

# Isolation and Molecular Detection of Pasteurellosis from Pneumonic Sheep in Selected Areas of Amhara Region, Ethiopia: An Implication for Designing Effective Ovine Pasteurellosis Vaccine

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**Introduction:** Pneumonic pasteurellosis mainly caused by bacterial species of *Mannheimia*, *Pasteurella*, and *Bibersteinia* causes a significant financial loss to the sheep production sector through reduced productivity and high mortality. There is a dearth of information on the major agents involved in the disease in the Amhara region, Ethiopia. Therefore, the aim of this study was to isolate and molecularly confirm *Mannheimia*, *Pasteurella*, and *Bibersteinia* from nasal swabs of sheep suspected of pneumonic pasteurellosis in selected areas of the Amhara region.

**Methods:** Isolation and phenotypic characterization were performed using microbiological and biochemical testing according to standard methods. Molecular confirmation of isolates was done through amplification of virulence associated genes, *PHSAA* and *Rpt2*, of *Mannheimia hemolytica* causing multiplex PCR.

**Results:** Accordingly, 46 out of 141 (32.62%) samples were presumably identified as *M. hemolytica* with no *Pasteurella multocida* and *Bibersteinia trehalosi*. Seven (n=7) out of the 46 isolates tested positive for either of the two virulence genes.

**Discussion and conclusion:** The finding of this study is indicative that *M. hemolytica* is the main bacteria linked with pneumonic pasteurellosis in the study area which suggests the need to develop a polyvalent vaccine including strains of *M. hemolytica* or its antigenic determinants. However, the role of other bacterial, viral, and parasitic agents in the cases investigated should also be considered.

**Keywords:** *Bibersteinia trehalosi*, isolation, *Mannheimia hemolytica*, *Pasteurella multocida*, *PHSAA*, pneumonic pasteurellosis, *Rpt2*, sheep

## Introduction

Ethiopia is known for its high and diverse sheep population with an estimate of 43 million sheep, of which 10.4 million are found in the Amhara region, one of the administrative regions in the country.<sup>1</sup> In addition to serving as source of immediate cash, sheep production provides an insurance in instances of low crop productivity due to erratic rainfall, severe erosion, frost, and water logging problems.<sup>2,3</sup> Thus, increasing the current level of sheep productivity is essential to meet the demands of the ever-increasing human population in the country.<sup>4</sup>

Of the several threats to the production sector, pneumonic pasteurellosis is a major concern for being economically devastating by causing reduced live weight, delayed marketing, increased treatment cost, and loss of animals in cases of outbreaks.<sup>5</sup> Clinically, an acute infectious disease is triggered when the immune system of animals is compromised by stress factors such as overcrowding, transportation, drought, extreme weather and other concurrent disease.<sup>6</sup> The main causative

agents of the disease are *Pasteurella multocida*, *Mannheimia hemolytica*, and *Bibersteinia trehalosi*, commensals of the upper respiratory tract of sheep, which descend to the lower respiratory tract when animals become immunosuppressed.<sup>7-9</sup>

Vaccination with inactivated *P. multocida* biotype A vaccine (National Veterinary Institute, Ethiopia) is currently practiced nationally on an annual basis to curb the impact of the disease. Nevertheless, outbreaks of pneumonic pasteurellosis are reported yearly in different parts of the country including the Amhara region. Among the possible reasons for limited vaccine efficacy, is a mismatch between the vaccinal and bacterial strains involved, particularly, when immunity against the disease is serotype-specific.<sup>10-12</sup> Despite few reports based on prospective clinical or bacteriological examinations, there are no studies conducted to isolate and molecularly confirm the causative agents involved in such types of outbreaks and supposed cases of pneumonic pasteurellosis in the Amhara region. Determination and characterization of the major pathogens involved is an essential input for vaccine producers to formulate effective vaccines.<sup>13</sup> Therefore, the aim of this study was to isolate and molecularly detect *M. hemolytica*, *P. multocida*, and *B. trehalosi* from pneumonic sheep.

