97%, respectively. The mentioned parameters for microbial culture compared to qPCR were 77%, 100%, 100%, and 85%, respectively.

Using the Kappa test, all three methods were evaluated for inter-rater reliability. The agreement between qPCR and modified RBT was 0.959 (95% CI: 0.896-1), between qPCR and culture was 0.792

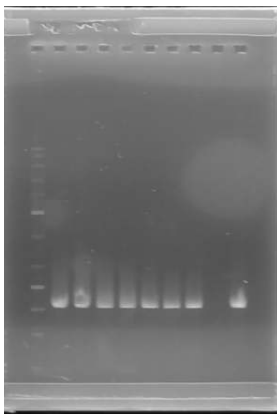

Figure 1.
PCR product of GAPDH gene. Lane 1: 100-bp DNA size marker (100-1500 bp); Lane 2-8: GAPDH gene; Lane 9: Negative control; Lane 10: Positive control

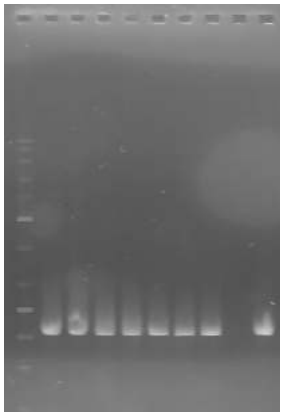

Figure 2.
PCR product of Brucella spp. Lane 1: 50-bp DNA size marker (50-1k bp); Lane 2-8: Brucella spp.; Lane 9: Negative control; Lane 10: Positive control

Table 1.
Prevalence of brucellosis in sheep with a history of abortion based on the positive results in each diagnostic test, n (%)

Sample (n)	Microbial culture	RBT	qPCR
100	34 (34%)	42 (42%)	44 (44%)

Table 2.
Data obtained from Rose Bengal Test, microbial culture, and qPCR, including true positive (a), true negative (d), false positive (b), and false negative (c) results

qPCR	Brucella (Modified RBT)		Total	Brucella (Microbial culture)	
	Negative	Positive		Negative	Positive
Positive	(a) = 42	(b) = 2	(a+b) = 44	(a) = 34	(b) = 10
Negative	(c) = 0	(d) = 56	(c+d) = 56	(c) = 0	(d) = 56
Total	(a+c) = 42	(b+d) = 58	n= 100	(a+c) = 34	(b+d) = 66

(95% CI: 0.667-0.897), and between modified RBT and culture was 0.831 (95% CI: 0.38-0.709). The positive and negative LR of the diagnostic tests used in this study were also evaluated (Table 3). A positive Rose Bengal or/and culture result is ∞ (infinity) times more likely to originate from an infected animal than from a healthy animal. Only 0.05 times as many animals with brucellosis as animals without the disease will provide a negative Rose Bengal result. An infected animal is 0.23 times more likely to have a negative culture result than a healthy animal.

Bruce-ladder Multiplex PCR

Regarding the capability of Bruce-Ladder multiplex PCR in identifying the Brucella species which are isolated in pure cultures, the test was performed on 32 extracted DNA samples of Brucella bacteria isolated from pure bacterial cultures. The patterns of produced fragments on the 1.5% agarose gel patterns were evaluated by Yoldi et al. All the isolates (Figure 3) were identified as B. melitensis (six amplicons with sizes of 152-bp, 450-bp, 587-bp, 794-bp, 1071-bp, and 1682-bp were multiplied).

Table 3.
Statistical parameters for modified RBT and microbial culture compared to qPCR for the diagnosis of brucellosis (95% CI

Statistic parameter based on qPCR	Value	
	Modified RBT	Culture
Sensitivity	95%	77%
Specificity	100%	100%
Positive Likelihood Ratio	∞	∞
Negative Likelihood Ratio	0/05	0/23
Positive Predictive Value	100%	100%
Negative Predictive Value	97%	85%
Accuracy	98%	90%
Kappa	95% CI: 0.896-1)0.959	95% CI: 0.709-0.38)0.831)
	(95% CI: 0.667-0.897)0.792	

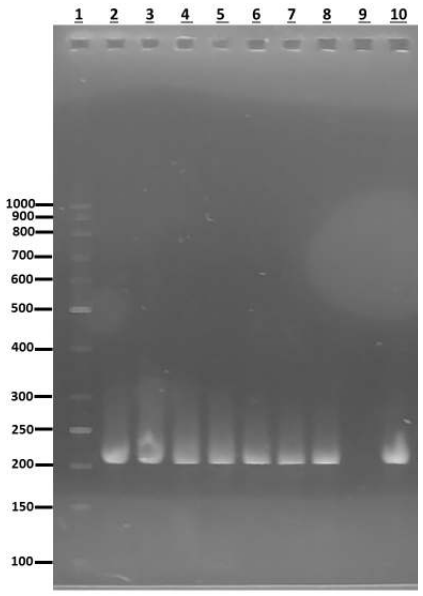

Figure 3.
Differentiation of B. abortus, B. melitensis, RB51, and Rev.1 vaccine strains by Bruce-ladder multiplex PCR. Lane 1: 100-bp Plus DNA size marker(100-3k bp); Lane 2: B. abortus; Lane 3: B. melitensis; Lane 4: B. abortus RB51 vaccine strain; Lane 5: B. melitensis Rev.1 vaccine strain

Discussion

Brucellosis due to B. *melitensis* is still a major problem for public health and also for sheep herds in several parts of the world, especially in the Middle East and the Mediterranean region. Most human cases of *brucellosis* around the world are infected with this species of Brucella. B. melitensis, the most important zoonotic pathogen between humans and animals, primarily infects sheep as its preferred host and transmits to humans mostly by consuming the milk and dairy products of sheep and goats which are unpasteurized, especially in endemic areas [8, 23-29]. Some clinical symptoms of brucellosis in sheep include abortion, stillbirth, retained placenta, weak lambs, and infertility which cause significant economic loss to the livestock industry [30]. In areas with a high prevalence of *brucellosis* (more than 5%), B. *melitensis* Rev. 1 strain vaccine is recommended on a large scale or/and for maiden ewes [28]. In the current study, sheep milk samples were directly subjected to molecular investigation for *Brucella spp*. DNA extraction was completed according to

Pokorska *et al.* [31], which showed the advantages of low cost, short time, and less volume of milk compared to many other methods. Studies on the prevalence of brucellosis in sheep have been conducted in Iran and other parts of the world using different methods and conditions of sheep (with a history of abortion or not). In the current research, the prevalence of brucellosis in ewes with a history of abortion was determined by three assays. In the milk culture, 34% of sheep were *Brucella*-positive, while serological methods and qPCR on milk samples determined the prevalence of *brucellosis* as 42% and 44%, respectively. As Al-Talafhah AH *et al.* [32] reported, monitoring the herd status in northern Jordan by RBT showed that 61% of all herds and 14% of sheep in each herd were positive for brucellosis. In another study conducted by Samadi A. et al. [33], 86 out of 188 (45.7%) samples of sheep with a history of abortion were positive for brucellosis using PCR. Zhang H *et al.* [34] reported that in the fetal tissues and milk of 120 sheep and cows, PCR for brucellosis was positive for 34 samples (28%). Therefore, there are some similarities and differences between the findings of this study and others. Differences in the prevalence of brucellosis can be due to variations in sample types or methods applied in each investigation. In a section of the study conducted by Hamadi et al. [17], blood and milk samples of 21 sheep were evaluated for brucellosis using RBT, culture, and PCR. Twenty samples were seropositive for RBT. *Brucella spp*. were isolated from 12 milk samples, while PCR detected Brucella spp. in ten milk samples. Eleven PCR-negative samples were positive in RBT, while a single Rose Bengal-negative sample was positive with PCR. In a study by Gupta et al. [6], out of 54 goat samples with a history of abortion, 32 serum samples were positive for SAT. Brucella genomic fragments were amplified in 48 milk samples, including 32 serum-positive specimens. It was found that PCR, as a controlled experiment, had a specificity of 100% and a sensitivity of 90%. Ilhan et al. [2] indicated that by examining the milk samples of sheep with a history of abortion, 8, 24, and 28 samples tested positive in culture, PCR, and MRT, respectively. Comparing MRT and PCR, 22 positive and 72 negative samples were common in both tests, and a coincidence of 96% was achieved. For PCR, the specificity and sensitivity were estimated at 100% and 81.3%, while for MRT, these indices were 75% and 75%, respectively. Altun *et al.* [35] evaluated 65 sheep milk samples for antibodies against *Brucella* with indirect ELISA and *Brucella* DNA with qPCR. According to their findings, 6.1% of the samples tested positive in both examinations. Lindahl et al. [16] examined blood samples with indirect ELISA and milk samples with qPCR from 570 non-vaccinated cattle. All serum-positive samples were also positive with qPCR, while 8.3% of seronegative cows tested positive for *Brucella spp.* DNA in their milk. In a study performed by Sabrina *et al.* [36], milk samples were obtained from 65 seronegative cows and tested for genomic fragments of Brucella with qPCR. Results revealed that 3.08% of cows tested positive for Brucella contamination. Zakaria [37] conducted research using 230 blood samples to establish the prevalence of brucellosis by RBT, modified in-house ELISA, and qPCR. The sensitivity and specificity of two serological tests were also calculated using qPCR as a standard. The overall prevalence of brucellosis was estimated at 53.9%, 75.2%, and 79.1% for ELISA, RBT, and qPCR, respectively. The sensitivity of RBT was 79.12%, and that of ELISA was 55.49%. In the present study, qPCR identified more positive samples (44 samples) than the culture method (34 samples) which indicates the higher sensitivity of qPCR than microbial culture for detecting brucellosis. Similar results were indicated in studies [37-39] comparing culture and conventional PCR on cow milk, which can be generalized to this study based on the higher sensitivity of qPCR than the conventional PCR. These results could be linked to the fact that in molecular methods, by targeting the genome of *Brucella*, both live and dead organisms could be detected, while in the culture method, only live organisms could be recognized by growing on a culture medium. Since a small number of *Brucella* organisms can cause the disease, the molecular approach seems more suitable than the culture method for identifying brucellosis in infected animals for the control and eradication purposes.

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No serological test has been specially defined for B. melitensis infection in sheep. It is commonly assumed that the serological tests used for identifying B. *abortus* in cows are sufficient to diagnose B. melitensis infection in sheep and other small ruminants, such as RBT which is widely used to diagnose *brucellosis* in sheep while it is mainly designed for B. *abortus*. Standardizing the antigens is a major challenge that affects the sensitivity of RBT. The antigen standardization conditions that seem suitable for detecting B. *abortus* in cows are insufficient for B. *melitensis* diagnosis in sheep [20, 40]. Moreover, RBT has specific limitations, including anti-complementary activity, the prozone effect that requires heat-inactivated serum [41], and low sensitivity confirmed in culture-proven cases [15, 42]. The Rose Bengal serology test used in this study demonstrated negative results for two sheep, while qPCR detected Brucella genomic fragments in the milk of these sheep. The results were similar to those reported by Leal-Klevezas et al. [38]. Despite it is recommended [40] that increasing the volume of serum can enhance the sensitivity of the RBT, the result of the present study showed that this Modified RBT can still have false negatives compare to the presented qPCR. This finding alarms and confirms that the sensitivity of RBT when testing blood samples of sheep requires improvement. However, modifying the antigen used in RBT by reducing the pH or cell concentration of the antigen may enhance the RBT sensitivity to an acceptable level when using sheep serum.

The qPCR protocol proposed in our study demonstrated advantages over the conventional microbial culture method, including higher speed and greater sensitivity. Moreover, there is no requirement for live *Brucella* organisms in this method which reduces the chance of infection transmission to laboratory staff and increases safety. Finally, it is recommended to use qPCR to diagnose or confirm the presence of B. melitensis in sheep milk as a stand-alone method or in combination with other techniques as a part of control and prevention programs. Although estimating the prevalence of brucellosis was not the main objective of our study, the results revealed that despite vaccination and other control measures over the years, clinical brucellosis still exists in sheep in various parts of the country and is one of the main causes of both sheep abortion and human brucellosis. This study was conducted on a small population of sheep with a history of abortion. Consequently, further extensive research at the national level is required to target the whole population of traditional and nomadic herds [26] using qPCR alongside other diagnostic methods as a sensitive, accurate, rapid, and easy technique. That can prevent the remaining infected sheep from being a false negative source of contamination in the herd.

Conclusion

One of the main measures of the control and prevention program for brucellosis is identifying infected animals. Screening is the first and most important step in the test-and-slaughter strategies. The discrepancy between the serological methods and qPCR highlights the need for additional diagnostic strategies to detect serologically false negative animals in screening, control, and eradication programs for brucellosis. However, in countries with limited resources, test-and-slaughter cannot be implemented. Therefore, identifying infected animals in herds allows farmers to take appropriate protective measures to reduce the spread of the disease.

Materials and Methods

Sampling

A total of 200 milk (n = 100) and blood (n = 100) samples were collected from ewes of different flocks with a history of abortion, aged 3-5 years, vaccinated with Rev.1 vaccine at the age of 6 months, which had not received any antibiotic or corticosteroids for at least one month before sampling. Following disinfection with 70% alcohol, blood specimens were taken from the jugular veins using 5 mL sterile syringes and were collected in tubes without anticoagulant. Before collecting milk samples, each teat was washed with warm water and wiped with a disposable towel. Initially, the first squirts of milk were disposed of. Then, about 10 mL of milk was collected from every teat in a sterile 50 mL Falcon tube. To prevent cross-contamination, the gloves were changed after each sampling. After taking the specimens under hygienic conditions, they were kept on ice and transferred to the laboratory within a maximum of 3 hours. The milk in the falcon tubes was divided into two sterile 15 mL tubes under laboratory conditions, one of which was used right away for microbial culture, while the second tube was stored at a temperature of -80°C for conducting molecular experiments in the future. It should be noted that all the manipulations of the samples and cultures in the laboratory were performed in a class II biological safety cabinet and national and international guidelines for dealing with Brucella-contami-

nated materials were followed.

Serological Test

Serological tests are a part of control and eradication programs for the detection of B. melitensis infection in ruminants.

Rose Bengal Test

To reduce false negative results, modified RBT, introduced by Blasco et al. [40], was used to increase sensitivity without affecting specificity [40, 43]. Briefly, 75 µL of the sera obtained from the studied ewes were mixed with 25 µL of Brucella antigen (Razi Vaccine & Serum Research Institute, Iran) at room temperature on a flat white ceramic plate and gently shaken for 4 minutes. Any agglutination that appeared during this time was recorded as a positive reaction.

Serum Agglutination Test and 2-MercaptoEthanol Test

The Wright and 2-ME tests were applied to confirm the positive results of modified RBT. For SAT, serum samples were prepared using a solution of sodium phenol chloride with a dilution ratio of 1:80, mixed with an equal volume of Brucella antigen (Wright Tube Kit®, Pasteur Institute, Iran) resulting in a 2-fold dilution. After incubating samples for 24 hours at 37°C, they were examined for agglutinated particles, and serum titers of 1:80 or higher were considered positive. The 2-ME test was performed for SAT-positive serum samples, with a 1:4 ratio of serum and the 2-ME solution mixed and incubated at 37°C for an hour. Next, a solution of sodium phenol chloride with a dilution of 1:80 was added, resulting in a 2-fold dilution of the reactions. After incubating for 24 hours at 37°C and resting for 1 hour at room temperature, the serum specimens were examined. A positive result was reported for the 2-ME test when the serum titers were 1:40 or greater.

Microbial Culture and Bacterial Isolation

Samples and Brucella strains were cultured in the CITA selective culture medium described by De Miguel et al. [44], which was also recommended by the World Organisation for Animal Health (OIE) for the isolation of *brucella* isolates, especially smooth Brucella species, such as B. *melitensis* and B. *abortus*. Briefly, the CITA selective medium consists of blood base agar plates containing 5% sterile sheep serum and is supplemented with antimicrobial agents as follows: antifungal agents amphotericin B (4 mg/L) and nystatin (100 000 IU/L) (Solarbio Science & Technology Co., Beijing, China), as well as antibiotics vancomycin (20 mg/L), colistin (7.5 mg/L), and nitrofurantoin (10 mg/L) (Solarbio Science & Technology Co., Beijing, China). Milk specimens were centrifuged at 3000 ×g for 15 min at 4°C. Afterwards, loopfuls of both cream and sediment were used for simultaneous inoculation onto two CITA plates. The plates were then incubated in two different atmospheres: aerobic and with 8%-10% carbon dioxide (Microbiology Anaerocult c®, Merck, Darmstadt, Germany) at 37°C for up to 16 days. The plates were evaluated for bacterial growth every 3 days starting from day 4, and if no growth was observed after day 16, the culture was reported as a negative result. In the case of bacterial growth observation, a pure culture was prepared for further phenotypic and molecular confirmation. Phenotypic characteristics for confirming *Brucella. spp*, such as colonial morphology, bacterial morphology, gram staining, catalase, oxidase, and urease activity were recorded.

Molecular Tests

In parallel to the microbial culture, the molecular method using the DNA extracted from the isolated strains was applied for genotypic identification using genus-specific primers for genus detection and Multiplex Bruce-ladder PCR for the diagnosis of Brucella species.

DNA Extraction

DNA was extracted from pure cultures using the modified boiling method introduced by Queipo-Ortuño et al. [45]. In summary, the bacteria obtained from pure culture were washed twice with Tris-HCL-EDTA buffer and centrifuged at 15000 ×g for 10 min. Approximately 600 µL of the top layer of the second centrifugation was removed, and the tube with the remaining material was incubated in a water bath at 100°C for 10 min. After keeping it on ice for 10 min, the tube was centrifuged at 15000 ×g for 10 min. The supernatant was separated and placed at -20°C for further use. To perform DNA extraction from milk samples, we followed the method previously described by Pokorska *et al.* [31]. In brief, 10 mL of milk collected during sampling was centrifuged at 7000 ×g for 10 min at 4°C. The liquid layer on the top of the tube along with the fat from the milk was removed, and the remaining pellet at the bottom of the tube with its supernatant liquid was transferred to a sterile 2 mL tube. The mixture then underwent the process of centrifugation at 5000 ×g for 3 min at 4°C, and the liquid layer on top was removed. The pellet was washed with 1 mL of buffer (15 mM Tris-HCl (pH 7.4-7.6), 25 mM NaCl, 5 mM $MgCl_2$, 15 mM Na_2HPO_4, 2.5 mM EDTA, 1% sucrose) by centrifuging at 5000 ×g for 3 min at 4°C, and discarding the supernatant liquid. This step was repeated until the supernatant liquid became clear. Then, 1 mL of lysis buffer (pH 8.8; 6% SDS, 3 mM $MgCl_2$, 15 mM Tris-HCl, 0.5% DMSO, 6% acetone) was added to the pellet obtained from the preceding step and incubated at 65°C in a water bath for about 60-90 min until the pellet was dissolved entirely. Next, the mixture was cooled at room temperature, and 450 µL of precipitating buffer (2.35 M NH_4Cl, 1.15 M NaCl, 38% ethanol pH: 5) was added. After Vortexing and centrifuging at 16000 ×g for 8 min at 10°C, the liquid on the surface was transferred to a new tube, and 600 µL of 100% isopropanol was added. The tube was then centrifuged at 10000 ×g for 8 min, and the remaining liquid on the surface was removed. The DNA pellet obtained was washed twice with 70% ethanol and air-dried. Next, the DNA pellet was dissolved in 100 µL of TE buffer (pH 8.0, 10 mM Tris, 1 mM EDTA). Quality and quantity assessment of DNA extracted from milk was beyond the main objectives of this study. However, To confirm the successful DNA extraction process from milk samples, the primers described by Kadivar et al. [46] were applied to amplify a 467 bp sequence of a housekeeping gene known as the glyceraldehyde-3-phosphate dehydrogenase (NC_056056.1). Calibrated 1% agarose electrophoresis (XM_060411591.1)) and Green Viewer safe stain (0.01 v/v) were used to assess the PCR products. A 100-bp DNA ladder (100-1500 bp) (Cat No.YT8503, Yekta Tajhiz Azma, Tehran, Iran) was used as a DNA marker. The sample was stored at -20°C for further examination if the result was positive.

