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Molecular Detection and Characterization of Haemoplasmas in Different Animal Species in Egypt

Sabry I. Eissa, Abdelaziz E.E, Hassan A.M., Yousreya H. M., Sahar E. Ouda,* Laila M.El shabiny.

Department of Mycoplasma, Animal Health Research Institute,Agricultural Research Center(ARC), Giza, 12618, Egypt.

BACKGROUND: *Hemotropic mycoplasmas* (HM) the non-cultivable bacteria cause infectious anemia in many animals and humans.

Objective: Molecular detection, partial sequencing and phylogenetic analysis of the 16S rRNA of haemoplasmas in some animal species in Egypt.

Method: Blood samples with anticoagulant were collected from jugular vein of camels, sheep, goats, dogs and cats. DNA was extracted and different PCR assays were applied and sequenced.

Results: *Candidatus M. haemoovis* was detected in 10% of sheep in Beni- Swef governorate and in 14.46% of goats in Gharbia and the PCR resulted in the specific fragment at 506 bp. Samples from both sheep and goats showed high similarity with the homologous species on GenBank. *Candidatus Mycoplasma haemolamae* of camels CMhl, was detected in Shalateen in 8.33% of examined camels andshowed a specific fragment at 310pb. Haemoplasma incidence in cats was 32% in Giza governorate. *Candidatus M haemominutum* (CMhm) and *Candidatus M. turicensis* (CMt) were detected in 16% of the examined cats and gave a specific fragments (202 bp) and (138 bp) respectively. *Mycoplasma haemofelis* could not be detected at all. *Candidatus M. haemominutum* (CMhm) showed high similarity with the homologous species on GenBank. *Candidatus Mycoplasma haematoparvum* (CMhp)was detected in dogs in Dkahlia governorate with 22% incidence and gave specific band at 380bp.

Conclusion: Haemoplasmas are circulating in different animal species in Egypt and PCR technique is the accurate method for detection of haemoplasma and sequencing is confirmatory to the results.

Keywords : Haemoovis, CMhl, CMhm, CMhp, PCR, Egypt.

Introduction

Haemotropic mycoplasmas (HM) are small pleomorphic, wallless, non-cultivable bacteria causing infectious anaemia in a wide range of animals [1]. They attach to the surface of red blood cells and cause deformation and damage to them [2]. In animals, disease symptoms due to haemotropic mycoplasma infection are commonly accompanied with stressors such as poor diet, pregnancy,lactation, or concurrent infections with more virulent pathogens or with drug-induced or virus-induced immunosuppression [3]. To isolate and culture HM, all attempts have failed, so that the analysis of biological features and pathogenesis of the bacteria is limited [2, 4]. The recent development of molecular techniques, as PCR amplification based on the bacterial 16S rDNA gene, has greatly enhanced the molecular characterization of these causative agents. This diagnostic approach has resulted in the investigation of several novel haemoplasma species in different hosts [5- 7]. The first molecular evidence of the occurrence of "*Candidatus* M *haemovis*" in sheep and goats was in China [8]. In Ohio of Canada, *Mycoplasma haemolamae* was detected from *Lama pacos, Lama glama,* and *Lama vicugna* [9]. In Iran, two haemotropic mycoplasmas were found in dromedary camels that are phylogenetically linked to two swine pathogenic species [10]. In Iraq [11], it was concluded that hemomycoplasmosis was circulating in the environment, and the Iraqi local *Mycoplasma hemolamae* isolates was genetically related to NCBI BLAST *Mycoplasma hemolamae* UK isolate (JF495171.1). In cats, infection with *Candidatus*

*Corresponding author: Sahar E.Ouda, E-mail: seoa200@yahoo.com. Tel.:00201100494624
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M. haemominutum does not usually cause anemia, but its co-infection with feline retroviruses can cause anemia. In 1942, feline haemoplasmas in South African cats was first reported. In 1928, *Mycoplasma haemocanis* [*Haemobartonella canis*] was first described in a splenectomized dog in Germany, and until 1939, the name *Bartonella canis* was proposed. After that, Tyzzer and Weinman created the new genus Haemobartonella, and subsequently *M. haemocanis* has been proposed as the species name since 2002 [12]. In host peripheral blood smears, haemoplasma can be seen as a pleomorphic bacterium with coccoid and ring forms that either singly or in chains. It is distributed worldwide with prevalence from 0.5% to 40%. The infections by haemoplasma in dogs are chronic and sub-clinical though infected animals can show heamolytic anaemia, especially when they are immunodepressed or concurrently infected with other pathogen species [12].*Mycoplasma haemocanis* (Mhc) infection causes clinically significant anemia in splenectomized dogs, whereas latent infection can cause subclinical anemia [13].*Candidatus mycoplasma hematoparvum* (CMhp) was first reported in association with anemia in dogs undergoing chemotherapy for leukemia and having their spleens removed [5]. The diagnosis of hemoplasma infection is currently based on the visualization of coccoid bacteria associated with red blood cells in a blood smear, together with the results of specific polymerase chain reaction (PCR) assays.

