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Comparative studies on diagnostic methods of feline haemotropic mycoplasmosis

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Abstract

Mycoplasma haemofelis and *Candidatus Mycoplasma haemominutum* (formerly known as *Haemobartonella felis*) are the causes of hemotropic mycoplasmosis in cats. The haemoplasma organisms attach on to the surface of the red blood cells and have the potential to cause severe alterations in the shape of RBC's, resulting in anaemia. Among the cats presented to Veterinary Hospital, Veterinary College, Bengaluru, Karnataka, seventy eight cats were suspected for Haemotropic Mycoplasmosis and were included for detailed study. Detailed clinical examination was done for all the suspected cats. Peripheral blood smear stained with Giemsa stain were examined for primary identification of the organisms. Among seventy eight cats screened, 12 (15 %) animals were found positive for haemotropic mycoplasma organisms by blood smear examination. The blood samples were further subjected to DNA extraction followed by Polymerase Chain Reaction for confirmation of Haemoplasmosis. The occurrence of *Mycoplasma haemofelis* and *Candidatus Mycoplasma haemominutum* was found to be five per cent and thirteen per cent respectively. Polymerase chain reaction was considered to be more sensitive technique for diagnosis of Haemotropic Mycoplasmosis than cytology as PCR assay was based on amplification of 16S rRNA gene of haemoplasma and different species of mycoplasmas can be differentiated by PCR.

Keywords: Haemoplasma, *Mycoplasma haemofelis*, Molecular detection, Cytological examination, Haemotropic Mycoplasma

Introduction

Feline Haemotropic Mycoplasmosis or Hemobartonellosis, also known as feline infectious anaemia (FIA), is a clinically important disease in cats caused by Feline haemoplasmas viz., *Mycoplasma haemofelis* (Mhf), *Candidatus Mycoplasma haemominutum* (CMhm) and *Candidatus Mycoplasma turicensis* (CMt). The disease has been recognised since 1942 which may be acute or chronic. Although the organism produces anaemia in most of the cats, it causes a wide range of clinical signs, which vary from reduced appetite to severe pyrexia, vascular collapse and sometimes death. The disease is caused by Mycoplasma organisms that infects erythrocytes which are characterised by cell wall-deficient, uncultivable bacteria that colonise on the surface of erythrocytes and infect a wide range of vertebrate hosts. The organisms are pleomorphic, rod-shaped, spherical or ring-shaped and are found individually or in chains across the surface of Red Blood Cells. Haemotropic mycoplasmas were previously known as Haemobartonella and *Eperythrozoon* spp., but now they are reclassified within the genus Mycoplasma based on 16S rRNA gene sequencing. The organisms have worldwide prevalence and arthropod vectors are considered to be the important mode of transmission. However, the infection can also spread through the fight injury between cats and during blood transfusion (Tasker and, 2002) [1]. Cytological identification and molecular diagnosis of haemotropic mycoplasmosis is described in this article.

Materials and Methods

A total of 78 cats with clinical signs like anorexia, pyrexia, pale or icteric mucous membranes, weight loss, tiredness etc., which were presented to Veterinary College Hospital, Bengaluru formed the study group. Detailed clinical examination of suspected cases was done. Blood samples were collected from suspected cats under aseptic conditions from cephalic or femoral vein and subjected for laboratory examination like haematology and serum biochemical analysis. A thin peripheral blood smear stained with Giemsa stain was observed under oil immersion objective for primary identification of haemoplasma.

Further, EDTA treated blood samples were subjected to molecular diagnostic technique such as conventional Polymerase Chain Reaction. Isolation of DNA was done from whole blood using commercially available kits (NcleoSpin® Blood Quick Pure kit. MACHEREY-NAGEL) using protocol recommended by the manufacturer.

Polymerase chain reaction amplification was carried out in 20µl reaction mixture containing 12µl EmeraldAmp® GT PCR Master Mix, Takara, 4µl of nuclease free water, 1µl of each forward and reverse primers and 2µl of extracted DNA.

The cycling was carried out in Veriti™ Thermal Cycler, Applied Biosystems, by using the primers with the details mentioned in

Table 1 and the protocol mentioned in Table 2. The positive DNA of *Mycoplasma haemofelis* and *Candidatus Mycoplasma haemominutum* was subjected for sequencing at Indus Biosolutions., Bengaluru and the resultant DNA sequence was almost 100% similar to the sequences reported earlier and was used as positive control for the above PCR reaction.