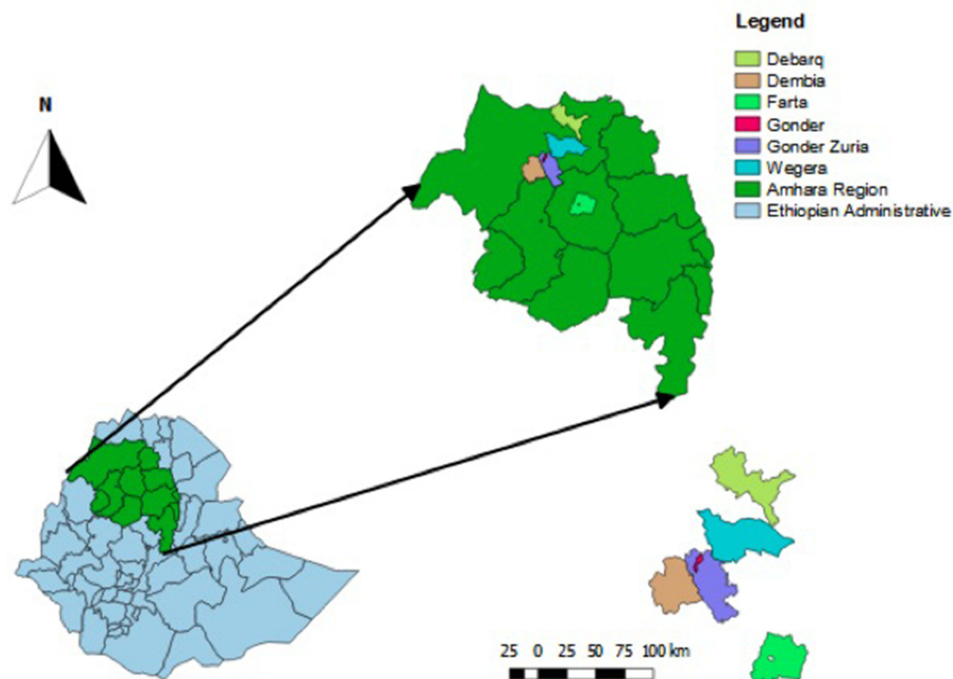
## Materials and Methods

### Study Area

The study was conducted in selected districts and towns of central, north, and south Gondar zones of the Amhara region, which includes Gondar town, Debarq town, Dembia, Gondar Zuria, Wegera, and Farta districts (Figure 1).

### Study Animals

Animals included in this study were sheep presented to the veterinary clinics found in the study sites having signs of respiratory problems suspected as pneumonic pasteurellosis. Therefore, sheep manifesting the clinical signs: coughing, dyspnea, lethargy, oculonasal discharges (serous to mucopurulent), and fever were considered for bacteriological sampling irrespective of their age, sex, breed, physiological, and vaccination status. Animals were handled and approached humanely, and have received the standard of veterinary care during their visits to the clinics.



**Figure 1** Map of the study sites. Amhara region, northwest of Ethiopia and the various towns and districts within the Amhara region included in the study.

## Study Design and Sampling Technique

A cross-sectional study design and purposive method of sampling was employed with the objective of isolating and identifying *M. hemolytica*, *P. multocida*, *B. trehalosi* from sheep suspected of having pneumonic pasteurellosis from November 2019 to May 2020. The study districts were selected purposively based on prior information on the problem and availability of sheep population. A total of 141 sheep fulfilling the clinical diagnostic criteria were sampled from the different study sites as follows: Gondar town (n=24), Debarq town (n=25), Dembia district (n=27), Gondar zuriya district (n=10), Wegera district (n=23), and Farta district (n=32).

## Sampling Procedure

During sample collection each animal was individually identified and restrained by an assistant. External nares were decontaminated using a cotton soaked with 70% ethanol prior to sampling.

Next, sterile cotton-tipped swabs moistened with tryptone soy broth (Oxoid, Hampshire, England) were inserted into the nasal cavities of each sheep and the mucus surface was rubbed by rotating the swabs on both nostrils gently. The swabs were allowed to remain in contact with the secretions for up to one minute and were placed back into labeled sterile universal tubes containing 3 mL of sterile tryptone soy broth (Oxoid). Labeled samples of nasal swabs were transported in icebox to the veterinary microbiology laboratory of the University of Gondar (UoG), College of Veterinary Medicine and Animal Science (CVMAS) for microbiological analysis. Samples were either processed immediately upon arrival or maintained and preserved at +4°C until further process.<sup>14</sup>