In the present study, Molecular detection, partial sequencing of the 16S rRNA were used as a molecular approach for phylogenetic analysis and genetic characterization of the haemoplasmas in sheep, goats , camels, dogs and cats in Egypt.

Material and Methods

Animals

Studied animals were brought from different localities of Egypt to the branches laboratories of Animal Health Research Institute(AHRI) located in different governorates showed signs of anemia and emaciation or apparently healthy

Sample collection

A total of 378 blood samples were collected on EDTA from jugular veins (120 camels, 100 sheep and 83goats), and from caudal veins of 50 dogs and 25 cats.The collected samples were stored in a cooler box and transported to the Mycoplasma Laboratory in the main branch of AHRI for DNA extraction.

Extraction of DNA

DNA was extracted from blood samples using Genomic DNA isolation kit (GeneDirex, Cat. no. NA023-0100 (Taiwan).

Polymerase chain reaction (PCR):

-*Detection of haemoplamas in sheep and goat blood samples using a nested PCR assay*.

The first PCR reaction was carried out using the primers, F; 5'-GGATAGCAGCCCGAAAGG-3' and R: 5'-GCAGCCCAAGGCATAAGG-3'. The cycling temperatures were initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 61°C for 30 s, and 72°C for 1min, with a final step of extension at 72° for 7 min. The second reaction of the nested PCR utilized the primers, F:5'-CTACGGGAAGCAGCAGTG-3' and R:5'-CTCGACCTAACATCAAATACCT-3' under the conditions of initial denaturation at at 94°C for 5 min, followed by35 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 45 s, with a final extension step at 72°C for 7 minutes [8].

-*PCR detection of Candidatus M. haemolamae in camel blood samples [14]*

Two primers namely CMhl –F: 5'- TAG ATT TGA AAT AGT CTA AAT TAA -3' andCMhl –R: 5'-AAT TAG TAC AAT CAC GAC AGA ATC -3' were utilized to amplify camel mycoplasma DNA. The amplification conditions consisted of an initial denaturation at 94°C for 10 min, followed by 32 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min with a final extension step of 72°C for 7 min.

- *PCR detection of cat haemoplasmas*

-Different primers were used in a multiplex PCR to detect haemoplasmas in cats blood [3]with modification:

F-5' ATGCCCTCTGTGGGGGATAGCCG'3 *Mycoplasma haemofelis*

R-5' ATGGTATTGCTCCATCAGACTTTCG'3 common reverse primer *Candidatus M.haemominutum*

F-5' CTGGGAAACTAGAGCTTCGCGAGC'3 specific forward (CMhm primer).

-Primer used for *Candidatus M turicensis* primer:CMt

F-5'AGAGGCGAAGGCGAAAACT'3

R-5'CTACAACGCCGAAACACAAA'3

PCR amplification with modification from (3): The PCR reaction included 35 cycles of denaturation (94 °C for 45 s, annealing using a temperature gradient (53 °C to 60 °C for 45 s), and polymerization (72 °C for 45 s).

-Primer used for haemolpasma in dog strain Haemocanis (Mhc) with modification from Barker *et al.,* [15]:

F-5' GTGCTACAATGGCGAACACA'3, R-5' TCCTATCCGAACTGAGACGAA'3

Primer used for haemolpasma in dog strain *Candidatus Mycoplasma haematoparvum* (CMhp) according to the reference [15]:

F-5lGGAGAATAGCAATCCGAAAGGl3, R-5lGCATTTACCCCACCAACAACl3

PCR amplification with modification from Barker *et al.,* [15]: The PCR reaction included one cycle of 5 min at 95°C, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 61°C for 45 s, and extension at 72°C for 60 s. Final extension at 72°C for 10 min.