PCR and Bruce-ladder

The *Brucella spp*. molecular confirmation was conducted on the DNA samples, which were extracted from Brucella genus identified positive bacteriologically using genus-specific primers (Metabion International AG, Planegg, Germany) according to the procedures proposed by Richtzen et al. [47] and calibrated 1% agarose electrophoresis was used with a 50-bp DNA size marker (50-1k bp) (DNA ladder III®, Cat No. S-5092-100, Dena Zist Asia, Mashhad, Iran). The characteristics of the primers applied in the current study are presented in Table 4. Furthermore, considering the capability of the Bruce-ladder multiplex PCR in identifying Brucella species (B. *melitensis*, B. *abortus*, B. *ovis*, B. *melitensis*, B. *canis,* B. *neotomae*, B. *pennipidialis*, and B. *ceti*) and

vaccine strains (B. abortus S19 vaccine strain, B. abortus RB51 vaccine strain and B. *melitensis* Rev.1 vaccine strain), detection of Brucella species was carried out on *Brucella* genus identified positive using genus-specific primers and the Bruce-ladder multiplex PCR as described by García-Yoldi D *et al.* [48]. In summary, using a thermocycler device (Gene Atlas 322®, Astec Co., Fukuoka, Japan) with 20 µL mixture containing 10 µL of Taq 2x Master Mix Red (Ampliqon A/S, Odense, Denmark), 4 µL of a primer mixture (Metabion International AG, Planegg, Germany), 1 µL of template DNA, and 5 µL of UltraPure™ DNase/RNase-free distilled water, PCR was performed. The PCR program included an initial denaturation at 95°C for 7 min, followed by 25 cycles of 35 sec of template denaturation at 95°C, 45 sec of annealing at 64°C, and 180 sec of extension at 72°C, with a final extension at 72 °C for 6 min. PCR products were analyzed by calibrated 1.5% agarose electrophoresis with Green Viewer safe stain (0.01 v/v) and a 100-bp Plus DNA size marker (100-3k bp) (DNA ladder II®, Cat No. S-5091-100, Dena Zist Asia, Mashhad, Iran). Moreover, the B. melitensis Rev.1 vaccine strain, B. abortus RB-51 vaccine strain (used in the vaccination program of the Iranian Veterinary Organization), and B. melitensis strain were used as positive controls.

qPCR Design and Setup

To detect most variants of Brucella, primers were designed for the conserved region of the complete genome sequence of B. *ceti* (NC_022905.1), B. *abortus* (NC_007618.1), B. *melitensis* (NC_003317.1), B. canis (NC_010103.1), B. *microti* (NC_013119.1), B. *neotomae* (NZ_UIGH01000001.1), B. *ovis* (NC_009505.1), and B. *suis* (NC_004310.3) by beacon designer (version 8.10, Premier Biosoft, USA) (Table 4). Using the Basic Local Alignment Search Tool from the GeneBank database and Snapgene software (version 3.2.1, USA), the in silico specificity was examined. The qPCR was conducted using a 10-µL mixture containing 5 µL of Real Q Plus 2x Master Mix Green (Ampliqon A/S, Odense, Denmark), 1 µL of reverse and forward primers (Metabion International AG, Planegg, Germany), 1 µL of template DNA, and 3 µL of UltraPure™ DNase/RNase-free distilled water. Amplification and detection were performed using a real-time device (mic-PCR®, Applied Biomolecular Systems Co., Australia). The activation step was carried out at 95°C for 15 min, and the template was subjected to a total of 35 cycles comprising 30 sec of denaturing at 95°C and 30 sec of annealing at 60°C. After completing the annealing step, melting curve analysis was performed within the temperature range of 65°C-95°C. The baseline and threshold were set using the auto baseline and threshold feature in mic-PCR® Software v2.6.4 (Applied Biomolecular Systems Co., Australia). Before data analysis, the melting curve (Figure 4) was recorded for each reaction, and by examining these curves, the accuracy of the peak related to the desired DNA fragment and the absence of primer dimers was confirmed. Moreover, in all qPCRs in our study, if the cycle threshold (Ct) values were 35 or lower,

Table 4.
Characteristics of the primers used in the PCRs

Primer Pair	Primer Name	Sequence (5'to 3')	Amplicon Size (bp)
GAPDH		F:TGGCAAAGTGGACATCGTTG	467
		R:TGGCGTGGACAGTGGTCATAAGTC	
Genus Brucella		F: TGGCTCGGTTGCCAATATCAA	223
		R: CGCGCTTGCCTTTCAAGGTCTG	
qPCR		F: TCCTCGGTCCAGACATAG	142
		R: GCGATGATTTATTCCGTATCCa	

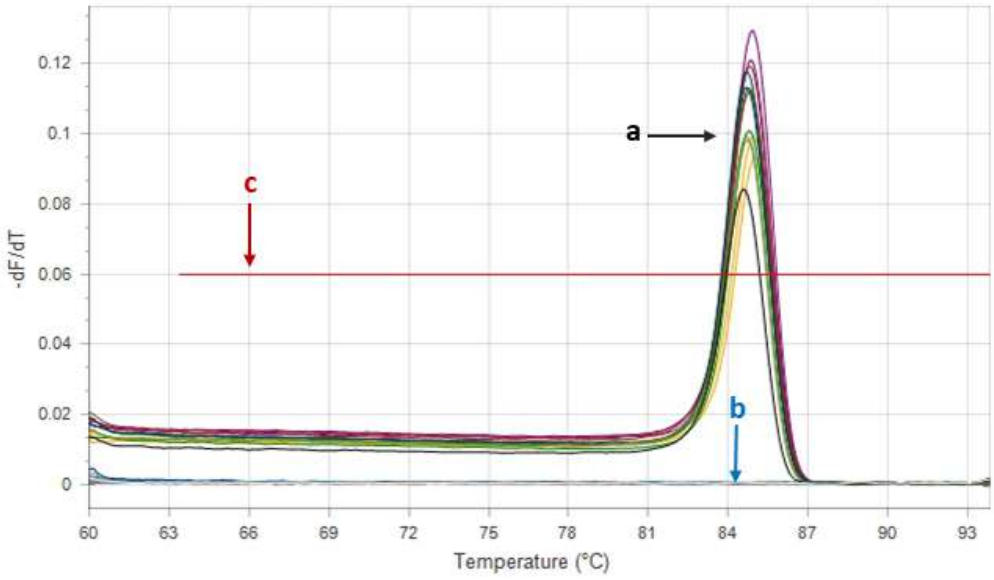

Figure 4.
Melting curve analysis for Brucella spp. in qPCR; a: Positive control and positive unknown samples; b: Negative control and negative unknown samples; c: Threshold line

they were considered positive. All samples were tested twice, and if the qPCR results for both times were positive, that sample was reported positive for the presence of Brucella spp.

Statistical Analysis

Contingency 2 × 2 tables were created to determine the sensitivity, specificity, PV+, PV-, and LRs of positive and negative test results for Rose Bengal and bacterial culture tests, where the result of qPCR was considered standard. The agreement between the tests was evaluated using Cohen's Kappa statistics. According to Landis *et al.* [49], the interpretation of the agreement varied depending on the estimated Kappa values. In detail, when the values were 0-0.20, the agreement was considered slight, but for values above 0.80, it was deemed almost perfect. When the Kappa values were 0.21-0.40, the agreement was considered fair, whereas values of 0.41-0.60 corresponded to a moderate level of agreement. Similarly, a substantial level of agreement was interpreted for values 0.61-0.80. The SPSS software version 16.0 was used for statistical analysis.

Statement of Animal Rights

This study with grant number 3/57600 received ethical approval from the Committee on Research Ethics IR.UM.REC.1401.063, which adheres to the ethical guidelines of research from the School of Veterinary Medicine, Ferdowsi University of Mashhad.

Authors' Contributions

Rahmani.Hk., Hashemi.K., Mirshokraei.P. Conceived and designed the experiments: Aminzadeh. M.J. prepared samples. Aminzadeh. M.J., Khaleghnia.N. Performed the experiment and contributed to preparing reagents, materials and analysis tools. Azizzadeh.M. analyzed data and results. Aminzadeh.M.J and Khaleghnia.N. Wrote the manuscript.

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Competing Interests

The authors declare no conflict of interest.

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The antimicrobial activity of peppermint (Mentha piperita) and pennyroyal (Mentha pulegium) essential oil on three mastitis-causing pathogens in milk

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ABSTRACT

Bovine mastitis causes a lot of economic losses, and the appearance of resistant strains of bacteria has led to the use of alternative natural bioagents for treatment. It is generally believed that high levels of fat and/or protein in foods may protect bacteria against the effects of essential oils (EOs). The purpose of this paper was to investigate the effect of EOs of Mentha piperita (*peppermint*) and *Mentha pulegium* (pennyroyal) on three bovine mastitis bacteria (*Escherichia coli, Streptococcus agalactiae, and Staphylococcus aureus*) in milk. Gas chromatography/mass spectrometry was used for the analysis of EOs. Antibacterial effects of the EOs on bacteria were evaluated with minimum bactericide concentration (MBC), minimum inhibitory concentration (MIC), and time-kill assay. Major components of peppermint EO were carvone (63.02%) and limonene (24.48%), and those of pennyroyal EO were pulegone (48.16%), eucalyptol (14.57%), and piperitenone (10.09%). The MIC and MBC were 0.62% and 1.25% for pennyroyal, 0.31-1.25% and 0.62-2.5% for peppermint, 0.31-0.62% and 0.62-2.5% for peppermint and pennyroyal, respectively. At 6-h, the bacterial reduction of treatments compared to the control group was significant for *E. coli* and *S. agalactiae* bacteria. The S. agalactiae and S. aureus counts significantly decreased in the peppermint and pennyroyal group at 24-h. In conclusion, peppermint and pennyroyal EO showed an antibacterial effect on these three bacteria and can be evaluated as an adjunct or alternative to antibiotics in the treatment of bovine mastitis.

Keywords

Antibacterial effect, mastitis, Mentha piperita, Mentha pulegium, milk

Abbreviations

EOs: Essential oils
MIC: minimum inhibitory concentration
MBC: minimum bactericidal concentration

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E. coli: Escherichia coli
S. aureus: Staphylococcus aureus
S. agalactiae: Streptococcus agalactiae

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Introduction

Bovine mastitis is a universal disease caused by many agents and affects the quality and quantity of milk. Mastitis is the cause of most economic losses in dairy cattle in many countries [1]. For a long time, antibiotics have been the most important cure and control program for mastitis. The appearance of resistant strains of common bacteria in lactating animals has been due to the usage of antibiotics [2]. EOs are secondary metabolites of plants and possess antimicrobial activity. After long-term use, no side effects in humans and resistance in bacteria have been observed, making them a potential weapon in the fight against bacterial diseases [3]. Antimicrobial effect of EOs is not due to one specific mechanism because there are several different chemical groups in the structure of EO. Hydrophobicity of essential oils or their components helps them to target the cell membranes of bacteria that contain lipid. This property increases the permeability of membranes, thus contents of cell leak [4].

The Mentha species is the most common plant for medicinal and health purposes [5]. Most studies have shown the antibacterial ability of Mentha pulegium (*pennyroyal*) and *Mentha piperita* (*peppermint*), two of the Mentha species, against different bacteria [6,7].

Escherichia coli, Staphylococcus aureus, and *Streptococcus agalactiae* cause various diseases. Moreover, these bacteria are the most important causes of bovine mastitis [8]. The most common treatment for mastitis is intramammary antibiotics. Nutrients of milk, such as fat and protein, may interfere with the antibacterial agents and decrease the bioavailability of EOs [9]. It is essential to examine the antibacterial activity of essential oils in milk before using them as an intramammary infusion. In previous studies, we tested the antimicrobial effects of essential oils from four other plants on these bacteria in milk [10,11]. Although many studies have been conducted on the antibacterial effect of various EOs in laboratory media, few studies exist investigating this antimicrobial effect in milk. Therefore, in this study, we decided to study the impact of peppermint and pennyroyal essential oils in milk.

Result

GC/MS analysis

The chemical constituents of peppermint and pennyroyal EO are given in Tables 1 and 2. Eight constituents were determined in peppermint, which represented 98.57% of the total oils. Carvone (63.02%) and limonene (24.48%) were found as

Table 1.
Chemical composition (relative % of peak area) of essential oil of peppermint determined by GC-MS analyses.

No.	Components	Retention time (min)	Area sum%
1	(-)-β-Pinene	4.931	0.91
2	D-Limonene	6.007	24.48
3	p-Menth-8-en-2-one, trans-	10.177	2.88
4	(±)-Pulegone	11.23	2.13
5	(-)-Carvone	11.438	63.02
6	Carvyl acetate E	14.357	1.08
7	(-)-β-Bourbonene	15.039	0.88
8	Caryophyllene	15.939	3.19

Table 2.
.Phytochemical components (relative % of peak area) of pennyroyal essential oil assessed by GC-MS analyses.

No.	Components	Retention time (min)	Area sum%
1	3-Carene	4.105	1.58
2	Sabinen	4.817	1.43
3	β-Pinene	4.936	2.12
4	β-Myrcene	5.103	0.76
5	Cymene	5.892	0.8
6	D-Limonene	6.003	2.37
7	Eucalyptol	6.08	14.57
8	1-Cyclohexene-1-methanol, α,α,4-trimethyl-	8.939	2.61
9	Menthone	9.092	0.79
10	.p-Menthan-3-one	9.323	0.78
11	endo-Borneol	9.516	0.96
12	Isopulegon	9.592	0.92
13	L-.alpha.-Terpineol	10.112	0.95
14	Pulegone	11.285	48.16
15	Carvone	11.381	1.55
16	Piperitone	11.64	1.45
17	Thymol	12.601	1.02
18	Piperitenone	13.837	10.09
19	Terpinyl acetate	14.058	1.01
20	3-Methoxy-5-propylphenol	14.395	4.17
21	Caryophyllene	15.942	1.42

the major components. GC/MS analysis of the pennyroyal EO showed 21 various constituents (99.51% of its chemical composition). Three major compounds were: pulegone (48.16%), eucalyptol (14.57%), and piperitenone (10.09%).

MIC and MBC

The effects of peppermint, pennyroyal EOs, and positive control (lincospectinomycin) on bacteria are declared in Table 3. The antibacterial effect was shown on the microorganisms. The MIC and MBC were 0.62% and 1.25% for pennyroyal, 0.31-1.25% and 0.62-2.5% for pep-

Table 3.
MIC and MBC of peppermint and pennyroyal essential oils against bacteria compared with a positive standard antibiotic (lincospectinomycin) in milk

	Bacterium	MIC (%V/V)	MBC (%V/V)
Peppermint	Escherichia coli	1.25	2.5
	Staphylococcus aureus	0.62	1.25
	Streptococcus agalactiae	0.31	0.62
Pennyroyal	Escherichia coli	0.62	1.25
	Staphylococcus aureus	0.62	1.25
	Streptococcus agalactiae	0.62	1.25
Peppermint+pennyroyal	Escherichia coli	0.62	1.25
	Staphylococcus aureus	1.25	2.5
	Streptococcus agalactiae	0.31	0.62
Lincospectinomycin	Escherichia coli	0.078	0.156
	Staphylococcus aureus	0.039	0.078
	Streptococcus agalactiae	0.039	0.078

MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration.

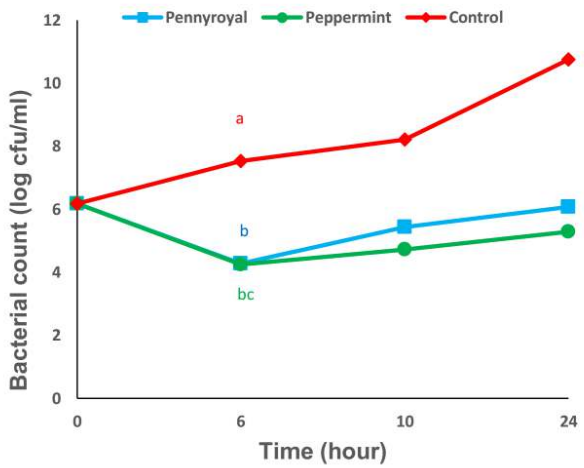

Figure 1.
Time-kill curve for *E. coli* exposed to 0% (◆, control) and sub-MIC of peppermint (●) and pennyroyal (■) EOs in milk.
[a-c] Values with different letters are significantly ($p < 0.05$) different within the same time.

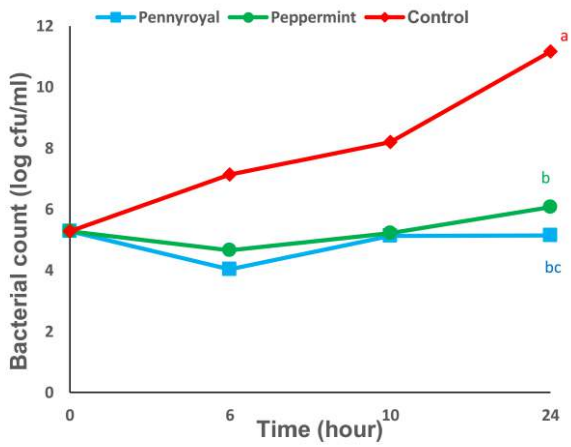

Figure 2.
Time-kill curve for *S. aureus* exposed to 0% (◆, control) and sub-MIC of peppermint (●) and pennyroyal (■) EOs in milk.
[a-c] Values with different letters are significantly ($p < 0.05$) different within the same time.

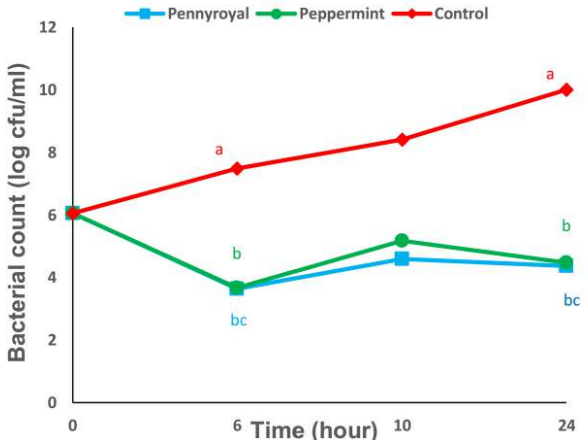

Figure 3.
Time-kill curve for *S. agalactiae* exposed to 0% (◆, control) and sub-MIC of peppermint (●) and pennyroyal (■) EOs in milk.
[a-c] Values with different letters are significantly ($p < 0.05$) different within the same time.

permint, 0.31-0.62% and 0.62-2.5% for peppermint and pennyroyal, respectively.

Time kill assay

The influence of peppermint and pennyroyal on the bacteria is depicted in Figures 1, 2, and 3. The bacterial counts of the treatment and control groups were initially about 6.0 log10cfu/ml for all three bacteria. At 6-h, the bacterial reduction of treatments compared to the control group was significant for *E. coli* (p = 0.00) and *S. agalactiae* (p = 0.01) bacteria. The *S. agalactiae* (p = 0.00) and *S. aureus* (p = 0.01) counts significantly decreased in peppermint and pennyroyal group at 24-h.

Discussion

In our study, the main components of peppermint were carvone (63.02%) and limonene (24.48%). The *Mentha piperita* EO extracted from Poland included menthyl acetate (19.2%), menthone (22.7%), and menthol (29.0%) [12]. In another study from Saudi Arabia, the main constituents of peppermint were menthofuran (6.88%), menthyl acetate (8.95%), menthone (24.56%), and menthol (36.02%) [5]. Major chemical compositions of peppermint EO from Brazil were terpin-4-ol (8.00%), 3-octanol (10.1%), carvone (23.42%), and linalool (51.0%) [13]. In several studies from Iran, major components of peppermint were reported as 1,8-cineole (2.15%), menthyl acetate (4.61%), menthofuran (6.49%), and menthol (25.16%) [14]; menthofuran (11.18%), menthyl acetate (15.1%) and menthol (53.28%) [6]; isomenthone (10.3%), trans-carveol (14.5%), pipertitinone oxide (19.3%), and α-terpene (19.7%) [15]. The major components of peppermint in our study were similar to a previous study from Iran [15] but different from other Iranian studies [6, 14].

Carvone is an oxygenated monoterpene and limonene is a monoterpene hydrocarbon. Most of the antimicrobial activity in the oils has been attributed to the oxygenated monoterpenes [16]. The mechanism of monoterpene's antimicrobial action is mainly associated with the lipophilic nature of their aglycones, which allows them to rapidly cross biological membranes and then interact with a plethora of biomolecules [17].

Major constituents of pennyroyal in the present study were pulegone (48.16%), eucalyptol (14.57%), and piperitenone (10.09%). These results are similar to EO from Iran [18] that report (E)-p-mentha-2-en- 1-ol (12.157%), 3,3'-dimenthol (18.859%), and cis-pulegone oxide (45.676%) as major components and essential oil from Portugal [19] represents pulegone (86.64%), isomenthone (4.60%), and piperitenone (2.58%) as main components. Also, another study from Portugal reported the main components of pennyroyal as neo-menthol (9.2%), pulegone (23.2%), and menthone (35.9%) [20]. The difference in principle components may be the result of climate, the effect of sunlight, and geographical conditions.

The strong antibacterial activity of pulegone have been demonstrated against a set of bacteria, including *S. typhimurim* and *E. coli*. Cytotoxicity of this essential oil appears to include a bacterial membrane damage that occurs when the essential oil passes through the cell wall and cytoplasmic membrane, and disrupts the structure of their different layers of polysaccharides, fatty acids and phospholipids [20].