## Bacteriological Assessment

Nasal swabs were incubated in tryptone soy broth (Oxoid) for 24 h at 37°C after which they were streaked into blood agar containing 5% defibrinated sheep blood (Titan Biotech Limited, Bhiwadi, India). Blood agar plates were then incubated at 37°C for 48 h. Subsequently, from culture-positive plates, typical colonies were Gram stained to determine staining property and cellular morphology under light microscope. Those colonies showing a Gram reaction and colony characteristics indicative of *Pasteurella* were subcultured on blood and MacConkey agar for further analysis. The presence of growth in blood agar without hemolysis and lack of growth in MacConkey is considered suggestive of *P. multocida*. Colonies were presumptively identified to be *M. hemolytica* and *B. trehalosi*, when they grow on blood agar (round, translucent and grayish colonies) with typical  $\beta$ -hemolytic pattern, and grow on MacConkey agar appearing pink pinpointed colonies, an indicator of lactose fermentation.<sup>15</sup>

## Biochemical Identification

Pure cultures of single colony type from both blood and MacConkey agars were subcultured on nutrient agar-slants at 37°C for 24 h for a series of primary and secondary biochemical tests. The biochemical tests were done as described previously.<sup>15</sup> Finally, colonies presumably identified as *M. hemolytica* were subcultured on 8 mL nutrient broth and preserved using 98% 2 mL glycerol for transportation into NVI, Bishoftu, Ethiopia for molecular identification.

## Molecular Detection

### Extraction of Bacterial Deoxyribose Nucleic Acid

Bacterial DNA extraction was conducted at the molecular biology laboratory of NVI, Ethiopia. A few colonies from the phenotypically characterized pure cultures of *M. hemolytica* grown on nutrient agar for 24 to 48 h were transferred into 1.8 mL Eppendorf tubes.<sup>16</sup> Bacterial DNA was extracted using Qiagen DNeasy Blood and Tissue Kit as per manufacturer's instructions (Qiagen, German town, MD, USA).

### Detection of Virulence Genes of *M. hemolytica* Using Multiplex PCR

Multiplex PCR (mPCR) was performed by simultaneously amplifying the two virulence-associated genes of *M. hemolytica*, i.e. *PHSSA* (*P. hemolytica* serotype specific antigen), a serotype specific antigen of *M. hemolytica*, and *Rpt2*, a gene coding for methyltransferase as reported previously.<sup>16</sup> The primer design and size of amplified products is provided in Table 1. The reaction mixture for the PCR comprised: 10  $\mu$ L of IQ Supermix (Bio Rad, USA) (DNA polymerase, dNTPs and buffer), 2  $\mu$ L

**Table 1** Primers Sequences Used in the Amplification of *PHSSA* and *Rpt2* in Molecular Detection of *M. hemolytica*

Target Genes	Primer	Sequence (5'→ 3')	Amplified Products	Reference
<i>PHSSA</i>	Forward	5' -TTCACATCTTCATCCTC-3'	325 bp	16
	Reverse	5'-TTTTTCATCCTCTTCGTC-3'		
<i>Rpt2</i>	Forward	5'-GTTTGTAAGATATCCCATTT-3'	1022 bp	16
	Reverse	5'-CGTTTTCCACTTGCGTGA-3'		

(5 pM/μL) of each primer pairs, 3 μL of nuclease-free water and 4 μL DNA template, totaling a reaction volume of 25 μL. Regarding the PCR condition, initial denaturation was performed at 95°C for 3 min, after which 35 cycles of each at 95°C for 1 min were completed. Then, annealing took place at 48°C for 1 min followed by an initial extension at 72°C for 30 seconds, and a final extension cycle at 72°C for 5 min.<sup>16</sup> Three reaction tubes: with DNA extraction control, without the DNA template (nuclease-free water), and with a DNA template from reference *M. hemolytica* from NVI culture pool (MH-NVI) were included as extraction, negative and positive controls respectively.

### Visualization of PCR Products Through Agarose Gel Electrophoresis

Agarose gel (2% w/v, 0.5× Tris borate EDTA buffer) stained with gel red was utilized in the analysis and detection of the PCR products. Each PCR product (5 μL) were mixed with 6× loading dye and loaded into a separate well of the preprepared gel while 1 kb plus DNA molecular markers were loaded onto the first and last lane and run at 120 V for 60 min on electrophoresis apparatus (EC 2060, USA). Finally, the different bands were visualized under UV transilluminator and photographed in a gel documentation system (UVI TEC, UK).