Nucleotides Sequencing:

PCR products for sequencing were purified using the Purification Kit; Gene JET PCR purification Kit K0701(Thermo Scientific,Litwania).The PCR product of random selected three isolates for 16S ribosomal RNA for haemoplasma were two isolates for haemoplasma ovis 16S, and one for haemoplama of cat Sequenced by GATC Company using Applied Biosystems 3130 Genetic Analyzer (ABI, USA) using forward and reverse primers, combining old Sanger method with new 454 technology.The Edited sequences of the haemoplasma isolate were characterized using EMBL's European Bioinformatics Institute (EMBL-EBI) multiple sequence allignment tool CLUSTAL OMEGA .Phylogenetic tree was created to determine the genetic relatedness of the haemoplasams using Neighbour-joining tree without distance corrections. Sequence alignment using : CLUSTAL O(1.2.4) multiple sequence alignment.

Homology between the isolates and other selected reference isolates was assessed using a sequence identity matrix. - created by Clustal2.1

Results

Incidence of haemoplasma detected in different animal species

As depicted in table (1), out of 378 blood samples of different animal species, 49 (12.96%) were positive for haemoplasmas employing specific PCR amplification assays. Of 100 sheep samples, 10 were positive for *Candidatus* M. haemoovis (10%), all sheep belonged to Beni Suef governorate. The same species was deteted in 12 out of 83 blood samples of goats from Gharbia governorate (14.46%). Of 120 camels at Shalateen region, the Egyptian south border, 12 had *Candidatus M. haemolamae* in their blood (10%).

Concerning pets, *Candidatus* M. haematoparvum (CMhp) was detected in 11 of 50 blood samples (22%) of dogs at Dakahlia governorate. In cats from Giza governorate, 8 out of 22 had haeoplasma in their blood (32%) of which 4 had *Candidatus* M. haemominutum (MChm) (16%) and 4 (16%) had *Candidatus* M. turicensis (Table 1).

The specific PCR products are illustrated in Figures 1-5.

TABLE 1. Incidence of haemoplasma detected in some animals in Egypt

Species	Animals No.	No. of Positive animals	Percentage of positive %	Type of detected Haemoplasma	Governorate	Accession No.
Sheep	100	10	10%	*Candidatus* M.haemoovis	Beni- Suef	OQ310852 M Ovis
Goats	83	12	14.46%	*Candidatus* M.haemoovis	Gharbia	OQ546593 M Ovis 2
Camels	120	12	10%	*Candidatus* M.haemolamae	Shalateen	ND
Dogs	50	11	22%	*Candidatus* M.haematoparvum (CMhp)	Dakahlia	ND
Cats	25	8	32%	*Candidatus* M.haemominutum (MChm) 4/8(16%)	Giza	OQ565624
				Candidatus M. turicensis (CMt) 4/8 (16%)		ND
Total	378	49	12.96%			

ND: Not Done

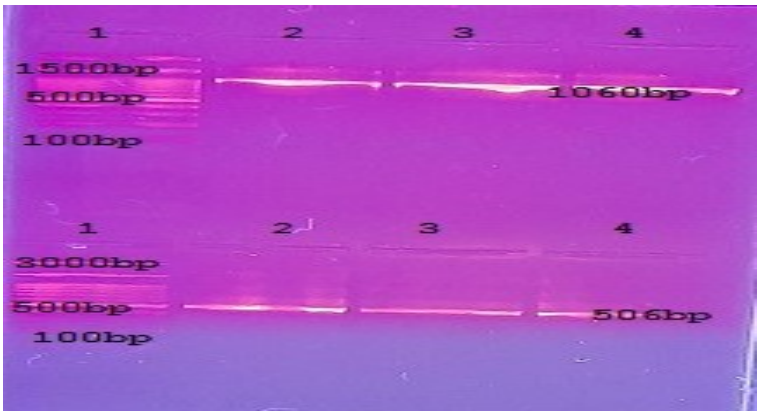

Fig. 1. Upper part: PCR for haemoplasma from sheep and goat at 1060bp, Lower part: Nested PCR for haemoplasma from sheep and goat at 506bp, Lane 1: 100 bp ladder, lanes 2-3 positive sheep samples lane 4: positive goat sample.

