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ISSN 2618-639X • EISSN 2619-905X
actaveteurasia.istanbul.edu.tr

ACTA VETERINARIA EURASIA

Formerly Journal of the Faculty of Veterinary Medicine İstanbul University
Official Journal of İstanbul University-Cerrahpaşa Faculty of Veterinary Medicine

VOLUME: 45 ISSUE: 1

'19

ACTA VETERINARIA EURASIA

Formerly Journal of the Faculty of Veterinary Medicine İstanbul University

İstanbul Üniversitesi-Cerrahpaşa Veteriner Fakültesi adına sahibi / Owner on behalf of the İstanbul University-Cerrahpaşa Faculty of Veterinary Medicine: Güven Kaşıkçı • Sorumlu Yazı İşleri Müdürü / Responsible Manager: Serhat Alkan • Yayın türü / Publication Type: Yerel süreli / Local Periodical • Basım yeri / Printed at: Hamdioğulları İç ve Dış Ticaret A.Ş. Zübeyde Hanım Mah. Elif Sokak No.7/197 Altındağ, Ankara, Türkiye. Tel: +90 (542) 695 77 60 • Basım tarihi / Printing Date: Ocak 2019 / January 2019

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Acta Veterinaria Eurasia (Acta Vet Eurasia) aims to contribute to the literature by publishing manuscripts at the highest scientific level on all fields of veterinary medicine. The journal publishes original articles, reviews, case reports, short communications, and letters to the editor that are prepared in accordance with the ethical guidelines.

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The target audience of the journal includes specialists and professionals working and interested in all disciplines of veterinary medicine.

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Address: İstanbul University-Cerrahpaşa Faculty of Veterinary Medicine, 34320 Avcılar, İstanbul, Turkey
Phone: +90 (212) 473 70 70/17260
Fax: +90 (212) 473 72 40
E-mail: actavet@istanbul.edu.tr

Publisher: AVES
Address: Büyükdere Avenue, 105/9 34394 Mecidiyeköy, Şişli, İstanbul, Turkey
Phone: +90 212 217 17 00
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E-mail: info@avesyayincilik.com
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Editor in Chief: Serhat Alkan
Address: İstanbul University-Cerrahpaşa Faculty of Veterinary Medicine, 34320 Avcılar, İstanbul, Turkey
Phone: +90 (212) 473 70 70/17260
Fax: +90 (212) 473 72 40
E-mail: actavet@istanbul.edu.tr

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Contents

Original Articles

Original Article Acta Vet Eurasia 2019; 45: 1-8

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Immunohistochemical Evidence of Multiple Viral and Bacterial Associations in Caprine Pneumonia in Nigeria: Implications for Vaccines

Theophilus Aghogho JARIKRE, Benjamin Obukowho EMIKPE

Department of Veterinary Pathology, Faculty of Veterinary Medicine, University of Ibadan, Ibadan, Nigeria

Cite this article as: Jarikre T.A., Emikpe B.O., 2019. Immunohistochemical Evidence of Multiple Viral and Bacterial Associations in Caprine Pneumonia in Nigeria: Implications for Vaccines. Acta Vet Eurasia 2019; 45: 1-8.

ORCID IDs of the authors: T.A.J. 0000-0001-8222-198X; B.O.E. 0000-0003-2458-6504.

Abstract

Caprine pneumonia is a major cause of economic loss and the conventional vaccines are not optimal in protecting goats. A better understanding of the associations of respiratory pathogens may help improve our knowledge for vaccination to effectively control caprine pneumonia. One hundred and fifty goats (140 pneumonic and 10 normal) were examined for various lung pathologies using standard gross and histologic techniques. Antigens of parainfluenza 3 virus (PI3V), respiratory syncytial virus (RSV) and peste des petits ruminants virus (PPRV) and bacterial antigens of *Mannheimia haemolytica* (*M. haemolytica*) and *Pasteurella multocida* (*P. multocida*) were demonstrated immunohistochemically in the lungs. The data of goats positive and negative for the viral and bacterial antigens were analysed using descriptive statistics.

Viral antigens were detected in 113 (81%) of the pneumonic lungs (100 as single, 11 dual and 2 triple). Bacterial antigens were detected in 120 (86%), *M. haemolytica* in 47 (34%), *P. multocida* in 59 (42%) and combined bacterial antigens in 14 (10%) of the pneumonic lungs. Multiple agents were detected in 108/140 positive cases; virus-bacterium association was observed in 106/108. PPRV antigens alone were observed in 15 cases. PPRV coexisted most frequently with *M. haemolytica* (n=20), *P. multocida* (n=13), PI3V with *P. multocida* (n=18), and RSV with *M. haemolytica* (n=9). The lesions corresponded to cranioventral (n=45), diffuse (n=75), and lobar consolidations (n=20) manifested as fibrinous bronchopneumonia (n=22), suppurative bronchopneumonia (n=20), bronchointerstitial pneumonia (n=61), interstitial pneumonia (n=25) and bronchiolitis (n=12). Thus, multiple infections are involved in pneumonia, hence we must consider combined vaccination strategies incorporating multiple antigens for adequate control of caprine pneumonia.

Keywords: Goat pneumonia, pathogens, *Pasteurella, Mannheimia*, PI3V, PPRV, RSV

Introduction

Pulmonary lesions in livestock arise due to infection in the respiratory mucosa and complications arising from myriad factors including host immune response, management, or environmental conditions (Chakraborty et al., 2014; Lacasta et al., 2008). The role of multiple pathogens forms the concept of polymicrobial diseases in man and livestock. These refer to infections by different viruses and bacteria synergism, fungi and parasites, and opportunistic infections secondary to immunosuppression (Hodgins et al., 2002). These multiple infections may manifest as severe lesions with poor prognosis. In small ruminants, peste des petits ruminants virus (PPRV) induced pneumonia was complicated by *M. haemolytica* (Ackermann and Brogden, 2000; Emikpe and Akpavie, 2012; Gonzalez and Maheswaran, 1993; Shoo, 1989). However, the role of other bacterial complications of respiratory viral infections other than PPRV has not been well established especially in our environment.

PPRV and *M. haemolytica* co-infection may have enhanced the virulence of *M. haemolytica* in the dwarf goat (Emikpe and Ak-

Address for Correspondence: Theophilus Aghogho JARIKRE • **E-mail:** get2theo@yahoo.com

Received Date: 23 March 2018 • **Accepted Date:** 11 March 2019 • DOI: 10.26650/actavet.2019.409009

Available online at actaveteurasia.istanbul.edu.tr

pavie, 2011). More so, the association of PPRV with other respiratory viruses and bacteria has received little attention, hence this study investigates the association of multiple viral and bacterial agents in caprine lungs from our environment. Understanding of the multiple infections will improve our knowledge on the need for multivalent vaccines for adequate control of caprine pneumonia.

Materials and Methods

Ethical approval
The study was approved by University of Ibadan Animal Care Use and Research Ethics Committee with accession number (UI-ACUREC/17/0060). The guidelines for the care and use of animals in research were strictly followed.

Study population and lung samples
This study was conducted for 72 weeks between March 2014 and August 2015, allowing for seasonal variation (wet and dry). The goats were sourced from various regions in Nigeria; 150 indigenous goats were randomly selected from a total of 700 comprising West African Dwarf, Red Sokoto and Sahelian breeds (Jarikre et al 2016).

The sex of the goats were determined physically and the age by the dentition as described by Lasisi et al. (2002). The general body conditions were evaluated and scored systemically as described by Battaglia (2001) on a scale of 1-5 (amount of fat and muscle at key anatomical points); 1=very thin (poor), 2=thin (fair), 3=normal (good), 4=fat (obese) and 5=very obese. However, all goats were scored as good, fair, or poor. All goats were screened for pulmonary lesions grossly and histologically following standard procedures as described by Jarikre et al. (2016).

Pathology and immunohistochemistry
Grossly the lungs were examined for changes in consistency, texture, color, and severity as described by Lopez (2012). Sections from all the lung lobes (apical, middle and caudal) were taken from both lungs into sample bottles with adequate volume of fixative for histological processing. The stepwise protocol for histological processing of the lung tissues was done using the automatic tissue processor with the lung tissue sectioned at a thickness of 4 µm. Morphological diagnosis was made from each goat and the pulmonary lesions were grouped according to the morphological changes. Thin sections of the lungs were further cut, floated, and mounted on 3-aminopropyltriethoxysilane (APES) charged glass slides in quintuple with additional six sets as primary antibody controls.

Parainfluenza 3 (PI3) virus monoclonal antibody (Cat No: MA5-27876) and respiratory syncytial virus (RSV) polyclonal antibodies (Cat No: PA1-73019), were sourced from Thermo Scientific USA (thermofisher.com/antibodies). They were supplied in cold chain under optimal conditions.

PPRV, *M. haemolytica*, and *P. multocida* polyclonal antibodies were raised individually in rabbits using the PPRV lineage 1 (Nig/75) and formalin-killed whole bacteria injected with Freund's complete adjuvant (FCA), respectively, into New Zealand white rabbits in duplicates (n=6) following standard procedures. The specificity and sensitivity of the bacterial antibodies were duly verified in our previous studies (Jarikre et al., 2018).

The detection of antigens with specific antibodies in the paraffin-embedded tissues followed standard procedures. The slides were rehydrated and antigens were unmasked via heat-induced retrieval. Primary antibody concentration for each of the monoclonal antibodies was set at 4 µg/ml while 1:100 dilutions were set for the polyclonal antibodies. The avidin-biotin-peroxidase kit (LOT: 2775482, IHC Select Detection System, HRP/DAB, Merck, Germany) was used for the staining following the instructions in the manual. The morphologically established normal goat lungs were used as negative controls, while negative sera were used as controls on the pneumonic lung tissues.

Photomicrographs of the tissues (images) were taken using a computer enabled digital AmScope camera (MU 900) and ToupView 3.2 software connected to the Olympus CX21 Microscope (Olympus Co., Tokyo, Japan).

Statistical analysis
The number and percentages of the caprine lung samples positive and negative for the three different viral antigens and the two bacterial antigens and cases of multiple infections were determined.

Results

Animals and clinical findings
In all (150), 22 goats were one-year old, 59 were two-year old, 57 were three-year old, and 12 were four-year old. Fifty-seven were from dry season (October to March) and 93 from wet season (April to August). Nine of the goats were female (7%) and 141 were males (93%). The breeds were as Red Sokoto breed (n=81), West African Dwarf breed (n=47), and Sahelian (n=22).

Clinically, the signs observed included dullness, mucopurulent oculo-nasal discharges, dyspnea, and tachypnea. Thirty-five (23%) of the goats were in good body condition, seventy-four (49%) apparently fair body condition, and forty-one (28%) in poor body condition.

Lesions and immunohistochemistry
The consolidation pattern of the lungs included cranio-ventral, lobar, and diffuse. Of the 150 goats, 61 had broncho-interstitial pneumonia (Figure 1a), 25 had interstitial pneumonia (Figure 1b), 42 had bronchopneumonia (Figure 1c), 12 had bronchiolitis, and 10 were normal caprine lung samples.

Immunohistochemically, 140 (93%) of the caprine lung tissues were positive for the antigens of the different

pathogens and 10 (7%) were negative. Viral (PPR, PI3 & RSV) antigens were detected in 113 (81%) caprine lung tissues and bacterial (*M. haemolytica* & *P. multocida*) antigens were in 120 (86%) of the lungs. Thirty-seven (25%) of the caprine lung tissues were negative for the viral antigens and 30 (20%) were negative for the bacterial antigens (Table 1).

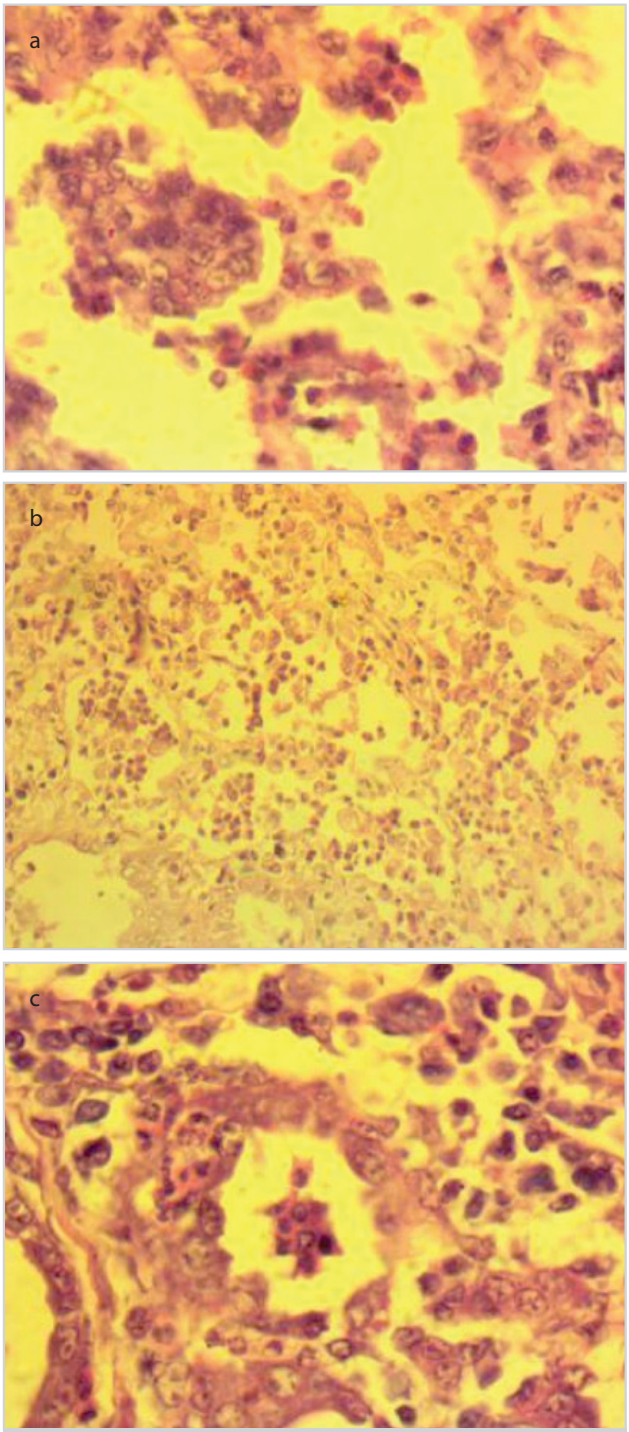

Figure 1. a-c. Photomicrographs of the lungs showing broncho-interstitial pneumonia with syncytial giant cells (a), interstitial pneumonia (b) and bronchopneumonia (c). HE x400

More than one pathogen were detected in 108/140 pneumonic goats. There were viral-bacterial antigens in 106/108 of the pneumonic goats. PPRV and *M. haemolytica* co-infection had the highest incidence (20/106); others included PI3V-*P. multocida* (18/106), PPRV-*P. multocida* (12/106), PI3V-*M. haemolytica* (10/106) and RSV-*M. haemolytica* (9/106).

PPRV was the single viral antigen (Figure 2a) in 15 (10%) animals. The antigens of other pathogens including PI3V (Figure 3a, 3b), RSV (Figure 4a, 4b), and *M. haemolytica* (Figure 5a, 5b) were demonstrated in less than five animals except for *P. multocida* antigen (Figure 6a, 6b) which was present in 19 goat lungs (Table 1). The photomicrographs of primary and secondary antibodies, and DAB controls (Figure 7a, 7b) are demonstrated.