### Data Management and Analysis

Data collected were coded and entered into the Microsoft Excel spreadsheet. Frequency and percentages were used to describe the isolation rate and the distribution across different study sites.

## Results

### Bacteriological and Biochemical Characteristics of Isolates

In this study, *M. hemolytica* was found to be the predominantly identified bacterial species (32.62%, 46/141) from cases of ovine pneumonic pasteurellosis throughout the study areas. Isolation rates as per study sites were as followed: 20.83% (5/24), 29.6% (8/27), 2% (2/10), 39.1% (9/23), 36% (9/25), and 40.6% (13/32) for Gondar town, Debark town, Dembia, Gondar Zuria, Wegera, and Farta districts, respectively (Table 2).

Isolates phenotypically identified as *M. hemolytica* were gram-negative, short ovoid rods arranged in chains with an occasional tendency of bipolar staining. In addition, these isolates were able to grow on blood agar with complete zones of hemolysis. Growth on MacConkey agar was also observed with varying degree of lactose fermentation evident by pinpoint red colonies. Biochemically, isolates were positive for catalase and oxidase while testing negative for methyl red, urease, citrate, and indole tests. Sugar fermentation profile indicated that the isolates were able to metabolize different sugars such as arabinose, maltose, sucrose, glucose, sorbitol and lactose but not trehalose. In this study, *P. multocida* and *B. trehalosi* were not identified as none of the colonies fulfilled the characteristic growth of these species on MacConkey and blood agar, and on the variety of biochemical tests as well (Table 3).

### Molecular Confirmation of Isolates

All of the isolates (n=46) presumably identified as *M. hemolytica* through microbiological and biochemical analysis were further subjected to mPCR test, amplifying *PHSSA* and *Rpt2* genes. Seven (n=7) out of the 46 isolates (15.21%) tested positive for either of the genes, i.e. three (n=3) of them were positive for both *PHSSA* (serotype A1) and *Rpt2* genes; two (n=2) of them tested positive only for *Rpt2* gene; while the remaining samples (n=2) tested positive only for *PHSSA* gene (Figure 2).

**Table 2** Frequency and Isolation Rate of Presumptive *M. hemolytica* in Different Study Areas

Study Sites	Number of Samples Examined	Isolated Bacterial spp.		
		<i>M. hemolytica</i>	<i>P. multocida</i>	<i>B. trehalosi</i>
Gondar town	24	5 (20.83%)	–	–
Dembia district	27	8 (29.6%)	–	–
Gondar Zuria district	10	2 (2%)	–	–
Wogera district	23	9 (39.1%)	–	–
Debark town	25	9 (36%)	–	–
Farta district	32	13 (40.6%)	–	–
Total	141	46 (32.62%)	–	–

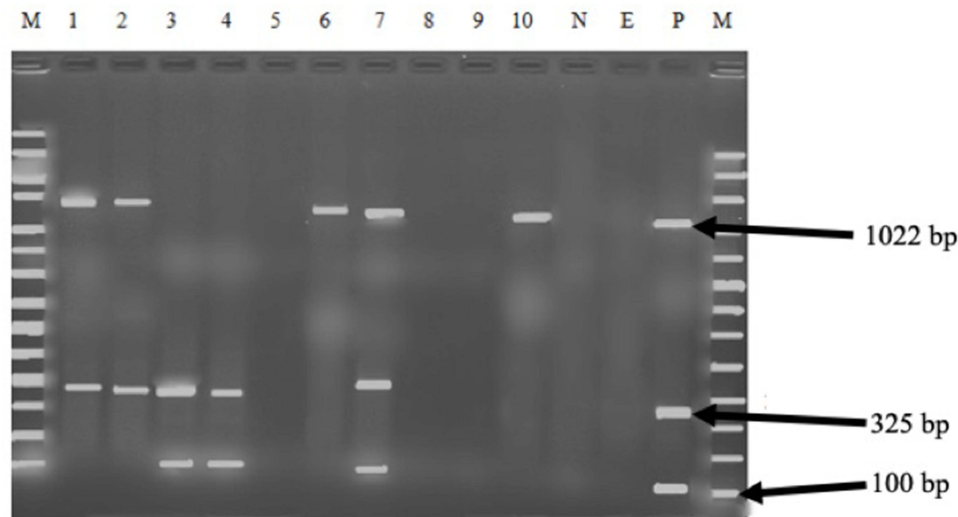
**Table 3** Biochemical Characteristics of Isolates from Cultures of Nasal Swabs of Sheep Affected with Pneumonic Pasteurellosis