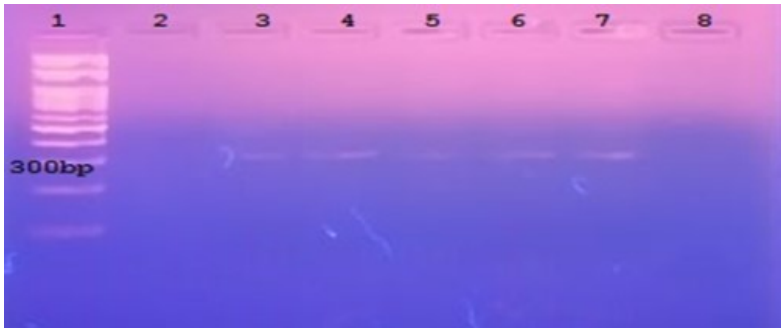

Fig. 2. A. Lane 1: DNA ladder100bp; lanes 2and 8 negative samples; lanes3-7 positive samples for camel haemoplasma 310bp

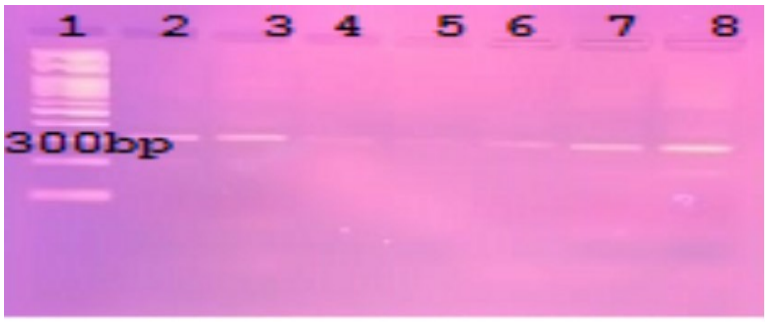

Fig. 2.B. Lane 1: DNA ladder100bp; lanes 2-8 positive samples for camel haemoplasma 310bp

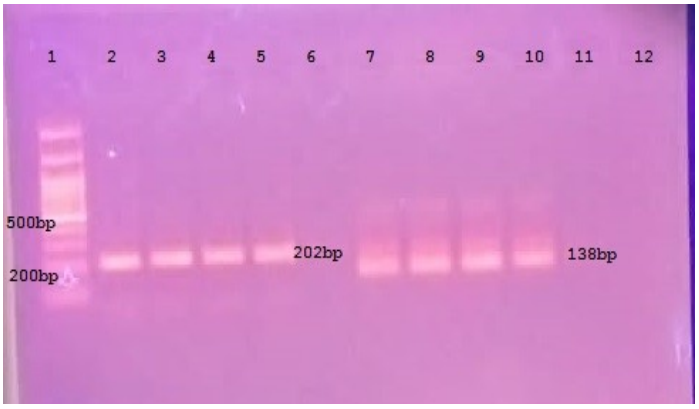

Fig. 3. Lane 1:100bp DNA ladder; lanes 2-5positive samples for *Candidatus M. haemominutum* of cat at 202bp , lanes 7-10 positive sample for *M.turicensis* (CMt) at 138bp.

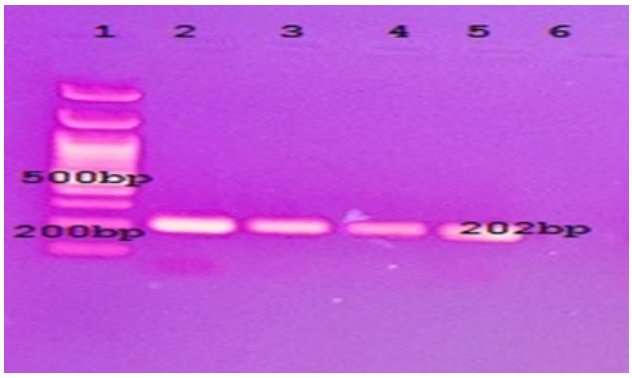

Fig. 4. Lane 1:100bp DNA ladder; lanes 2-5positive samples for *Candidatus M. haemominutum* of cat at 202bp.

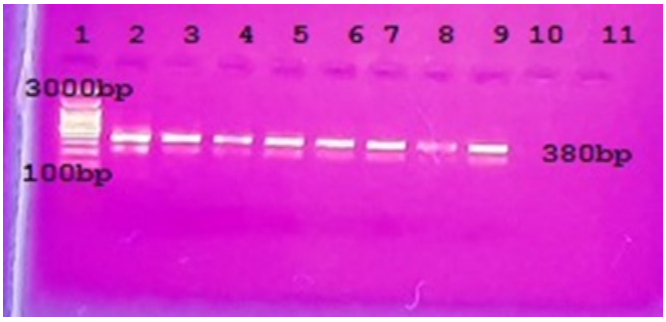

Fig. 5.A. Lane 1: DNA ladder100bp; lanes 2-4 positive samples for Haemoplasma of dog at 380bp

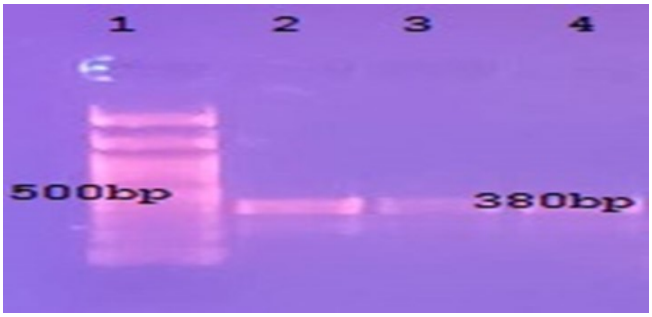

Fig. 5.B. Lane 1: DNA ladde100bp;lanes 2-4 positive samples for Haemoplasma of dog at 380 bp.