To the best of our knowledge, there is no study about the antibacterial effects of peppermint and pennyroyal oil against these three bacteria in milk. Nevertheless, various MIC and MBC values have been declared against S. aureus and *E. coli* in laboratory mediums. In one study, the MIC value ranged from 0.39 to 3.12 mg/ml, and the MBC value ranged from 0.78 to 12.48 mg/ml for peppermint against multidrug-resistant strains of *S. aureus* [12]. In other studies, MIC values of peppermint were reported against reference strains of S. aureus 0.5 mg/ml [21], 0.75 ± 0.03 µg/ml [5], 0.10% [22] and against *E. coli* 0.20 ± 0.09 µg/ml [5] and 0.25% [22]. The MIC and MBC of pennyroyal oil reported by Luis and Domingues (2021) against E. coli and S. aureus were %8. In another study, MIC and MBC of pennyroyal against *S. aureus* and *E. coli* were reported 0.004% and 0.0005%, respectively [23]. In our study, the MIC of peppermint against *E. coli* and S. aureus was 1.25% and 0.62%, respectively and the MIC of pennyroyal on S. aureus and E. coli was 0.62% which was higher than most of other studies in the laboratory mediums.

Based on findings of the present study, the antibacterial activity of peppermint and pennyroyal was lower than lincospectin in milk, but in a previous study, the antibacterial effect of peppermint was the same (Streptococcus agalactiae) or higher (*S. aureus* and *E. coli*) than lincospectin and pennyroyal effect on Streptococcus agalactiae was higher than lincospectin in laboratory synthetic media [24].

According to the MIC and MBC results of the present study, *E. coli* (Gram-negative bacteria) was more resistant than *S. aureus* and *S. agalactiae* (Gram-positive bacteria) to peppermint EO. The resistance of the three bacteria to pennyroyal EO was the same. The resistance of Gram-negative bacteria is due to the outer membrane surrounding the cell wall which restricts the diffusion of hydrophobic

compounds through the lipopolysaccharide. In addition, the periplasmic space contains enzymes, which are able to break down foreign molecules introduced from outside [25].

Significant reductions in bacterial population were observed at 6 and 24-hour in the time-kill assay. The antibacterial activity of peppermint and pennyroyal oil appears promising based on their activity at sub-MIC concentrations. To the best of our knowledge, we did not find any other studies comparing the impact of these essential oils on the bacteria in milk.

Though much information exists about the antibacterial activity of EOs, many studies have been done in model broth systems. The essential oil concentrations for antibacterial effect are higher in complex foodstuffs such as vegetables, dairy products, fish, and meat than in laboratory media [26]. For the application of essential oils in the treatment of bovine mastitis, oils must have miscibility in milk and obtain in vivo antibacterial effect in milk. In this study, we investigated the antibacterial effect of peppermint and pennyroyal EOs using the MIC&MBC method and time-kill assay in milk. According to MIC and MBC results, the antibacterial effects of our EOs were lower in milk than those effects in laboratory mediums in other studies. In a few studies, the activity of essential oils in milk and laboratory medium has been compared. Zhue et al. (2016) studied the antibacterial effect of Cinnamon cassia on major bacterial bovine mastitis in culture media and its miscibility in milk [9]. They showed that 4 MBC of oil of C. cassia was eliminated and had similar activity against S. aureus and E. coli 29 in milk within 8 hours [9]. In another study, milk composition, especially fat content, decreased the antibacterial efficacy of eugenol in milk [27].

In conclusion, peppermint and pennyroyal essential oils exhibited antibacterial properties against the three bacteria tested, indicating potential as a supplement or alternative to antibiotics in treating bovine mastitis.

Materials and Methods

Essential oils Analysis

EOs of peppermint and pennyroyal were obtained from Dorrin Golab Company, Kashan, Iran. Chemical composition determination was conducted with gas chromatography coupled to GC/MS (Model 5977A, Agilent Technologies, USA) using an HP-5MS capillary column with an internal diameter of 0.25 mm and film thickness of 0.25 µm. The temperature of the injection port was set to 260 °C, while the oven temperature was programmed to increase from 50°C to 250°C at a rate of 4°C per minute. Helium was used as the carrier gas, with an injection volume of 1µl and a flow rate of 1 mL/min.

Cultures and Medias

The antibacterial effects were assessed against three major mastitis reference bacteria purchased from Persian Type Culture Collection, Tehran, Iran (PTCC) as a lyophilized culture: one gram-negative bacteria (Escherichia coli ATCC 25922) and two gram-positive bacteria (Streptococcus agalactiae ATCC 13813 and *Staphylococcus aureus* ATCC 9144). Cultures were prepared with twice the growth of the lyophilized cultures in tryptic soy broth (TSB) (Biolife, Milano, Italy) for 18 - 20 h at 37 °C. Sterile glycerin (1:5) was used for dilution of the cultures, and these cultures were kept at - 20 °C. Stock cultures were obtained by twice culture in TSB and streaking on tryptic soy agar (TSA) (Biolife, Milano, Italy) slant. After incubation at similar conditions, these cultures were kept at 4 °C and subcultured once a month [28].

Inoculum Preparation

Bacterial inoculum was obtained by transferring cells from working cultures to TSB tubes, and after incubation for 18 h at 35 °C, the subcultures were performed. The optical density (OD) (absorbance) of the bacterial broth cultures was adjusted to an optical density at 600 nm of 0.1 with a spectrophotometer (Libra S12, Biochrom Ltd., Cambridge, London). The cell concentration of these cultures was 1.64 × 1011 cfu/ml of S. agalactiae, 3.4 × 1010 cfu/ml of S. aureus, and 2.4 × 1011 cfu/ml of *E. coli* [28].

Preparation of Milk

Free antibiotic residue milk was obtained and sterilized with autoclavation (121 °C, 15 min) [4].

Determination of MIC and MBC

DMSO (Sigma, Germany) was used to increase the solubility of EOs in milk with the preparation of a 1:1 dilution of the oils. Antibacterial analysis was done using this dilution. Autoclaved milk was applied as the growth medium rather than the laboratory medium. MIC was determined using double serial dilutions (10, 5, 2.5, 1.25, 0.62, 0.31, 0.15, and 0.07%) of the oils in milk. These tubes containing 1 mL of liquid were vortexed for 90 s. 100 µl of 1:400 dilution of bacterial inoculums were inoculated in tubes, and after 15 s vortex, the cultures were incubated at 37 °C for 24 h. After the vortex, eight dilutions (10-fold) were made with 0.1 mL of culture and normal saline. Eighths of a TSA plate were inoculated with dilutions and incubated at 37 °C for 24 h. Then, bacterial counts were

determined. The lowest concentration that did not result in the growth of bacteria after subculture on TSA was regarded as MBC. MIC was considered the Ante-MBC concentration [9].

For assessment of synergism/antagonism in antibacterial action between the EOs of peppermint and pennyroyal, the essential oils were mixed volume to volume (1:1). Some controls were employed in experiments. The negative control was milk to assess autoclavation. Positive control was milk containing the bacteria to evaluate the growth of bacteria in the milk. Also, bacterial culture and DMSO without the EOs were other positive controls. Another control was the antibiotic Linco-Spectin (Linco-Spectin 5% + spectinomycin 10%) (Lincoject, Rooyan Darou, Semnan, Iran) to compare the antibacterial activity of essential oils. Each treatment was done in duplicate. Each trial was repeated at two separate times.

Time-kill assay

Sub-MIC of EOs and each bacteria were inoculated to milk. Inoculated media lacking in EO were regarded as control. After 0, 6, 10, and 24 h of incubation at 37 °C nine 10-fold dilutions using sterile normal saline were created. TSA plates were used to plate on dilutions, and after incubation for 24 h at 37 °C, a count of bacteria in dilutions was carried out. Each trial was repeated at two separate times. Plotting log10 cfu/ml versus time (hour) was used to construct curves [9,28].

Statistical analysis

The data were analyzed utilizing one-way ANOVA along with Tukey's test employing the SPSS (18) software (IBM Corp., Armonk, NY, USA) at p <0.05).

Authors' Contributions

R.R.: study design and preparation of manuscript. S.N.: conduct the study and data collection. J.B.K.: analysis and interpretation of the data.

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Competing Interests

The authors declare that there is no conflict of interest regarding to publication of this paper.

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Prevalence of Coxiella burnetii infection and risk factors in aborted sheep and goats in Kerman province, south-east of Iran

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ABSTRACT

Coxiellosis, also known as Q fever, is a zoonotic disease caused by Coxiella burnetti and has become a significant cause of small ruminant abortion globally. Q fever is endemic in Iran, but there is a dearth of epidemiological data regarding the true prevalence of *C. burnetii* in some areas of Iran. Small ruminants, mainly goats and sheep, are considered the primary reservoir for human infection, posing a considerable threat to human health. Theses reservoirs can shed the bacterium into vaginal mucus.The objective of this study is to examine the prevalence of coxiellosis through the detection of the *IS1111* gene of C. burnetii using Real-time PCR and also to identify the related risk factors (such as the location of livestock, age, species, and parity) associated with the disease in the vaginal discharge of small ruminants residing in Kerman province, located in the southeast of Iran. During the winter of 2019 and autumn of 2020, a total of 134 vaginal samples from aborted small ruminants (70 samples from sheep and 64 from goats) were gathered from 32 herds located in different areas of Kerman province. The results showed that 26 samples, comprising 14 from goats (21.88 %) and 12 from sheep (17.14 %), were positive for coxiellosis. According to the findings of our study, the detection of *C. burnetii* showed that coxiellosis is circulating in the studied area. Additionally, our analysis revealed no statistically significant association between the prevalence of Q fever and small ruminants' location, number of parturition, and age that were examined as potential risk factors.

Keywords

Q fever; Coxiellosis; Real-time PCR; Small ruminants; Prevalence; Kerman province

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Abbreviations

SCV: small cell variant
LCV: large cell variant
POMP: Polymorphic Outer Membrane Proteins
PBS: phosphate-buffered saline
PCR: Polymerase chain Reaction
CDC: Centers for Disease Control and Prevention

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Introduction

Coxiellosis, also known as Q fever, is a zoonotic disease with a global distribution and is caused by the pleomorphic Gram-negative intracellular bacterium Coxiella burnetti (*C. burnetii*) [1,2]. This pathogen is classified within the Coxiella genus, Coxiellaceae family, and Gammaproteobacteria class based on 16S rRNA sequence analysis [3,4]. C. burnetii can endure environmental conditions for prolonged periods throughout its developmental cycle. This non-replicating form of the disease plays a crucial role in spreading the infection to humans and animals [1,5–7]. Upon entry into host cells, the spore-like form of *C. burnetii*, known as the small cell variant (SCV), transforms into the actively replicating large cell variant (LCV) before ultimately being released from the host cell as SCV once again [7–10]. Due to its capacity for rapid dissemination over long distances in a short period, C. burnetii has been included in the "B" list of potential biological weapons by the Centers for Disease Control and Prevention (CDC) [11]. The zoonotic nature of Q fever has been recognized by its inclusion in the OIE Animal Health Code, with Member Countries required to report any incidence of the disease to the OIE [10]. This pathogen exhibits a broad host range, affecting humans and numerous animal species, such as avian and mammalian species, with ruminants being the principal reservoirs [10–12]. Livestock serves as the primary reservoir of the microorganism, which can lead to Q fever outbreaks in humans [13].

In humans, acute infection typically presents as a self-limiting, influenza-like illness, whereas endocarditis is the predominant symptom in chronic cases. However, untreated acute infections may progress to chronic infections in 1-5% of cases [7,13,14]. Coxiellosis in small ruminants is usually asymptomatic, but chronic infection may result in reproductive disorders, such as stillbirth, weak offspring, infertility, endometritis, milk yield losses, and abortion [10,13,15].

The occurrence of abortion in animals following colonization of the placental macrophages by *C. burnetii* is attributed to the organism's high tropism for reproductive organs. Consequently, vasculitis, diffuse inflammation, necrosis, and calcification in the inter-cotyledonary region may ensue [15]. Notably, the pathogenicity of *C. burnetii* is influenced by virulence factors such as lipopolysaccharide (LPS) and host factors such as pregnancy-related immunosuppression [7,10,15]. In addition, the high affinity of the bacterium for the placenta results in a higher incidence of infection in females than males [16].

Phase I of *C. burnetii*, the virulent form characterized by smooth full-length LPS, undergoes phase variation to Phase II (rough LPS) after multiple passages in cell culture. Phase II is less contagious than Phase I [7,10].

In humans and small ruminants, inhalation of contaminated aerosols is the primary route of infection, with the bacterium being excreted by infected animals via various bodily fluids, such as urine, milk, vaginal discharge, fetal fluids, and abortion products [12,17]. Goats and sheep have been shown to shed the organism in their vaginal mucus for weeks [8,18]. Other transmission modes include consuming contaminated milk and dairy products, handling contaminated tissues, direct contact, and sexual and vertical transmission [13,19,20].

Effective control and prevention of C. burnetii infection are critical due to the organism's high environmental resistance, low infectious dose (less than ten bacteria), multiple transmission routes, presence in different reservoirs, and rapid spread over long distances via wind [7,11,13]. Laboratory tests, including staining, serological, and molecular tests, are more useful than clinical diagnosis for identifying C. burnetii, as no specific clinical signs are associated with this disease. This underscores the importance of appropriate diagnostic measures in the early detection and management of C. burnetii infection [21,22].

C. burnetii is challenging to identify using traditional staining techniques, and serological assays have limitations in detecting the bacterium in shedder animals [5,12,23]. The OIE recommends using Polymerase chain reaction (PCR) as a highly sensitive and specific method for detecting *C. burnetii*. PCR amplifies specific DNA sequences from the bacterium, enabling accurate detection and surveillance of the infection in humans and animals. The PCR-based method has become essential for diagnosing and monitoring C. burnetii infection, especially when traditional diagnostic approaches are inadequate [24].

Studies have demonstrated that molecular identification tests such as PCR and quantitative Real-time PCR (qPCR) are highly effective in detecting *C. burnetii* in abortion materials due to multiple copies of the bacterial genome's insertion sequence and no need for prior isolation or cultivation. They can potentially improve the accuracy and efficiency of disease diagnosis in both veterinary and human medicine [5,12,25].

The PCR-based assay has been shown to have high specificity for detecting *C. burnetii* in vaginal abortion samples without cross-reacting with other bacteria present [26]. However, inadequate epidemio-

Abbreviations-Cont'd

LPS: Lipopolysaccharide
qPCR: quantitative Real-time PCR
ELISA: Enzyme-Linked Immunosorbent Assay.

logical studies and a lack of information about Q fever have resulted in misdiagnosis and the re-emergence of the disease in different regions of Iran [11]. To address these problems this study was conducted to determine the prevalence of *C. burnetii* in small ruminants of Kerman province using real-time PCR. This research aimed to recognize the risk factors linked with coxiellosis to enhance the control and management of Q fever in the targeted area. Using PCR-based assays in disease surveillance and diagnosis can improve the accuracy and efficiency of disease management strategies in veterinary and human medicine.

Result

Our investigation involved collecting 134 samples, comprising 70 from aborted sheep and 64 from aborted goats, of which 26 samples (19.4%) tested positive for coxiellosis using the Real-time PCR method. The prevalence of infection was 21.87% (14 out of 64) and 17.14% (12 out of 70) in goat and sheep samples, although the difference was not statistically significant. We also examined the prevalence of *C. burnetii* infection by analyzing independent variables, including the location of livestock, parity, age, and species of small ruminants, as presented in Table 1. It is also revealed that the prevalence of coxiellosis did not exhibit any significant association with age categories in sheep or goats ($p < 0.05$). No significant correlation was observed between the prevalence of coxiellosis and parity in either goats or sheep ($p < 0.05$). However, we observed a rise in the prevalence of *C. burnetii* infection in sheep as the number of parturitions increased.

Our investigation found a variation in the prevalence of coxiellosis across the studied regions, ranging from 0% in Kerman to 30% in Baft for goats and 0% in Bam to 22% in Kerman for sheep. However, we did not observe a significant correlation between the location and the occurrence of infected animals, as determined by a p-value of less than 0.05 (Table 2).

Table 1.
Prevalence of infection *C. burnetti* by independent variables included the location of livestock, animals, parity and age in sheep and goat.

Variable	Different groups in each variable	No. of animals (%)	Test for C. burnetii (PCR)		Prevalence (%)	Odds Ratio	95 % CI	P-value
			+ve	-ve				
Animals	Sheep	70 (52.24)	12	58	17.14	1.00		0.64
	Goat	64 (47.76)	14	50	21.88	1.35	0.56-3.02	
Parity	<2	59 (44.03)	10	49	16.94	1.00		
	2-4	40 (29.85)	8	32	20	1.22	0.42-3.23	0.90
	>4	35 (26.12)	8	27	22.86	1.45	0.49-3.91	0.66
Age	<2	46 (34.33)	7	39	15.22	1.00		
	2-4	49 (36.57)	12	38	24.49	1.76	0.60-4.55	0.41
	>4	39 (29.10)	7	31	17.95	1.26	0.42-3.73	0.92
Location of livestock	Baft	60 (44.78)	15	45	25	1.00		
	Bam	10 (7.46)	1	9	10	0.33	0.02-2.11	0.52
	Bardsir	25 (18.65)	5	20	20	0.75	0.27-2.15	0.83
	Kerman	14 (10.45)	2	12	14.29	0.5	0.10-2.19	0.61
	Shahr-e-babak	25 (18.65)	3	22	12	0.41	0.12-1.47	0.30

CI=95% confidence interval
P-value for difference of prevalence of groups belonging to each variables
Significant variables based on $p < 0.05$

Table 2.
Relationship between positivity for *C. burnetii* in vaginal samples from aborted small ruminants of small ruminants and main risk factors such as province, number of parturition, and age.

	Category	Goat	Prevalence	CI	P-value	Sheep	Prevalence	CI
Parity	<2	7/31	22.58%			3/28	10.71%	
	2-4	3/14	21.42%	0.23-3.69	0.76	5/26	19.23%	0.43-8.09
	>4	4/19	21.05%	0.26-3.90	0.82	4/16	25%	0.64-12.11
Age	<2	5/20	25%			2/26	7.69%	
	2-4	7/27	25.92%	0.26-3.80	0.79	5/22	22.72%	0.60-18.88
	>4	2/17	11.76%	0.07-2.55	0.55	5/22	22.73%	0.60-18.88
Location of livestock	Baft	8/26	30.76%			7/34	20.58%	
	Bam	1/4	25%	0.05-5.79	0.72	0/6	0%	0.00-2.78
	Bardsir	3/15	20%	0.14-2.75	0.70	2/10	20%	0.17-5.14
	Kerman	0/5	0%	0.00-2.138	0.38	2/9	22.22%	0.20-6.26
	Shahr-e-babak	2/14	14.28%	0.07-1.74	0.44	1/11	9%	0.03-2.97

CI=95% confidence interval
P-value for difference of prevalence of groups belonging to each variables
Significant variables based on $p < 0.05$

Discussion

Q fever is a re-emerging zoonotic disease caused by C. burnetii. In recent outbreaks, C. burnetii has been identified as one of the responsible agents for abortion in small ruminants [3,8,27]. The disease was initially discovered in 1935 among abattoir workers in Australia and has since been reported in various parts of the world [28]. The first case of Q fever in Iran was documented in 1952 [11].

Over the past few years, Q fever has been identified as a significant cause of abortion in small ruminants, resulting in substantial economic losses for affected communities [3,6]. There is also a concern that ruminants infected with C. burnetii may serve as a potential source of human infection, posing a risk to public health [13].

The significance of coxiellosis is being overlooked in Iran [14]. The primary data on the disease in small ruminants predominantly consists of serological information, and the lack of a highly sensitive detection method hinders the conduction of molecular surveys [15,18]. The precise identification of C. burnetii using molecular techniques is a crucial factor in preventing the spread of this pathogen amongst both humans and ruminants, highlighting the importance of developing more accurate detection methods [29].

The most effective methods for diagnosing coxiellosis from vaginal mucus involve directly detecting the bacterium through PCR or the Real-time PCR approach [3,18]. Vaginal mucus is a significant route for shedding C. burnetii by infected ovine and caprine into the environment.

This study aimed to determine the prevalence of coxiellosis in vaginal samples from aborted ovine and caprine from ovine and caprine in Kerman province using the Real-time PCR method, which is a highly sensitive and accurate diagnostic approach. The overall prevalence rate was approximately 20%, with 17% in sheep and 22% in goats. These findings are consistent with previous studies conducted in Iran, which detected C. burnetii DNA in aborted fetuses at rates of 17.95% (20.43% in goats and 15.47% in sheep) using nested and RT-PCR [30], and prevalence rates of 17.3%, 20.8%, and 16.6% in sheep in different regions of Iran [5,31,32].

The frequency of coxiellosis in our study was found to be higher than the rates reported in western Iran (0.9% in sheep) and southern Iran (2% in ruminant fetuses) [25,33]. However, the frequency of coxiellosis in our survey was lower than that reported in other studies conducted in Iran, including those in Ardabil, Mazandaran, the southeast, and northeast regions of Iran [2, 34-36], as well as a serological survey in southeast Iran [37]. A review of coxiellosis cases in Iran between 2000 and 2015 reported 27% and 33% infection rates in sheep and goats, respectively [20].

Variations in the reported prevalence rates of coxiellosis in different surveys may be attributed to several factors, including differences in animal management practices, timing and method of sampling, and type of diagnostic test used to detect the bacterium [6,10,12,17,18,38]. In a recent study, the prevalence

of infection is higher in goats than in sheep, which is consistent with most available data in Iran and other countries [11,16,20,39–44]. The difference in prevalence rates between the two species may be due to differences in their immune response or shedding of the bacterium during two consecutive delivery periods in goats [6,11]. Although our study demonstrated that the prevalence of infection is higher in goats than in sheep, it was not significant. A similar trend has been reported in animals with a history of abortion in Iran [17].