Table 1. Name and number of agents causing pneumonia, morphological lesions, and the lesion score in 140 cases of caprine pneumonia observed in Nigeria

Agent (n)	Morphological change (lesion score)
PI3 (4)	Bronchiolar epithelial necrosis (1)
PI3 + Mh (10)	Bronchopneumonia moderate diffuse (2)
PI3 + Pm (18)	Bronchiolar epithelial necrosis (1)
PI3 + Mh + Pm (2)	Bronchopneumonia moderate diffuse (2)
RSV + Mh (9)	Bronchopneumonia moderate diffuse (2)
RSV + Pm (3)	Bronchiolar epithelial necrosis (1)
RSV + Mh + Pm (9)	Bronchopneumonia moderate diffuse (2)
PPR (15)	Bronchointerstitial pneumonia (3)
PPR + Mh (20)	Diffuse fibrinous severe bronchopneumonia (5)
PPR + Pm (12)	Bronchointerstitial pneumonia (3)
PPR + Pm + Mh (4)	Diffuse fibrinous severe bronchopneumonia (5)
PI3 + RSV + Mh (1)	Diffuse fibrinous severe bronchopneumonia (4)
PI3 + RSV + Pm (1)	Diffuse fibrinous severe bronchopneumonia (4)
PI3 + RSV + PPR (1)	Bronchopneumonia moderate diffuse (3)
PI3 + RSV + PPR + Pm (1)	Bronchointerstitial pneumonia (4)
Mh (4)	Fibrinous bronchopneumonia
Pm (18)	Bronchopneumonia moderate diffuse (2)
Mh + Pm (4)	Diffuse pleuropneumonia (5)
Total: 140	

Lesion score:
1= degeneration of epithelial cells
2= degeneration and necrosis of epithelial cells + a few neutrophils
3= degeneration and necrosis of epithelial cells + giant cells + a few neutrophils
4= degeneration and necrosis of epithelial cells + giant cells + inflammatory cells
5= suppurative/purulent inflammation

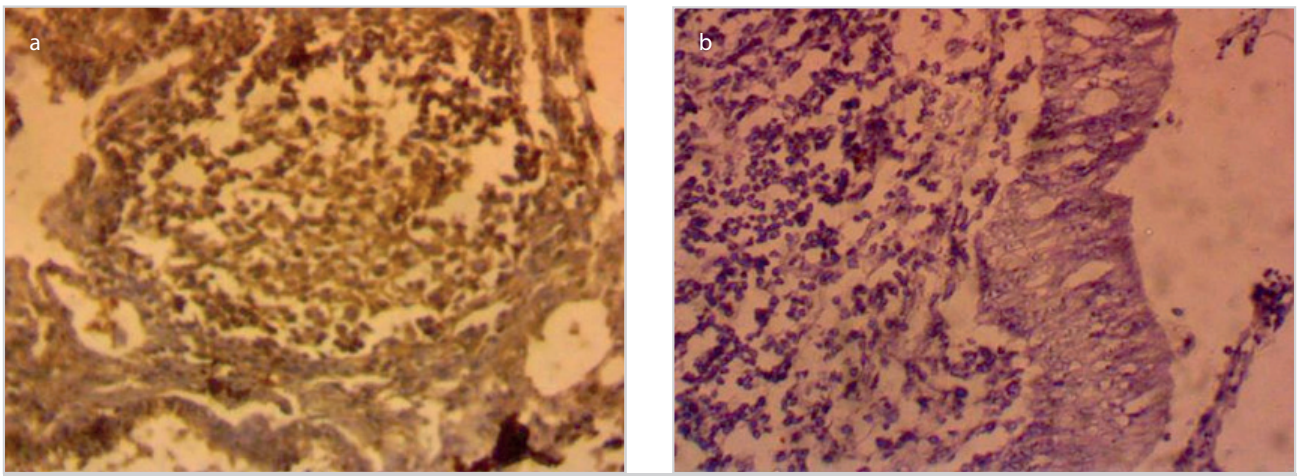

Figure 2. a, b. Photomicrograph of pneumonic goat lung immunostained with antibody to PPRV antigens (a) in bronchus-associated lymphoid tissue and same tissue (b) without antibody (control). ABC HRP/ Haematoxylin counterstain x400

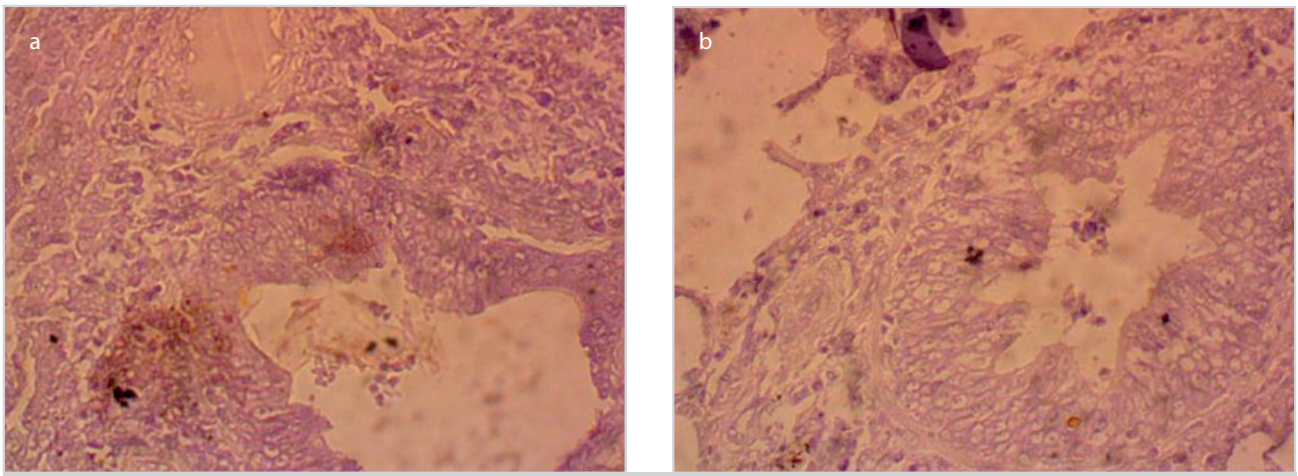

Figure 3. a, b. Photomicrograph of pneumonic goat lung immunostained with antibody to PI3V antigens (a) in hyperplastic bronchiolar epithelium and same tissue (b) without antibody (control). ABC HRP/ Haematoxylin counterstain x400

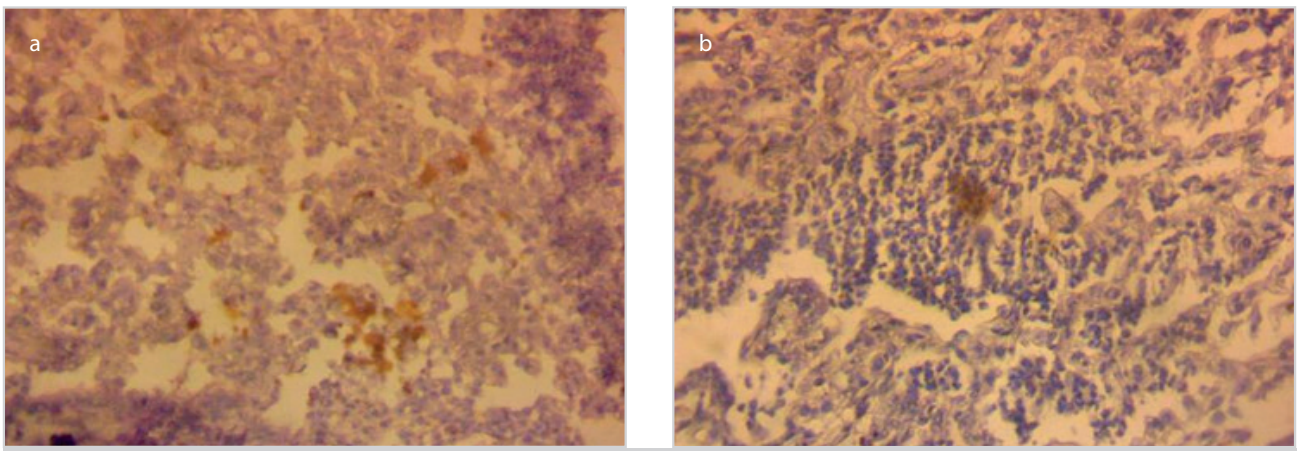

Figure 4. a, b. Photomicrograph of pneumonic goat lung immunostained with antibody to RSV antigens (a) in pneumocytes & macrophages and same tissue (b) without antibody (control). ABC HRP/ Haematoxylin counterstain x400

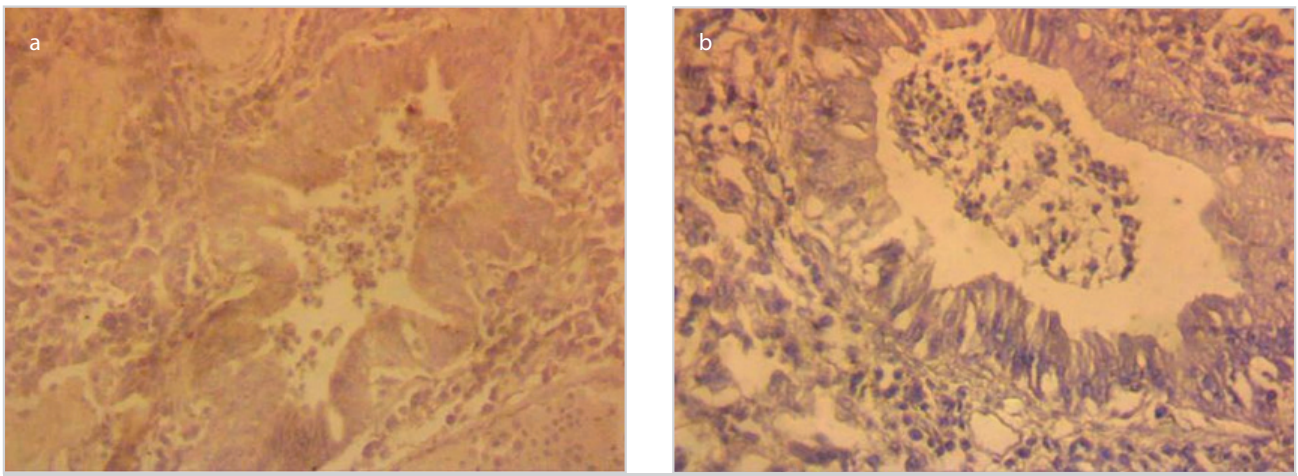

Figure 5. a, b. Photomicrograph of pneumonic goat lung immunostained with antibody to *M. haemolytica* antigens (a) in exudate and same tissue (b) without antibody (control). ABC HRP/ Haematoxylin counterstain x400

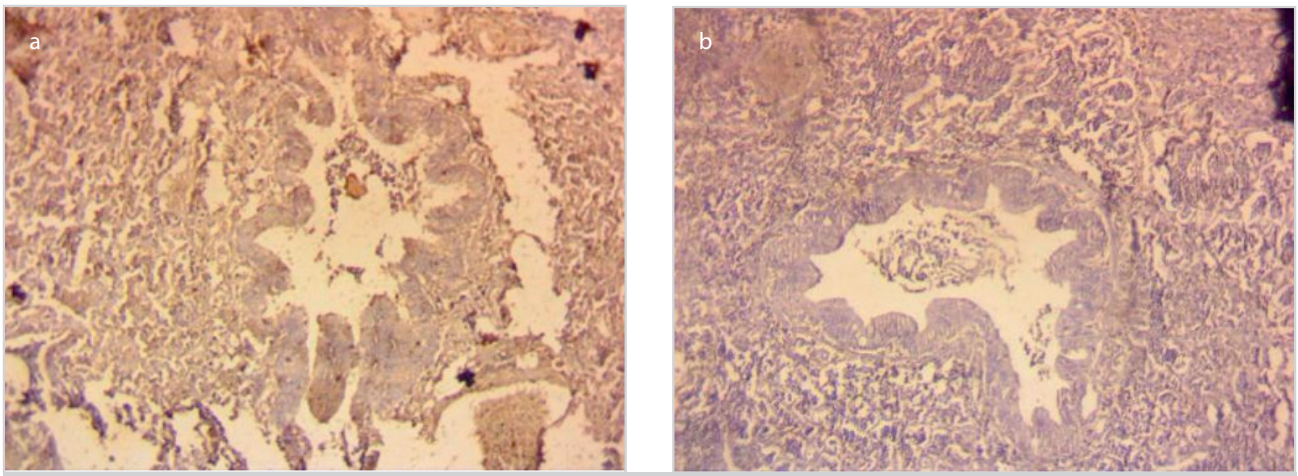

Figure 6. a, b. Photomicrograph of pneumonic goat lung immunostained with antibody to *P. multocida* antigens (a) in exudate and same tissue (b) without antibody (control). ABC HRP/ Haematoxylin counterstain x400

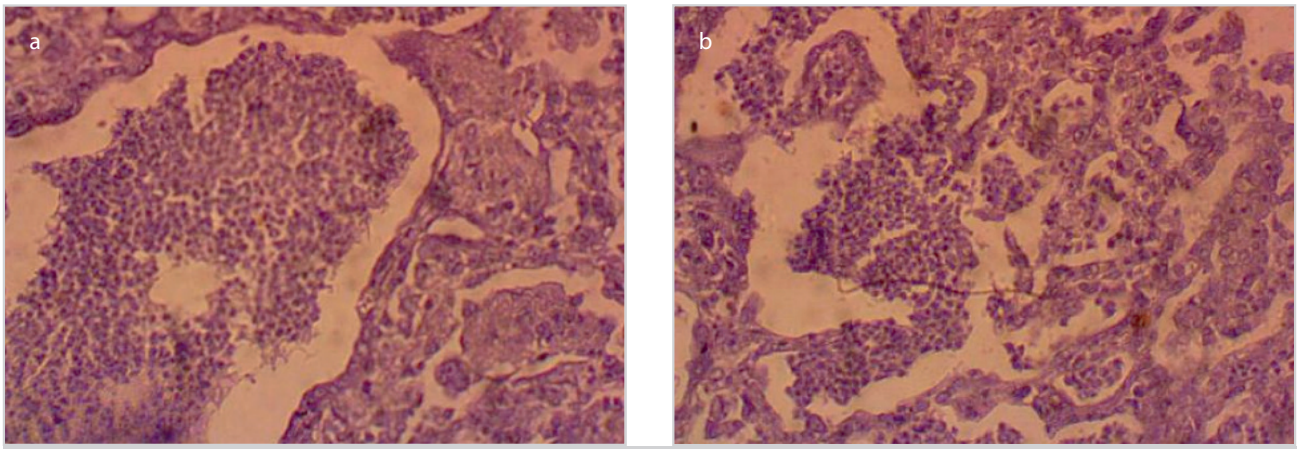

Figure 7. a, b. Photomicrograph of pneumonic goat lung used as secondary antibody (a) and DAB (b) controls. ABC HRP/ Haematoxylin counterstain x40

The specific immunostainings were characterised by the presence of light to dark brown stained antigens demonstrated in: bronchial, bronchiolar epithelial cells, macrophages, leukocytes, pneumocytes, giant cells and the desquamated bronchial and bronchiolar epithelial cells. The intensity varied with the distribution of the lesion with very slight immunostaining in the pneumocytes, interstitial macrophages, and in blood vessels; while the bronchial, bronchiolar epithelium and the luminal exudates with macrophages in the bronchial associated lymphoid tissue stained strongly. The morphological changes observed in the airways and air spaces varied from degeneration and necrosis to inflammation with presence of giant cells (Table 1).

Discussion

This study elucidates the association of respiratory viruses and bacteria in caprine pneumonia in Nigeria. It showed the prevalence of single to multiple microbial respiratory infections in goats from our environment. Single infections of PPRV, and *P. multocida* ranked highest in precipitating lesions after the dual infections of the respiratory pathogens in the pneumonic caprine lungs. This further confirms the susceptibility of goats to pneumonia as compared to sheep (Cutlip et al., 1996; Dassanayake et al., 2013; Emikpe et al., 2013), which showed that one to two respiratory pathogens are enough to produce a disease in goats. Furthermore, our findings supported the reports of Brown et al. (1991) and Saliki et al. (1994) on the role of PPRV in caprine pneumonia while also highlighting the nature or pattern of the pulmonary lesions which included broncho-interstitial respiratory viral pneumonia of goats (Kumar et al., 2004), and bronchopneumonic lesions in respiratory bacterial pneumonia in goats (Haritani et al., 1987; Yener et al., 2009).

However, it has now become evident that other pathogens other than PPRV and *M. haemolytica* are also involved in pneumonia complex of goats, which may have contributed to the endemicity of caprine pneumonia. Similar reports on PI3 virus (Fulton et al., 2000), RSV (Sharma and Woldehiwet, 1990), adenovirus (Davies et al., 1982), bovine viral diarrhea virus (Gånheim et al., 2003), and herpes viruses (Narita et al., 2000) have indicated these agents as aetiology of pneumonia in ruminants. Recently, we also reported the detection of other respiratory viral infections other than PPR, which included PI3V and RSV in pneumonic lungs of goats in Nigeria (Jarikre and Emikpe, 2017).

It is necessary therefore to further investigate the exact interaction of these pathogens in caprine pneumonia to avoid the unnecessary abuse of antibiotics and elusiveness to control (Hodgins et al., 2002). It is worthy of note that virus-virus association, bacterium-virus association, and single infections are common in goats which further underscored the susceptible nature of goats to pneumonia.

Our findings on multiple respiratory pathogen infections in goats further agree with observations from pneumonia of alpaca neonates (Cirilo et al., 2012; Guzmán et al., 2013; Rosadio et al., 2011). The mechanisms involved in multiple pathogen infections include immunosuppression of the host due to morphological, functional or stress induced changes and colonization of pathogen to mucosa surface followed by inflammatory reactions (Hodgins et al., 2002). Respiratory viruses enable colonization of bacteria to mucosal epithelium which is followed by a severe inflammation. The influence of transport stress, helminthosis, population density, and age has been incriminated in the multiple infection phenomena (Adeyemi et al., 2017; Brogden et al., 1998; Jasni et al., 1991; Zamri-Saad et al., 1996). The high prevalence of *M. haemolytica* and *P. multocida* in the goats in this study may not be unconnected to the influence of stress and the possible reversal of virulence in the respiratory tract as highlighted in experimental pasteurellosis of goats by Zamri-Saad et al. (1989) and Zamri-Saad et al. (1991).