Sequencing and Phylogenetic analyses of three haemoplasmas strains

The 16S rRNA gene PCR products of two *M. heamovis* (one from sheep and the other from goat) were sequenced and deposited on the GenBank with the accession numbers OQ310852 and OQ546593, respectively.The molecularly detected *Candidatus M. haemominutum* was deposited with the accession no. OQ565624. The molecularly detected camel haemoplasma and dog haemoplasma are under progress for sequencing and deposition on the GenBank.

Phylogenetic tree for *Candidatus M. haemovis* from sheep and goats

Figure 1 illustrates the phylogenetic tree for the two *Candidatus M. haemovis* strains detected in sheep and goats. Table (2) shows the identity percentages of the aligned sequences between the two stains and with the published sequences. From the table, the similarity between the two strains detected in this study was 99.32%. Meanwhile, the homology between either of the local strains and the similar published caprine and ovine haemoplsmas' sequences exceeded 99% in all comparisons.

Concerning *Candidatus M. haemominutum* sequence alignment, the homology was 100% between strain detected in this study and the similar published sequences of feline haemoplasmas (Table 3).

TABLE 2. Similarity between *M. Haemovis* from Egypt and other countries

Accession no.	Strain name	Animal species	Country	Year of detection	Similarity to OQ310852	Similarity to OQ546593
OQ310852	Egypt field 1	Sheep	Egypt	2023	100%	99.32%
OQ546593	Egypt field 2	Goat	Egypt	2023	99.32%	100%
OP851563	TN-TVM-16	goat	India	2022	99.34%	99.34%
MG018459	TITAHS-T58	Tick infesting (Goat) *Capra aegagrus*	India	2018	99.76%	99.34%
MG018457	DHEKOHS-D19	*Haemaphysalis bispinosa* tick infesting Capra aegagrus Haemaphysalis bispinosa	India	2018	99.34%	99.34%
MH379799	MOBRRS01	Sheep	Brazil	2018	99.76%	99.34%
MF377463	**Mycoplasma ovis isolate ada54**	Sheep	Turkey	2017	99.27%	99.34%
KU983746	Zhumadian 83	Sheep	China	2016	99.51%	99.01%
KU983745	Yunnan 611	Goat	China	2016	99.76%	99.34%
KU983740	Anyang 407	Sheep	China	2016	99.76%	99.34%
KJ458989	MOTNSH01	small ruminants	Tunisia	2015	98.99%	98.99%
CP006935	Michigan	Sheep	USA	2013	99.02%	98.36%
GU230142	TX1294-B	Homo sapiens veterinarian	USA	2010	99.32%	99.01%

TABLE 3. Similarity between *M. Haemminutum1* from Egypt and others countries:

Accession No	Strain name	Host	Country	Year of detection	Similarity
OQ565624	Candidatus Mycoplasma haemominutum isolate Haemminutum1	Cat sample from blood	Giza Governorate Egypt	2023	100%
ON202710	Candidatus Mycoplasma haemominutum isolate 2012/V.45	*Felis silvestri* Sample from spleen	Germany	2022	100%
KF743739	Candidatus Mycoplasma haemominutum clone FC30	Felis catus (cat)Sample from blood	USA	2017	100%
KY780179	Candidatus Mycoplasma haemominutum isolate 224	Domestic cat	Brazil	2017	100%
KR905456	Candidatus Mycoplasma haemominutum isolate 36/09*	Cat	Southern Italy	2016	100%
KM275256	Candidatus Mycoplasma haemominutum isolate E3_CMhm	domestic cat	Brazil	2015	100%
JQ689946	Candidatus Mycoplasma haemominutum **strain Taiwan** 170	Cat	central Taiwan	2012	100%
EU839985	Candidatus Mycoplasma haemominutum strain IT238_17	cat	Northern Italy	2008	100%
EF198145	Candidatus Mycoplasma haemominutum isolate H14_CMhm	feral cats	Korea	2008	100%
DQ825457	Candidatus Mycoplasma haemominutum isolate Z7	Eurasian lynx wild felid cat species	Switzerland	2007	100%
AY529633	Candidatus Mycoplasma haemominutum	Yamaguchi cat no. 6424	Japan	2004	100%
AY150983	Candidatus Mycoplasma haemominutum isolate UK no. 4	UK no. 4 cat	UK	2003	100%