The prevalence of coxiellosis in small ruminants varies across different countries, as reported in several studies [1,6,45–51,9,12,13,19,24,26,38,41]. High rates of infection have been documented in Kosovo (66%) and Ethiopia (64%) [27,52], while lower rates of positivity have been observed in Brazil (8%) based on placental samples [15]. Various factors contribute to the prevalence of coxiellosis in small ruminants, including farm-associated factors (such as livestock location and herd size), animal-associated factors (such as age, breed, and parity), animal management practices, type of sample collected, and diagnostic method used [12,18,26,32,45]. This variability has been reported in studies conducted in different parts of the world, including those in Turkey, Egypt, Brazil, Germany, and Pakistan.

Regarding localities, climate change may play a role in the emergence of vector-borne diseases [10]. Additionally, ticks, which are carriers of C. burnetii in animals, may contribute to the regional prevalence of the infection [11]. Airborne transmission has also been suggested as a significant factor in the regional spread of the bacterium, as reported previously [13,53]. Severe winds and the agent's spread to different regions may also influence the emergence of new diseases [11]. Although some surveys demonstrated a significant relationship between location and the rate of C. burnetti infection [2,34,35,47,50,51,54], our study findings suggest that the geographical region could not be a significant factor in the prevalence of coxiellosis in ovine and caprine. Similarly, a study conducted by Roshan et al. (2018) in Sistan and Baluchestan reported that the location of livestock was not a significant risk factor for the epidemiology of *C. burnetii* in aborted fetuses of sheep.

Previous studies have shown a significant positive association between increasing age and a higher prevalence of coxiellosis in small ruminants, as reported in studies conducted in various countries [2,6,17,32,36,39,42,51,54,55]. However, in the present study, we did not observe a significant correlation between age and the prevalence rate of the infection. Similar findings have shown no significant association between Q fever seropositivity and the age of small ruminants [19,34,47,56].

Our study did not find a significant association between parity and the prevalence of C. burnetii in sheep and goats. Similarly, studies conducted by Kayedi et al. (2017) and Esmaeili et al. (2019) in Iran reported that parity was not a significant risk factor for the epidemiology of C. burnetii in goats and sheep. In contrast to our findings, a study conducted by Roshan *et al.* (2018) in the Sistan region of southeastern Iran reported a decrease in the prevalence rate of coxiellosis with increasing parity in aborted fetuses of ewes. Also, a statistically significant difference between parity and prevalence of infection was reported in goats in Ethiopia [52].

Conclusion

Our findings suggest that *C. burnetii* infection is present in the vaginal discharge from aborted small ruminants of small ruminants in Kerman City, southeast Iran. However, the prevalence rate of the disease varies across different animal species with different ages and parity, as well as across different provinces within Kerman. These results may benefit veterinary organizations and practitioners in controlling and preventing future disease outbreaks. Further research is necessary to gain a comprehensive understanding of the epidemiology of the disease in other regions of Iran and to ensure accurate surveillance of this zoonotic agent. It is important to note that controlling coxiellosis in small ruminants is critical for preventing Q fever in humans.

Materials and Methods

Sample collection

A total of 134 vaginal samples from aborted sheep (n = 70 samples) and aborted goats (n = 64 samples) were collected from 32 herds in Kerman province between the autumn of 2019 and the winter of 2020. The samples were collected 24 hours after the abortion and were dissolved in a phosphate-buffered saline (PBS) solution. The resulting solutions were transported on ice to the Microbiology Laboratory of the Shahid Bahonar University of Kerman-Iran and stored at -20 °C until further DNA extraction. Genomic DNA was extracted from approximately 200 µL of the abortion fluid using the Roche High Pure PCR template preparation kit, following the manufacturer's instructions. The isolated DNA was stored at -20°C for subsequent Real-time PCR analysis.

A questionnaire was created to gather information on several risk factors, including the location of small ruminants, the age of ewes and goats, and parity. According to the classification in a previous study [57], small ruminants were divided into three groups according to age: under two years, two to four years, and more than four years.

Real-time PCR

For detection of C. burnetti via Real-time PCR, the IS1111 insertion sequence gene (the repetitive transposon-like region) was amplified in LightCycler 96® System (Roche, Germany) by forward primer -AAAACGGATAAAAAGAGTCTGTGGTT), and

reverse primer (CCACACAAGCGCGATTCAT).

A reaction mixture with a total volume of 20 µL was prepared to perform real-time PCR. The mixture consisted of 10 µL of 2x Real Q Master Mix (Ampliqon, Denmark), 800 nM of each primer, 3.4 µL of sterile distilled water, and 5 µL of DNA template. The reaction was carried out using the following program: preincubation at 95°C for 15 minutes, followed by 45 cycles of 15 seconds at 95°C, 20 seconds at 54°C, and 20 seconds at 72°C. DNA from the Nine Mile strain (RSA 493) and molecular-grade water were used for positive and negative controls, respectively. If the threshold cycle (Ct) value of the IS1111 gene was less than 29, the sample was considered positive for coxiellosis.

Statistical analysis

The information gathered from the questionnaire and diagnostic results underwent statistical analysis using GraphPad Prism 9.0 software (Graph-Pad Software, San Diego, CA). The data were analyzed using Chi-Square tests with Yates's correction. The aim was to determine risk factors associated with a particular outcome. The analysis involved calculating the odds ratio for a range of factors, such as animal species, individual age, number of parturition, and location of the livestock. The results were presented as odds ratios and 95% confidence intervals, with a P-value of less than 0.05 considered statistically significant.

Authors' Contributions

MS: Conceptualization, Data curation, Investigation, Writing – original draft preparation; MG: Conceptualization, Methodology, Validation, Resources, Project administration, Supervision, Visualization, Writing – review & editing; MK: Conceptualization, Validation; EM: Methodology, Investigation; MAS: Formal analysis, Writing – original draft preparation, Writing – review & editing. All the authors read and approved the final manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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Global Mycobacterium avium subsp. paratuberculosis Research Trends: A Network and Bibliometric Analysis

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ABSTRACT

MAP is the causative agent of paratuberculosis and has also been implicated in the etiology of Crohn's Disease in humans. Therefore, the importance of studies on this subject increases as MAP causes many economic losses by causing disease in cattle and is important for public health. The aim of this paper was to map the global scientific landscape related to MAP research. The WOS database was queried for publications bearing the title "Mycobacterium avium subsp. paratuberculosis" during January 2001-December 2021 employing the R bibliometrix program. A total of 1775 articles were reviewed in this bibliometric analysis. Notably, the majority of these articles originated from the USA (n = 536, 30.541%). The year 2013 emerged as the most productive year for publications. In terms of research fields, veterinary science (n = 730, 41.6%) was the leading research area. These studies were conducted by a diverse array of researchers, including veterinarians, physicians, and other experts working to define MAP. The sustained increase in the number of publications on paratuberculosis underscores the ongoing global interest in this factor. The determination of effective control strategies for paratuberculosis is important for the food sector and public health. Therefore, a research collaboration between countries should be established in this regard.

Keywords

Mycobacterium avium subsp. Paratuberculosis, Scientometrics, Publication, Bibliometric, VOSviewer software

Abbreviations

MAP: Mycobacterium avium subsp. paratuberculosis
WOS: Web of Science
SCIE: Science Citation Index Expanded
ESCI: Emerging Sources Citation Index
Hirsch index: h-index

Number of Figures: 6
Number of Tables: 5
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Introduction

Mycobacterium avium subsp. paratuberculosis (MAP) is a Gram-positive bacterium with a size of 0.5×1.5 microns as short thick rods. MAP is acid-fast, immobile, non-spore-forming, aerobic, non-encapsulated, and an obligate intracellular bacterium. It grows very slowly in media and forms a visible colony in 8-24 weeks [1]. Due to its lipid-rich cell wall, MAP can survive for over a year in cattle feces and soil, approximately 160 days in river waters, 9 months in pool waters, and at least one year at -14°C [2]. MAP is the etiological agent of paratuberculosis (Johne's disease), an infectious disease characterized by chronic gastroenteritis, diarrhea, and emaciation in domestic and wild ruminants [3, 4]. Animals usually contract MAP through the consumption of contaminated feed, water, and colostrum. In infected animals, it progresses with diverse symptoms, such as severe diarrhea, cachexia, rough fur, dry skin, severe anemia, submandibular edema, and a decrease in milk yield [5]. Paratuberculosis has been reported in many countries with a high prevalence [6-8]. There is a hypothesis that MAP, the causative agent of paratuberculosis, may also be involved in Crohn's disease in humans [9]. Crohn's disease is a chronic inflammatory condition affecting the human gastrointestinal tract from the mouth to the anus, and its exact cause remains undetermined [3]. The similarity of the clinical and pathological findings between paratuberculosis in animals and Crohn's disease in humans supports the idea that MAP might be involved in Crohn's disease [10, 11]. Studies have shown the presence of MAP DNA in the intestines of individuals with Crohn's disease, strengthening the notion that MAP could play a role in this disease [12, 13].

It has been suggested that the transmission of MAP from infected animals to humans may occur through the consumption of contaminated meat, meat products, dairy products, and water [14]. Both milk and feces from dairy cows with clinical and subclinical paratuberculosis can lead to foodborne contamination [15]. Studies have reported the presence of MAP in milk, dairy products, meat, meat products, baby foods, and river waters [16-20]. The fact that MAP causes great economic losses in the ruminant/livestock industry worldwide, harming animal welfare, and its role in Chron's disease in humans reveals the importance and seriousness of this bacterium. Recently, there have been increasing reports on the role and importance of MAP in public health [21].

The aim of this study was to investigate the importance of publications in this field and to perform a bibliometric analysis to review relevant trends and clusters. This analysis will provide a better understanding of the direction for future scientific and clinical research.

Result

Document Analysis

In our bibliometric analysis of MAP studies conducted during 2001-2021, we identified 1755 articles, 96.467% of which were published in SCIE-indexed journals, and 3.533% were published in ESCI-indexed journals. English was the predominant language, ac-

Table 1.
Number and rates of articles by languages.

Languages	Record Count	% of 1.755
English	1722	98.120
Spanish	13	0.741
German	10	0.570
Portuguese	5	0.285
Indonesian	2	0.114
Polish	2	0.114
Italian	1	0.057

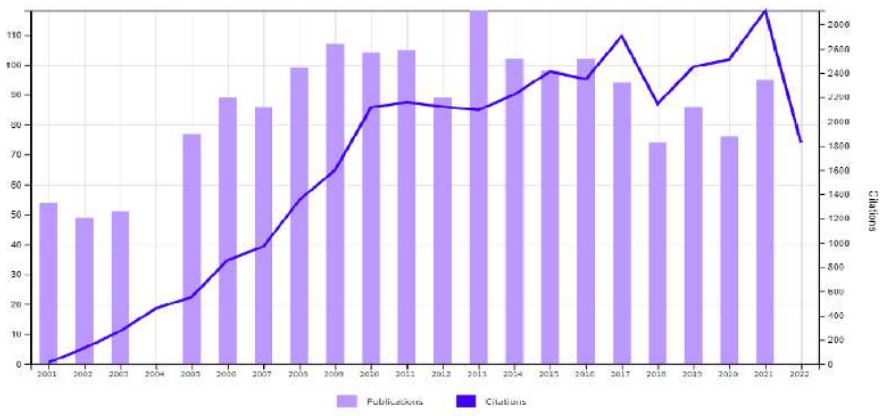

Figure 1.
Publication and citation frequency of *Mycobacterium avium subsp. paratuberculosis* by year.

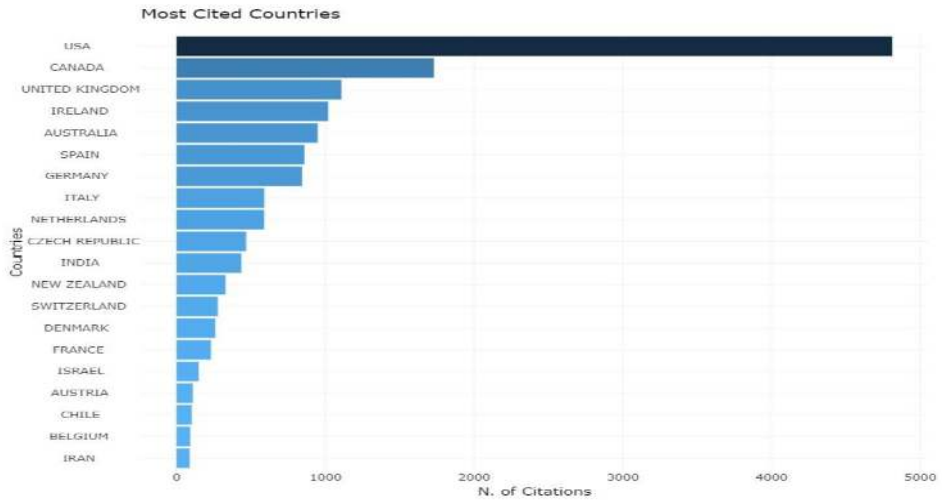

Figure 2.
Countries most cited in *Mycobacterium avium subsp. paratuberculosis* studies

Figure 3.
Global article map of Mycobacterium *avium subsp. paratuberculosis*
*4 record(s) (0.228%) do not contain data in the field being analyzed

counting for 95.448% of the papers (Table 1).

Citation Distribution by Year

The analyzed articles amassed a total of 36 237 citations, resulting in an average h-index of 72. Notably, 2013 had the highest number of papers, while 2021 had the highest number of citations (Figure 1). The United States was the most cited country (Figure 2).

Distribution of Publications by Country

The leading countries in terms of MAP-related publications were the USA (n = 536), Canada (n = 167), and Australia (n = 141). MAP-related publications emanated from 75 different countries across the globe (Table 2 and Figure 3).

Most Active Research Areas

The articles were primarily related to the research fields of Veterinary Sciences (n = 730), Microbiology (n = 519), and Immunology (n = 287) (Table 3).

Table 2.
Most productive countries on MAP research.

Countries/Regions	Record Count	% of 1.755
USA	536	30.541
Canada	167	9.516
Australia	141	8.034
Germany	133	7.578
India	127	7.236
Italy	116	6.610
Spain	84	4.786
Netherlands	79	4.501
England	67	3.818
New Zealand	55	3.134
Czech Republic	53	3.020
North Ireland	51	2.906
Scotland	45	2.564
France	44	2.507
Ireland	39	2.222
South Korea	39	2.222
Egypt	37	2.108
Iran	37	2.108
Chile	35	1.994
Argentina	34	1.937
Brazil	31	1.766
Japan	31	1.766
Denmark	29	1.652
Norway	23	1.311
Greece	22	1.254

Table 3.
Research areas of the publications on MAP

Research Areas	Record Count	% of 1.755
Veterinary Sciences	730	41.595
Microbiology	519	29.573
Immunology	287	16.353
Agriculture	226	12.877
Food Science Technology	169	9.630
Biotechnology Applied Microbiology	158	9.003
Infectious Diseases	134	7.635
Science Technology Other Topics	81	4.615
Biochemistry Molecular Biology	79	4.501
Gastroenterology Hepatology	55	3.134
Research Experimental Medicine	38	2.165
Genetics Heredity	28	1.595
Cell Biology	22	1.254
Public Environmental Occupational Health	15	0.855
Zoology	15	0.855
Neurosciences Neurology	14	0.798
Pathology	14	0.798
Life Sciences Biomedicine Other Topics	12	0.684
Chemistry	10	0.570
Reproductive Biology	8	0.456
General Internal Medicine	6	0.342
Engineering	5	0.285
Environmental Sciences Ecology	4	0.228
Mathematical Computational Biology	4	0.228
Pharmacology Pharmacy	4	0.228

*Showing 25 out of 53 entries, 5 record(s) (0.285%) do not contain data in the field being analyzed.

Most Prolific Affiliations

The organization with the most extensive research output on MAP, contributing 168 articles, was the United States Department of Agriculture (USDA) (Table 4).

Most Prolific Journals

The Journal of Dairy Science stood out as the journal with the highest number of MAP-related papers, with a total of 102 publications on this subject (Table 5).

Keyword Occurrence

Our bibliometric analysis highlighted the most frequently used keywords depicted in Figure 4. Keywords are integral to bibliometric analysis, aiding in the identification of primary topics and themes within a specific research area. Search engine algorithms rely on keywords to detect relevant literature. In our keyword visualization analysis, colors indicate preferred keywords, with red representing particularly popular ones. Keywords displayed in larger font sizes had a higher frequency of occurrence in the articles (Figure 2).

Bibliographic Coupling Between Countries

In the network visualization of bibliographic coupling between countries, the size of the bubbles was directly proportional to the volume of research conducted by each country. The width of the lines connecting countries indicates the strength of their coupling, while the line colors signify the cluster to which each country has been assigned. For this research, we considered a

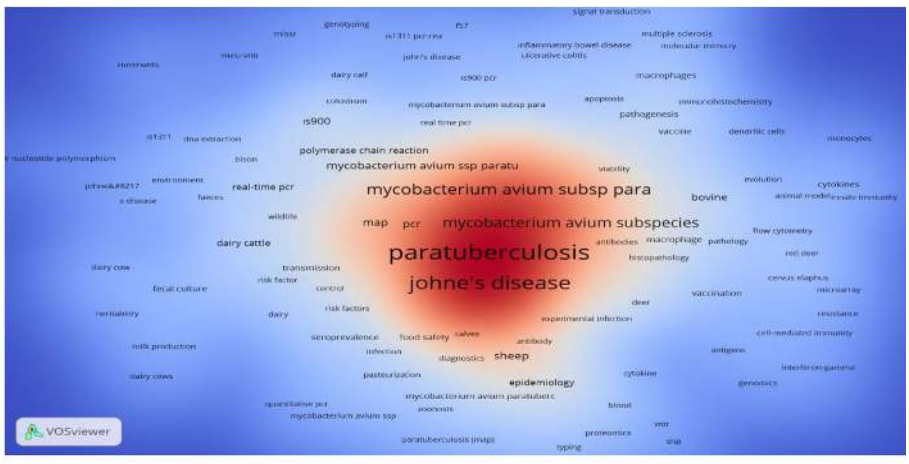

Figure 4.
Keyword visualization map of articles containing *Mycobacterium avium subsp.* paratuberculosis at least 5 times
*** Colors demonstrate preferred keywords, especially red show popular keywords.. Keywords represented with larger circle size or font size had a relatively higher occurrence in the articles.

Table 4.
List of the top affiliations.

Affiliations	Record Count	% of 1.755
United States Department of Agriculture USDA	168	9.573
University of Wisconsin System	88	5.014
University of Wisconsin Madison	87	4.957
University of Sydney	84	4.786
Indian Council of Agricultural Research Icar	81	4.615
University of Minnesota System	76	4.330
University of Minnesota Twin Cities	76	4.330
University of Sassari	58	3.305
Cornell University	53	3.020
Czech Veterinary Research Institute	53	3.020
Iowa State University	53	3.020
Icar Central Institute for Research on Goats	51	2.906
University of Guelph	48	2.735
University of Pennsylvania	48	2.735
University of Calgary	46	2.621
Queens University Belfast	45	2.564
Icar Indian Veterinary Research Institute	39	2.222
Utrecht University	39	2.222
Egyptian Knowledge Bank Ekb	37	2.108
University of Veterinary Medicine Hannover Foundation	36	2.051
Justus Liebig University Giessen	35	1.994
Universidad Austral De Chile	35	1.994
Agresearch New Zealand	34	1.937
Inrae	33	1.880
Friedrich Loeffler Institute	31	1.766

*Showing 25 out of 1.201 entries, 753 record(s) (42.906%) do not contain data in the field being analyzed.

Discussion

The results of bibliometric analysis in the field of diseases have increasingly captured the attention of scientists in recent years [22, 23]. Bibliometric analysis provides a scientific map of various diseases. In the realm of health sciences, it evaluates how a particular disease has evolved over the years and how the inclination to research that disease has shifted, employing a variety of indicators. Bibliometric analysis serves as an analytical method unveiling the broader intellectual landscape surrounding the disease while helping to identify particularly influential articles [23, 24].

In our bibliometric analysis of MAP studies spanning from 2001 to 2021, we discovered 1775 articles, 96.467% and 3.533% of which were published in SCIE-indexed and ESCI-indexed journals, respectively. These papers collectively received 36 237 citations, resulting in an average h-index of 72. While the year 2013 had the highest number of articles, 2021 saw the highest number of citations. A steady increase in the number of citations persisted until 2017; however, in recent years (2018-2020), the average number of citations has decreased. This phenomenon might be attributed to older publications being cited more frequently than newly published articles within that year [25].