It is now clear that caprine pneumonia is a complex of multiple pathogens in Nigeria, a fact which may have contributed to the elusiveness of caprine pneumonia control. However, in Sub-Saharan Africa and part of Asia, the role of PPRV cannot be over emphasized (Emikpe et al., 2011; Ozkul et al., 2002).

Since vaccination is the most effective control measure; the appropriate vaccine candidate to use may be a challenge due to variation of pathogens and the different associations observed. The timing, delivery system, route and other factors are therefore also critical for optimal immune response and protection.

It is therefore important to note that different interactions occur in caprine pneumonia but the nature of these interactions need to be investigated for caprine pneumonia control. A combination of vaccines that could utilize the possible interaction mechanisms should be employed.

Ethics Committee Approval: Ethics Committee approval was received for this study from the University of Ibadan Animal Care Use and Research Ethics Committee with approval number UI-ACUREC/17/0060 on 14-07-2017.

Author Contributions: Concept – T.A.J.; Design – B.O.E.; Supervision – B.O.E.; Resources – T.A.J.; Materials – B.O.E.; Data Collection and/or Processing – T.A.J.; Analysis and/or Interpretation – T.A.J., B.O.E.; Literature Search – T.A.J.; Writing Manuscript – T.A.J.; Critical Review – B.O.E.; Other – B.O.E.

Acknowledgements: The authors appreciate the support of Nwagbara Ambrose in histological processing of tissues.

Conflict of Interest: The authors have no conflict of interest to declare.

Financial Disclosure: The authors declared that this study has received no financial support.

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A Survey Study on Self-Evaluations of Small Pet Practitioners about Exotic Pets in Istanbul in 2016

Belgi Diren SIĞIRCI, Serkan İKİZ, Baran ÇELİK, Seyyal AK

Department of Microbiology, İstanbul University-Cerrahpaşa, Faculty of Veterinary Medicine, İstanbul, Turkey

Cite this article as: Sığırcı, B.D., İkiz, S., Çelik, B., Ak, S., 2019. A Survey Study on Self-Evaluations of Small Pet Practitioners about Exotic Pets in Istanbul in 2016. Acta Vet Eurasia 2019; 45: 9-15.

ORCID IDs of the authors: B.D.S. 0000-0001-7153-7428; S.İ. 0000-0001-6502-0780; B.Ç. 0000-0001-9122-0284; S.A. 0000-0002-6687-8401.

Abstract

Exotic pet animal ownership is on the rise all over the world. Regardless of being companion animals which are important subjects in veterinary medicine, they also play a role in the transmission of diseases to other animals and human beings. Therefore, veterinarians are expected to have the knowledge and good practice in exotic pet medicine. This survey was performed among small animal practitioners with the aim of identifying their self-evaluation of competency and knowledge about the exotic pet medicine in Istanbul. As a data collection tool, a three-part questionnaire developed by the researchers was used in the current study. The first part of the survey covered demographic variables of respondents; the second part consisted of 6 questions, which examine the self-evaluation of small animal practitioners on competency and knowledge about the exotic pet medicine practice. The third part consisted of 8 Likert type questions about the husbandry, transmission, prevention, diagnosis and therapy of the diseases for the four different exotic pets including fish, turtle, other reptiles and bird. The results showed that approximately 80% of the respondents consider that exotic pet disease is essential regarding veterinary medicine. Thirty-five percent of the veterinarian said that they were not sure about "what is an exotic pet disease" while, 53% of them responded that question, as they did not have any knowledge about the exotic pet diseases. For the 91.4% of the clinics investigated, the exotic animals as a patient were 1-10% or less than 1% of all the patients. It has shown that 42.4%, 32%, 16.9%, and 8.7% of the exotic pets' species examined were birds, turtles, the other reptiles, and 8.7% fish, respectively. Ninety percent of the veterinarians consider that they did not get enough education about the exotic pet animal practice during their undergraduate study at the Faculty of Veterinary Medicine, Istanbul. It has been determined that more than half of the participants (65%) were considered themselves as having adequate knowledge of the husbandry, transmission, prevention, diagnosis and therapy of the diseases of the birds. However, they did not have enough experience about turtle, other reptiles and fish. As a conclusion, the results indicated the importance of education, specialisation and practice on potential exotic pet species.

Keywords: Exotic pets, İstanbul, self-evaluation, survey, Veterinary Medicine

Introduction

Humans have been living with animals continually since ancient times; they domesticated them at first, benefited from some of their products, and took them home and bought them as friends (Öner and Şahin, 2009). The definition of exotic in "Merriam-Webster's Collegiate Dictionary" is as: introduced from another country, not native to the place where found; foreign, alien; and strikingly, excitingly or mysteriously different or unusual (Merriam-Webster, 2003). An exotic pet is a rare or unusual animal pet, or an animal kept within individual households, which is generally thought of as a wild species not typically held as a pet (Schuppli and Fraser, 2000). According to Mitchell and Tully (2009), the history of exotic pets has begun to keep and breed goldfishes as aesthetic purposes in China in Sung Dynasty (960-1279). The population of exotic pets

Presented in: This study was presented in 3rd International VETistanbul Group Congress, 17-20 May 2016, Bosnia and Herzegovina.

Address for Correspondence: Belgi Diren Sığırcı • **E-mail:** belgis@istanbul.edu.tr

Received Date: 13 June 2018 • **Accepted Date:** 27 November 2018 • DOI: 10.26650/actavet.2019.433657

Available online at actaveteurasia.istanbul.edu.tr

is increasing worldwide. Members of this new pet group are birds, reptiles, fish, small mammals and rodents (such as rats, hamsters and ferrets) (Ebani and Fratini, 2005). Additionally, exotic animals were not only imported as pet animals but also were imported for zoos, scientific education and research and protection programs (Marano et al., 2007). Mayer and Martin (2005) indicated that exotic pets had been used for various scientific studies throughout more than 100 years.

Generally, exotic pets are imported from overseas countries. Moreover, many of them get acquired illnesses during long shipments (Marano et al., 2007). Veterinarians do not have sufficient knowledge about the disease, behaviour, natural history and physiology of these exotic species (Mayer and Martin, 2005). However, regardless of their keeping as companion animals, not only exotic pets are essential subjects in veterinary medicine, but also play a role in the transmission of diseases to other animals and human beings (Chomel et al., 2007). It is estimated that 75% of emerging infectious diseases occurring today are the zoonosis. Exotic pets or wildlife species carry most of this zoonosis. Veterinarians could be held legally responsible for the transmission of zoonosis to staff or clients (Souza, 2011). There are differences between pet animals such as cats, dogs and exotic pets (anatomical, physiological, etc.) and this can lead to differences in medicine. Thus occurs a new field of Veterinary Medicine (Öner and Şahin, 2009). On the other hand, working with exotic and wild animals is generally hazardous, and holding of exotic animals is a more challenging business than other pet and farm animals (Miller and Fowler, 2014).

A majority of the drugs should be administered parenteral or enteral to the exotic pet, are given empirically due to the lack of pharmacokinetic data. In this respect, it has been benefited from the field experience of the physicians, the dose rates used in other pet animals or the treatment forms created by trial and error. Drugs used in specific doses as a result of experimental studies and post-operative treatments cause the animals to get back to their normal health but are at risk for a variety of reasons, such as side effects, application revolutions and the need to use drugs in large volumes. Therefore, veterinarians are expected to have the knowledge and good practice in exotic pet medicine (Öner and Şahin, 2009).

In recent years, there is an interest and sensitivity on exotic pets. Day by day, more exotic pets are brought to the veterinary faculties and veterinary clinics for treatment. According to Fowler's wild animal medicine, there are 6.200 species of Anurans, ten large families of Caudate, 322 species of turtles and tortoise. With the addition of other Amphibian, reptiles, birds and mammalian species, exotic animal's medicine is a vast ocean. Therefore, it is either not easy to know or to educate the complete exotic animal medicine to students. However, in determined species, primary medicine and selected diseases are necessary to educate (Miller and Fowler, 2014). For further education and practice needs specialisation. Association of Exotic Mammal Veterinarians (AEMV) and the Association of Reptilian and Amphibian Veterinarians (ARAV) are founded to address those issues stated above and help improve the knowledge in the field of exotic animal practice (AEMV, 2000; ARAV, 1990).

The European College of Zoological Medicine (ECZM) was established to help further progress in research and practice to benefit the health and well-being of free-ranging and captive non-domesticated animals. The establishment organised summer courses, workshops and approved residency programs for veterinarians who need specialisation on exotic and zoo medicine area (ECZM, 1993). In Turkey, for the first time, Department of Wildlife and Ecology was launched in Afyon Kocatepe University, Faculty of Veterinary Medicine in Turkey in 2014. Then, Department of Wildlife Disease and Ecology was established in Kafkas University Faculty of Veterinary Medicine in 2015 and Istanbul University Faculty of Veterinary Medicine in 2018. In this context, The Chamber of Veterinary Surgeons, Istanbul was organised first time "Wild Animal Medicine and Exotic Congress in 2014 and "1st Exotic and Wildlife Medicine Workshop with International Participation" in 2017, in İstanbul.

This survey was performed among small animal practitioners with the aim of identifying their self-evaluation of competency and knowledge about the exotic pet medicine. Additionally, the influences of determined demographic characteristics were also investigated.

Materials and Methods

Questionnaire

A total of 174 small animal practitioners from different geographical and socioeconomic regions of Istanbul was used as the population pool of the questionnaire used in the current survey. The study was conducted in this city because of the density of the human population covered by the province of Istanbul (approximately 15 million), the animal population, and high-volume of the pet trade, having the biggest air border gates of Turkey, their proximity to the western land boundaries and being of major seasonal migration routes.

As a data collection tool, a three-part questionnaire developed by the researchers was used. The first part of the questionnaire covered demographic variables of respondents including gender, age, work experience, the potential of the exotic pet and the exotic pet species coming for the examination. Part two consisted of 6 questions, which examine the self-evaluation of small animal practitioners on competency and knowledge about the exotic pet medicine practice. Each question was assessed using a 5-point Likert scale (1=strongly disagree; 2=disagree; 3=neither agree nor disagree; 4=agree; 5=strongly agree). The third part consisted of 8 Likert type questions about the husbandry, transmission, prevention, diagnosis and therapy of the diseases for the four different exotic pets including fish, turtle, other reptiles and bird.

Statistical analysis

The demographic characteristics of small animal practitioners were presented with frequencies and percentages. To describe the responses to Likert-type questions, median, mean and standard deviation values for each item were calculated, as well as frequency and percentage values for each Likert item.

Non-parametric statistical tests were selected to determine the effects of demographic characteristics on responses to Likert type items. Mann-Whitney U test was used for demographic variables with two levels (i.e. gender), whereas Kruskal Wallis test was utilised when demographic characteristics had three or more levels (i.e. age, work experience, the potential of the exotic pet and the exotic pet species coming for the examination) (Özdamar, 2003).

Results

Of the veterinarians participating in the survey, 42% were female, 58% were male, and the average age was 35.7. The work experience level of the majority of clinician veterinarians was determined as 6-15 years (36.2%). When the exotic pet potential of the clinics evaluated, 48.3% is 1-10 percent of all of the patients, and 43.1% is 1 or less than 1 percent. It is examined that 42.4% of the exotic pets' species coming for the examination is the bird, 32% is the turtle, 16.9% is other reptiles and 8.7% is fish. The demographic information of respondents summarised in Table 1.

Table 1. Demographic information of respondents

Items	Frequency, n	Percentage, %
Gender		
Male	101	58
Female	73	42
Ages		
23-30 years old	53	30.5
31-40 years old	82	47.1
41-50 years old	33	18.9
51-69 years old	6	3.5
Work experience		
0-5 years	58	33.3
6-15 years	63	36.2
16-25 years	45	25.9
Higher than 26 years	8	4.6
Potential of the exotic pets		
Less than 1%	75	43.1
1-10%	84	48.3
11-50%	12	6.9
Higher than 51%	3	1.7
The exotic pet species coming for the examination		
Turtle (Chelonians)	128	32.0
Other reptiles	68	16.9
Fish	35	8.7
Bird	170	42.4

Approximately 80% of the respondents consider that exotic pet disease is essential regarding veterinary medicine. Thirty-five percent of the veterinarian said that they were not sure about "what are the exotic pet diseases" while, 53% of them responded that question, as they did not have any knowledge about the exotic pet diseases. Ninety % of the veterinarians consider that they did not get enough education on the exotic pets and diseases during their graduate study in Faculty of Veterinary Medicine. The rates of the veterinary that choose the answers to "disagree" to questions that investigated their self-evaluation of knowledge on transmission, control and prevention of exotic pet diseases, were considerably higher. The veterinarians considered that they did not have enough knowledge about the husbandry, transmission, prevention, diagnosis and therapy of the diseases of the exotic pets including turtle (Chelonians), other reptiles and fish. It has been determined that more than half of the participants (65%) be considered themselves as having adequate knowledge of the husbandry, transmission, prevention, diagnosis and therapy of the diseases of the birds. The descriptive statistics related to responses of respondents to questions summarised in Table 2.

The effects of demographic characteristics on averages of Likert-type responses concerning gender, work experience, the potential of the exotic pets and the exotic pet species were summarised in Table 3.

Discussion

Exotic pet medicine is one of the fastest growing disciplines in veterinary medicine worldwide. A perception that is widespread in the exotic pet medicine is the lack of clinical information for veterinarians. Donnelly (2004) elaborated on two critical points:

There are significant differences between the medical problems seen in domestic animals and those in laboratory animals.

There is an inconsistency between what is described in the current literature and what is seen in the clinical specimens of exotic domestic animals.

Mayer and Martin (2005) highlighted that although exotic animal medicine is an area of growth within veterinary medicine, there is nevertheless a general lack of training opportunities and pharmacological data concerning exotics and that a general absence of context-specific literature can be problematic. Also, they added that one of the most significant problems facing the exotic animal veterinarian is the use of drugs in species for which no pharmacological data are available.

Table 2. Descriptive statistics related to responses of respondents to questions

	Questions	Disagree strongly n (%)	Disagree n (%)	Undecided n (%)	Agree n (%)	Agree strongly n (%)	Mean Score (SD)	Median
	Q1- I consider that exotic pet disease is important in terms of veterinary medicine.	2 (1.1)	11 (6.3)	21 (12.1)	50 (28.7)	90 (51.7)	4.23 (0.97)	5
	Q2- I consider that I have enough knowledge about what are the exotic pet diseases	41 (23.6)	51 (29.3)	60 (34.5)	18 (10.3)	4 (2.3)	2.39 (1.02)	2
	Q3- I consider that I got enough courses about the exotic pets and diseases in my graduate Faculty of veterinary medicine	123 (70.7)	34 (19.5)	13 (7.5)	1 (0.6)	3 (1.7)	1.43 (0.79)	1
	Q4- I consider that I have enough knowledge about transmission routes of the exotic pet diseases from animal to human beings	39 (22.4)	56 (32.2)	44 (25.3)	26 (14.9)	9 (5.2)	2.48 (1.14)	2
	Q5- I consider that I have enough knowledge about prevention of myself in terms of the exotic pets' diseases	34 (19.5)	63 (36.2)	35 (20.1)	29 (16.7)	13 (7.5)	2.56 (1.19)	2
	Q6- I consider that I have enough knowledge about prevention of animals in terms of the exotic pets' diseases	42 (24.1)	57 (32.8)	39 (22.4)	27 (15.5)	9 (5.2)	2.44 (1.16)	2
Turtle	Q7- I have enough knowledge about husbandry.	34 (19.5)	49 (28.2)	43 (24.7)	39 (22.4)	9 (5.2)	2.65 (1.17)	3
	Q8- I have enough knowledge about feeding.	31 (17.8)	44 (25.3)	43 (24.7)	46 (26.4)	10 (5.7)	2.77 (1.18)	3
	Q9- I have enough knowledge about transmission r outes to animal.	32 (18.4)	57 (32.8)	44 (25.3)	34 (19.5)	7 (4)	2.58 (1.11)	2
	Q10- I consider that I have enough knowledge about prevention of animals in terms of diseases.	29 (16.7)	49 (28.2)	52 (29.9)	37 (21.3)	7 (4)	2.67 (1.10)	3
	Q11- I have enough knowledge about the contamination routes of infected animals (from animal to environment).	26 (14.9)	55 (31.6)	43 (24.7)	43 (24.7)	7 (4)	2.71 (1.11)	3
	Q12- I consider that I have enough knowledge about the clinical diagnosis of diseases.	34 (19.5)	47 (27)	58 (33.3)	30 (17.2)	5 (2.9)	2.56 (1.07)	3
	Q13- I know which samples and at which conditions should be sent to the laboratory in suspected cases<	47 (27)	44 (25.3)	35 (20.1)	36 (20.7)	12 (6.9)	2.55 (1.27)	2
	Q14- I consider that I have enough knowledge about treatment.	28 (16.1)	47 (27)	54 (31)	39 (22.4)	6 (3.4)	2.70 (1.09)	3
Reptile	Q15- I have enough knowledge about husbandry.	63 (36.2)	54 (31)	29 (16.7)	23 (13.2)	5 (2.9)	2.15 (1.13)	2
	Q16- I have enough knowledge about feeding.	59 (33.9)	51 (29.3)	36 (20.7)	22 (12.6)	6 (3.4)	2.22 (1.14)	2
	Q17- I have enough knowledge about transmission routes to animal.	61 (35.1)	58 (33.3)	30 (17.2)	17 (9.8)	8 (4.6)	2.15 (1.14)	2
	Q18- I consider that I have enough knowledge about prevention of animals in terms of diseases.	58 (33.3)	49 (28.2)	39 (22.4)	21 (12.1)	7 (4)	2.25 (1.16)	2
	Q19- I have enough knowledge about the contamination routes of infected animals (from animal to environment).	56 (32.2)	56 (32.2)	36 (20.7)	20 (11.5)	6 (3.4)	2.21 (1.12)	2
	Q20- I consider that I have enough knowledge about the clinical diagnosis of diseases.	62 (35.6)	52 (29.9)	35 (20.1)	20 (11.5)	5 (2.9)	2.16 (1.12)	2
	Q21- I know which samples and at which conditions should be sent to the laboratory in suspected cases	67 (38.5)	48 (27.6)	26 (14.9)	23 (13.2)	10 (5.7)	2.20 (1.24)	2
	Q22- I consider that I have enough knowledge about treatment.	63 (36.2)	46 (26.4)	32 (18.4)	28 (16.1)	5 (2.9)	2.22 (1.18)	2
Fish	Q23- I have enough knowledge about husbandry.	48 (27.6)	55 (31.6)	37 (21.3)	26 (14.9)	8 (4.6)	2.37 (1.16)	2
	Q24- I have enough knowledge about feeding.	45 (25.9)	42 (24.1)	45 (25.9)	32 (18.4)	10 (5.7)	2.54 (1.21)	2.5
	Q25- I have enough knowledge about transmission routes to animal.	45 (25.9)	50 (28.7)	44 (25.3)	26 (14.9)	9 (5.2)	2.44 (1.17)	2