Phylogenetic tree for *Candidatus M. haemovis* from sheep and goats:

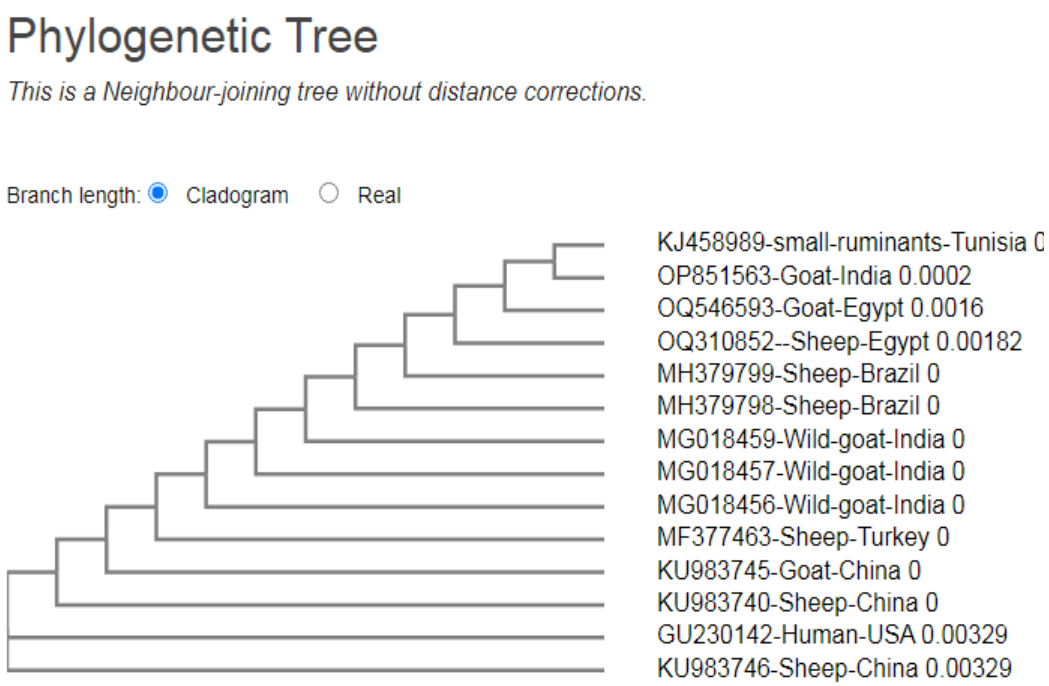

Fig. 6.The phylogenetic tree *M. heamovis* from sheep accession no. OQ310852 from Beni-Swaf and *M. heamovis* from goat accession no.OQ546593 from Gharbia.

Phylogenetic tree for *Candidatus M. haemominutum*:

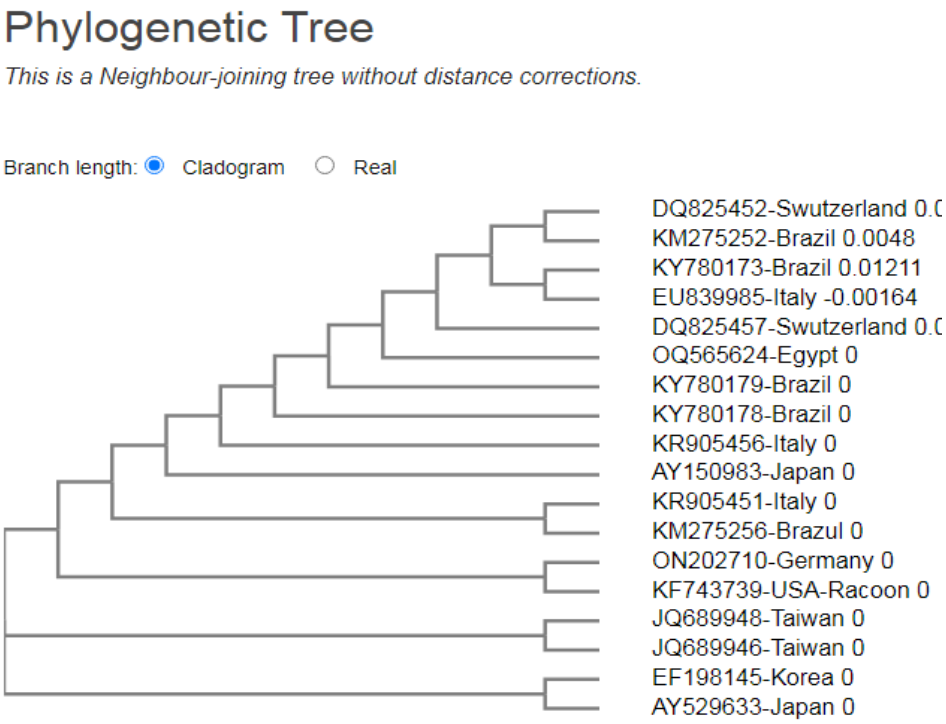

Fig. 7. The phylogenetic tree *of Candidatus haemominutum* (Egyptian Haemminutum1) accession No. OQ565624

Discussion

In the current study, molecular screening for the incidence of haemoplasmas in different animal species in Egypt was carried out for the first time The tested sheep and goat blood samples were 100 from sheep at Beni- Swef governorate and 83 from goats at Gaharbia governorate were collected during March 2020-October 2021.

Candidatus M. haemovis was detected in both sheep and goats by nested PCR with the sizes 1060bp and 506bp (Fig. 1) as previously recorded [8]. A higher haemoplasma detection rate was recorded in goats (14.46%) and it was deposited on the Gen Bank with the accession no. OQ54693 (M. ovis 2) followed by sheep (10%) deposited on the Gen Bank with the accession no. OQ310852(M. ovis). The phylogenetic analysis of the nucleotide sequences obtained from sheep and goats showed nearly 98.99 to 99.76% similarity to *Candidatus* M. haemoovis detected in China [8], Japan [16], Hungary [17] and *Candidatus* M. haemoovis detected in Tunis 2015 and to *Candidatus* M. haemoovis detected in Human in US in 2010 (Figure 6).

Concerning the incidence of Haemoplasma of camel in Egypt, it was detected in Shalateen with 8.33% of 120 examined imported camel samples by 16S rRNA PCR. The positive samples showed amplicons at position 310 bp as in figure (2-AandB), which agrees with a preceding report [18,19] in England and [10] in Iraq. *Mycoplasma haemolamae*, the most common species in camelids, is extremely common in different parts of the world, including Africa, the Americas, Southern Europe and Central Asia, and has circulated in different parts of Iraq [20] and it is transmitted mechanically by different species of ticks [14] .

Feline haemoplasmas are *M. haemofelis, M. turicensis* and *M. haemominutum*.

In the present study, we found that; Haemoplasma incidence in cats in Giza governorate Egypt was 20%, *Candidatus* M. heamominutum (CMhm) 12% was detected at position 202 bp by 16S rRNA figures (3and 4). Also, M. turicensis (CMt) 8% as in figure (3) was detected at 138 bp as mentioned by [21],while *Mycoplasma haemofelis*; the most pathogenic feline haemoplasma which causes fatal hemolytic anemia [22] could not be detected at all. *Candidatus* M. haemominutum (CMhm) sequence was deposited on the GenBank with accession No. OQ565624 (table 2). The infection rates by haemoplasma in cats were lower than that found in China during a nationwide epidemiological survey of haemoplasma as 26.4% of cats tested were infected with one or more species of haemoplasma (21% M. haemominutum (CMhm), 6.7% (CMt) and Mhf was 5.1%). The rate of detection in China was higher due to large-scale survey on a large number of cats all over the country. In Italy haemoplasma infection was 33.1% with 22.3% (CMt) and 10.8% for (Mhf) (23).

The Phylogenetic analysis revealed that, *Candidatus* M. haemominutum (CMhm) detected in Giza was 100% similar to that of Brazil KY78010179, 99% similar to that of Italy KR905453 and 99% similar to that of Taiwan(JQ689946) and Korea (EF 198145). Thess results are in accordance with [24]. Nearly complete 16S rRNA gene sequences for feline and canine plasma isolates from Europe, Australia, Africa, and Asia showed almost 100% identity with previously reported sequences for isolates from the United States. By using 16S rRNA PCR, haemoplasma of the dog strain *Candidatus Mycoplasma haematoparvum* (CMhp)was found in Dakahlia governorate as the amplicon was detected at position of 380 bp as in figure(5) with 24% incidence as described by [15]. Our findings confirmed that haemoplasmas are worldwide re-emerging zoonotic pathogens that affect livestock, wildlife, pet animals and humans causing serious and economically important disease problems as previously mentioned by [25]and [26].