Our findings revealed that a substantial portion of MAP publications originated from the United States, accounting for 30.541 articles. This is hardly surprising, as the USA, similar to the case of tuberculosis, allocates more resources to research and development related to paratuberculosis than most other countries [26]. In terms of the sheer number of publications, Canada ranks second with 9516 articles, while Australia se-

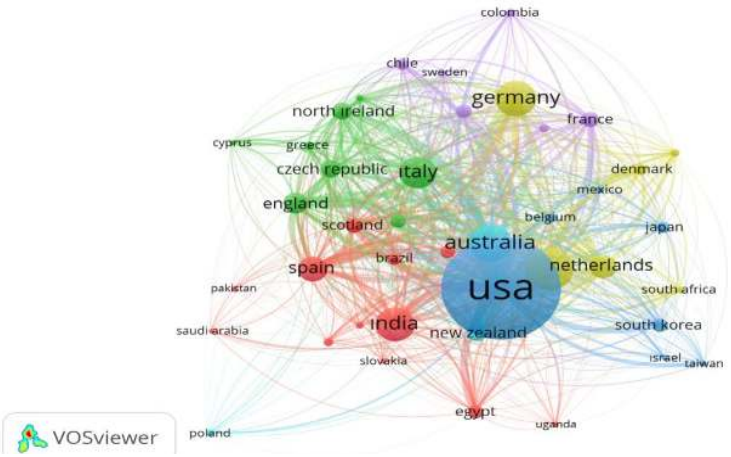

Figure 5.
Countries with at least 5 publications and 5 citations are shown on the map. *Collaboration is shown with lines linking nations. Stronger cooperation is indicated by thicker lines. Countries with a bigger circle or text size had a higher level of international cooperation.

cures the third position with 8349 articles. The presence of these countries in the top three may signify the widespread prevalence of this factor within their regions. Germany, as the fourth most productive country, with 7578 publications, can be linked to its substantial research funding initiatives. India, an Asian nation, also occupies a prominent position in the top five for productivity in MAP research. An overarching examination of the worldwide distribution of publications by country underscores the necessity for the development and implementation of global strategies to combat paratuberculosis.

Reviewing the leading journals based on the number of published articles, we find that the Journal of Dairy Science, ranked first, with an impact factor of 4.225. It is worth noting that the official publication of the American Dairy Science Association is not only indexed in WOS but is also included in various databases, such as Agricola, Biological Abstracts, Biological and Agriculture Index, BIOSIS Database, CABI Abstracts, Current Contents, Elsevier Bibliographic Databases, PubMed, and Scopus. When examining the most cited publications, the article titled 'The Complete Genome Sequence of MAP' by Li L. et al. published in 2005, has garnered 353 citations.

In our co-citation analysis among countries, an interesting trend emerges; the countries ranking in the top ten for article publication, including the USA, Australia, Germany, India, Netherlands, Italy, and Spain, are also prominent in joint citation analysis for MAP.

The keywords "paratuberculosis" and "Johne's disease" were the most frequently encountered in our bibliometric analysis of the number of publications, which aligns

Table 5.
List of journals that published the greatest number of articles on MAP

Journals	Record Count	% of 1.755
Journal of Dairy Science	102	5.812
Veterinary Microbiology	94	5.356
Veterinary Immunology and Immunopathology	74	4.217
Plos One	58	3.305
Applied and Environmental Microbiology	57	3.248
Preventive Veterinary Medicine	52	2.963
Journal of Clinical Microbiology	42	2.393
Journal of Veterinary Diagnostic Investigation	38	2.165
Infection and Immunity	37	2.108
Clinical and Vaccine Immunology	32	1.823
Veterinary Research	26	1.481
Journal of Microbiological Methods	22	1.254
Vaccine	22	1.254
Small Ruminant Research	21	1.197
Tropical Animal Health and Production	21	1.197
Journal of Wildlife Diseases	19	1.083
American Journal of Veterinary Research	18	1.026
Canadian Veterinary Journal Revue Veterinaire Canadienne	18	1.026
International Journal of Food Microbiology	18	1.026
Veterinary Record	18	1.026
BMC Microbiology	17	0.969
BMC Veterinary Research	17	0.969
Gut Pathogens	17	0.969
Journal of Applied Microbiology	17	0.969
Research in Veterinary Science	17	0.969

*Showing 25 out of 329 entries

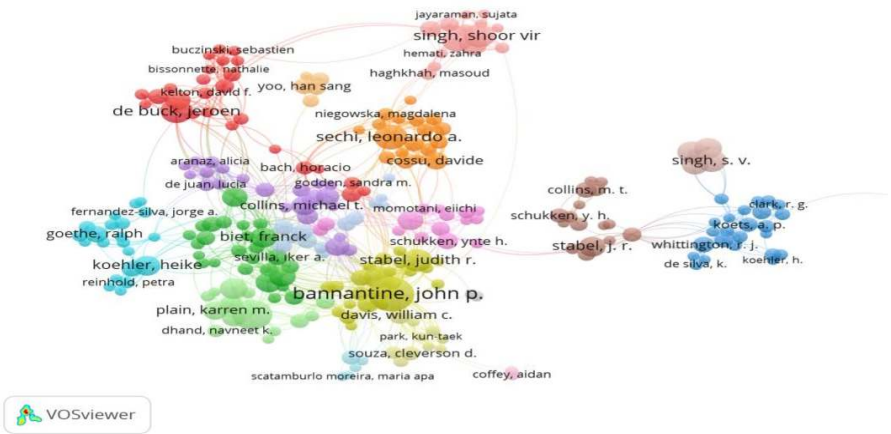

Figure 6.
Authors with at least 10 publications and 100 citations are shown on the map. **Citations are shown by lines linking authors. Authors with a greater circle size or font size had a higher number of citations.

with previous research confirming these data [27]. The fact that the agent is pathogenic for both animals and humans makes these keywords the most commonly used.

The varying budgets between countries and universities influence the number of research projects funded. The role of financial institutions in advancing science and research is crucial [28]. The results of the present research showed that the USA ranks in the top three financial institutions contributing to MAP research, which is consistent with other studies [29].

Analyzing the institutions where research published during 2001-2021 was conducted, the United States Department of Agriculture, the University of Wisconsin System, and the University of Wisconsin Madison emerged as the top three funders of paratuberculosis research. The significance of these data is expected to increase over time, encouraging funders to be more diligent in using official fund names [30]. In line with the mentioned argument, a book by Kazda et al. (2009) documented the prevalence of mycobacteria and their impact on the health of animals and humans, highlighting financial losses in ruminants due to MAP, food, and environmental contamination [31].

The majority of publications were in English (98.12%), and other languages included Spanish (0.74%), German (0.57%), Portuguese (0.28%), Indonesian (0.11%), Polish (0.11%), and Italian (0.05%). These findings in our bibliometric analysis align with a previous study [27]. The prevalence of English in publications can be attributed to the widespread acceptance of English in the scientific community and the fact that many publishers primarily accept articles in English, increasing the international accessibility of research findings.

The current study, conducted by veterinarians, physicians, and food researchers, aims to define MAP. It presents up-to-date data and sheds light on the boundaries and trends in paratuberculosis research from the past to the present. The continuous increase in the number of publications addressing paratuberculosis signifies that this factor remains a topic of active research worldwide. Given the importance of determining effective control strategies for paratuberculosis in both the food industry and public health, it is crucial to foster research collaborations between countries. This bibliometric analysis provides a substantial period within the scientific literature for assessing paratuberculosis, offering diverse data for future research endeavors.

In conclusion, this study provides up-to-date data on MAP, providing important information about the frontiers and trends in MAP research from past to present were presented. The steady rise in the number of publications on paratuberculosis reflects the continued global interest in this factor. Establishing effective control strategies for paratuberculosis is vital for the food industry and veterinary medicine as well as public health, highlighting the need for international research collaborations. This bibliometric analysis provides an important timeframe in the scientific literature for evaluating paratuberculosis and provides diverse data for future research in veterinary and human medicine.

Limitations

In this study, we examined the current status and prospects of MAP research through bibliometric analysis. However, there are several limitations to this analysis. Firstly, the scope of this bibliometric analysis was limited to publications published and indexed in the WOS database, potentially overlooking reports from other databases. Despite these limitations, the study provided valuable insights into MAP trends and identified areas with information gaps.

Secondly, considering that some authors may have multiple names or variations in name spellings, standardizing author names and terms based on VOSviewer results may not be entirely accurate. This could potentially lead to inaccurate results for certain authors. Despite these drawbacks, this article offers a foundational overview of MAP research.

Materials and Methods

Data Collection

Ethics approval was not required for this study as no human or animal participants were included. To retrieve Mycobacterium avium subsp. paratuberculosis publications, the WOS database (Clarivate PA, USA) was used. To evaluate the impact of scientific research effectively, the h-index was introduced as an alternative to traditional bibliometric indicators. Data for this research were sourced from the WOS database as of November 28, 2022. Information from WOS, including publication titles, authors' names, publication years, research countries, affiliated organizations, journal names, keywords, abstracts, and citation data for each record, was saved as TXT files and imported into Microsoft Office Excel 2019 (Los Angeles, USA). The research materials were accessed through Çanakkale Onsekiz Mart University's online library and digital resources.

Comprehensive Overview of the WOS Database

The WOS database was used to determine the research location or country, the type of study, authorship, and the number of citations. Only studies published during 2001-2021 were considered within the designated time frame. As the publication process for 2023 investigations has not been completed yet, those belonging to 2022 and 2023 were not included in the study. English was the selected search language, and h-index was employed as an indicator of publication impact.

Search Strategy

Aydemi ME. et al., IJVST 2023; Vol.15, No.4
DOI: 10.22067/ijvst.2023.83873.1289

The search terms (keywords) for this study were "Mycobacterium avium paratuberculosis" (Title) OR "Mycobacterium avium subspecies paratuberculosis" (Title) and Article (Document Types) OR Science Citation Index Expanded (SCI-EXPANDED) OR Emerging Sources Citation Index (ESCI) (WOS Index) and 2001-2021 (Publication Years). The selected timeframe was 2001-2021, allowing us to observe bibliometric developments over the last two decades. This research exclusively included research articles, while letters, reviews, editorials, and other types of articles were excluded. The WOS publications saved as TXT files were imported into Microsoft Office Excel 2019 alongside document categories, publication years, author names, journals, affiliations, keywords, group authors, and citations.

Network Analysis

In this bibliometric study, VOSviewer (version 1.6.10, Leiden University, The Netherlands) was employed for data importation and also to reveal future trends, collaboration networks, and significant findings. In addition, authorship, links, keywords, citations, and thematic words were collected and reviewed using this software. This tool allowed for the analysis of keywords, co-occurrences, citations, co-authorships, and co-citations.

Authors' Contributions

M.E.A. and S.K.A. conceived and planned the experiments. S.A. carried out the analysis. M.E.A., S.K.A. and S.A. contributed to the interpretation of the results. M.E.A. took the lead in writing the manuscript. All authors provided critical feedback for nalysis and manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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Online supplemental material

Supplementary Figure 1. A Three-field Plot (Sankey diagram) of country, keyword, and cited journals for the ten most researched topics.
Supplementary Figure 2. Trend topic

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RESEARCH ARTICLE

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Isolation, Antimicrobial Resistance, and Virulence Genes of Thermophilic Campylobacter Species from Backyard Ducks in Amol, Northern Iran

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ABSTRACT

Domestic poultry are considered natural reservoirs for the transmission of *Campylobacter* spp., mainly *C. jejun*i and *C. coli*, to other birds and humans. This study aimed to determine the Campylobacter infection status in backyard ducks in Iran. A total of 100 cloacal swabs were obtained from apparently healthy backyard ducks in different rural areas of Amol, a city in northern Iran. Bacterial isolation was based on traditional culture procedures, and genus and species identification were performed using an mPCR. All isolates were examined for antimicrobial resistance to seven antibiotics by Kirby Bauer's disk diffusion test. The virulence-associated genes *cadF, iamA, pldA, cdtA, cdtB, cdtC,* and *wlaN* were detected as well. Out of the 27 *Campylobacter* isolates recovered, 19 (70.4%) were *C. coli*, and 3 (11.1%) were C. jejuni. The remaining five isolates (18.5%) were not identified. All (100%) isolates showed resistance to ciprofloxacin. Most isolates were resistant to ampicillin, tetracycline, and nalidixic acid. The resistance rate to amoxicillin-clavulanic acid and erythromycin was moderate but was relatively low to gentamicin. Moreover, over two-thirds of the isolates were MDR. All virulence genes, except *iamA*, were variably detected. The *cadF* and *pldA* genes had the highest (92.6%) and lowest (7.4%) positivity rates, respectively. In addition, a statistically significant association was observed between *Campylobacter* spp. and most of the critical virulence genes ($p < 0.05$). Our findings imply that backyard ducks should be paid attention to as a major source of human campylobacteriosis.

Keywords

Duck, Campylobacter, Antimicrobial resistance, Virulence, Iran, Food-borne disease

Number of Figures: 2
Number of Tables: 6
Number of References:: 60
Number of Pages: 12

Abbreviations

FQs: fluoroquinolones
CDT: cytolethal distending toxin
LOSSIAL: sialylated lipo-oligosaccharide
AMR: antimicrobial resistance

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Introduction

Campylobacter species are generally considered a component of the normal gut flora of poultry [1]. Many species of domestic poultry and wild birds may be infected with thermophilic *Campylobacter* spp., mainly *C. jejuni* followed by *C. coli* and rarely *C. lari* [1, 2]. Ducks could be also a reservoir of Campylobacter spp. [3]. Recently, high rates of *Campylobacter* infection have been reported in domestic duck flocks in South Korea and Malaysia [4, 5]. In commercial poultry flocks, *Campylobacter* is not found in the first 2-3 weeks of age. This initial lag phase is probably related to maternal immunity [2]. Horizontal transmission from the environment, including contaminated water, feed, fomites, wild birds, other farm animals, rodents, and insects, is the major source of *Campylobacter* colonization. Vertical transmission of *Campylobacter* is unlikely, and the eggs are not contaminated [1, 2]. Despite extensive colonization in the cecum, colon, and cloaca (up to 109 colony-forming units/g feces), Campylobacter infections produce mild or no clinical diseases in poultry [1-3].

Food-borne bacterial pathogens are the most important etiologic agents of human gastroenteritis in the United States of America and worldwide [6]. *Campylobacter* causes more than 800 000 food-borne illnesses and 8000 hospitalizations in the USA each year [2]. The majority (50%-80%) of human campylobacteriosis cases occur through the ingestion of contaminated poultry products [7]. In a study in the United Kingdom, 50.7% of duck meat samples were infected with *Campylobacter*, which was comparable to chicken meat contamination (60.9%) [8]. Human infections are usually recognized by fever, diarrhea (watery/bloody), nausea, and abdominal pain after an incubation period of 2-5 days [1, 2]. In addition, GBS/acute neuromuscular paralysis may occur as a post-infection disease in 0.1% of the infected individuals and eventually causes respiratory compromise and death [1]. Usually, *Campylobacter* enteritis is a self-limiting infection, but antimicrobial therapy is needed in severe cases or immune-compromised patients [9]. Fluoroquinolones (e.g., ciprofloxacin) and macrolide antibiotics (e.g., azithromycin and erythromycin) are the appropriate medications. Tetracycline and gentamicin are occasionally used as alternative agents to treat systemic infection in humans [6]. Today, these antibiotics are utilized in food animals as a growth promotor or a therapeutic medicine. In recent years, an increase in drug-resistant *Campylobacter* isolates, particularly to FQs, has been observed in poultry, which poses a threat to public health [9]. Moreover, the Centers for Disease Control and Prevention classified antimicrobial-resistant *Campylobacter* strains under "microorganisms with a threat level of serious" and estimated that the resistance rate to FQs, macrolides, and tetracyclines among *Campylobacter* isolates is 22%, 2%, and 49%, respectively, in the U.S. annually [6, 9].

The pathogenesis of campylobacteriosis is not well understood. However, some of the putative virulence genes of Campylobacter which are associated with adhesion, colonization, invasion, and toxin production and are needed to induce infection have been investigated [7]. The *cadF* (*Campylobacter* adhesion to fibronectin) gene is responsible for adhesion and colonization. The pldA (Phospholipase A) and invasion-associated marker iam genes are involved in invasion [1, 7]. Among several different cytotoxins in *Campylobacter*, CDT, a tripartite toxin, which is encoded by three related genes, namely *cdtA*, *cdtB*, and *cdtC*, has been characterized in detail. Two heterodimeric subunits CdtA and CdtC are responsible for holotoxin binding to the cell membrane, and *CdtB* is an enzymatically active subunit [7]. The *wlaN* gene encodes the β-1,3-galactosyltransferase enzyme that is responsible for sialylated lipo-oligosaccharide (LOSSIAL) production, an essential pathogenic factor of GBS [10].

Duck rearing is a main part of poultry production in some countries of the world, such as China, France, South Korea, and Malaysia [4, 11, 12]. As a result, the highest consumption of duck meat (over 80%) has been reported in Asian countries [12]. In Iran, this industry has also been thriving and providing a part of human needs. As mentioned above, extensive research has been conducted on *Campylobacter* spp. infection in chickens, but the relationship between ducks and food-borne pathogens has been poorly investigated [11]. Therefore, the current study was performed to determine the infection status of Iranian backyard ducks to thermophilic campylobacters, and also the antibiotic resistance and virulence genes of the obtained isolates.

Abbreviations-Cont'd

MDR: multidrug-resistant
rs: Spearman's rank correlation coefficient
mPCR: multiplex polymerase chain reaction
ATCC: American-type culture collection
CLSI: Clinical and Laboratory Standards Institute
GBS: Guillain-Barre syndrome

Result

Infection rate

The results of mPCR are presented in Figure 1. In addition, the geographic distribution of thermophilic *Campylobacter* spp. in different backyard flocks is shown in Table 1. Out of the 28 backyard duck flocks examined, 17 (60.7%) were positive for thermophilic

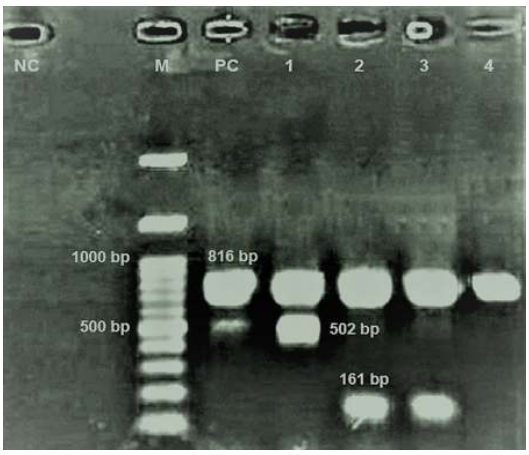

Figure 1.
Multiplex PCR assay for the identity of the *16S rRNA* gene (816 bp) for *Campylobacter* genus, the cj0414 gene (161 bp) for *C. jejuni,* and the ask gene (502 bp) for *C. coli.* Lane NC: Negative control (deionized water), Lane M: 100-bp DNA ladder, Lane PC: Positive control (*Campylobacter coli* ATCC 43478), Lane 1: *C. coli* isolate, Lane 2: a 161-bp amplified fragment of the *cj0414* gene was sequenced and confirmed as *C. jejuni* isolate, Lane 3: *C. jejuni* isolate, and Lane 4: *Campylobacter* spp. isolate (unidentified).

Table 1.
Regional distribution of thermophilic *Campylobacter* species isolated from backyard ducks in Amol villages.

Rural region	No. of flocks sampled	No. of positive flocks (%)	Isolated Campylobacter
North	11	2 (18.2)	*Campylobacter coli*
West	7	5 (71.4)	*C. coli and* other spp.
South	4	4 (100.0)	*C. coli and* other spp.
East	6	6 (100.0)	*C. coli and C. jejuni*
Total	28	17 (60.7)	*C. coli, C. jejuni, and* other spp.

Campylobacter species. Moreover, out of 100 cloacal samples tested for *Campylobacter* spp., 27 (27%) were infected. The majority of the isolates (19/27, 70.4%) were *C. coli*, while only three isolates (11.1%) were *C. jejuni*. The remaining five (18.5%) isolates were thermophilic *Campylobacter* spp., but have not been identified. None of the swab samples had mixed *Campylobacter* infection. In the present study, *C. lari* was not detected.

Antimicrobial resistance

All 27 *Campylobacter* isolates were examined for resistance to seven antibiotics belonging to five antibiotic classes. As shown in Table 2, all Campylobacter isolates were resistant to ciprofloxacin (100%). Moreover, most strains exhibited resistance to ampicillin (81.5%), tetracycline (77.8%), and nalidixic acid (74.1%). Resistance to amoxicillin/clavulanic acid and erythromycin was moderate at 51.9% and 44.4%, respectively, whereas resistance to gentamicin was relatively low (25.9%). Moreover, no statistically significant association was found between *Campylobacter* spp. and resistance to tested antibiotics (Table 2). Thirteen AMR patterns were observed in Campylobacter isolates, eight of which were MDR, and 19 out of 27 Campylobacter isolates (70.4%) were found to be MDR (Table 3).

Virulence genes

The results of PCR are presented in Figure 2. In

Table 2.
Antimicrobial resistance rate of *Campylobacter spp.* in backyard ducks.