Table 2. Descriptive statistics related to responses of respondents to questions (continued)

	Questions	Disagree strongly n (%)	Disagree n (%)	Undecided n (%)	Agree n (%)	Agree strongly n (%)	Mean Score (SD)	Median
	Q26- I consider that I have enough knowledge about prevention of animals in terms of diseases.	47 (27)	53 (30.5)	44 (25.3)	22 (12.6)	8 (4.6)	2.37 (1.14)	2
	Q27- I have enough knowledge about the contamination r outes of infected animals (from animal to environment).	44 (25.3)	57 (32.8)	41 (23.6)	23 (13.2)	9 (5.2)	2.40 (1.15)	2
	Q28- I consider that I have enough knowledge about the clinical diagnosis of diseases.	53 (30.5)	61 (35.1)	35 (20.1)	18 (10.3)	7 (4)	2.22 (1.11)	2
	Q29- I know which samples and at which conditions should be sent to the laboratory in suspected cases	67 (38.5)	56 (32.2)	23 (13.2)	19 (10.9)	9 (5.2)	2.12 (1.18)	2
	Q30- I consider that I have enough knowledge about treatment.	52 (29.9)	62 (35.6)	36 (20.7)	16 (9.2)	8 (4.6)	2.22 (1.11)	2
Bird	Q31- I have enough knowledge about husbandry.	12 (6.9)	18 (10.3)	52 (29.9)	69 (39.7)	23 (13.2)	3.41 (1.06)	4
	Q32- I have enough knowledge about feeding.	9 (5.2)	12 (6.9)	44 (25.3)	79 (45.4)	30 (17.2)	3.62 (1.01)	4
	Q33- I have enough knowledge about transmission routes to animal.	9 (5.2)	15 (8.6)	50 (28.7)	80 (46)	20 (11.5)	3.5 (0.98)	4
	Q34- I consider that I have enough knowledge about prevention of animals in terms of diseases.	12 (6.9)	16 (9.2)	51 (29.3)	71 (40.8)	24 (13.8)	3.45 (1.06)	4
	Q35- I have enough knowledge about the contamination routes of infected animals (from animal to environment).	8 (4.6)	16 (9.2)	48 (27.6)	74 (42.5)	28 (16.1)	3.56 (1.01)	4
	Q36- I consider that I have enough knowledge about the clinical diagnosis of diseases.	12 (6.9)	17 (9.8)	57 (32.8)	71 (40.8)	17 (9.8)	3.36 (1.02)	4
	Q37- I know which samples and at which conditions should be sent to the laboratory in suspected cases	24 (13.8)	32 (18.4)	44 (25.3)	53 (30.5)	21 (12.1)	3.08 (1.23)	3
	Q38- I consider that I have enough knowledge about treatment.	12 (6.9)	12 (6.9)	57 (32.8)	74 (42.5)	19 (10.9)	3.43 (1.01)	4

A survey related to exotic pet ownership and care was conducted by the UK-based Royal Society for the Prevention of Cruelty to Animals (RSPCA) with 190 veterinarians, and a report was prepared. In the report, it has been criticised for exotic pet owners and pet shops, and it has been determined that disregarding animal breeding or abandoning animals is not a serious risk factor. However, it has been reported that animal owners do not have sufficient experience in animal welfare, nutrition and care, which is a problem for animal welfare. As a result of the study, it was determined that less than half of the veterinarians treat exotic, 33% referred the cases to other veterinarians, and 20% neither treated nor referred. It was pointed out that there is not enough number of veterinarians trained for the treatment of exotic animals (RSPCA, 2003).

In this study, it has been determined that less than half of the veterinarians are not sure about "what is the exotic pet diseases" while 53% of them responded to that question with a score demonstrating a complete lack of knowledge regarding exotic pet diseases. Ninety percent of the practitioners consider that they did not get enough courses about exotic pets and their diseases before graduating from the veterinary faculty.

Similar to the other studies (Mayer and Martin, 2005; RSPCA, 2003), in this study, it has been shown that the veterinarians did not have enough knowledge about the husbandry, transmission, prevention, diagnosis, and therapy of the diseases of the exotic pets including the turtle, other reptiles and fish. It has been determined that more than half of the participants have adequate knowledge about the husbandry, transmission, prevention, diagnosis, and therapy of the diseases of the birds. All of the veterinarians who answered the questionnaire took "poultry diseases" lessons which were the transmission, prevention, diagnosis, and therapy of the avian diseases had been taught, that is why awareness of exotic bird diseases was found higher than another type of the exotic pet diseases.

Vander Veen and Schulte (2005) sent an informal questionnaire to 11 accredited American Veterinary Medical Association (AVMA) veterinary technology schools in the United States. They detected that 7 out of 11 schools offer exotic medicine courses in addition to laboratory animal courses and three schools stated that they were looking into courses in exotic pet medicine (including husbandry, nutritional information and common diseases associated with specific exotic species). In conclusion, they pointed out that although some faculty or

Table 3. The effects of demographic characteristics on averages of Likert-type responses concerning gender, work experience, potential of the exotic pets and the exotic pet species

			Turtle			Reptile			Fish			Bird	
Item	**Subgroup**	**Median**	**MS (SD)**	**p**	**Median**	**MS (SD)**	**p**	**Median**	**MS (SD)**	**p**	**Median**	**MS (SD)**	**p**
Gender	Male	20	20.73 (0.78)	0.295	16	16.78 (0.82)	0.116	18	19.43 (0.87)	0.210	28	26.76 (0.79)	0.211
	Female	23	21.89 (0.84)		16	18.72 (0.95)		16	17.71 (0.88)		30	28.41 (0.79)	
Work Experience	0-5 years	20.5	20.98 (0.99)	0.946	16	16.22 (0.98)	0.352	18	19.15 (1.07)	0.585	29	27.98 (0.98)	0.756
	6-15 years	22	20.98 (0.97)		17	18.52 (1.10)		16	18.76 (1.07)		30	27.41 (0.88)	
	16-25 years	21	21.33 (1.06)		16	17.2 (1.13)		16	17.55 (1.21)		26	26.46 (1.19)	
	Higher than 26 years	19.5	24.12 (3.78)		21	22.5 (4.23)		20.5	21.62 (3.23)		27	29.5 (3.34)	
Potential of the exotic pets	Less than 1 %	16	17.52 [c] (0.8)	<0.001	11	13.38 [c] (0.77)	<0.001	16	17.64 (0.91)	0.300	26	25.13 [c] (0.9)	<0.001
	1-10 %	24	22.89 [b] (0.69)		18	19.40 [b] (0.75)		18	19.04 (0.86)		30	28.72 [b] (0.73)	
	Higher than 11 %	31	30.33 [a] (1.83)		32	28.53 [a] (2.43)		19	22.2 (2.98)		32	31.93 [a] (1.86)	
Turtle examination	The practitioners who do not tread	16.5	17.08 (0.91)	<0.001	12.5	13.91 (0.92)	0.001	16	17.30 (1.05)	0.247	25	25.02 (1.09)	0.006
	The practitioners who tread	24	22.70 (0.67)		17	18.92 (0.75)		18	19.21 (0.76)		30	28.32 (0.65)	
Reptile examination	The practitioners who do not tread	19	19.74 (0.72)	0.003	15.5	14.89 (0.66)	<0.001	16.5	18.34 (0.78)	0.509	27.5	26.69 (0.71)	0.038
	The practitioners who tread	24	23.51 (0.89)		20.5	21.80 (1.04)		18.5	19.27 (1.05)		31	28.63 (0.92)	
Fish examination	The practitioners who do not tread	21	20.97 (0.65)	0.499	16	17.11 (0.69)	0.110	16	17.56 (0.65)	0.002	28	27.38 (0.61)	0.485
	The practitioners who tread	19	22.17 (1.29)		17	19.51 (1.4)		24	23.25 (1.56)		31	27.71 (1.43)	
Bird examination	The practitioners who do not tread	22	21 (5.91)	0.968	16.5	18.25 (5.03)	0.968	10	17 (7.72)	0.324	25	24.25 (2.68)	0.270
	The practitioners who tread	21	21.22 (0.58)		16	17.58 (0.63)		18	18.75 (0.62)		29	27.52 (0.58)	

[#]Mann Whitney U test for two subgroups and Kruskal-Wallis test for more than two subgroups were used for statistical comparisons.
[a,b,c] Differences between groups carrying different letters in the same column are significant ($p<0.05$).

staffs have minimal training in avian and pocket pet medicine, there are no specialist trained personnel in all types of exotic medicine.

The Veterinary Faculties in Turkey have not provided adequate information on exotic pet diseases so far. Also, most of the faculties have changed their curriculum due to the growing interest in exotic pets and the exotic pet diseases course started to be taught as mandatory. In İstanbul University-Cerrahpaşa, Faculty of Veterinary Medicine, there has been an exotic pet track, which is comprised of three years of track based elective lessons. Moreover, it has been aimed to provide in-depth knowledge of topics such as mammals, fish, wildlife, reptiles and birds.

In this study, it was observed that there was no statistical difference for the competency and knowledge about the exotic pet species between male and female when gender. The potential of the exotic pets had a significant influence on all of the questions except the questions about the fish breeding and treatment, while the effect of work experience was not significant. One explanation could be that practitioners, in general, do not

handle the fish individually because they treat the fish through water of the aquarium. For the other species, they need to handle the patients individually.

There was no statistically significance between accepting and treating exotic pet species and work experience. When the potential of the exotic pets is evaluated according to animal species, it is found that knowledge is increasing in parallel significantly.

The threshold rate of exotic pets among other species is found at 10%. It is found that the practitioners who treat turtle and other reptiles evaluated their selves as more informed than other practitioners for all other exotic species. The practitioners, who treat fish declared themselves more informed about fish care and treatment than others who do not significantly. The possible explanation for this specific result could be the influence of work experience. Furthermore, the practitioners could improve their ability according to the potential of species. Vice versa, the patients' owners may choose the practitioner who is good at pet species.

Conclusion

Shortly, because of the significant increase in the population of exotic pets, it is necessary to increase the knowledge of persons in the field and organisations such as veterinarians, feed companies, pharmaceutical industry and pet products sector to address issues in the practice. Apart from the mission that incumbent on the universities, the chamber of veterinary surgeons and the associations also have great missions. Our results indicated the importance of education, specialisation and practice on potential exotic pet species. Besides, the veterinary faculties in Turkey should introduce exotic pet medicine to their syllabus. Seminars and workshops for veterinarians who lack the knowledge on exotic pets, should be continued.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – B.D.S., S.İ.; Design – B.D.S., B.Ç.; Supervision – B.D.S., S.İ.; Resources – B.D.S., B.Ç.; Materials - B.D.S.; Data Collection and/or Processing – B.D.S.; Analysis and/or Interpretation – B.D.S., B.Ç.; Literature Search – B.D.S., S.İ., S.A.; Writing Manuscript – B.D.S., S.İ.; Critical Review – S.İ., S.A.

Conflict of Interest: The authors have no conflict of interest to declare.

Financial Disclosure: This research was supported by Scientific Research Projects Coordination Unit of Istanbul University (Project number BEK-2016-21151).

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Mollusks (*Gastropoda*) as Intermediate Hosts of Cattles' Trematodes (*Trematoda*) in Conditions of Dnipro Basin's Small Ponds (Northern Ukraine)

Diana Valeriyivna FESHCHENKO[1], Tetiana Ivanivna BAKHUR[2*], Biricik Halil SELÇUK[3], Anatoliy Anatoliyovych ANTIPOV[2], Oksana Anatoliyivna ZGHOZINSKA[1], Oksana Anatoliyivna DUBOVA[1], Valentina Oleksandrivna YEVSTAFYEVA[4], Volodymyr Petrovich GONCHARENKO[2], Raisa Volodymyrivna SHAHANENKO[2], Volodymyr Sergiyovych SHAHANENKO[2], Vitaliy Vasyliovych MELNYCHUK[4]

[1]Department of Parasitology, Veterinary-Sanitary Expertise and Zoohygiene, Zhytomyr National Agroecological University, Zhytomyr, Ukraine
[2]Department of Parasitology and Pharmacology, Bila Tserkva National Agrarian University, Bila Tserkva, Kiev, Ukraine
[3]Department of Laboratory Animals, Afyon Kocatepe Üniversitesi, Afyon, Turkey
[4]Department of Parasitology and Veterinary-Sanitary Expertise, Poltava State Agrarian Academy, Poltava, Ukraine

Cite this article as: Feshchenko, D.V., Bakhur, T.I., Biricik, H.S., Antipov, A.A., Zghozinska, O.A., Dubova, O.A., Yevstafyeva, V.O., Goncharenko, V.P., Shahanenko, R.V., Shahanenko, V.S., Melnychuk, V.V., 2019. Mollusks (Gastropoda) as Intermediate Hosts of Cattles' Trematodes (Trematoda) in Conditions of Dnipro Basin's Small Ponds (Northern Ukraine).Acta Vet Eurasia 2019; 45: 16-21.

ORCID IDs of the authors: D.V.F. 0000-0002-4811-2488; T.I.B. 0000-0001-8271-8267; B.H.S. 0000-0003-4974-1611; A.A.A. 0000-0003-3955-3377; O.A.Z. 0000-0003-4622-6307; O.A.D. 0000-0002-8303-573X; V.O.Y. 0000-0003-4809-2584; V.P.G. 0000-0002-7279-6146; R.V.S. 0000-0002-5848-1367; V.S.S. 0000-0002-3484-2962; V.V.M. 0000-0003-1927-1065.

Abstract

The article presents the data on distribution and defeat of gastropod mollusks by parasitic trematodes in biotopes of small reservoirs (rivers, lakes and swamps) of the Dnipro basin of northern regions of Ukraine. During the 2016-2017 years, at the following areas were collected and identified: *Lymnaea* (L.) *stagnalis* (Linnaeus, 1758); *L. truncatula* (Müller, 1774); *Planorbis* (P.) *corneus* (Linnaeus, 1758); *P. planorbis* (Linnaeus, 1758); *Viviparus contectus* (Millet, 1813); *Valvata piscinalis* (Müller, 1774) and *Succinea pfeifferi* (Rossmässler, 1834). The microscopic study of the mollusks' liver allowed us to detect the presence of pathogens of cattle trematodoses inside a certain number of the snails – *Fasciola hepatica* (Linnaeus, 1758) and *Paramphistomum* sp. (Fischoeder, 1901). At biotopes of small rivers, 8.3% of mollusks *L. truncatula* species, 23.5% of *L. stagnalis* and 5.7% of *P. corneus* were affected. At lakes and swamps, the number of affected L. truncatula was 36.3%, and *L. stagnalis* – 13.7%. It was determined the defeat of ruminants with fasciolosis and paramfistomatoses in designated regions. It testifies to the formation of sustainable natural foci of these invasions.

Keywords: Cattle, *Fasciola hepatica*, Gastropod mollusks, *Paramphistomum* sp., trematodes

Introduction

The parasitic system is a special biosystem based on the trophic relationship between the parasite and the host (Beklemishev, 1970). The functioning of parasitic systems, in particular, the regulation of the relationship between their members, is ensured by a whole complex of environmental factors (Antipov et al., 2018; Kennedy, 1978; Krasnoshchekov, 1996).