Bacteriological/Biochemical Attributes	Expected Reactions of		
	<i>M. hemolytica</i>	<i>P. multocida</i>	<i>B. trehalosi</i>
Beta-hemolysis	+	–	+
Growth on MacConkey	+	–	+
Catalase	+	+	–
Oxidase	+	+	+
Indole	–	+	–
Urease	–	–	–
Citrate	–	–	–
Methyl red	–	–	–
Glucose	+	+	+
Lactose	+	–	–
Sucrose	+	+	+
Sorbitol	+	+	+
Arabinose	+	–	–
Trehalose	–	–	+
Maltose	+	–	+
<b>Number of isolates fulfilling the identification criteria</b>	<b>n=46</b>	<b>Nil</b>	<b>Nil</b>

Notes: "+" = positive reactions; "–" = negative reactions for the respective tests.

## Discussion

Ovine pasteurellosis causes a significant harm to the sheep production sector affecting the livelihood of communities that rely on sheep rearing as well as industries that process sheep meat and their products at large.<sup>17</sup> *Pasteurella multocida* and *Mannheimia hemolytica* are the main causative agents of the disease responsible for 30% of deaths in feedlot cattle and acute outbreaks in sheep population with consequence of massive mortality all across the globe.<sup>9</sup> Several biotypes and serotypes of these bacterial species have been reported with varied distribution across different areas.<sup>6,18</sup> The aim of this study was to isolate *Pasteurella*, *Mannheimia*, and *Bibersteinia* involved in pneumonic pasteurellosis of sheep in selected areas of the northwest Amhara region, Ethiopia. The finding of this study indicated that *M. hemolytica* is the major bacteria associated with cases of pneumonic pasteurellosis in the study areas. Bacteriological and biochemical methods were employed in the isolation and presumptive identification of the bacteria species. Of the 141 cultures of nasal swabs, 46 (32.62%) were tentatively identified as *M. hemolytica*. The microbiological and biochemical features of the isolates, and the clinical feature of affected sheep in the present study are consistent with *M. hemolytica* and pneumonic pasteurellosis described elsewhere.<sup>19,20</sup>



**Figure 2** Multiplex PCR amplification of *PHSSA* and *Rpt2* genes from cultures of nasal swabs of sheep affected with pneumonic pasteurellosis in 2% agarose gel. M; 1 kb plus DNA molecular markers. Lanes 1–4, 6, 7, and 10 positive samples. Lanes 5, 8, and 9 negative samples. **Abbreviations:** N, negative control; E, extraction control; P, positive control.

This finding agrees with previous studies, which reported that *M. hemolytica* is the most common agent isolated in cases of ovine pneumonic pasteurellosis.<sup>13,21,22</sup> The recovery rate of *M. hemolytica* in this study is lower than reports of Legesse et al (2018)<sup>13</sup> in central Ethiopia (34.21%); Aschalew (1998)<sup>23</sup> in North Shewa (35%); Mekonnen<sup>24</sup> in Arsi (48%); Alemneh et al<sup>25</sup> in Fogera wereda (79.5%); Abera et al<sup>26</sup> in Bedelle districts (46%); and Kaoud et al<sup>27</sup> in Egypt (52%). This variation could arise from various factors including difference in sample size and season of sample collection, geographical variation, site of sampling, and sensitivity of tests used. In fact, Ali and Al Balaa<sup>28</sup> reported a significant increase in isolation rate of *M. hemolytica* in summer and winter as compared to spring. Contrary to our findings, Marru et al,<sup>21</sup> Deressa et al,<sup>22</sup> Abera et al,<sup>26</sup> and Tesfaye<sup>29</sup> reported to isolate either *B. trehalose* or *P. multocida* or both, none of which were recovered in the present study. Similarly, variation in sample size, geographical areas, animal factors (age for example), and management practice between the study sites could explain why those species were not isolated in the current study. Of note, Alemneh et al<sup>25</sup> reported a significantly higher frequency of infection with pneumonic pasteurellosis in winter and spring compared to autumn and summer. Age-wise, *B. trehalosi* is reported to cause a septicemic disease in young lambs which often emerges in the form of severe outbreaks.<sup>30</sup> The application of monovalent vaccine derived from *P. multocida* biotype A (NVI, Ethiopia) in our study areas may have lessened the contribution of *P. multocida*, thus, its isolation from diseased animals.