Antimicrobial class	Antimicrobial	No. of resistant Campylobacter isolates (%)			Total (n=27) No. (%)	*p*-Value
		C. coli (n=19)	*C. jejuni* (n=3)	Other spp. (n=5)		
Fluoroquinolones	CIP	19 (100.0)	3 (100.0)	5 (100.0)	27 (100.0)	*NC*
	NAL	15 (78.9)	1 (33.3)	4 (80.0)	20 (74.1)	0.297^{ns}
Macrolides	ERY	7 (36.8)	1 (33.3)	4 (80.0)	12 (44.4)	0.227^{ns}
Tetracyclines	TET	15 (78.9)	1 (33.3)	5 (100.0)	21 (77.8)	0.119^{ns}
β-Lactams	AMP	17 (89.5)	1 (33.3)	4 (80.0)	22 (81.5)	0.072^{ns}
	AMC	10 (52.6)	1 (33.3)	3 (60.0)	14 (51.9)	1.000^{ns}
Aminoglycosides	GEN	3 (15.8)	1 (33.3)	3 (60.0)	7 (25.9)	0.092^{ns}

Abbreviations: CIP: Ciprofloxacin, NAL: Nalidixic acid, ERY: Erythromycin, TET: Tetracycline, AMP: Ampicillin, AMC: Amoxicillin/clavulanic acid, GEN: Gentamicin, *NC*: Not calculated, and ns: Not statistically significant (represents no significant association between *Campylobacter* spp. and AMR; $p > 0.05$)

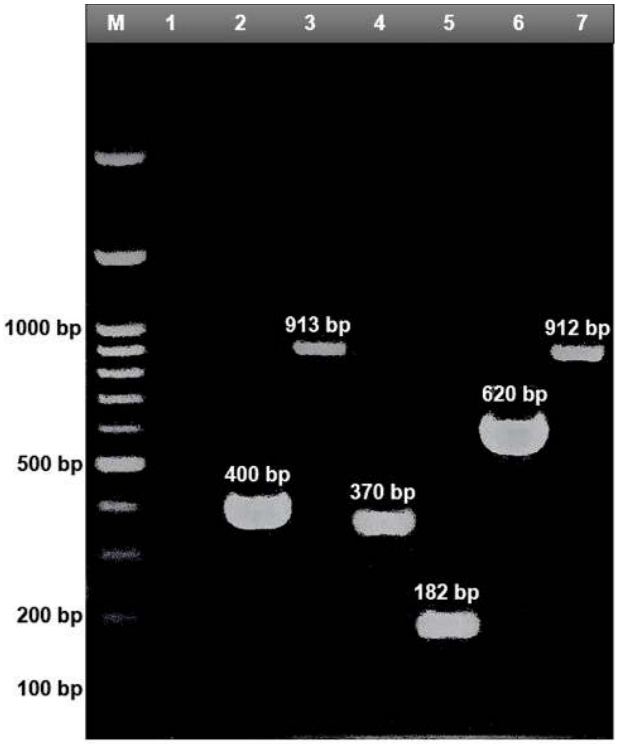

Figure 2.
Amplified PCR products of virulence genes (except the *iamA* gene) among *Campylobacter* isolates from backyard ducks. Lane M: 100-bp DNA ladder, Lane 1: negative control (deionized water), Lane 2: *cadF* gene positive, Lane 3: *pldA* gene positive, Lane 4: *cdtA* gene positive, Lane 5: *cdtC* gene positive, Lane 6: *cdtB* gene positive, and Lane 7: *wlaN* gene positive.

total, 27 *Campylobacter* isolates were screened for the presence of seven putative virulence and toxin genes, and the details of our findings are summarized in Table 4. The *cadF* (adhesion) gene with a positivity rate of 92.6% was the most prevalent gene. All C. coli and *C. jejuni* isolates were positive for this gene. Regarding invasion-related genes, 2 (7.4%) of the isolates carried *pldA*, while *iamA* was not detected in any of the isolates. Among the genes encoding *CDT, cdtA, cdtB,* and *cdtC* were present in 25.9%, 85.2%, and 29.6% of the isolates, respectively. Moreover, 25.9% of stains possessed the *cdtABC* gene cluster. The *wlaN* gene associated with LOSSIAL production was found in 14 (51.9%) of the isolates. A statistically significant association was observed between *Campylobacter* spp. and the majority of virulence-related genes (*cadF, cdtA, cdtB, cdtC, cdtABC,* and *wlaN*) ($p < 0.05$). Six virulence gene patterns (genotypes) were found in 25 of 27 *Campylobacter* isolates (92.5%) (Table 5).

Statistical analysis of phenotypic antimicrobial resistance with virulence genes

There was no significant correlation between phenotypic resistance to antibiotics and genotype (virulence genes) in *Campylobacter* spp. isolated from backyard ducks ($rs = -0.35$, $p = 0.08$).

Table 3.
Antimicrobial resistance (AMR) patterns in *Campylobacter* isolates from backyard ducks.

Antibiotic resistance pattern	No. of *Campylobacter* isolates in a given AMR pattern (%)			
	C. coli (n=19)	*C. jejuni* (n=3)	Other spp. (n=5)	Total (n=27)
CIP	---	2 (66.7)	---	2 (7.4)
CIP-NAL	1 (5.3)	---	---	1 (3.7)
CIP-TET	1 (5.3)	---	---	1 (3.7)
CIP-NAL-TET	---	---	1 (20.0)	1 (3.7)
CIP-NAL-AMP-AMC	3 (15.8)	---	---	3 (11.1)
CIP-NAL-TET-AMP*	3 (15.8)	---	---	3 (11.1)
CIP-ERY-TET-AMP*	1 (5.3)	---	---	1 (3.7)
CIP-NAL-TET-AMP-AMC*	4 (21.1)	---	---	4 (14.8)
CIP-ERY-TET-AMP-AMC*	1 (5.3)	---	---	1 (3.7)
CIP-NAL-ERY-TET-AMP*	2 (10.5)	---	---	2 (7.4)
CIP-ERY-TET-AMP-GEN*	1 (5.3)	---	1 (20.0)	2 (7.4)
CIP-NAL-ERY-TET-AMP-AMC*	---	---	1 (20.0)	1 (3.7)
CIP-NAL-ERY-TET-AMP-AMC-GEN*	2 (10.5)	1 (33.3)	2 (40.0)	5 (18.6)
Total	19 (100.0)	3 (100.0)	5 (100.0)	27 (100.0)
No. of MDR* isolates (%)	14 (73.7)	1 (33.3)	4 (80.0)	19 (70.4)

* MDR pattern: resistance of *Campylobacter* isolates to at least three antimicrobial classes

Table 4.
Frequency of virulence genes in *Campylobacter* isolates from backyard ducks.

Campylobacter Species	No. of isolates	No. of positive isolates for a specific gene (%)							
		cadF	*iamA*	*pld*A	*cdt*A	*cdt*B	*cdt*C	*cdt*ABC	*wla*N
C. coli	19	19 (100.0)	0	1 (5.3)	2 (10.5)	18 (94.7)	3 (15.8)	2 (10.5)	13 (68.4)
C. jejuni	3	3 (100.0)	0	1 (33.3)	3 (100.0)	3 (100.0)	3 (100.0)	3 (100.0)	1 (33.3)
Other spp.	5	3 (60.0)	0	0	2 (40.0)	2 (40.0)	2 (40.0)	2 (40.0)	0
Total	27	25 (92.6)	0	2 (7.4)	7 (25.9)	23 (85.2)	8 (29.6)	7 (25.9)	14 (51.9)
p-Value		0.037*	*NC*	0.242 ns	0.003*	0.016*	0.007*	0.003*	0.010*

Abbreviations: *NC*: Not calculated, and ns: Not statistically significant
* indicates a statistically significant association between *Campylobacter* spp. and virulence genes ($p < 0.05$)

Table 5.
Virulence gene patterns (genotypes) in *Campylobacter* isolates from backyard ducks.

Virulence gene pattern	No. of Campylobacter isolates in a given genotype (%)			
	C. coli (n=19)	*C. jejuni* (n=3)	Other spp. (n=5)	Total (n=27)
cadF	1 (5.3)	---	1 (20.0)	2 (7.4)
cadF-cdtB	4 (22.0)	---	---	4 (14.8)
cadF-cdtB-wlaN	11 (57.9)	---	---	11 (40.7)
cadF-cdtB-cdtC-wlaN	1 (5.3)	---	---	1 (3.7)
cadF-cdtA-cdtB-cdtC	1 (5.3)	2 (66.7)	2 (40.0)	5 (18.5)
cadF-pldA-cdtA-cdtB-cdtC-wlaN	1 (5.3)	1 (33.3)	---	2 (7.4)
Total	19 (100.0)	3 (100.0)	3 (60.0)	25 (92.5)

Discussion

Backyard poultry can serve as a transmission source for a variety of food-borne pathogens, including thermophilic *Campylobacter* spp., for other bird and human populations because biosecurity practices in backyard flocks are commonly not monitored and executed [13]. There is little data on the on-farm prevalence of *Campylobacter* in domestically reared duck flocks. Therefore, this research aimed to estimate the infection rate, AMR, and genes associated with the virulence of *Campylobacter* spp. among backyard ducks in Iran.

In the present study, thermophilic *campylobacters* were confirmed using both the standard culture methods and mPCR in 27% (27/100) of the cloacal samples of backyard ducks. This finding was in agreement with the *Campylobacter* infection rate in chicken meat samples in Iran, which was reported at 28.9% (26/90) [14], while lower isolation rates of *Campylobacter* spp. were identified in the urban duck fecal samples in Iran (17.3%) [15] and in turkey, game bird (pheasant and quail), and duck cecal samples in Canada (11.9%, 4.5%, and 3.4%, respectively) [16]. On the other hand, a higher level of *Campylobacter* infection was found among the duck and goose intestinal samples in Iran (34.2%) [17] and in mallard duck and white-fronted goose cloacal samples in Poland (32.8% and 45.5%, respectively) [18]. Although *C. jejuni* was reported as the prevailing species in most studies, the most prevalent species identified in the current study was *C. coli* (70.4%). This finding was in accordance with the research completed in Spain and Germany [19, 20]. The results of this study showed that *Campylobacter* infection is highly prevalent in ducks and these hosts can be considered the main source of *Campylobacter* spp. (both *C. jejuni* and *C. coli*) and a possible risk of human campylobacteriosis.

In this research, the resistance rate to ciprofloxacin was 100%, and high resistance to nalidixic acid was observed among *Campylobacter* isolates (74.1%), while *C. coli* (78.9%) was more resistant to nalidixic acid than *C. jejuni* (33.3%). Similarly, in a study by Wysok *et al.* (2020), most of the *Campylobacter* strains

from domestic goose cecal samples revealed resistance to ciprofloxacin (92%) and nalidixic acid (88%) [21]. In another study, high resistance to ciprofloxacin was found among human isolates in Europe, China, and Korea [22]. Contrarily, previous studies reported relatively low resistance to ciprofloxacin and nalidixic acid among *Campylobacter* strains [23, 24]. Overall, the very high resistance rate to FQs in this study may result from the expansive usage of this class of antimicrobials, such as enrofloxacin and sarafloxacin, to treat certain infections (e.g. *Escherichia coli*) in the poultry industry [25].

In the present study, 44.4% of *Campylobacter* isolates (36.8% *C. coli* and 33.3% *C. jejuni*) indicated moderate resistance to erythromycin. Our result was similar to those of Wei et al. (2014) [4] and Ghoneim et al. (2020) [26]. On the other hand, all *Campylobacter* strains isolated from layer hen cloacal swabs and farm environment samples in Tunisia were resistant to erythromycin [27]. Overall, the findings of this research showed that the use of macrolides (e.g., spiramycin and tylosin) for therapeutic purposes and growth promotion in commercial poultry has caused the high prevalence of macrolide-resistant *Campylobacter* isolates in backyard flocks [6].

High resistance to tetracycline was shown in *Campylobacter* strains (77.8%) in this research, while *C. coli* was more resistant to this antibiotic compared to *C. jejuni* (78.9% *vs*. 33.3%). Similarly, high resistance to tetracycline was reported in Iran (70.6%) [28] and China (nearly 100%) [29]. Conversely, a study in Belgium showed relatively low or moderate resistance to tetracycline (48.3%) among *Campylobacter* strains isolated from the intestinal samples of international travelers [30]. Our findings indicated that tetracyclines should be used cautiously to treat animals and humans.

In the current research, the rate of gentamicin-resistant *Campylobacter* strains was relatively low (25.9%) and C. jejuni (33.3%) exhibited higher resistance to gentamicin than *C. coli* (15.8%). Similarly, Qin et al. (2012) reported a relatively low rate of resistance against gentamicin (>20%) among *Campylobacter* isolates from broiler chickens [31]. In another study, relatively low resistance to gentamicin was estimated among Campylobacter strains obtained from human and chicken sources in the U.S. [25]. Consequently, aminoglycosides (e.g., gentamicin) can be utilized to treat acute and systemic *Campylobacter* infections in humans and to prevent bacterial infections in various avian species [6, 25].

In our study, 81.5% of *Campylobacter* isolates (89.5% of *C. coli vs*. 33.3% of *C. jejuni)* were resistant to ampicillin. These results were similar to those of Giacomelli *et al.* (2014) [32] and Casagrande Proietti et al. (2020) [33]. However, beta-lactams, such as penicillin, are the most commonly used antibiotics for turkeys in Germany [34]. On the other hand, 51.9% of Campylobacter strains (52.6% *C. coli* and 33.3% *C. jejuni*) demonstrated moderate resistance to amoxicillin/clavulanic acid (co-amoxiclav) in this investigation, which was similar to previous estimates by Jehanne et al. (2021) in France [35] and Hadiyan et al. (2022) in Iran [36]. As a result, oral beta-lactams, such as co-amoxiclav, can be an appropriate choice to treat human *Campylobacter* infection due to the rising resistance of *C. jejuni* and *C. coli* to FQs, erythromycin, and tetracycline [25].

Overall, *C. coli* strains identified in the present research had more resistance to important antibiotics than *C. jejuni* isolates. Furthermore, 70.4% of *Campylobacter* isolates exhibited resistance against three or more antimicrobial classes. In this study, the prevalence rate of MDR was much higher for *C. coli* than for *C. jejuni* (73.7% *vs*. 33.3%), which was similar to the results of an investigation conducted in China [37]. Determining the virulence factors of *Campylobacter* is very important to better understand the infection rate [18]. Therefore, several critical virulence genes were identified in this research.

The prevalence of *cadF*, the most prevalent virulence gene, among Campylobacter isolates obtained from backyard ducks was 92.6% (25/27), which enhances the ability of *Campylobacter* to attach to host fibronectin and colonization of the intestine [7]. This result was similar to the research conducted by Kim et al. (2019) in South Korea (93.3%) [38] and Rossler et al. (2020) in Argentina (92%) [39], while the low prevalence rate of the cadF gene was detected in broiler chickens in South Africa (23.1%) [40]. In general, the prevalence of virulence genes responsible for adhesion in *Campylobacters* is very high regardless of the source and geographic area [18].

Both the invasion-associated marker (*iam*) and *pldA* genes encode pathogenic factors related to the *Campylobacter* invasion of intestinal epithelial cells. Moreover, the *pldA* gene encodes an outer membrane protein, phospholipase A, that is involved in hemolytic activity [7]. In this study, none of the *Campylobacter* isolates had the *iamA* gene, which was consistent with the results of a previous investigation in Brazil [41]. In addition, 7.4% (2/27) of the isolates possessed the *pldA* gene in our study. Similarly, a low frequency of the *pldA* gene was found among strains isolated from duck samples in South Korea (3.6%) [42] and individuals with diarrhea in Iran (15%) [43]. In contrast, the high prevalence rates of *iam* and *pldA* genes have been reported in earlier studies [44, 45]. The reasons for the considerable diversity of *iam* and *pldA* genes are not yet elucidated [41].

CDT is a key marker for *Campylobacter* pathoge-

nicity in humans, which is produced by three linked genes named *cdtA, cdtB*, and *cdtC*. The *CdtB* subunit has type I DNase activity and causes cell cycle arrest at the G2/M phase, while CdtA and CdtC subunits are responsible for the binding of *CDT* and its internalization into host cells. Ultimately, *CDT* leads to distention and cell death. However, the role of this toxin during Campylobacter colonization in the avian hosts is unclear [46]. The presence of three cdt genes is necessary for the function of CDT holotoxin [7, 46]. In this investigation, a relatively low frequency of the *cdtABC* gene cluster among backyard duck *Campylobacter* isolates was detected (25.9%), while all *C. jejuni* strains possessed the *cdtABC* genes. A similar rate of *cdtABC* was reported among all *Campylobacter* isolates from American crows in the U.S. and healthy pet birds in Iran, with a range of 20%-33% [47, 48], while the higher frequency of the *cdtABC* cluster was previously detected in Ireland (86%) [49] and Spain (100%) [50]. In general, the prevalence of CDT (*cdtABC*) genes in different research is highly variable, which may be due to heterogeneity in the genetic reservoir of *Campylobacter* strains [51].

The *wlaN* gene is responsible for the biosynthesis of sialylated lipo-oligosaccharide (LOSSIAL), which has a structure similar to human GM1 ganglioside. The LOSSIAL factor may cause autoimmune diseases, such as GBS polyneuropathy, following Campylobacter infection [10]. In this research, 51.9% of the *Campylobacter* strains had the *wlaN* gene, while this gene was more prevalent among *C. coli* isolates than *C. jejuni* (68.4% vs. 33.3%). The frequency of wlaN was 10% in wild birds in South Korea [52], 36% in human and broiler chicken sources in Egypt [53], and 44% in human stool samples in Hungary [54], which was lower than our result. Previous investigations have shown no association between the source of isolates and the presence of the *wlaN* gene [10].

Virulence gene patterns in the current study demonstrated that C. jejuni isolates carried more virulence factors than C. coli. In other words, *C. jejuni* strains are likely more pathogenic. Finally, we indicated a statistically significant association between *Campylobacter* spp. and the presence of virulence genes, especially genes related to the production of cytotoxins (CDT) and the occurrence of GBS. *Campylobacter* isolates obtained from backyard ducks can be a threat to food hygiene and human health.

In conclusion, the current study highlights that backyard ducks harbor commensal thermophilic campylobacters and can be regarded as potential reservoirs of *Campylobacter* infection for other hosts. Awareness of owners' backyard poultry flocks about the risk of the transmission of zoonotic diseases, including campylobacteriosis, hygiene and biosecurity measures (e.g., cleaning and disinfection, daily water and food change, rodent and insect control, and keeping wild birds away from backyard flocks), veterinary care, and antibiotic monitoring are essential for improving husbandry practices and avian health in backyard flocks, and decrease the prevalence of zoonotic pathogens between commercial and backyard poultry farms, leading to reduced infection in humans.

Materials and Methods

Sample collection

This study was conducted on June 2021-July 2022 in different geographical regions of the rural area of Amol (a city in northern Iran). A total of 100 healthy ducks from 28 backyard flocks were tested for *Campylobacter* infection. A cloacal swab sample was taken from all birds. To do this, the vent was cleaned with disinfectant iodine solution (10%) and a swab was inserted into the cloaca and was rotated. The samples were stored in a Cary-Blair transport medium (12.6 g/991 ml; HiMedia, India) at 4°C and were directly transmitted to the laboratory. The swabs were examined 4 h after sampling.

Bacterial examination

The swab samples were cultured in enrichment Preston broth consisting of Preston broth base (25 g/945 ml; MilliporeSigma, USA), *Campylobacter* selective supplement IV, modified with polymyxin B [2500 IU/500 mL], rifampicin [5 mg/500 mL], trimethoprim lactate [5 mg/500 mL], and amphotericin B [5 mg/500 mL] (MilliporeSigma, USA), and 5% lysed sheep blood (Zistroyesh, Iran). The inoculated broths were incubated at 37°C for 4 h, followed by 44 ± 4 h at 42°C in a microaerobic chamber (85% nitrogen, 10% carbon dioxide, and 5% oxygen) (Anaerocult® C; MilliporeSigma, USA). One loop full of enriched sample (1 µl) was cultivated on Preston selective agar containing *Campylobacter* agar base (19.75 g/500 mL; HiMedia, India), 5% defibrinated sheep blood, and mentioned antibiotics at the same doses. Bacterial cultures were incubated in a microaerobic environment at 42°C for 48 h. Subsequently, suspected colonies of *Campylobacter* were purified on brain heart infusion agar (52 g/L; MilliporeSigma, USA) with 5% sheep blood. Plates were incubated for 24 h at 42°C in a similar atmosphere. Preliminary recognition of *Campylobacter* isolates was performed based on colony characteristics (greyish, round, flat, and shiny with a regular edge), examination of typical cellular shapes ("S" or "seagull-like"), rapid darting motility using the phase-contrast microscopy, and biochemical reactions consisting of oxidase test (tetramethyl-p-phenylene-diamine), catalase test (3% H_2O_2), and glucose fermentation. Finally, a molecular assay was performed to confirm presumptive colonies [1].

DNA extraction

A pure single colony of each *Campylobacter* isolate was suspended in 200 µl sterile deionized H_2O. Bacterial DNA was prepared by boiling at 95°C for 15 min. The samples were centrifuged at 11000 rpm for 2.5 min and the supernatants were stored at -20°C until utilization [55].