Trematodes (*Treamentoda: Digenea*) are extremely common in tropical and subtropical climates (including Bolivia, Ecuador, Peru, Cuba, Egypt, Turkey, Iran), as well as in the European Union, Russia and Ukraine. Intermediate hosts of trematodes are freshwater gastropods (Caminade et al., 2015; Cañete et al., 2004; Caron et al., 2014; El-Shazly et al., 2012; Gorokhov et al., 2010; Khoramian et al., 2014; Lopez et al., 2012; Novobilský et al., 2013; Rondelaud et al., 2015; Stadnichenko, 2006).

Trematodes, which parasitize in the organs of the digestive system of cattle (in particular in the duodenum, the liver and the sections of the multi-chamber stomach) are an important epizootic danger to the cattle breeding of these countries (Munguía-Xóchihua et al., 2007). In this context, separate representatives of the following species should be singled out: *Fasciola hepatica* (*F. hepatica*) (Linnaeus, 1758), *Paramphistomum ichikawai* (Fukui, 1922), *Paramphistomum cervi* (Schrank, 1790) and *Liorchis scotiae* (Willmott, 1950).

Gastropods mollusks (*Gastropoda*) the most numerous class in the *Mollusca* type (Linnaeus, 1758), which has about 60.000-75.000 species (Zhadin, 1926). Among the gastropods, there are very few real parasites, for example: *Eulima bilineata* (Alder, 1848), parasite of bottom marine animals (*Ophiuroidea*; Grey, 1840) from the type of echinoderms (*Echinodermata*; Klein, 1734) (Nekhaev, 2011).

Address for Correspondence: Tetiana Ivanivna BAKHUR • **E-mail:** fly_13@ukr.net
Received Date: 6 September 2018 • **Accepted Date:** 14 February 2019 • DOI: 10.26650/actavet.2019.18009
Available online at actaveteurasia.istanbul.edu.tr

However, many species of gastropods are included to the life cycle of helminths and the epizootic chain of domestic animals' invasive diseases, in particular *Fasciola* and *Paramphistomum* invasion of cattle.

According to their biology, freshwater mollusks are natural inhabitants of large and small ponds involved in water purification and are indicator species for determining the degree of anthropogenic loading of biotopes. The fauna of mollusks depends to a large extent on the propagation of certain types of higher vegetation and depth of water bodies, velocity, temperature and pH of water in the pond (Bennema et al., 2011; Sargison et al., 2016; Zhytova and Kornyushyn, 2017).

In Ukraine, the northern Polissya regions, geographical center of which is in the territory of Kyiv and Zhytomyr regions, are characterized by the largest species diversity of gastropods in the small rivers of the Dnipro basin (Zhytova and Kornyushyn, 2017). It causes a severe enzootic situation with *Trematoda* invasions of large and small ruminants in this zone.

Figure 1. Points of mollusks' collecting at nothern regions of Ukraine (Dnipro basin)

Figure 2. Shells of *Lymnaea truncatula* (Muller, 1774)

So, the study of the epizootology of ruminant animals' trematodoses and freshwater fauna is closely interrelated and the provision of quality products and profitability of cattle breeding in endemic areas is impossible without comprehensive knowledge about ecology of the gastropods.

Materials and Methods

The research protocol of the current study was approved by the Ethic Committee of the Zhytomyr National Agroecological University (Approval number: 2016/07).

Freshwater mollusks were collected during 2016-2017 from river basins and their tributaries-rivers Teteriv and Sluch (Zhytomyr region), Bucha, Ros, Skvira (Kiev region). Particular attention was paid to the study of the malakofauna of lakes and swamps, which are located on the territory of these regions. Points of mollusks' collecting are shown on the map (Figure 1).

In general, more than one thousand specimens of molluscs from the family *Lymnaeidae* (2 species), *Valvatidae* (1 species), *Planorbidae* (2 species), *Viviparidae* (1 species) and *Succineidae* (1 species) were investigated. Cameral processing of materials was carried out in accordance with the recommendations of Zdun (1961). The molluscs were harvested using common methods (Stadnichenko, 2006). Identification of the species of mollusks was carried out according to external conchological features (Stadnichenko, 2004).

For histological study of the most common species of mollusks – *Lymnaea stagnalis* (*L. stagnalis*) (Linné, 1758), *Planorbarius corneus* (*P. corneus*) (Linné, 1758), hepatopancreas was taken from which were made preparations for parasitological microscopic examination. The presence of partenites (rediae) and larvae (cercariae) in the body of mollusks was determined using a microscope "XS-6320 (MICROmed, Poltava, Ukraine)". The intensity of the invasion was assessed visually according to the following criteria: weak-larvae defeat less than 1/10 volume of hepatopancreas; average-from 1/10 to 1/2; and strong – more than 1/2.

Results and Discussion

From the biotopes of groundwater ponds of the Dnipro basin in Kiev and Zhytomyr regions, we collected and identified 6 species of freshwater mollusks: *L. stagnalis* (Linnaeus, 1758); *L. truncatula* (Müller, 1774); *P. corneus* (Linnaeus, 1758); *P. planorbis* (Linnaeus, 1758); *Viviparus contectus* (Millet, 1813); *Valvata* (*V.*) *piscinalis* (Müller, 1774), and 1 terrestrial species – *Succinea* (*S.*) *pfeifferi* (Rossmässler, 1834).

Dominant species were *L. truncatula, L. stagnalis,* and *P. corneus* with a population density of 4-9 specimens/m^2 in the spring, in the summer of 14-20 specimens/m^2, and in the autumn, 1-3 specimens/m^2. Other species occurred singly, 1-3 specimens/m^2. For trematodes (*Trematoda: Digenea*) of ruminants, as intermediate hosts can serve: *L. truncatula* – for *Fasciola hepatica*

(Figure 2); *L. stagnalis* (Figure 3), *L. truncatula* and *P. corneus* (Figure 4) – for pathogens *Paramphistomidae* sp.; *S. pfeifferi* (Figure 5), sometimes may be an intermediate host of the dicrocoeliosis' pathogen (*Dicrocoelium lanceatum*) (Charlier et al., 2016; Dreyfuss et al., 1999; Faltýnková et al., 2008; Hodasi, 1972; Vignoles et al., 2016; Zhytova and Kornyushyn 2012).

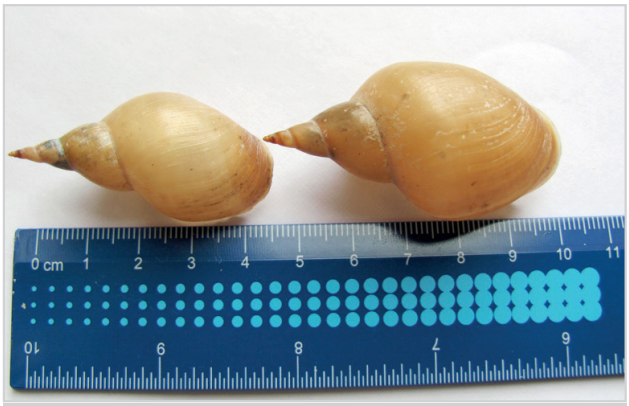

Figure 3. Shells of *Lymnaea stagnalis* (Linnaeus, 1758)

Figure 4. Shells of *Planorbis corneus* (Linnaeus, 1758)

Figure 5. Shells of *Succinea pfeifferi* (Rossmässler, 1834)

Mollusk *V. piscinalis* can be the first intermediate host for trematodes *Ichthyocotylurus pileatus* (Rudolphi, 1802) and *Diplostomum baeri* (Dubois, 1937), the additional hosts of which are freshwater fish, and the definitive – gulls. According to the fact that there is no literature data about the distribution of ruminant trematodes' larvae in *V. piscinalis*, microscopic examination of these mollusks' hepatopancreas was not carried out. Our studies have confirmed that the ratio of species of molluscs in a certain area and the level of their invasion by partenites and larvae of the trematodes depend on the biotope's characteristics.

Thus, the malakofauna of small flowing (rivers, their tributaries and floodplains) and standing reservoirs (lakes, swamps) of the Dnipro basin of northern Ukraine differed considerably.

The data obtained is illustrated in Table 1, from which it is evident that part of the representatives of the species *L. truncatula* (up to 36.3%) was affected by rediae and cercariae of *F. hepatica* (Figure 6); up to 23.5% of *L. stagnalis* mollusks contained parthenogenetic genera of both *F. hepatica* and *Paramphistomum* sp. in hepatopancreas; some specimens of gastropods of the

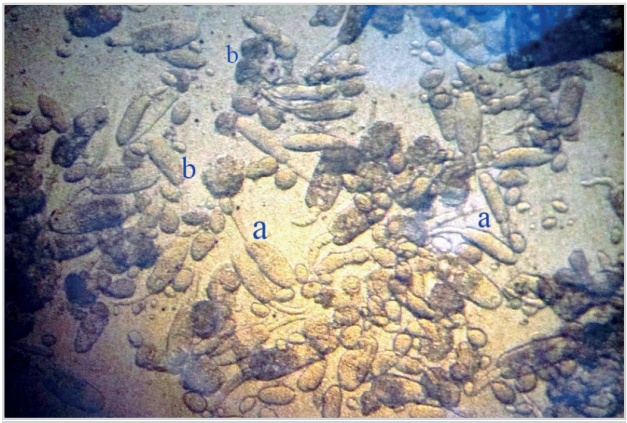

Figure 6. a, b. Parasitic trematode larvae (Trematoda: Digenea) in the liver of mollusk *Lymnaea truncatula* (Muller, 1774): a) mature cerkarias; b) young cerkarias

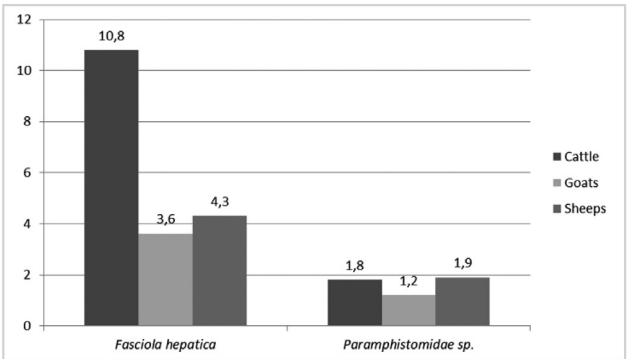

Figure 7. Average extensiveness of the invasion of large and small cattle by trematodes in the territory of Kyiv and Zhytomyr regions during 2016-2017, %

Table 1. Parasitologic study of mollusks' hepatopancreas (selected from ponds, Dnipro basin, Northern Ukraine)

Species of Mollusks	Extensiveness of the Invasion, %		Level of the Invasion	
	Biotopes of Lakes and Swamps	Biotopes of Rivers	Biotopes of Lakes and Swamps	Biotopes of Rivers
L. truncatula (Müller, 1774)	*F. hepatica*			
	36.3	8.3	Average	Weak
L. stagnalis (Linnaeus, 1758)	*F. hepatica* and *Paramphistomidae* sp.			
	13.7	23.5	Weak	Strong
P. corneus (Linnaeus, 1758)	*Paramphistomidae* sp.			
	0	5.7	0	Weak
P. planorbis (Linnaeus, 1758)	*Paramphistomidae* sp.			
	0	0	0	0
S. pfeifferi (Rossmässler, 1834)	*Dicrocoelium lanceatum*			
	0	0	0	0

species *P. corneus* (5.7%) were invasive solely by *Paramphistomum* sp. trematodes.

Taking into account that *L. truncatula* mollusks are biological hosts exclusively for *F. hepatica*, as well as the fact that they are widely distributed in the ponds of Ukrainian Polissya and the damage by the trematodes' partenites and larvae (especially in biotopes of lakes and swamps), there is a high risk for ruminants about fasciolosis throughout the studied area.

In contrast, the populations of *L. stagnalis* species of mollusks, which are often infected with rediae and cercariae of *F. hepatica* and *Paramphistomidae* sp., are more numerous near rivers than near standing water bodies.

Thus, in the northern regions of Ukraine at ponds of Dnipro basin, the predominant number of cases of cattle contamination by pathogens of paramfistomoses occurs near the rivers, and invasion by fascioles – near any ponds (both flowing and standing).

To establish the scale of the damage by the ruminant animals' trematodes in the investigated area, we have analyzed the official data of the state veterinary service in the Kyiv and Zhytomyr regions for 2016-2017. The analysis results are presented in Figure 7. The high extensiveness of ruminal invasion with *Paramphistomidae* sp. (1.2-1.9% of all examined animals), and especially *F. hepatica* (3.6-10.8%), confirm the obtained data on the widespread distribution of the diseases in the northern regions of Ukraine.

It is known that 25% invasion of malakofauna with helminths in a particular region is quite sufficient for the preservation and spread of the disease among the favorable domestic animals (Beesley et al., 2017). Just in one mollusc can develop at the same time more than 100 trematodes' cercariae. This is confirmed by our own research, according to which, up to 11% of cattle in the territory of Kyiv and Zhytomyr regions are having fasciolosis. It is not a critical indicator, but it is also not a reason to ignore the problem, since the stationary troubles of farms bring not less economic damage than sudden outbreaks of especially dangerous diseases.

To disrupt the stability of the parasitic system (trematodes-mollusks-ruminants), a sharp change in external environmental factors may occur. For example, the subletal for gastropods is the temperature of water above 27°C, depending on the type of mollusk (Afanasyev, 1993), due to the population of mollusks in the waters of Central Europe in the summer, it is sometimes significantly reduced. However, representatives of the subclass *Pulmonata* (Cuvier, 1817), adapted to the sharp fluctuations of temperature, falling into the summer (aestivation period) or winter (hibernation period) lethargy.

In most of the water systems of the Dnipro basin in the north of Ukraine, conditions for the development of freshwater mollusks are quite favorable. The climate of this area is mild, moderately continental. Endemic species of molluscs are evolutionally adapted to possible short-term critical climatic oscillations, as evidenced by the seasonal fluctuations of the number of gastropods in the studied ponds.

Thus, the presence of a permanent population of gastropod mollusks, affected by trematodes' pathogens, in small ponds of the north of Ukraine creates a permanent natural reservoir of fasciolosis and paramfistomatoses. This does not let to allow the cattle to be protected from further invasion without using of complex control measures against helminths.

Conclusion

In the biotope of natural ponds of the Dnipro basin at the northern regions of Ukraine, there are 7 species of gastropods, which are intermediates of ruminant's trematodes: *L. stagnalis,*

L. truncatula, P. corneus, P. planorbis, Viviparus contectus, V. piscinalis and *S. pfeifferi*. The first three species are dominated by numbers: from 4 to 9 specimens/m^2 in spring, from 14 to 20 per m^2 in summer, and from 1 to 3 per m^2 in autumn.

Intraspecies indices of mollusks' invasiveness by partenites and larvae of the trematodes varied depending on the type of ponds. More often (23.5%) and more intensively the *L. stagnalis* population was affected by rediae and cercariae of *F. hepatica* and *Paramphistomum* sp., while in *P. corneus* (5.7%) was found only *Paramphistomum* sp. in rivers' biotopes. Indices of damage to mollusks of the species *L. truncatula* by the *F. hepatica* trematodes increased in lakes and swamps (36.3%).

Ethics Committee Approval: Ethics Committee approval was received for this study from the Ethics Committee of Zhytomyr National Agroecological University, Ukraine (Approval number: 2016/07).

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – D.V.F., T.I.B., A.A.A.; Design – D.V.F., O.A.Z., R.V.S.; Supervision – D.V.F., A.A.A., V.P.G.; Resources - V.O.Y.; Materials – D.V.F., O.A.D., V.V.M.; Data Collection and/or Processing – T.I.B., V.P.G., V.S.S.; Analysis and/or Interpretation – D.V.F., T.I.B., B.H.S.; Literature Search – D.V.F., O.A.Z., O.A.D., V.S.S.; Writing Manuscript – D.V.F., T.I.B.; Critical Review – A.A.A., B.H.S.

Acknowledgements: The authors are grateful to Associate Professor Zhitova O.P. from Zhytomyr National Agroecological University for thematic advisory support.

Conflict of Interest: The authors have no conflict of interest to declare.

Financial Disclosure: The authors declared that this study has received no financial support.

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Effects of Mediterranean Mussel Shell (*Mytilus galloprovincialis*) on Performance and Egg Quality in Laying Quails

Kadir Emre BUĞDAYCI[1], Hıdır GÜMÜŞ[1], Mustafa Numan OĞUZ[1], Fatma KARAKAŞ OĞUZ[1], İskender GÜLLE[2]

[1]Department of Animal Nutrition and Nutritional Diseases, Faculty of Veterinary Medicine, Mehmet Akif Ersoy University, Burdur, Turkey
[2]Department of Hydrobiology, Faculty of Arts and Science, Mehmet Akif Ersoy University, Burdur, Turkey

Cite this article as: Buğdaycı, K.E., Gümüş, H., Oğuz, M.N., Karakaş Oğuz, F., Gülle, İ., 2019. Effects of Mediterranean Mussel Shell (Mytilus galloprovincialis) on Performance and Egg Quality in Laying Quails. Acta Vet Eurasia 2019; 45: 22-29.