Conclusion

Haemoplasmas are circulating in Egypt in sheep, goats, imported camels, cats and dogs. The infected animals showed various clinical manifestation or being apparent healthy. PCR technique is a useful method for detection of haemoplasmas and sequencing is confirmatory to the results. Ideal control measures are needed to minimize and eliminate internal diseases in different animals. This work is just a screening for presence of hasemoplasmas in Egypt so; further investigation on a large scale is recommended.

Acknowledgements

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Conflict of interest

There is no competing of financial interests exists.

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Self-funding

Ethical approval

This research was performed under the approval of The Institutional Animal Care and Use Committee (ARC-IACUC) of the Agricultural Research Center, organized and operated in accordance with the World Organization for Animal Health (OIE) and the Eighth Edition of its

Care Guidelines and Use of Laboratory Animals (2011).IACUC Protocol Number:

ARC	AHRI	23	32

Author`s contributions

Eissa S. I. putting the idea of work ,molecular identification, sequencing of haemoplasma and share in writing the manuscript**, Abdelaziz E.E and Yousreya H. M.,** collecting samples share in molecular identification of haemoplasma**, Hassan A.M.,** sequencing of haemoplasma and analysis of data **Sahar E.Ouda,*** molecular identification, sequencing of haemoplasma and analysis of data and writing and reviewing manuscript.

Laila M.El shabiny putting the idea of work, sequencing of haemoplasma follow up the work, share in writing and reviewing the manuscript

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الكشف الجزيئي وتوصيف الهيموبلازما في بعض أنواع الحيوانات المختلفة في مصر

صبرى اسماعيل عيسى ، عبد الواحد محمود حسن ، عيد السعيد عبد العزيز ، يسرية هاشم محمد[1] ، سحر السيد عودة و ليلى مصطفى الشعبينى

قسم الميكوبلازما، معهد بحوث صحة الحيوان ،مركز البحوث الزراعية، 12618، الجيزة، مصر

الخلفية: الميكوبلازما هيموجروب (HM) البكتريا الغير قابلة للزرع تسبب فقر الدم المعدي في العديد من الحيوانات والبشر.

الهدف: الكشف الجزيئي والتسلسل الجزئي والتحليل الوراثي للحامض النووى الريبوزى S16 من الهيموبلازما في بعض انواع الحيوانات المختلفة في مصر.

الطريقة: تم جمع عينات دم تحتوي على مضادات التخثر من حبل الوريد من الإبل والأغنام والماعز والكلاب والقطط. استخراج الحمض النووي، وتم تطبيق فحوصات تفاعل انزيم البلمرة المتسلسل المختلفة ثم التسلسل الجينى.

النتائج: تم اكتشاف Candidatus M.haemovis في 10% من الأغنام بمحافظة بني سويف وفي 14.46% من الماعز بالغربية وأعطى قطعة محددة عند 506 نقطة أساس، وأظهر كلا النوعين تشابهًا كبيرًا مع الأنواع المتماثلة في بنك الجينات. تم اكتشاف Candidatus Mycoplasma haemolamae في الهجن CMhl في شلاتين في 8.33% من الإبل المفحوصة وأعطى قطعة محددة بتركيز pb310. بلغت نسبة الإصابة بالهيموبلازما في القطط 32% في محافظة الجيزة. تم اكتشاف Candidatus M. haemominutum (CMhm) و Candidatus M.turicensis (CMt) في 16% من القطط التي تم فحصها.

وأعطى شظية محددة في الموضع 202 نقطة أساس و138 نقطة أساس على التوالي. لم يتم اكتشاف الميكوبلازما الدموية على الإطلاق. أظهرت Candidatus M. haemominutum (CMhm) تشابهًا كبيرًا مع الأنواع المتماثلة في البنك العالمى للجينات. تم اكتشاف Candidatus Mycoplasma haematoparvum (CMhp) في الكلاب بمحافظة الدقهلية بنسبة إصابة 22% وأعطى نطاق محدد عند 380 نقطة أساس.

الخلاصة : تنتشر الهيموبلازما في بعض الأنواع من الحيوانات المختلفة في مصر، وتعتبر تقنية تفاعل انزيم البلمرة المتسلسل هي الطريقة الدقيقة للكشف عن الهيموبلازما، كما أن التسلسل الجينى يؤكد النتائج.

الكلمة الدالة: Haemoovis، CMhl، CMhm، CMh