Genus and species identification

A mPCR was performed following the method described previously by Yamazaki-Matsune *et al.* (2007) [56]. The target genes of *16S rRNA* for the *Campylobacter* genus, *ask* (aspartokinase) for *C. coli*, *glyA* (serine hydroxymethyltransferase) for *C. lari*, and *cj0414*

Table 6.
Antimicrobial resistance (AMR) patterns in Campylobacter isolates from backyard ducks.

Target gene	Primer	Sequences (5'- 3')	Annealing temperature (°C)	Size (bp)	Reference
16S rRNA (Campylobacter)	C412F	GGATGACACTTTTCGGAGC	58	816	[56]
	C1228R	CATTGTAGCACGTGTGTC			
ask (C. coli)	CC18F	GGTATGATTTCTACAAAGCGAG	58	502	[56]
	CC519R	ATAAAAGACTATCGTCGCGTG			
glyA (C. lari)	CLF	TAGAGAGATAGCAAAAGAGA	58	251	[56]
	CLR	TACACATAATAATCCCACCC			
cj0414 (C. jejuni)	C-1	CAAATAAAGTTAGAGGTAGAATGT	58	161	[56]
	C-3	CCATAAGCACTAGCTAGCTGAT			
cadF	cadF-F2B	TTGAAGGTAATTTAGATATG	45	400	[55]
	cadF-R1B	CTAATACCTAAAGTTGAAAC			
iamA	iamA F	GCGCAAAATATTATCACCC	52	518	[59]
	iamA R	TTCACGACTACTATGCGG			
pldA	pldA-84	AAGCTTATGCGTTTTT	45	913	[55]
	Pld-981	TATAAGGCTTTCTCCA			
cdtA	DS-18	CCTTGTGATGCAAGCAATC	49	370	[55]
	DS-15	ACACTCCATTTGCTTTCTG			
cdtB	cdtB-113	CAGAAAGCAAATGGAGTGTT	51	620	[55]
	cdtB-713	AGCTAAAAGCGGTGGAGTAT			
cdtC	cdtC-192	CGATGAGTTAAAACAAAAAGATA	47	182	[55]
	cdtC-351	TTGGCATTATAGAAAATACAGTT			
wlaN	wlaN F	AGGGTTTTAATAGTTGCAATTTCTC	50	912	[60]
	wlaN R	ATGAAATTTTTAATATCTTTACG-GAATTAA			

(oxidoreductase) for *C. jejuni* were amplified using specific primer sets (Table 6). Briefly, the amplification reaction was performed in a 25 µl final volume, including 2 µl DNA of bacteria, 12.5 µl PCR Master Mix 2X (Sinaclon, Iran), 0.5 µl forward and reverse primers (Sinaclon, Iran), and 6.5 µl distilled deionized H_2O. The mPCR was performed by a thermocycler (Bio-Rad, USA) according to the following program: an initial 15 min denaturation at 95°C, 35 cycles of denaturation (95°C, 0.5 min), annealing (58°C, 1.5 min), extension (72°C, 1 min), and a final step of 7 min at 72°C. Amplified products (10 µl each) were run on electrophoresis 1.5% agarose gel stained with DNA-safe stain (Sinaclon, Iran) in 1X tris-acetate-EDTA buffer and were seen under UV light. The 100-bp DNA ladder (Sinaclon, Iran) was employed as a molecular weight standard. *C. coli* strain ATCC 43478 and sterile deionized H_2O were utilized as positive and negative controls, respectively. Moreover, one of the *C. jejuni* isolates obtained in this study was subjected to sequencing of a 161-bp PCR amplicon of the *cj0414* gene by the Sanger sequencing method (Codon Genetic Group, Iran). Based on nucleotide BLAST analysis, the sequence data of the *cj0414* gene and *C. jejuni* strain 2016-IZSVE-19-111250 (GenBank: CP053659.1) from Italy were 99.24% identical.

Antibiotic susceptibility testing

The antimicrobial sensitivity of identified *Campylobacter* strains was assessed using Kirby Bauer's disk diffusion test according to the CLSI guideline for fastidious organisms [57]. The used antibiotic disks (Padtanteb, Iran) consisted of ciprofloxacin (CIP, 5 µg), nalidixic acid (NA, 30 µg), erythromycin (E, 15 µg), tetracycline (TE, 30 µg), ampicillin (AM, 10 µg), amoxicillin/clavulanic acid (AMC, 20/10 µg), and gentamicin (GM, 10 µg). Bacterial colonies were suspended in nutrient broth (25g/L; MilliporeSigma, USA) to acquire a McFarland turbidity of 0.5. The prepared suspensions were cultured on Mueller-Hinton agar media (38g/L; MilliporeSigma, USA) containing 5% defibrinated sheep blood, and were incubated microaerobically at 42°C for 24 h. The zone of bacterial growth inhibition was measured for each antibiotic and evaluated under interpretive criteria provided by CLSI. Acquired resistance to at least one drug in three or more antibiotic classes was considered MDR [58].

Detection of virulence genes

The genomic DNA was amplified by PCR to detect genes involved in adhesion (*cadF*), invasion (*iamA* and *pldA*), production of toxin (*cdtA, cdtB, cdtC*), and biosynthesis of sialylated lipooligosaccharide (wlaN) using the primer sets listed in Table 6. The reaction mixture (25 µl) consisted of 2 µl DNA of bacteria, 12.5 µl PCR Master Mix 2X (Sinaclon, Iran), 1 µl of each primer (Sinaclon, Iran), and 8.5 µl deionized H_2O. The PCR assays were completed according to the following conditions: initial denaturation at 95°C for 5 min, 35 cycles of denaturation (95°C, 1 min), annealing at a temperature specific to the primer pair for 1 min, extension (72°C, 1 min), and a final extension step at 72°C for 6 min. The PCR product of each gene (10 µl each) was electrophoresed on 1.5% agarose gel stained with DNA-safe stain (Sinaclon, Iran) in 1X TAE buffer and was visualized under UV light. Sterile deionized H_2O was used as the negative control. The amplicon size was determined using the 100-bp DNA ladder.

Statistical analysis

Statistical analysis was performed by SPSS 23. In this study, the correlation between phenotypic resistance to antibiotics and genotype (virulence genes) was determined using Spearman's correlation coefficient. Furthermore, Fisher's exact test was used to evaluate the association between *Campylobacter* spp. and phenotypic resistance to antibiotics and virulence genes. p-value < 0.05 was considered statistically significant.

Authors' Contributions

H.G., and R.A.J. conceived and planned the experiments. H.G., R.A.J., D.G., and R.K. carried out the experiments. H.G. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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Case Report

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Sperm cells in peritoneal fluid of a ram with obstructive urolithiasis: First report

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ABSTRACT

Detection of sperm in the peritoneal fluid of animals is unusual and has not been reported in the literature. In this report, we describe the presence of sperm cells in the peritoneal fluid of a two-year-old ram. The ram was presented with dyspnea, reduced rumen contractions, a mild degree of dehydration, cyanotic mucosa, difficulty in standing, and anuria. Ancillary diagnostics, including ultrasonography, radiography, complete blood cell count, and abdominocentesis were performed. In the peripheral blood sample, no blood parasites were observed, although thrombocytosis with toxic neutrophilia, and atypical lymphocytes were seen in the blood smear. In radiographs, urolithiasis was confirmed just on the sigmoid flexure position. Ultrasound examination revealed a distended bladder and large amounts of free fluid within the peritoneal cavity. The electrocardiogram analysis showed the absence of P waves, bradycardia, wide QRS complexes, ST-segment elevation, and tented T waves. In the peritoneal fluid smears, a large number of spermatozoa, and increased inflammatory cells were observed. It was concluded that the trauma or rupture in colliculus seminalis, ductus deferens, or the urethra with urinary stones resulted in leakage of spermatozoa to the peritoneal cavity. To our knowledge, this is the first report of the presence of spermatozoa in the peritoneal fluid of large animals.

Keywords

Spermatozoa, peritoneal effusion, sheep, urogenital, urolithiasis

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Abbreviations

BCS: Body condition score
EDTA: Ethylenediaminetetraacetic Acid
WBC: White Blood Cell
Hct: Hematocrit
ECG: Electrocardiogram

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Case Description

*I*n autumn 2021, a 2-year-old ram (Ghezel breed) from a sheep herd was referred to the veterinary teaching hospital of the School of Veterinary Medicine, Urmia University, Iran. The main complaints of the owner were the complete and sudden loss of appetite, painful and difficult urination, and urine dribbling in the past three days. The owner of the animal had used dexamethasone at an unknown dose before referring to the hospital.

The body condition was good (BCS: 3). The physical examination revealed a heart rate of 62 bpm, rectal temperature of 36.9 C°, arrhythmia heard on cardiac auscultation, dyspnea, cyanotic mucosa, and reduced rumen contractions. Attempts to take a urine sample were unsuccessful.

In the peripheral blood sample taken from the ear vein, no blood parasites were observed. Blood samples were collected from the jugular vein in vacuum tubes with 10% EDTA for a complete blood cell count. The results of the blood sample were as follows: WBC 14300 /µl, segmented neutrophils 8400 /µl, lymphocyte 1500/µl, monocyte = 1000/µl, Hct 33%, and thrombocytosis with toxic neutrophilia and atypical lymphocyte were seen on the blood smear. Urinary stones were observed only in radiographs, and due to the lack of access to the stones inside the urinary tract, identification of these stones was impossible.

An ECG was recorded using a base-apex lead. A portable single-channel electrocardiograph (Nihon Kodhen, Japan) was used for recording. The lead selector was positioned on lead I, and the ECG was recorded with the paper speed of 25 mm sec-1 and sensitivity of 10 mm = 1 mV.

The electrocardiogram analysis revealed an absence of atrial electrical activity (no discernible P waves), bradycardia (ventricular rate≈ 60 bmp), and ST segment depression. These ECG changes might be attributable to hyperkalemia (Figure. 1-A).

Transabdominal ultrasound examination revealed the thickness of the bladder and proximal urethra wall, distended bladder with urine, and large amounts of free fluid within the peritoneal cavity. Ultrasound-guided abdominocentesis was carried out, and smears prepared from the recovered fluid revealed numerous inflammatory cells and abundant numbers of spermatozoa (Figure 1-B).

Radiographs were carried out from the pelvis and abdominal cavity in order to seek a more detailed examination of the urinary tract and urethral path. Radiographs showed two radiopaque round calculi with dimensions of 4.2 mm lodged in the sigmoid flexure. Details of the radiographic findings are presented in Figure 2.

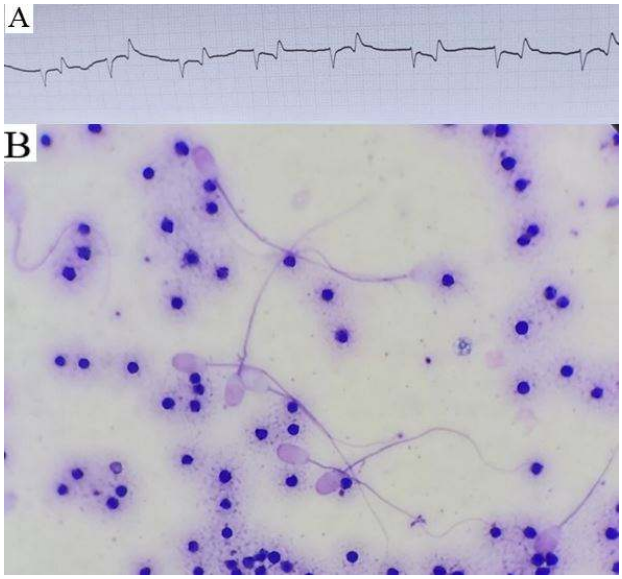

Figure 1.
A: ECG recorded from a 2-year-old ram with obstructive urolithiasis. Bradycardia, absence of P waves, wide QRS complexes, and depression of ST segment are seen in the figure. These findings might indicate increased serum potassium concentration, which is one of the most common laboratory abnormalities with uroperitoneum. Base-apex lead 25 mm/s, 10 mm=1 Mv. B: In the peritoneal fluid cytology (Wright-Giemsa stain; 100×), a large number of atypical cells containing basophilic granules and a large number of sperm were observed.

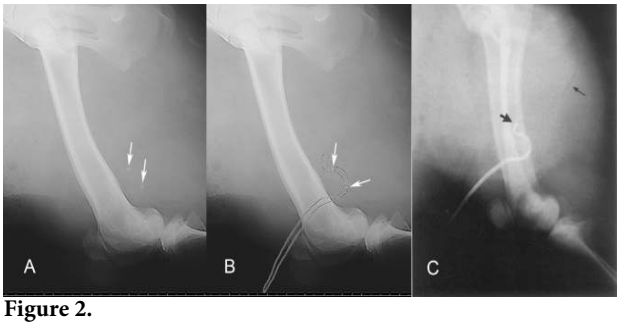

Figure 2.
A: lateral plain Radiography in a ram suspected to urethral calculi. Two radiopaque round matter with dimensions of 4.2 mm (stone) are seen (with arrow) just on the sigmoid flexure position. B: The same radiograph A in which the urethral track was drawn according to radiograph C.1 Radiopaque materials (stones) are marked with dotted lines and located (with an arrow) at the sigmoid flexure of the urethra. C: Radiograph 3. Positive contrast urethrography in a Nubian goat [1].

Discussion

The Presence of spermatozoa in the peritoneal fluid has been extremely rarely reported, with only one case report of a 52-year-old man [2-3]. To the best of our knowledge, this report of sperm cells in peritoneal fluid from a ram is the first documented case in the veterinary medicine field.

The total number of WBCs, segmented neutrophils, and monocytes was more than the reference range. The maximum range for these parameters in sheep was reported as 8000/µl, 6000/µl, and 750/µl respectively. Neutrophilia or neutrophilic leukocytosis with the presence of toxic neutrophils was seen in this case. Extreme neutrophilia, exceeding upper reference limits, is usually seen in inflammation. In the acute stage of the inflammatory response, neutrophilia occurred with an increase along with a left shift and toxic changes. Although, the number of lymphocytes was lower than the normal range. Lymphopenia may occur with stress conditions, most commonly as a response to glucocorticoid secretion or corticosteroid administration, acute viral or bacterial infections, and endotoxemia. Atypical lymphocytes may be seen in response to antigenic stimulation because toxic neutrophils concurrently were detected in leucogram, which may appear as a response to infectious agents' antigens [4,5].

Various pathologic conditions cause fluid accumulation (effusion) in the peritoneal cavity [6 -9]. In cases with protein-losing enteropathy or nephropathy, lymphatic obstruction, and portal hypertension, transudates resulting from excess diffusion of water or lymph from the vascular space are a result of abnormalities of hydraulic or oncotic pressure [10]. Although inflammatory processes are caused by bacteria, some viruses, protozoa, parasites, neoplasms, foreign bodies, or uroperitoneum increase vascular permeability so that plasma exits the vasculature, often along with leukocytes [11, 12]. Effusions may be caused by a rupture of visceral organs, including those resulting from urinary tract rupture, biliary leakage, and gastrointestinal rupture. In early phases of the uroperitoneum, peritoneal effusion will have the character of urine as very low TP and total nucleated cell count, but with time and irritation to the peritoneum will take on characteristics of exudates with increased TP and total nucleated cell count, which may be diluted by high volumes of urine leakage [12]. Additional but less common effusions include lymphorrhage from lymphatic leakage in which multiple pathophysiologic processes alter the character of the peritoneal fluid [12].

The precise mechanism by which sperm enters the peritoneal cavity in male animals and humans remains unknown. In a report from a human with sperm in peritoneal fluid, specimen contamination during sample collection and processing, bladder rupture with retrograde ejaculation, sperm entering into the abdominal cavity, and incidental injury of vas deferens during paracentesis were considered as potential explanations for this observation. But none of these explanations could be plausible in our case. We aspirated peritoneal fluid from two different regions too far from the genital tract. In addition, sampling was performed using percutaneous ultrasound-guided abdominocentesis to avoid accidental puncture of intraabdominal structures; therefore, it seems unlikely that the peritoneal sample was contaminated with spermatozoa from vas deference or another storage site of spermatozoa.

The patient's history and clinical signs, along with ultrasonographic and radiographic findings, were consistent with obstructive urolithiasis and blockage of the urethra with stones and hydroperitoneum. However, the presence of spermatozoa within the peritoneal fluid is an abnormal finding and has not been reported so far from male animals following urolithiasis or any other disease. Uroperitoneum usually occurs secondary to rupture of the bladder; however, in our case, ultrasonography confirmed a distended and likely unruptured bladder.

In the male reproductive system, only the vas deferens (inside the scrotum or spermatic cord) is located inside the abdomen after originating from the epididymis and before entering the pelvic area and connecting with the pelvic urethra. This duct, together with the epididymis and its ampoule, is the main storage of sperm. Therefore, its rupture, along with the spermatic cord may cause sperm to enter the peritoneal cavity. Attributing the rupture of the urethra (in the pelvic part or its flexure) as the origin of the entry of sperm into the peritoneal fluid is not consistent with its anatomical position because only a small part of the urethra is inside the pelvis, and the other parts do not have a direct connection with the abdominal cavity to drain the sperm into the abdominal cavity [13]. On the other hand, with the possibility of pelvic urethral rupture, the entry of sperm from these parts into the peritoneal fluid is not easily conceivable because it is unlikely that the urethra has a sufficient population of sperm (sperm enters the urethra from its reservoir during ejaculation) unless there is an abnormal situation that causes a large number of sperm to enter the urethra or bladder by itself. Searching for sperm in urine was not possible. Otherwise, it could have helped to clarify this issue. It is also difficult to imagine the simultaneous occurrence of several unlikely possibilities without providing evidence. Likely, trauma or rupture in colliculus seminalis or ductus deferens and

pelvic urethral may be the origin of sperm leakage in the peritoneal cavity.

We assume that the lodging and passage of stones from colliculus seminalis and proximal or intrapelvic urethra damaged its wall (necrosis and rupture), and leakage of urine and spermatozoa to the peritoneal cavity occurred. In the geographic region covered in this report, the breeding season in sheep flocks starts in late summer and extends to the autumn. It is probable that due to the coincidence of mating season and increased pressure in the urethra following stone lodging, retrograde ejaculation resulted in leakage of urine and spermatozoa from the proximal urethra into the peritoneal cavity. However, due to the owner's disagreement to perform exploratory laparotomy or necropsy, we could not determine the exact origin of the injury or the leakage. If the animal's general condition is stable, surgery and removal of stones can be the correct method in dealing with similar cases.

It was concluded that trauma in the ductus deferens and or the perforation with urinary stones in the colliculus seminalis area or the pelvic segment of the urethra is the most likely cause of the appearance of spermatozoa in the peritoneal fluid.

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Conflicts of Interest

The authors declare that there is no conflict of interest.

Authors' Contributions

G.J., B.D., S.H., and Y.N. contributed to the sample preparation. G.J., B.D., S.H., and Y.N. contributed to the interpretation of the results. G.J. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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Errata

The Iranian Journal of Veterinary Science and Technology publishes corrections when they are of significance to patient care, scientific data or record-keeping, or authorship, whether that error was made by an author, editor, or staff during processing of the article. Errata also appear in the online version and are attached to files downloaded from ijvst.um.ac.ir. (More information on: https://publicationethics.org/case/corrigendum-or-erratum)

In the article entitled "Therapeutic Effects of ADU-S100 as STING Agonist and CpG ODN1826 as TLR9 Agonist in CT-26 Model of Colon Carcinoma" by Sare Hajiabadi; Soodeh Alidadi; Mohammad Mehdi Ghahramani Senoo; Zohreh Montakhab Farahi; Hamid Reza Farzin; Alireza Haghparast, which had appeared in Vol.15. No.2, 2023/ doi:10.22067/ijvst.2023.80505.1223, there was an error on page 36. Specifically, in the acknowledgement section, the sentence that had been read "Financial support was also received from the Iranian Biotechnology Initiative Council" should have been read "Financial support was also received from Iran National Science Foundation (INSF)".

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مریم گلی بنفش نقایص شناختی را متعاقب هیپوپرفیوژن مزمن مغزی در موش بهبود می بخشد

یلدا گلریز[1]، امیر افخمی گلی[2]، حمیدرضا صادق نیا[3]، حسین کاظمی مهرجردی[4]*

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چکیده

هیپوپرفیوژن مزمن مغزی (CCH) ناشی از بیماری های عروق مغزی، به عنوان عامل اولیه اختلال شناختی در سال های اخیر در نظر گرفته شده است. در این فرآیند، استرس اکسیداتیو نقش مهمی ایفا می کند و به نورون های هیپوکامپ آسیب می رساند. مطالعات نشان داده است که مریم گلی به واسطه ترکیبات پلی فنولی دارای فعالیت آنتی اکسیدانی بوده و باعث از بین بردن رادیکال های آزاد می شود. این مطالعه با هدف بررسی اثر مریم گلی بنفش در هیپوپرفیوژن مزمن مغزی در مدل موش انجام شد. از یک هفته قبل از عمل جراحی تا ۱۴ روز بعد، به نیمی از موش‌ها عصاره ی الکلی مریم گلی بنفش به صورت روزانه و به نیمی دیگر نرمال سالین خورانده شد. هیپوپرفیوژن مغزی با بستن دو طرفه شریان های کاروتید مشترک القا شد. شناخت موش-ها یک هفته پس از عمل جراحی در ماز آبی موریس مورد بررسی قرار گرفت. آنالیز این تست نشان دهنده ی تفاوت معناداری میان گروه جراحی شده با گروه کنترل از نظر زمان تاخیر فرار، مسافت شنا کردن تا یافتن صفحه پنهان و زمان سپری کردن در ربع هدف بود ($P < 05/0$). نتایج این مطالعه نشان داد عصاره ی گیاه مریم گلی بنفش می تواند ضایعات شناختی ناشی از کاهش خون رسانی مغزی در رت که به دنبال حضور رادیکال های آزاد اکسیژن و تخریب هیپوکمپ رخ می دهد را کاهش دهد.