ORCID IDs of the authors: K.E.B. 0000-0002-1715-6904; H.G. 0000-0001-7077-1036; M.N.O. 0000-0001-8802-3423; F.K.O. 0000-0002-9077-8531; İ.G. 0000-0003-3298-3657.

Abstract

The effect of Mediterranean mussel shell *(Mytilus galloprovincialis)* (MMS) on performance, egg quality and some blood parameters were studied on quails *(Coturnix coturnix Japonica)* for a period of 10 weeks (13-23 weeks). A total of 90 quails were randomly separated into one control and two treatment groups. Each group was divided into six subgroups, each containing 5 animals. MMS was replaced with lime stone at the ratios of 50 and 100% in first (50% MMS) and second (100% MMS) group rations respectively. The diets were prepared to be isocaloric and isonitrogenous. Rations and water were given *ad libitum*. Ca source replacement did not significantly affect body weight, egg weight, egg yield, feed intake and feed conversion ratio of laying quails. The effects of MMS replacement on shape index, yolk index, yolk color, blood Ca, Mg, P levels with Mg and P levels of egg shell had no significance. Ca levels of egg shell decreased ($p<0.05$) in treatment groups however, the amount of crude ash of tibia was not altered. In sum, dietary MMS did not alter egg quality of laying quails. It may be concluded that MMS can be replaced with limestone in the diet of laying quails.

Keywords: Laying quails, mussel shell, performance, egg quality

Introduction

Blue mussel (*Mytilus galloprovincialis*) is cultivated or collected for human consumption in most of the coastal regions of Turkey. The production amount of all mussel species in Turkey from 2000 to 2016 differed from 9000 to 59000 ton annually, and the amount of blue mussel reached 12000 ton per year from 78 ton per year during these years (Anonymous, 2018). The shell percentages of mussels collected from different parts of Spain were determined between the ratio of 52 and 61% (Fuentes et al., 2009). Nearly 4500 to 28000 ton mussel shell waste was produced in Turkey annually. Marin and Luquet (2004) reported that 1-5% of the mollusc shell weight included organic matrix (aragonite, calcite, or in particular cases, vaterite). Other parts of shell were composed by calcium carbonate (Barros et al., 2009).

There were a number of studies on dietary organic Ca sources on layers except limestone (Ahmed et al., 2013; Çetin and Gürcan, 2006; Houndonougbo et al., 2012; Kismiati et al., 2012; Scheideler, 1998). There were also several studies (Ayaşan and Okan, 1999; Cufadar, 2014; Ertaş et al., 2006) on different sources (egg shell, oyster shell, mussel shell) and different sizes of dietary Ca in quails. Dietary replacement (25, 50, 75 and 100%) of limestone with eggshell (Gongruttananun, 2011) and limestone with eggshell and/or oyster shell (Cufa-

Presented in: A part of this manuscript was presented from "3rd International Vet Group Congress 2016, May 17-20, 2016 Sarajevo, Bosnia and Herzegovina".

Address for Correspondence: Kadir Emre BUĞDAYCI • **E-mail:** kebugdayci@mehmetakif.edu.tr

Received Date: 3 September 2018 • **Accepted Date:** 11 January 2019 • DOI: 10.26650/actavet.2019.18010
Available online at actaveteurasia.istanbul.edu.tr

dar, 2014) and limestone with mussel shell (Ertaş et al., 2006) were studied on layers. Gongruttananun (2011) and Cufadar (2014) reported that dietary replacement (50 and 100%) of limestone with eggshell and dietary oyster shell did not affect egg weight and egg production of layers respectively. On the other hand Ertaş et al. (2006) reported that egg production of quails improved when the ratio of 75% of limestone in the diet was replaced with mussel shell under heat stress conditions. Besides these investigations, the effects of dietary particle sizes of Ca sources were studied on layers (Safaa et al., 2008; Tunç and Cuhadar, 2015); and it was declared that dietary large particle sized of egg shell and oyster shell (Tunç and Cuhadar, 2015) or dietary replacement of fine lime stone to coarse lime stone or oyster shell (Safaa et al., 2008) did not affect laying performance of layers. It was reported that normal levels of dietary Ca (3% for laying quails and 3.75% for laying hens) with lower levels of dietary P (0.4% for laying quails and 0.53% for laying hens) has positive effect on egg production of layers without concerning source of mineral feed (Ahmad and Balander, 2003; Ayaşan and Okan, 1999). As reported by Lichovnikova (2007), there were also positive effects on egg shell weight and quality due to *ad libitum* oyster shell consumption. Some of these dietary Ca investigations was worked on especially late periods of layers (Cufadar, 2014; Safaa et al., 2008).

There were also several studies on the use of aquatic Ca sources on layers (Bugdayci et al. 2016; Pelícia et al., 2007). Bugdayci et al. (2016) declared that the replacement of cuttlefish bone with limestone at the levels of 50 and 100% did not affect productive performance and quality of eggs in japan quails. Moreover, Pelícia et al. (2007) used a source of marine calcium from marine alga (*Lithothamnium* sp.) in the diets of layers and reported that marine calcium could be replaced up to 45% of limestone without any effect on production and egg quality.

Recently, mussel has been cultivated for human diet. Many investigations have been carried out by using different sources and sizes of Ca in layers; however, experiments involving Mediterranean mussel (*Mytilus galloprovincialis*) shell (MMS) as a Ca source are lacking in literature. The availability of Ca content of MMS may be altered by salty water. This may have a positive effect on performance and egg quality for layers. There is a study which is carried out with fresh water mussel shell (*Unio elangatus eucirrus Bourguignat*) at the same dietary proportions as our study in quails. However, there is no study on the use of 50% and 100% MMS in laying quails. The aim of this investigation is to determine the possible effects of Mediterranean mussel shell on productive performance, internal – external egg quality and some blood parameters of japan quails.

Materials and Methods

Animals, management and rations

Protocols of animal use, animal care and research protocols were approved (2015-121) by local ethical committee of Burdur Mehmet Akif Ersoy University as required. 13 week-old 90 quail (*Coturnix coturnix japonica*) were used in the investigation. The quails were randomly allocated into one control and two treatment groups. Each group contained 30 quails. All groups were divided into six replicates (each contained 5 quails). Quails were kept in cages (45cmx50cmx22cm) in a windowed house. 8/16 h dark/light regimens were applied in the windowed house. Ration and water were given *ad libitum* during the 10 weeks of experiment. The ingredients and chemical composition of all groups' diet are presented in Table 1. The diets consisted of two levels of MMS (50 and 100%) by limestone in the diets of first and second treatment groups, respectively.

Basal diet's nutrient composition was determined by using AOAC (1990) directives. Titus and Fritz (1971) equation was used to estimate metabolizable energy of diets.

The $CaCO_3$ content of MMS was determined in the laboratory. For this purpose, weighed amounts of MMS were burned in an ash furnace, crumbed and waited in 37% HCl for 12 h. After the waiting period, the mixture of MMS and HCl was filtered from Whatmann paper. Paper was dried (105°C) in a stove (Memmert UE500, C593.0011, Germany) and burned (550°C) in ash furnace (Carbolite, S302RR, UK). The Ca level of MMS (38.5%) was determined by using the amounts of acid soluble ash (96.19%) of MMS and total Ca levels of group rations were illustrated in Table 1.

Traits recorded and methods applied

Animals were weighed individually at the beginning and at the end of the trial. Quails were weighted by using a precision balance (Model: HGM-20K-ER8412, 1g sensitivity, UWE CO, Taipei-Taiwan) and mortality was recorded as it occurred.

Eggs were gathered daily and egg production was stated on a hen-day basis. Laid eggs throughout the last two consecutive days of every week were weighed individually. For this purpose, precision balance (Model CP224S-14105100, 0.1 mg sensitivity, Sartorius AG, Göttingen-Germany) were used for determine the egg weight. Feed consumption was recorded weekly and calculated (g/day/quail). The feed efficiency was calculated in two type (g feed/g egg and g feed/dozen egg).

Egg quality

Internal egg quality were determined by the fallowing procedure. 12 eggs laid at 09.00 - 12.00 h were collected randomly from each group in total (2 eggs from each replicate) on

Table 1. Ingredients and chemical composition of control and treatment groups' diet

	Treatment groups		
Ingredients of diets, (%)	**Control**	**50% MMS**	**100% MMS**
Corn	45.55	45.55	45.55
Wheat	7	7	7
Soybean meal (48%)	18	18	18
Full fat soy	10	10	10
Sunflower meal (36%)	9	9	9
Vegetable oil	2.8	2.8	2.8
DCP	1.5	1.5	1.5
Limestone	5.4	2.7	-
Mediterranean mussel shell	-	2.7	5.4
Salt	0.3	0.3	0.3
Vit-Min complex*	0.25	0.25	0.25
DL-Methionine	0.1	0.1	0.1
L-Lysine	0.1	0.1	0.1
Analysed chemical composition of diets as feed, (g/kg)			
Crude protein	174.9	171.4	174.6
Eter extract	80.0	79.0	79.3
Crude fibre	40.7	41.4	40.9
Crude ash	100.1	98.8	102.8
Dry matter	918.4	918.2	919.1
Ca**	25.00	25.15	25.30
ME (kcal/kg)	2561.76	2529.37	2524.60

*Each kilogram of vitamin-mineral mix contains 12 000 000 IU A vit, 20 000 mg E vit, 50 000 mg Mn, 50 000 mg Fe, 50 000 mg Zn, 10 000 mg Cu, 800 mg L, 150 mg Co, 150 mg Se;
** Calculated levels

4th, 6th, 8th and 10th week of the experiment (as a total of 48 eggs per group throughout the experiment). Each egg was weighed with a precision balance individually and shape index of eggs were calculated (Shape index=(egg width/egg length)x100).

The egg content was broken onto a glass plate. Then, the height of the albumen and the yolk was measured with a tripod micrometer (Mitutoyo, No: 2050S-19, 0.01-20 mm; Kawasaki-Japan). Albumen length, albumen width and yolk diameter were measured with digital calliper.

Yolk index [(yolk height/yolk diameter) x 100], albumen index [(albumen height/average of albumen length and albumen width) x 100] and Haugh units [[100 x log (H+7.57-1.7W0.37)] where H is albumen height and W is egg weight] were calculated by using the values of albumen length, albumen width and yolk diameter (Card and Nesheim, 1972). Roche yolk color fan was used for scoring egg yolk colors of the treated eggs on a glass plate (Vuilleumier, 1969).

Internal and external quality analyses of egg were completed within the next 24 h after the eggs were gathered. Egg weights of treated eggs were used to evaluate of their quality individually.

Ca, P and Mg content of egg shells of control and treatment groups were detected by ICP-OES (Perkin Elmer ICPOES Optima 8000, USA). For this purpose, all treated egg shells were collected and mixed in a poll of their own subgroups. In sample preparation 0.3 g of shell ash and 6 ml HNO_3 (65%) treated in microwave (Milestone Stard D) according to the method of Al-Obaidi et al. (2012).

Serum analysis

At the end of the experiment, two quails from each replicate (12 from each group) were randomly selected and slaughtered. Blood samples were gathered at the slaughtering operation and centrifuged at 3000xg for 10 min. individually. Fresh serum samples were analyzed to determine of serum Ca, P and Mg levels by autoanalyser (Model: Gesan-Chem200, No:

Table 2. The effects of dietary treatments on initial and final body weights of laying quails (g)

	Dietary treatments			
	Control	**50% MMS**	**100% MMS**	**p**
Initial body weight (g)	274.93±4.11	270.26±5.44	270.03±4.45	0.709
Final body weight (g)	277.68±5.12*	283.48±6.64*	284.76±6.10	0.675

Control: 100% limestone; T1: 50% MMS + 50% limestone; T2: 100% MMS; n=30; *n=29; p<0.05

Table 3. The effects of dietary treatments on performance of laying quails (mean±SE)

	Dietary treatments			
	Control	**50% MMS**	**100% MMS**	**p**
Feed intake (g/day per quail)	34.68±1.12	33.29±1.25	33.54±0.64	0.615
Egg production (%)	92.06±3.68	93.46±2.92	93.52±3.64	0.944
Egg weight (g)	13.38±0.10	13.07±0.28	13.23±0.13	0.522
FCR* (kg feed / kg egg)	2.59±0.09	2.54±0.07	2.53±0.03	0.829
FCR (kg feed / dozen egg)	0.43±0.02	0.42±0.02	0.40±0.02	0.764

Control: %100 limestone; T1: 50% MMS + 50% limestone; T2: 100% MMS; FCR: Feed conversion ratio; n=6 per group.

Table 4. The effects of dietary treatments on egg traits of laying quails (mean ±SE)

	Dietary treatments			
	Control	**50% MMS**	**100% MMS**	**p**
Weight of treated eggs (g)	13.42±0.16^{a}	12.91±0.31^{b}	13.26±0.06^{a}	0.011
Crude ash of tibia (%)	64.14±1.38	63.55±1.56	61.81±2.69	0.688
Shape index (%)	77.32±0.41	77.29±0.44	76.31±0.51	0.223
Albumen high (mm)	5.08±0.12^{a}	4.54±0.40^{b}	4.87±0.42^{ab}	0.013
Albumen index (%)	11.77±0.33	10.53±0.27	11.07±42	0.057
Yolk index (%)	42.37±0.37	41.65±0.35	41.32±0.25	0.085
Haugh unit	91.10±0.67^{a}	88.61±0.65^{b}	90.36±0.64^{ab}	0.033
Egg yolk color[1]	10.08±0.12	10.07±0.12	10.08±0.13	0.996

Control: %100 limestone; T1: 50% MMS+50% limestone; T2: 100% MMS

^{a,b} Means within a row followed by the different superscripts differ significantly (p<0.05)

[1]Roche color scores are based upon Roche Color Fan Edition 1965, n=12

1102422, Campobello-Italy) using its commercial kit (Monoreagent-LR-C2230150V, Italy).

Determination of tibia ash

The right legs of slaughtered quails (12 from each group) were removed and placed in an autoclave for the separation of meat and bone. Separated tibias were dried at 105°C for 12 h in an incubator. Then, tibias were burned at 550°C for 5 h in an ash furnace.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) program (SPSS Inc., Chicago, USA) used for statistical analyses. Kolmogorov-Smirnov test used for checking the normality of the data. The differences among groups examined (One-way ANOVA). Duncan was used for the significance of mean differences between groups. Quality characteristics of egg were analyzed after detecting egg weight. Egg internal and external quality characteristics were analyzed after adjusting egg weight and values were given as conjectural marginal means and standard error of mean. p<0.05 was determined for the level of significance (Dawson and Trapp, 2001).

Results

Dietary MMS did not alter the final body weight (Table 2), productive performance, feed intake, egg weight and feed

Table 5. The effects of dietary treatments on Ca, Mg and P of egg shell ash (%) and Ca, Mg, P levels of blood serum (mg/dL) of laying quails, (mean±SE)*

	Dietary treatments			
	Control	**50% MMS**	**100% MMS**	**p**
Egg shell (%)				
Ca	41.77±2.01[a]	21.37±5.67[b]	19.73±4.53[b]	0.004
Mg	0.63±0.02	0.61±0.04	0.71±0.02	0.098
P	0.25±0.03	0.28±0.02	0.30±0.01	0.332
Blood serum (mg/dL)				
Ca	21.78±2.64	24.96±1.50	24.87±2.04	0.496
Mg	2.65±0.05	2.65±0.03	2.56±0.03	0.276
P	34.10±1.62	36.84±1.71	35.05±1.04	0.440

Control: %100 limestone; T1: 50% MMS + 50% limestone; T2: 100% MMS; n=6; $p<0.05$

conversion ratio of quails (Table 3) in the present study. Egg quality parameters results, such as shape index, albumen index, yolk index and egg yolk color were not altered by dietary MMS. However, albumen high and Haugh unit of treated eggs differed (Table 4). Calcium levels of egg shell were significantly decreased ($p<0.05$) by the replacement of lime stone with mussel shell in both ratios (Table 5). On the other hand, Mg and P levels of egg shell were not affected in the present study. Crude ash of tibia was not changed by dietary limestone replacement (Table 4). Dietary substitution of limestone with mussel shell did not alter Ca, Mg and P levels of blood (Table 5).

Discussion

In the present study, egg production of quails was not affected by the replacement of limestone with MMS. This result was similar to lime stone replacement with cuttlefish bone in laying quails (Bugdayci et al., 2016) and also similar with oyster shell and egg shell in laying hens (Cufadar, 2014). Beside this, Scheideler (1998) declared that the replacement of 25 of limestone with oyster shell did not affect egg production in laying hens. On the other hand, Ertaş et al. (2006) reported that the replacement of 75% of limestone with fresh water mussel shell in quail rations increased egg production. Hence, we can state that, the availability of fresh water mussel shells or salty water mussel shells as a Ca source may be different for quails.