In this study molecular confirmation of *M. hemolytica* was performed through mPCR, simultaneously amplifying the genes, *PHSSA* and *Rpt2*. Seven (n=7) out of the 46 the isolates were confirmed to harbor those genes, which is contrary to the studies of Legesse et al<sup>13</sup> and Hawari et al<sup>31</sup> which reported that all isolates identified to be *M. hemolytica* through microbiological and biochemical tests were all found to be positive for PCR detection.

The serotype specific antigen, PHSSA, is reported to play a role in conversion of commensal microbes into pathogenic in stressful situations indicating its involvement in pathogenesis of pneumonic pasteurellosis due to *M. hemolytica*.<sup>16,32</sup> In contrast, the species-specific *Rpt2* gene, coding for methyltransferase, plays an important role in protection of bacterial DNA from endonuclease cleavage by catalyzing the methylation of a specific DNA recognition sequence designating it a self-DNA. Thus, *Rpt2* takes part in modulation of type III restriction-modification system.<sup>16,33</sup> Therefore, *Rpt2* and *PHSSA* genes of *M. hemolytica* are appropriate molecular diagnostic targets.<sup>16</sup> As mentioned earlier, isolates (n=39) passing the phenotypic criteria of *M. hemolytica* tested negative for both of the virulence-associated genes. These isolates are likely to belong to serotypes other than A1 (as the PHSSA used was of A1 serotype) or nonpathogenic strains of *M. hemolytica*.<sup>34</sup>

Ovine pasteurellosis is a disease of multifactorial etiological agents. In addition to the external factors, the presence of different bacterial biotypes and serotypes with immunity that does not cross protect further complicates the problem. As mentioned earlier, presently, a monovalent vaccine based on *P. hemolytica* biotype A (NVI, Bishoftu, Ethiopia) is used in Ethiopia. Infection with one bacterial species or serotype does not confer protection against infection or disease by other bacterial species or serotypes.<sup>10–12</sup> Thus, vaccination with a monovalent *P. hemolytica* biotype A based vaccine is unlikely to provide adequate protection against diseases caused by *M. hemolytica* which is indicated to be the prevailing agent associated with pneumonic pasteurellosis in the study areas. Therefore, this suggests the need to develop a multivalent vaccine that incorporates appropriate bacterial strains of *M. hemolytica* or its antigenic determinants for enhanced vaccine effectiveness and disease control strategies.

## Conclusion

The current study reports the first attempt to isolate and molecularly detect *M. hemolytica* from pneumonic sheep in selected areas of the Amhara region, Ethiopia. Our finding is indicative of the significant association of *M. hemolytica* with pneumonic pasteurellosis in the study area. The current vaccination strategy using inactivated *P. hemolytica* biotype A vaccine could provide little-to-no protection against outbreaks of pneumonic pasteurellosis that may involve diverse serotypes of *M. hemolytica*, which may call for the need to develop a polyvalent vaccine including *M. hemolytica* or its antigenic determinants. This, in turn, could augment other control and prevention strategies in the region, thus, avoiding the drastic consequences of the disease. However, the study did not cover significant other parts of the region, which entails further studies to identify and molecularly (phylogenetically) characterize the etiological agents implicated in the disease. Finally, the involvement of other potential pathogens in the respiratory cases investigated in this study needs to be taken into consideration.

## Abbreviations

CVMAS, College of Veterinary Medicine and Animal Sciences; DNA, deoxyribonucleic acid; mPCR, multiplex polymerase chain reaction; NVI, National Veterinary Institute; PHSSA, *P. hemolytica* serotype specific antigen; UoG, University of Gondar.

## Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon a formal request.

## Ethical Approval and Consent to Participation

This study has obtained an ethical approval by institutional Review Board of UoG, CVMAS. Animals were approached with great care according to the guidelines for ethics of animal research. Moreover, animal owners were consented, and the benefits and outcomes of the study explained for the study participants.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The authors report no conflicts of interest in this work.

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