واژگان کلیدی

مریم گلی، رت، هیپوپرفیوژن، زوال

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بررسی میزان شیوع سرمی و فاکتورهای خطر برای عفونت با ویروس سینسیشیال تنفسی گاو، ویروس پاراآنفلوآنزای گاوی–۳ و آدنوویروس گاوی–۳ در گاوداری‌های شیری استان فارس (جنوب ایران)

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چکیده

در این مطالعه مقطعی، شیوع سرمی ویروس سینسیشیال تنفسی گاو (BRSV)، ویروس پاراآنفلوآنزای گاوی-۳ (BPIV-3) و آدنوویروس گاوی-۳ (BAV-3) و عوامل خطر مرتبط با آنها در گاوداری های شیری در جنوب ایران بررسی شد. نمونه های سرمی (۴۲۰ عدد) از ۳۶ گله گاو در مناطق شمالی، مرکزی و جنوبی منطقه مورد مطالعه جمع آوری شد. یک کیت تجاری سنجش ایمونوسوربنت مرتبط با آنزیم برای شناسایی آنتی بادی های این ویروس ها استفاده شد. آزمون مجذور کای و تجزیه و تحلیل رگرسیون لجستیک برای بررسی ارتباط بین عوامل خطر و شیوع سرمی ویروس ها استفاده شد. شیوع سرمی برای همه ویروس های مورد مطالعه در سطح گله ۱۰۰ درصد و برای BRSV، BPIV-3 و BAV-۳ به ترتیب ۷۶.۴۳درصد، ۷۶.۹۰درصد و ۹۲.۶۲درصد در سطح دام برآورد شد. در تجزیه و تحلیل رگرسیون لجستیک، سن برای همه ویروس‌ها، فصل برای BPIV-3 و BAV-3 و منطقه و نوع پرورش برای BAV-3 به طور معنی‌داری با شیوع سرمی در سطح دام مرتبط بود. ارتباط معنی‌داری از عفونت‌های دوگانه با ویروس‌های مورد مطالعه شناسایی شد. نتایج این مطالعه نشان می‌دهد که BRSV، BPIV-3 و BAV-3 در گله‌های گاو شیری در جنوب ایران بسیار شایع هستند و نیاز به برقراری یک برنامه کنترل را برجسته می کند.

واژگان کلیدی

شیوع سرمی، فاکتورهای خطر، BAV-3، BPIV-3، BRSV

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مقایسه روش های تشخیص بروسلوز در گوسفندهای با سابقه سقط به روش کشت باکتریایی، سرولوژی و qPCR

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۵ قطب علمی سقط جنین و مرگ‌ومیر نوزاد نشخوارکنندگان، بیمارستان تخصصی دانشکده دامپزشکی دانشگاه فردوسی، مشهد، ایران.

چکیده

بروسلوز از شایع‌ترین بیماری‌های مشترک انسان و دام است که موجب مشکلات قابل توجهی برای سلامت عمومی و صنعت دامپروری می‌شود. در این مطالعه تعیین میزان شیوع بروسلوز در سرم و شیر گوسفندان دارای سابقه سقط به‌کمک روش‌های کشت باکتریایی، رزبنگال و qPCR مورد بررسی قرار گرفت. سرم و شیر از ۱۰۰ گوسفند ۳ تا ۵ سال تهیه شد. رزبنگال تغییریافته بر روی سرم جهت تعیین میزان شیوع سرمی بروسلوز، کشت باکتری برای جداسازی باکتری و qPCR جهت شناسایی DNA باکتری بر روی شیر انجام شدند. میزان شیوع بروسلا برای تست‌های رزبنگال تغییریافته، جداسازی باکتری و qPCR به‌ترتیب ۳۲٪، ۴۲٪ و ۴۴٪ بدست آمد. با در نظر گرفتن qPCR بعنوان تست طلائی، رزبنگال تغییریافته دارای حساسیت ۹۵٪، ویژگی ۱۰۰٪، دقت۹۸٪، ارزش پیشگوئی مثبت۱۰۰٪ و ارزش پیشگوئی منفی ۹۷٪ بدست آمد. حساسیت، ویژگی، دقت، قدرت پیشگوئی مثبت و منفی بترتیب ۷۷٪، ۱۰۰٪، ۹۰٪، ۱۰۰٪ و ۸۵٪ برای کشت بدست آمد. میزان توافق بین qPCR و رزبنگال تغییریافته ۹۵۹/۰ (۹۵٪ CI:۰.۸۹۶-۱)، qPCR و کشت ۷۹۲/۰ (۹۵٪ CI:۰.۶۶۷-۰.۸۹۷) و بین رزبنگال و کشت ۸۳۱/۰ (۹۵٪ CI:۰.۷۰۹-۰.۳۸) بدست آمد. با توجه به نتایج بدست آمده جداسازی باکتری از شیر به دلیل هزینه بالا، حساسیت پائین، وقت گیر بودن و خطر آلودگی افراد جز در موارد خاص توصیه نمی‌شود، اگرچه از رزبنگال تغییر یافته شده می‌توان به‌عنوان یک روش روتین به‌علت مقرون بصرفه بودن و داشتن حساسیت و دقت بیشتر نسبت به جداسازی باکتری استفاده کرد. با این حال، qPCR بعنوان تست طلائی شناسایی بروسلوز با استفاده از شیر در گوسفندان دارای سابقه سقط توصیه می‌شود.

واژگان کلیدی

بروسلوز، رزبنگال تغییر یافته، qPCR و گوسفند

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اثر ضدباکتریایی اسانس نعنا و پونه بر سه باکتری اصلی ورم پستان در محیط شیر

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چکیده

ورم پستان گاو عامل خسارتهای اقتصادی زیادی است. ظهور سویه های مقاوم باکتری باعث تلاش برای یافتن جایگزین هایی برای آنتی بیوتیک ها شده است. عقیده عمومی این است که مقدار زیاد چربی و پروتئین در مواد غذایی ممکن است باکتری ها را در برابر اثر اسانس ها محافظت کند. هدف این مطالعه بررسی اثر ضد باکتریایی اسانس نعنا و پونه بر سه باکتری مهم عامل ورم پستان گاو شامل استافیلوکوک آرئوس، استرپتوکوک آگالاکتیه و اشریشیاکلی در محیط شیر بود. برای آنالیز اسانس ها روش گاز کروماتوگرافی و برای برررسی اثرات ضدباکتریایی روش های حداقل غلظت مهاری، حداقل غلظت کشندگی و نمودار رشد استفاده شدند. فراوانترین ترکیبات اسانس نعنا به ترتیب کارون (۶۳/۰۲ ٪)، لیمونن (۲۴/۴۸ ٪) و اسانس پونه به ترتیب پولگون (۴۸/۱۶ ٪)، اکالیپتول (۱۴/۵۷ ٪) و پیپرتنون (۱۰/۰۹ ٪) بودند. حداقل غلظت مهاری و کشندگی به ترتیب برای پونه ۰/۶۲ ٪ و ۱/۲۵ ٪ ، برای نعنا ۱/۲۵ - ۰/۳۱ ٪ و ۰/۶۲-۲/۵ ٪ و برای نعنا+پونه ۰/۳۱-۰/۶۲ ٪ و ۰/۶۲-۲/۵ ٪ بود. اسانس نعنا و پونه باعث کاهش معنی دار تعداد اشرشیاکلی و استرپتوکوک آگالاکتیه در ساعت ۶ و استافیلوکوک آرئوس و استرپتوکوک آگالاکتیه در ساعت ۲۴ شدند. بطور کلی اسانس نعنا و پونه علیه هر سه باکتری اثر ضدباکتریایی نشان داده و می توانند در مطالعات بالینی به همراه یا جایگزین آنتی بیوتیک ها مطالعه شوند.

واژگان کلیدی

اثر ضد باکتریایی، ورم پستان، نعنا، پونه، شیر

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شیوع تب کیو و عوامل خطر در گوسفند و بز سقط شده در استان کرمان، جنوب شرق ایران

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چکیده

بیماری کوکسیلیوز که به عنوان تب کیو نیز شناخته می شود، یک بیماری مشترک بین انسان و دام است که توسط باکتری کوکسیلا بورنتی ایجاد می شود و به یکی از علل مهم سقط جنین نشخوارکنندگان کوچک در سطح جهان تبدیل شده است. تب کیو در ایران بومی است، اما اطلاعات اپیدمیولوژیک در مورد شیوع واقعی کوکسیلا بورنتی در مناطق خاصی از کشور کم است. نشخوارکنندگان کوچک، به ویژه بزها و گوسفندها، مخزن اولیه عفونت انسان در نظر گرفته می شوند و تهدید قابل توجهی برای سلامت انسان به شمار می روند. این مخازن می توانند عامل عفونت را از طریق مخاط و ترشحات واژن پخش کنند. هدف از این مطالعه، بررسی میزان شیوع بیماری کوکسیلیوز با استفاده از روش Real-time PCR و شناسایی عوامل خطر مرتبط (مانند محل دام، سن، گونه و تعداد زایش) مرتبط با این بیماری در ترشحات واژینال نشخوارکنندگان کوچک ساکن در استان کرمان واقع در جنوب شرقی ایران بوده است. طی زمستان سال ۱۳۹۸ و پاییز سال ۱۳۹۹، در مجموع ۱۳۴ نمونه ترشحات سقط جنین از ۳۲ گله واقع در مناطق مختلف استان کرمان، شامل ۷۰ نمونه از گوسفند و ۶۴ نمونه از بز، جمع‌آوری گردید. تشخیص ژن IS1111 کوکسیلا بورنتی در این نمونه ها با استفاده از روش Real-time PCR انجام شد. بنابر نتایج این مطالعه، از میان نمونه های اخذ شده، تعداد ۲۶ نمونه شامل ۱۲ نمونه بزی (۲۱/۸۸ ٪) و ۱۴ نمونه گوسفندی (۱۷/۱۴ ٪) از نظر وجود بیماری کوکسیلوز، مثبت بودند. یافته‌های مطالعه ما نشان داد که بیماری کوکسیلوز شایع است و عامل آن در منطقه مورد بررسی در حال چرخش می باشد. علاوه بر این، تجزیه و تحلیل ما نشان داد که هیچ ارتباط آماری معنی‌داری بین میزان شیوع بیماری تب کیو و محل، میزان زایش و سن که به عنوان عوامل خطر بالقوه مورد بررسی قرار گرفتند در نشخوارکنندگان کوچک این استان وجود ندارد.

واژگان کلیدی

:تب کیو، بیماری کوکسیلیوز، Real-time PCR، نشخوارکنندگان کوچک، شیوع، استان کرمان

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Abstract in Persian

جداسازی، مقاومت ضد میکروبی و ژن های حدت گونه های کمپیلوباکتر گرمادوست از اردک های خانگی در آمل، شمال ایران

حسین گیلانی [۱]، رمضانعلی جعفری [۲]، داریوش غریبی [۳]، راحم خوشبخت [۴]، فروغ طلازاده [۲]

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چکیده

طیور اهلی به عنوان مخازن طبیعی برای انتقال گونه های کمپیلوباکتر، عمدتا کمپیلوباکتر ژژونی و کمپیلوباکتر کولای، برای سایر پرندگان و انسان ها محسوب می شوند. این مطالعه با هدف تعیین وضعیت عفونت کمپیلوباکتر در اردک های خانگی ایران انجام شد. در مجموع، ۱۰۰ سوآب کلوآک از اردک های خانگی به ظاهر سالم در مناطق مختلف روستایی آمل (شهری در شمال ایران) بدست آمدند. جداسازی باکتری بر اساس روش های معمول کشت انجام گرفت و سپس شناسایی جنس و گونه با استفاده از واکنش Multiplex PCR انجام شد. همه جدایه ها برای مقاومت ضد میکروبی به ۷ آنتی بیوتیک با استفاده از تست Kirby Bau-er's disk diffusion بررسی شدند. ژن های مرتبط با حدت *cadF, iamA, pldA, cdtA, cdtB, cdtC* و *wlaN* نیز شناسایی شدند. از ۲۷ جدایه کمپیلوباکتر بدست آمده، ۱۹ (۷۰/۴ ٪) کمپیلوباکتر کولای و ۳ (۱۱/۱ ٪) کمپیلوباکتر ژژونی بودند. ۵ جدایه باقی مانده (۱۸/۵ ٪) شناسایی نشدند. همه (۱۰۰٪) جدایه ها مقاومت به سیپروفلوکساسین را نشان دادند. اکثر جدایه ها به آمپی سیلین، تتراسایکلین و نالیدیکسیک اسید مقاوم بودند. میزان مقاومت به آموکسی سیلین-کلاولونیک اسید و اریترومایسین متوسط بود، اما نرخ مقاومت به جنتامایسین نسبتاً کم بود. هم چنین بیش از دو سوم جدایه ها به چند دارو مقاوم بودند. همه ژن های حدت به جز ژن *iamA* به طور متغیر شناسایی شدند. ژن های *cadF* و *pldA* به ترتیب بیش ترین (۹۲/۶ ٪) و کم ترین (۷/۴ ٪) فراوانی را داشتند. علاوه بر این، ارتباط آماری معنی داری بین گونه های کمپیلوباکتر و بیش تر ژن های مهم حدت مشاهده شد ($p < 0.05$). یافته های ما نشان می دهد، که اردک های خانگی باید به عنوان منبع اصلی کمپیلوباکتریوز انسانی مورد توجه قرار گیرند.

واژگان کلیدی

اردک، کمپیلوباکتر، مقاومت ضد میکروبی، حدت، ایران، بیماری منتقله از غذا

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Abstract in Persian

اولین گزارش مشاهده اسپرم در مایع صفاقی قوچ مبتلا به سنگ‌های ادراری

قادرجلیل زاده امین*[1]، بهرام دلیر نقده [1]، سید محمد هاشمی اصل[2]، یاسر نوظهور[1]

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چکیده

مشاهده اسپرم در مایع صفاقی حیوانات وضعیت غیرعادی است و تا کنون در متون علمی گزارش نشده است. شرح مورد: در این گزارش به تشریح وجود اسپرم در مایع صفاقی قوچ دو ساله پرداخته شده است. قوچ مذکور با نشانه های درمانگاهی تنگی نفس، کاهش انقباضات شکمبه، درجه خفیف کم آبی، مخاط سیانوتیک، مشکل در ایستادن و آنوری ارجاع داده شده بود. انواع روش های کمک تشخیصی شامل سونوگرافی، رادیوگرافی، شمارش کامل سلول های خونی و اخذ مایع صفاقی انجام شد. در نمونه خون محیطی، هیچ انگل خونی مشاهده نشد، اگرچه ترومبوسیتوز همراه با نوتروفیلی توکسیک و لنفوسیت‌های آتیپیک در اسمیر خون مشاهده گردید. در تصاویر رادیوگرافی، حضور سنگ ادراری فقط در موقعیت خم سیگموئید تایید شد. معاینات سونوگرافی، مثانه متسع همراه با حضور مقدار زیادی مایع آزاد در حفره صفاقی را مشخص نمود. تحلیل نوار قلبی (ECG)، عدم وجود امواج P ، برادی کاردی، کمپلکس های QRSپهن، ارتفاع بلند قطعه ST و امواج T را نشان داد. در گسترش تهیه شده از مایع صفاقی، تعداد زیادی اسپرم و سلول های التهابی مشاهده گردید. ممکن است که ضربه یا پارگی در کولیکولاس سمینالیس، مجرای دفران یا مجرای ادراری به دلیل حضور سنگ های ادراری، منجر به نشت اسپرم به حفره صفاقی شود. طبق اطلاعات ما، این اولین گزارش از حضور اسپرم در مایع صفاقی حیوانات است.

واژگان کلیدی

اسپرم، مایع صفاقی، گوسفند، دستگاه ادراری تناسلی، سنگ ادراری

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Guide for authors

SCOPE

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Gene names: The standard gene names, as provided by HGNC (HUGO Gene Nomenclature Committee) should be used. Gene names must be italicized. If the case of mammalian species and if gene names refer to rodent species, they must be upper case; if they refer to non-rodent species they must be written in capitals. If they refer to other species, they must written lower case. Protein names are written in capitals and are not italicized. As an example:

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Quantitative PCR: If the quantitative PCR method has been used, the related section in Materials and Methods must be written following the reference:

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The following information must be provided in the section:

Protocol for DNA/RNA extraction, including quantification and determination of purity;

Reverse transcription (if used): amount of RNA, concentration of all reagents: primers concentration (either random primers or oligonucleotides), reverse transcriptase and master mix components;

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References for the above example:

1. Hull J, Forton J, Thompson A. Paediatric respiratory medicine. Oxford: Oxford University Press; 2015.

2. Eckerman AK, Dowd T, Chong E, Nixon L, Gray R, Johnson S. Binan Goonj: bridging cultures in Aboriginal health. 3rd ed. Chatswood, NSW: Elsevier Australia; 2010.

3. Johnson C, Anderson SR, Dallimore J, Winser S, Warrell D, Imray C, et al. Oxford handbook of expedition and wilderness medicine. Oxford: Oxford University Press; 2015.

4. McLatchie GR, Borley NR, Chikwe J, editors. Oxford handbook of clinical surgery. Oxford: Oxford University Press; 2013.

5. Petitti DB, Crooks VC, Buckwalter JG, Chiu V. Blood pressure levels before dementia. Arch Neurol. 2005; 62(1):112-6.

6. Liaw S, Hasan I, Wade, V, Canalese R, Kelaher M, Lau P, et al. Improving cultural respect to improve Aboriginal health in general practice: a multi-perspective pragmatic study. Aust Fam Physician. 2015; 44(6):387-92.

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Written authorization from all authors for publication of the article is mandatory for IJVST to start the review process. This form entitled "Conflict of interest declaration and author agreement form" must be signed and completed by all authors. This statement and signatures certifies that all authors have seen and approved the manuscript being submitted. Also, the authors by signing this form warrant that the article is the Authors' original work, that the article has not received prior publication and is not under consideration for publication elsewhere, and that the corresponding author shall bear full responsibility for the submission.

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Publication Ethics

Ethical guidelines for Peer reviewers

Iranian Journal of Veterinary Science and Technology (IJVST) follows and adheres to COPE Ethical Guidelines for Peer Reviewers. IJVST peer reviews all submitted manuscripts with contents in the scope of the journal. The process has been explained in the section "Peer Review Process".

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PEER REVIEW PROCESS

Iranian Journal of Veterinary Science and Technology peer reviews all submitted manuscripts with contents within the scope of the journal.

Initial assessment

The submitted manuscript will be subjected to a primary review by the editor or a member of the editorial board for suitability and relevance of the findings to the scope of the journal and quality of the science presented in the paper (sufficient originality, having a message that is important to the general field of Veterinary Medicine, quality of data, novelty, English language, and overall manuscript quality) within two weeks. If the paper is evaluated to be relevant to the scope of the journal and having enough scientific rigor and novelty, it will be sent for the next stage. Otherwise, those manuscripts which are evaluated as not-appropriate in the initial review will be rejected at this stage.

Initial screen

The initial screen will be performed by the editorial office for the structure and format of the manuscript.

Peer review (double-blind)

The manuscripts which are found to be appropriate after the initial screen will be sent for external review by experts in the related field. We have prepared a checklist for reviewers that summarizes their evaluation of the manuscript. The items in this checklist are:

1. TITLE is clear and adequate
2. ABSTRACT clearly presents objects, methods, and results.
3. INTRODUCTION well-structured and provides a rationale for the experiments described.
4. MATERIALS AND METHODS are sufficiently explained and is detailed enough to be reproduced.
5. RESULTS are clearly presented and supported by figures and tables.
6. DISCUSSION properly interprets the results and places the results into a larger research context, and contains all important references.
7. Conclusions are logically derived from the data presented.
8. English Language/style/grammar is clear, correct, and unambiguous.
9. Figures and tables are of good quality and well-designed and clearly illustrate the results of the study.
10. References are appropriate.
11. Regarding this article are you concerned about any issues relating to author misconduct such as plagiarism and unethical behavior.
12. Comments on the importance of the article.

Final Decision

Based on the reviewers' recommendations a final decision is made by the editor and if needed the help of a member of the editorial board (depending on the field of study). Decisions will include accept, minor revision, major revision with and without re-review, and reject. We aim to reach a final decision on each manuscript as soon as their review results are available.

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