There are several investigations which reported that the effects of different Ca sources and different sizes did not change the egg production of layers (MacIsaac et al., 2016; Safaa et al., 2008; Tunç and Cuhadar, 2015). However, Pelicia et al. (2011) declared that increasing Ca levels (3 to 4.5%) of laying hen rations linearly decreased productive performance of laying hens. On the other hand, Pizzolante et al. (2006) reported that different dietary Ca levels (3.5 and 4.0) did not alter the productive performance of semi-heavy layers without concerning limestone particle composition. Studies also showed that laying performance of layers is related with the dietary ratio of Ca/P; dietary Mg and P levels as well as size with/or source of dietary Ca (Ahmad and Balander, 2003; Ayaşan and Okan, 1999; Skřivan et al., 2016).

The results of this study regarding feed intake and egg weight of quails agree with the report of Ertaş et al. (2006) who did not find any difference on feed intake and egg weight when 25, 50, 75 and 100% of fine limestone in the diet of laying quails was substituted with mussel shell. Similarly, Bugdayci et al. (2016) reported that replacement of limestone with the ratio of 50 and 100% of cuttlefish bone did not significantly affect the feed intake and egg weight of laying quails. On the other hand, the replacement (50%) of limestone with egg shell, the replacement (50%) of limestone with oyster shell (Cufadar, 2014), and the replacement (100%) of limestone with whelk shell in laying hen rations (MacIsaac et al., 2016) decreased feed intake without affecting egg weight. There are also several studies (Cufadar, 2014; MacIsaac et al., 2016) reported that alternative Ca sources could decrease feed intake of layers.

Cufadar (2014) reported that the replacement of the ratio of 50 and 100% proportions of oyster shell and egg shell did not alter the feed conversion ratio of laying hens, which is consistent with the relating result of this study. In addition to this report, MacIsaac et al. (2016) declared that the use of grounded whelk shell (100%) in the rations at high temperature conditions decreased feed conversion ratio of laying hens. The present study was completed during normal environmental temperature.

Quality parameters of treated eggs except albumen high and Haugh unit were not altered by the substitution of lime stone

with mussel shell; on the contrary, Wang et al. (2014) reported that large dietary particle Ca sources (oyster shell and limestone) increased albumen height and Haugh unit in layers. The replacement of limestone with mussel shell at the ratio of 50% had lower albumen high and Haugh unit when compared with control group ($p<0.05$). This result might be caused by the weights of treated group eggs which selected randomly (Table 4). The use of large particle Ca sources (oyster shell and limestone) in layer diets did not affect yolk color of eggs mentioned by Wang et al. (2014), which is similar with the present study.

Dietary MMS decreased the Ca levels of egg shell in the present study; however, Bugdayci et al. (2016) reported that dietary cuttlefish bone did not alter the Ca levels of egg shell in quails. This result may be caused by the difference of the Ca availability of dietary MMS and cuttlefish bone. Also, Wang et al. (2014) reported that large dietary particle Ca sources (oyster shell and limestone) increased shell content of phosphorus and magnesium when compared with small particle sizes. On the other hand, Skřivan et al. (2016) declared that dietary limestone size (fine or ground) did not affect shell percentages (weight of egg shell / weight of egg) of laying hens. Besides these results, Wang et al. (2014) reported that dietary oyster shell instead of limestone in laying ducks caused lower Ca accumulation in egg shell. It is clear that as an aquatic additive, mussel shell like cuttlefish bone (Bugdayci et al., 2016) or chitosan (Świątkiewicz et al., 2018) had different effects on bio-mineralization of organism. Bugdayci et al. (2016) reported that dietary replacement of Ca source with an aquatic one did not change the amount of crude ash of egg shell and tibia in laying quails. However, Świątkiewicz et al. (2018) declared that dietary chitosan supplementation increased egg shell thickness of laying hens at late production period. There were several studies declaring the egg shell mineral content alteration fed with different dietary Ca sources and different size of these sources (de Witt et al., 2009; Tunç and Cuhadar, 2015).

The amount of crude ash of tibia was not affected in the study; and this result was similar with the results regarding the use of egg shell as a Ca source and the use of different amounts (3.5-4.7%) of Ca in laying hen in Gongruttananun (2011) and An et al. (2016) respectively. Wang et al. (2014) also declared that the use of dietary small particle sized Ca source in laying ducks did not affect the ash content of tibia in laying ducks. Similarly, Pelicia et al. (2011) reported that increasing Ca levels of laying hen rations from 3.0 to 4.5% did not alter tibia ash of layers at the age of 35 weeks old. It is clear that the size or the source or the dietary amount (not less than 3.5%) of Ca additives did not effect on total mineral content of tibia for laying ducks (Wang et al., 2014) and hens (An et al., 2016; Gongruttananun, 2011). However, Tunç and Cuhadar (2015) reported that the levels of Ca, P and Mg of the tibia changed with different sources and proportions of dietary Ca in laying hens.

Dietary substitution of limestone with MMS decreased the levels of egg shell Ca content; however, blood Ca levels were not affected as well as blood levels of Mg and P. This result may be caused by the source of available Ca. On the other hand, Pelicia et al. (2011) reported that the blood Ca levels of laying hens were affected by the dietary Ca levels of layers. In the present study, the levels of analyzed dietary Ca in control and treatment groups were similar. For this reason, blood Ca levels of quails may not have been altered. Besides these, the source and the proportions of Ca did not affect egg production and crude ash of tibia in the present study.

Bugdayci et al., (2016) and Gongruttananun, (2011) reported that 50% and 100% dietary limestone replacement with eggshell or cuttlefish bone did not alter the blood Ca levels of laying quails and laying hens respectively. These reports were similar to the results of the present study. However, the levels of calcium in the blood and eggshell were not strictly correlated to each other. This may be caused by the availability of different Ca sources.

Related with the production of Mediterranean mussel, the amounts of mussel shell by-product can reach to 28000 tons annually. This amount of mussel shell could be brought into the economy. If the MMS is considered as a source of calcium in animal feeding, the environmental pollution also will decrease. In the present study the results point out that total substitution of limestone with MSS in the diet does not have negative effect on the laying performance of quails or quality parameters of eggs.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – K.E.B., M.N.O.; Design – K.E.B., M.N.O.; Supervision – F.K.O.; Resources – İ.G.; Materials – K.E.B., M.N.O.; Data Collection and/or Processing – K.E.B., M.N.O., H.G.; Analysis and/or Interpretation – K.E.B., M.N.O.; Literature Search – K.E.B.; Writing Manuscript – K.E.B.; Critical Review – M.N.O.

Acknowledgements: The authors are thankful to Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine.

Conflict of Interest: The authors have no conflict of interest to declare.

Financial Disclosure: the authors declared that this study has received no financial support.

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Molecular Epidemiology and Risk Factors Assessment of *Anaplasma* spp. on Dairy Cattle in Southwest of Iran

Vahid NOAMAN[1], Morteza MORADI[2]

[1]Veterinary Medicine Group, Department of Animal Science Research, Isfahan Agricultural and Natural Resources Research and Education Center, Agricultural Research, Education and Extension Organization (AREEO), Isfahan, Iran

[2]Faculty of Veterinary Medicine, Islamic Azad University, Boroujerd Branch, Boroujerd, Iran

Cite this article as: Noaman, V., Moradi, M., 2019. Molecular Epidemiology and Risk Factors Assessment of *Anaplasma* spp. on Dairy Cattle in Southwest of Iran. Acta Vet Eurasia; 45: 30-36.

ORCID IDs of the authors: V.N. 0000-0002-3002-2417; M.M. 0000-0002-7713-9567.

Abstract

The present study was carried out to determine *Anaplasma* species and potential risk factors associated with molecular prevalence of *Anaplasma* spp. among dairy cattle in southwest of Iran. A total of 88 samples out of 200 generated an expected amplicon of 866 bp from *Anaplasma marginale* msp4 gene. Six samples that were identified as A. *marginale* gave also positive results for A. *phagocytophilum* 16S rRNA gene with specific nested polymerase chain reaction (nPCR). The multivariate analysis of risk factors revealed that the cattle of mountain regions were significantly (p=0.0001) at higher risk as compared to the plain regions. Cattle <1 year age and the latitude 32-33°C were significantly at lower risk (p<0.01). The cattle with low milk yield were significantly (p=0.002) at lower risk. Low hygienic farms were significantly (p=0.011) at higher risk as compared to good and normal hygienic farms. Distance from other farms (<1Km) was another important risk factor which showed significant association with the occurrence of *Anaplasma* infection (p=0.021). The results of this study can be used in strategic planning for prevention and control of bovine anaplasmosis in dairy cattle in the southwest of Iran.

Keywords: *Anaplasma,* cattle, Iran, molecular epidemiology, risk factors

Introduction

Anaplasmosis, theileriosis, and babesiosis are the most important tick-borne diseases in dairy cattle of tropical and subtropical regions of the world (Kocan et al., 2015). In cattle, anaplasmosis caused by different genres of *Anaplasma* (Rickettsiales: *Anaplasmataceae*) including *Anaplasma marginale* (*A. marginale)*, *A. phagocytophilium*, *A. central* and *A. bovis* (Kocan et al., 2015).

Bovine anaplasmosis caused by *A. marginale* is the most prevalent and pathogenic forms of the disease in Iran. In affected animals, anemia, icterus, fever, abortion, lethargy weight loss and death are the most prevalent clinical signs of the disease. Infected animals with *A. marginale* remain as carriers throughout their lifetime. Under some circumstances such as stress or other predisposing factors can induce anaplasmosis in carriers or persistent infected animals (Kocan et al., 2010; Noaman and Bastani, 2016).

A. phagocytophilum is a wide range host organism and can cause tick borne fever in dairy cattle and the other ruminants. The disease is characterized by fever, abortion, reduced fertility, reduced milk yield, leukopenia and inclusions in circulating neutrophils. The infections commonly have not any observable symptoms except combined with other pathogens. *A. phagocytophilum* is also considered as a zoonotic agent and can cause human granulocytic anaplasmosis (Bakken and Dumler, 2015; Noaman et al., 2016). Giemsa-stained blood smears and serological tests like competitive enzyme-linked immunosorbent assay and immunofluorescent antibody have been used widely in epidemiological researches. However, they have not sufficient sensitivity and specificity for the determination of early infections, true negative and carrier animals (Aubry and Geale,

Address for Correspondence: Vahid Noaman • **E-mail:** v.noaman@areeo.ac.ir

Received Date: 8 September 2018 • **Accepted Date:** 11 January 2019 • DOI: 10.26650/actavet.2019.18014
Available online at actaveteurasia.istanbul.edu.tr

2011; Noaman and Shayan, 2010a). Polymerase chain reaction (PCR), nested polymerase chain reaction (nPCR) and restriction fragment length polymorphism based on the 16S rRNA and major surface proteins are capable to identify low levels of *Anaplasma* spp. in persistent infected animals (Noaman, 2013a ; Quiroz-Castaneda et al., 2016).

Several factors such as type of livestock, breed, sex, age, milk yield, herd size, interaction with wildlife, stress management, pasture type, presence of vectors, ecological and climatic conditions and socio-economic factors may play important roles in the epidemiology of anaplasmosis. However, in different regions, there are different risk factors associated with the presence of anaplasmosis (Amorim et al., 2014; Atif, 2015).

The diversity of climate in Iran can cause the diversity of tick species and subsequently, tick-borne diseases (Noaman, 2012; Noaman et al., 2017; Walker, 2014). Four *Anaplasma* genres including *A. marginale*, *A. centrale*, *A. phagocytophilium* and *A. bovis* have been recognized in Iranian cattle based on molecular assays (Noaman, 2013b; Noaman et al., 2009; Noaman and Shayan, 2009; Noaman and Shayan, 2010b). Khuzestan province is located in the southwest of Iran with tropical climate where the tick-borne diseases (especially anaplasmosis) are important in livestock. In Iran, anaplasmosis has been usually detected in blood smears using traditional Giemsa staining. However, this method only suitable in acute anaplasmosis and has no ability in detection of carrier animals and epidemiological studies. The goals of this study were to recognize the *Anaplasma* species in cattle using molecular method and to assess the risk factors affecting the epidemiology of *Anaplasma* spp. in tropical region of Iran.

Materials and Methods

Study area

The province of Khuzestan is located in the southwest of Iran, borders Iraq and the Persian Gulf and occupies an area of 63.213 km^2. It is located between 48°E and 49.5°E longitudes and between 31°N and 32°N latitudes (Figure 1). Topographic elevations in the province vary between zero and 3740m (above MSL). The climate of this area varies from arid to humid. The northern parts of the province experience cold weather, whereas the southern parts have tropical climate. Summer season is from April to September, and winter is from October to March. The annual mean of maximum summer temperatures in the province is about 50°C (in July), and annual mean of minimum winter temperature is 9°C (in December). The average annual rainfall is 150-256 mm in the south and 995-1100 mm in the north, and about 70% of annual rainfall events occur from February to April. The annual evaporation is 2000-4000mm (Zarasvandi et al., 2011). Figure 1 shows the geographical situation of Khuzestan province in Iran.

Sampling

From 21 June 2010 to 20 December 2016, a total 200 blood samples were collected from healthy dairy cattles of Khuzestan province based on multistage random sampling method. Sampling was carried out in 22 countries including: Andimeshk, Dezful, Shosh, Gotvand, Anika, Shoshtar, Masjed-soleiman,

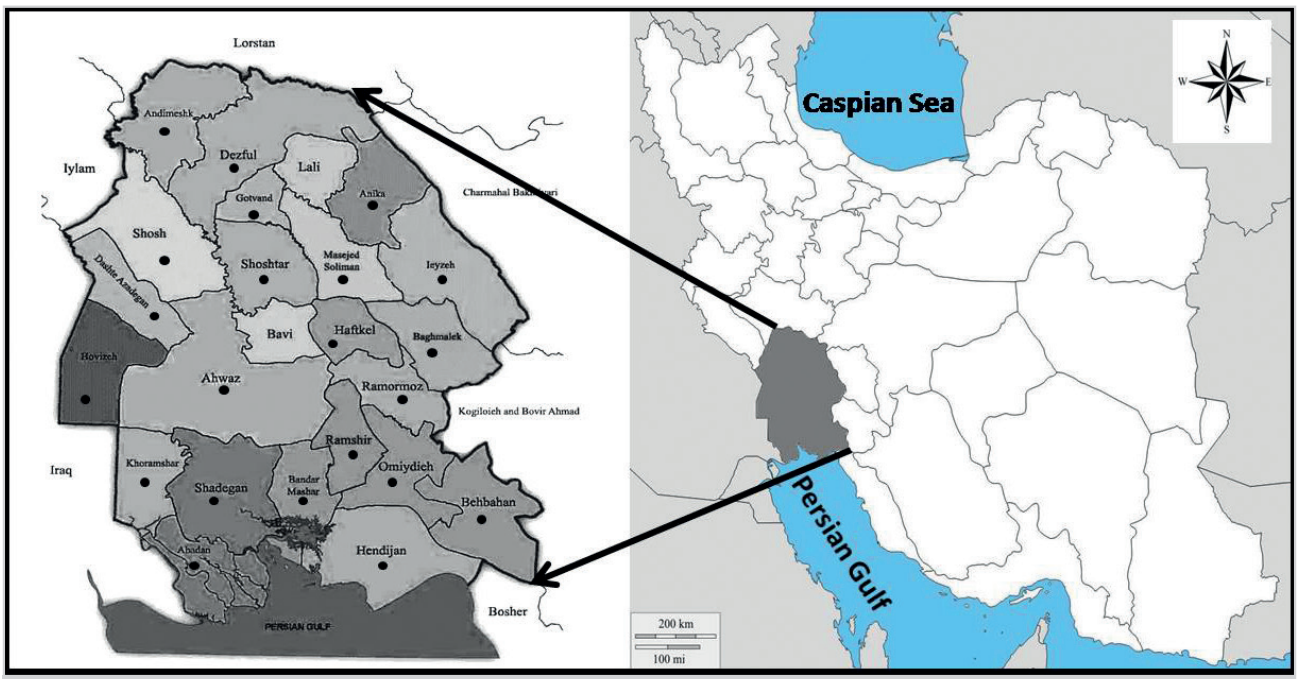

Figure 1. Geographical location of Khuzestan province in southwest of Iran. Sampled counties for *Anaplasma* spp. infection prevalence assessment are denoted by black circles

Leyzeh, Baghmalek, Haftkel, Ramhormoz, Ramshir, Dasht-Azadeghan, Ahwaz, Hoveizeh, Omidiyeh, Behbahan, Hendijan, Mahshahr, Shadegan, Abadan, Khoramshahr (Figure 1). Sample size was estimated based on a prevalence of 15%, a confidence level of 95%, and a precision of 0.5. In addition, a personal interview was conducted via a standardized questionnaire on farm management. Use of the chemical acaricides and kind of vectors (Tick/Mosquito) were recorded according to the farmer's statements.

The variables of climate, altitude, latitude, season, farm type, hygiene, vectors, use of acaricide, distance from other farms, farm density, race, age, sex and milk yield were recorded for each animal. Blood samples were taken from the jugular vein of each animal using vacuum tube containing the anticoagulant Ethylene Diamine Tetra-Acetic acid (EDTA), (Ava Co., Tehran, Iran). The blood samples were stored at -20˚C until DNA extraction.

DNA extraction

DNA was extracted using the DNA isolation kit [Molecular Biology System Transfer, Iran] according to the manufacturer's instructions. The qualification analysis was determined using spectrophotometer (Varian Medical Systems, Palo Alto, CA, USA) at wavelength of 260 and 280 nm. The purification of the extracted DNA was conducted by OD260/OD280 ratio. The quantification analysis of the extracted DNA was performed using 1.5% agarose gel electrophoresis.

PCR and specific nPCR

For the identification of all *Anaplasma* species, a first PCR was used to amplify almost a 1468 bp fragment of the 16S rRNA gene containing the hyper variable (V1) region. The first PCR was performed using the universal primers fD1 and Rp2, in 50 µL total volume including one time PCR buffer, 1.25 U Taq Polymerase (Cinnagen, Iran), 0.4 µM of each primer, 0.2mM of each dATP, dTTP, dCTP and dGTP (Cinnagen, Iran), 1.5mM $MgCl_2$ and approximately 100-500 ng extracted DNA in automated thermocycler (Bio-Rad T100, Bio-Rad Laboratories Inc., CA, USA) using the following program: 5 min incubation at 95°C to denature double strand DNA, 40 cycles of 45 s at 94°C (denaturing step), 45 s at 55°C (annealing step) and 1.5 min, at 72°C (extension step) (Weisburg et al., 1991).

Specific internal primer sets targeting the V1 region of the 16S rRNA were used to detect *A. bovis* and *A. phagocytophilum* (Barlough et al., 1996; Kawahara et al., 2006). Specific nPCR reactions were performed directly with 1 µL of the primary PCR product separately. The nPCR for *A. bovis* and *A. phagocytophilum* was performed in 25 µL total volume.

The nPCR for detecting *Anaplasma centrale* (Amori strain) was performed as described by Inokuma et al. (2001).

The *A. marginale msp4* gene was amplified by MSP45/MSP43 primers as reported previously by de la Fuente et al. (2002) in a 25 µL volum PCR. The PCR and nPCR products were analyzed on 2% agarose gel in 0.5 times Tris-Borate-EDTA buffer and visualized using ethidium bromide (Merck, Darmstadt, Germany) and UV-transilluminator (Vilber Lourmat, Marne-la-Vallee Cedex, France). The primers are listed in Table 1.

The PCR products were purified with a MBST Gel extraction Kit (MBST, Tehran, Iran) and submitted for sequencing to Pishgam Biotech Co. (Tehran, Iran). The PCR product was sequenced three times in one direction. The *A. marginale* and *A. phagocytophilum* 16S rRNA gene sequences were deposited to GenBank under accession numbers MG757665 and MG768969, respectively.

Categorization and classification of evaluated risk factors

Risk factors were categorized and classified as Climate (Mountain, Plain), Altitude (500-1000, <500), Latitude (32-33, <31),

Table 1. PCR and n-PCR tested including primers, annealing, cycling conditions and PCR product length

Name of primer	Publication references and Accession No. in GenBank	Nucleotid sequences	Annealing temp (C°)	No. of cycles	PCR-product
fD1	Weisburg et al., 1991	5`AGAGTTTGATCCTGGCTCAG 3`	55	40	1468 bp
Rp2	AF414399	5'ACAGCTACCTTGTTACGACTT3'			
Anaplasma phagocytophilum sense	Barlough et al., 1996	5'GTCGAACGGATTATTCTTTTATAGCTTGC 3'	50	35	926 bp
Anaplasma phagocytophilum Antisense	M73220	5'CCCTTCCGTTAAGAAGGATCTAATCTCC 3'			
Anaplasma bovis sense	Kawahara et al., 2006	5'CTCGTAGCTTGCTATGAGAAC3'	55	35	551 bp
Anaplasma bovis Antisense	U03775	5'TCTCCCGGACTCCAGTCTG3'			
Anaplasma centrale (Amori strain) sense	Inokuma et al., 2001	5'CAAATCTGTAGCTTGCTACGGA3`	54	35	403 bp
Anaplasma centrale (Amori strain) Antisense	AF283007	5' GAGTTTGCCGGGACTTCTTCT 3'			
MSP45	de la Fuente et al., 2002	5'GGGAGCTCCTATGAATTACAGAGAATTGTTTAC3'	56	35	866 bp
MSP43	AF393742	5'CCGGATCCTTAGCTGAACAGGAATCTTGC3'			

PCR: Polymerase Chain Reaction

Season (Fall, Summer), Farm type (Semi-Industrial, Traditional), Hygiene (Good, Low, Normal), Vectors (Mosquito, Tick), Use of acaricide (No, Yes), Distance from other farms (<1Km, >5Km), Farm density (High, Low, Normal), Race (Hybrid, Native), Age (<1 Year, 1-3 Years, 3-5 Years, >5 Years), Sex (Female, Male), Milk yield (High, Low, Normal, Without).

Statistical analysis

A multiple logistic regression was performed for analyzing risk factors by using Statistical Package for Social Services (SPSS Inc, Chicago, USA) version 18.0. Chi-square (χ^2) test was used to compare the variable factors in the cattle infected with *A. marginale* and *A. phagocytophilum*. A p value less than 0.05 was considered statistically significant.

Results

A total of eighty-eight samples out of two hundred examined generated an expected amplicon of 866 bp from *A. marginale msp4* gene. Following the first PCR for amplifying the 16S rRNA gene of all *Anaplasma* species, positive samples were examined by specific nPCR for detection of *A. phagocytophilum*, *A. bovis* and *A. centrale* (Amori strain). Six of eighty-eight positive samples were giving positivity for *A. phagocytophilum* with nPCR. No samples generated an expected amplicon of *A. bovis* and *A. central* in specific nPCR. The overall prevalence of *A. marginale* and *A. phagocytophilum* infections were 44% and 3% respectively. All infected cattle with *A. phagocytophilum* were also involved with *A. marginale*.

Multivariate analysis of risk factors revealed that cattle of mountain regions were significantly (p<0.0001; OR=1.18) at higher risk as compared to plain regions. Significant association was found among different ages (p<0.002). Cattle <1 year age was (p<0.02; OR=605.3) at lower risk as compared to 1-3, 3-5 and >5 year age. Significant association was found between different latitude (p<0.01), i.e. the latitude 32°-33° (p<0.003; OR=30.48) was at lower risk as compared to <31°. Cattle with low milk yield were significantly (p<0.002; OR=175.86) at lower risk as compared to high, normal and without milk yield.

Low hygienic farms were significantly (p<0.011; OR=0.013) at higher risk as compared to good and normal hygienic farms. Distance from other farms (<1Km) was another important risk factor which showed significant association with the occurrence of *Anaplasma* infection (OR=66.18, p=0.021) (Table 2). There was no significant association between altitude, season, farm type, vectors, use of acaricide, farm density, race and sex with the occurrence of *Anaplasma* infection.

The Chi-square test output showed that the *A. marginale* prevalence was significantly higher (p=0.006) in cattle at latitude <31° as compared to the latitude 32°-33°. The prevalence of *A. marginale* was higher (p<0.0001) in fall as compared to that in summer. Farms with normal hygienic level had significantly higher (p=0.0001) prevalence as compared to those in other hygienic levels.

Table 2. Multivariate analysis of risk factors associated with *Anaplasma* spp. in Khuzestan province, Iran

			Anaplasma spp. Positive			95% Confidence Interval for Odds ratio		
Category	**Level**	**Total N**	**Count**	**Row Total N %**	**p value**	**Odds ratio**	**Lower Bound**	**Upper Bound**
Climate	Mountain	20	12	60.0	0.0001	1.18	4.49	3.12
	Plain	180	76	42.2	-	-	-	-
Latitude	32°-33°C	56	16	28.6	0.003	30.48	3.18	291.33
	<31°C	144	72	50.0	-	-	-	-
Hygiene	Good	10	2	20.0	0.995	3.86	.000	.c
	Low	144	50	34.7	0.011	.013	.000	.372
	Normal	46	36	78.3	-	-	-	-
Distance from other farms	<1Km	196	86	43.9	0.021	66.18	1.90	2296.47
	>5Km	4	2	50.0	-	-	-	-
Age	<1Year	16	4	25.0	0.002	605.30	9.64	37981.46
	1-3Years	56	26	46.4	0.057	32.95	.905	1200.66
	3-5Years	46	26	56.5	0.096	.30	.077	1.23
	>5Years	82	32	39.0	-	-	-	-
Milk yield	High	24	12	50.0	0.162	19.33	.305	1225.55
	Low	80	28	35.0	0.002	175.86	6.51	4744.49
	Normal	24	12	50.0	0.824	.732	.047	11.40
	Without	72	36	50.0	-	-	-	-

c. Floating point overflow occurred while computing this statistic. Its value is therefore set to system missing.

The presence of mosquito vectors in farm was found to be significantly associated to the prevalence of *A. marginale* infection (p=0.002) than presence of tick vectors. Farms with acaricide treatment showed significantly a higher (p=0.007) prevalence of *A. marginale* infection as compared to other farms. No significant association was found between prevalence of *A. marginale* infection and climate, altitude, farm type, distance from other farms, farm density, race, age, sex and milk yield (p>0.05).

The highest prevalence of *A. phagocytophilum* was observed (p<0.0001) in fall as compared to summer significantly.

Farms with normal hygienic level had a higher (p<0.0001) prevalence of *A. phagocytophilum* infection as compared to other farms (good and low hygienic level).

The higher infection rates of *A. phagocytophilum* were observed in the farms with normal density (p=0.004) than the farms with low or high density.

Farms with acaricide treatment showed significantly higher (p=0.042) prevalence of *A. phagocytophilum* infection as compared to other farms. No significant association was found between prevalence of *A. phagocytophilum* infection and climate, altitude, latitude, farm type, vectors, distance from other farms, race, age, sex and milk yield (p>0.05).

Discussion

The present study is the first molecular epidemiology in Iran to estimate the overall prevalence for *Anaplasma* spp. and recognize risk factors significantly associated with highly infected animals. Since dairy cattle breeding in Khuzestan province is more common in the shape of semi-industrial and traditional type dairy farms, samples were collected from these farms. Molecular results showed that *Anaplasma* species were frequent and widely distributed in Khuzestan province of Iran. In another study, overall molecular prevalence for *Anaplasma* spp. has been recorded at 38.7% of cattle in the central region of Iran (Noaman et al. 2009).

In the climate category, cattle in mountain regions where the elevation is between 735-482 m above the sea level and average temperature is between 26.9°C-31.8°C, had 1.18 times higher positivity than cattle in the plain regions where the elevation is between 0-307 m above the sea level and average temperature is between 30.3°C-41.2°C. It can be predicted that microclimate in mountainous area is more suitable than plain areas for plant growth, cattle breeding and tick proliferation. Therefore, the presence of tick-borne disease agents such as *Anaplasma* species in mountainous cattle is more likely in these areas compared to plain areas (Dantas-Torres, 2015). Studies in other geographical regions (Central and South America) have also revealed that the tick borne diseases are detected at a higher altitudes (mountain) than where the diseases was present in the recent past (plain) (Estrada-Peña and Salman, 2013; Milner and van Beest, 2013).

Clinical signs of anaplasmosis mainly appear in cattle older than one year. However, cows of all ages are susceptible to anaplasmosis (Aubry and Geale, 2011; Atif, 2015; Kocan et al., 2010). The present study revealed that the group of cattle under one year age was at a lower risk compared to other age groups. The good level of protective colostral immunity and lower exposure to *Anaplasma* spp. vectors have impact on the lower risk of anaplasmosis in this age group. Relation of anaplasmosis with age in this study was supported by the finding of other researchers in Brazil, Bangladesh, India and Pakistan (Amorim et al., 2014; ; Atif et al., 2013; Rahman et al., 2015; Sharma et al., 2015).

The latitude 32°-33° was at lower risk when compared to <31° for *Anaplasma* spp. infections. The higher infection rate was seen in cattle of Ramshir, Omidiyeh, Behbahan, Hendijan, Mahshahr, Shadegan, Abadan and Khoramshahr at latitude <31°. Global warming may have different impact in the epidemiology of vector-borne diseases such as anaplasmosis. It has been demonstrated that the changes in climate including temperature levels can cause changes in the geographic distribution of ticks and other vectors in new latitudes (Milner and van Beest 2013). The *A. marginale* prevalence in cattle was significantly higher at latitude <31° than 32°-33°. The findings show that, vectors survived better at this latitude. The information on geographical latitude incidence patterns is important for the practitioners in planning the control. Since the disease occurred mainly in the southern parts of the province, vectors and transmission pattern in this region should be identified.

The present study confirmed that low yielding cattle have significantly lower risk when compared to high-yielding, moderate-yielding and non-milkers, in parallel with the results of da Silva and da Fonseca (2014). In another study, da Silva and da Fonseca (2014) found association between milk yield and seroprevalence for *A. marginale* in cattle. They observed that dairy cattle with higher milk production had 0.78 times chance to be more seropositive than animals with lower milk production. They suggested that lactation stress along with per parturient hormonal changes have some impact on immunosuppression status in animals and maintenance of anaplasmosis (da Silva and da Fonseca, 2014).

It may be expectable that farms in an isolated area and far from other farms are at very low risk of disease transmission. The present study showed that the farms with less than one km distance to each other played a main role as a risk factor which had significant association with the occurrence of anaplasmosis. There is no confirmed report about association between "distance between farms" and infection with *Anaplasma* species. To our knowledge, this is the first study that found "distance between farms" is an important risk factor of anaplasmosis.

Farms with good hygienic level had significantly lower prevalence than those in other hygienic levels. Previous studies indicated the hygienic management was one of the potential risk factors for anaplasmosis (Kispotta et al., 2017; Sajid et al., 2014).

There was any relation with the prevalence of anaplasmosis and the use of acaricide in the present study. The results of this paper disagree with Atif et al. (2013) who observed a significant relation between the moderate acaricide application within 60-90 days and seroprevalance to *A. marginale* in cattle.

Da Silva and da Fonseca (2013) observed a significant association between high animal density and high prevalence of anaplasmosis. In the current study, we found no evidence to suggest that farm density was associated with the prevalence of anaplasmosis.

Tick infestation is identified as an important risk factor which has significant association with the occurrence of *Anaplasma* infection (Atif et al., 2013; Costa et al., 2013; da Silva et al., 2014; da Silva and da Fonseca, 2014; Rahman et al., 2015;). Use of chemical acaricides and kind of vectors (Tick/Mosquito) were recorded according to the farmer's statements. Usually, acaricide spraying on the body of the cattle is a simultaneous method in case of the presence of the tick or mosquito on the skin of the cattle and thus, in these farms the livestock has been exposed to pathogens. This probably explains the higher prevalence of *A. marginale* in herds with acaricide treatment.

Only cattle in the rural areas of Gotvand and Shoshtar cities were infected by *A. phagocytophilum*. The climatic conditions in these areas are different from those in other Khuzestan zones. These areas have a lower average temperature and less than 100 meters altitude above sea level. The six positive cases were from traditional small scale cattle farms and pasture grazing was the main feed source. In pasture grazing feeding, the cattle have a great likelihood of tick infestation, so indoor housing hygiene and acaricide treatment do not have a great impact on control of tick-borne diseases in indoor housing.

Although season, race, sex, and vectors were reported as risk factors by Sajid et al. (2014) and Rahman et al. (2015), there was no significant association between these factors and infections with *Anaplasma* species.

Conclusion

The present study shows that in Khuzestan province the tropical region of Iran, infections were caused by *Anaplasma* spp. and the prevalence of anaplasmosis was 44%. The mountain regions, age, latitude, milk yield, farm hygiene and distance from other farms are the major risk factors associated with molecular prevalence to *Anaplasma* spp. in dairy cattle in Khuzestan, Iran. It can be a guide to strategic control programs for anaplasmosis in this area. There was no significant association between altitude, season, farm type, vectors, use of acaricide, farm density, race and sex with the occurrence of *Anaplasma* infection. Further studies are needed on the identification of biological and mechanical vectors of *Anaplasma* species in this region.

Ethics Committee Approval: Ethics Committee approval was received for this study from the Animal Ethics Committee of Agricultural Research, Education and Extension Organization (AREEO) (2016/48445/2).

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – V.N.; Design – V.N.; Supervision – V.N.; Resources - V.N.; Materials – V.N., M.M.; Data Collection and/or Processing – V.N., M.M.; Analysis and/or Interpretation – V.N., M.M.; Literature Search – V.N.; Writing Manuscript - V.N.; Critical Review – V.N.

Acknowledgements: We appreciate the farmers for their cooperation with this study.

Conflict of Interest: The authors have no conflict of interest to declare.

Financial Disclosure: This study was supported by the Isfahan Agricultural and Natural Resources Research and Education Center, Agricultural Research, Education and Extension Organization (AREEO), Isfahan, Iran (Project Number: 2015-48445).

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