Table 1: Primer details used for PCR

PCR	Target Gene	Primers	Product	Reference
<i>Mycoplasma hemofelis</i> (Forward)	16s rRNA	ATGCCCTCTGT GGGGGATAGCCG	273 bp	Watanabe <i>et al.</i> , 2008 [20]
<i>Mycoplasma hemofelis</i> (Reverse)	16s rRNA	ATGGTATTGCTC CATCAGACTTTCG	273 bp	Watanabe <i>et al.</i> , 2008 [20]
<i>Candidatus Mycoplasma hemominutum</i> (Forward)	16s rRNA	CTGGGAAAGTAG AGCTTCGCGAGC	202 bp	Watanabe <i>et al.</i> , 2008 [20]
<i>Candidatus Mycoplasma hemominutum</i> (Reverse)	16s rRNA	ATGGTATTGCTC CATCAGACTTTCG	202 bp	Watanabe <i>et al.</i> , 2008 [20]

Table 2: Protocol for species specific PCR for *Mycoplasma haemofelis* and *Candidatus Mycoplasma hemominutum*.

Step No.	Step	Temperature	Time
1	Initial denaturation	94 °C	5 Min
2	Denaturation	94 °C	45 Sec
3	Annealing	61 °C	45 Sec
4	Extension	72 °C	45 Sec
5	Steps 2 to 4 repeated 35 times		
6	Final extension	72 °C	7 Min
	Hold	4 °C	∞

Results and Discussion

Feline haemoplasma organisms are found worldwide. Reports of Feline Haemotropic Mycoplasmosis from India are scanty. Diagnosis of the disease was based on the demonstration of the organism in the Giemsa stained blood smears and by molecular methods.

Cytological examination was reported to be one of the preliminary methods for diagnosis of haemotropic mycoplasmosis. Most widely accepted and reviewed method for cytological diagnosis of haemoplasmosis is Giemsa staining technique. Haemoplasmas are visible as small coccoid organisms in the periphery of the red blood cells though it is reported as pleomorphic by many of the authors (Hackett *et al.*, 2006 and Sykes, 2010) [15, 16].

Among seventy eight cats screened by blood smear examination, 12 (15 %) cats were found positive for the presence of haemotropic mycoplasma organisms on the surface of RBCs. Alleman *et al.* (1999) [17] and Harvey and Gaskin (1977) [18] used the similar technique for identification of haemoplasma organisms.

Out of 12 samples found positive for haemoplasma in blood smear examination, only 8 samples were positive for haemoplasma by PCR and other 6 samples were found to be positive by PCR which were negative by blood smear examination. Hence it is hypothesized that Cytology includes a high rate of false positive results. The reason behind which may be because the dye sediments formed due to improper staining, water droplets, Howell jolly bodies and Pappenheimer bodies may get confused with bacterial organisms as observed by Fard *et al.* (2014). Tasker (2010) [2] reported that sensitivity of blood smear examination to be less than twenty per cent. Using PCR as the gold standard test, cytology had a

sensitivity of 27% and specificity of 89.74% (Ghazisaeedi *et al.*, 2014) [4]. In the present study, it was observed that Polymerase chain reaction is more sensitive than cytology. Similar observations were reported by Willi *et al.* (2007) [2].

The DNA extracted from blood samples of seventy eight cats with anaemia were subjected to conventional PCR for the confirmation of haemotropic mycoplasmosis. A total of fourteen samples were found positive by PCR assay with overall occurrence of 18 per cent whereas only eight animals were found positive by blood smear examination. Tasker and (2002) [1] and Tasker *et al.* (2003) recorded increased sensitivity of PCR than cytology for diagnosis of haemotropic mycoplasmosis.

The *Mycoplasma haemofelis* DNA was detected in 4 cats with five per cent occurrence and *Candidatus Mycoplasma haemominutum* DNA was identified in 10 cats with 13 per cent occurrence. Petry *et al.* (2020) [10], Salim *et al.* (2020) [9], Demkin and Kazakov (2021) [6] and Malangmei *et al.* (2021) [8] reported a lower *Mycoplasma haemofelis* occurrence. In comparison to the present study, higher prevalences of *Mycoplasma haemofelis* was reported by Ghazisaeedi *et al.* (2014) [4] and Do *et al.* (2020). Also, a higher incidence of *Mycoplasma haemofelis* (66.66 %) was reported than *Candidatus M. haemominutum* (33.33%) among infected cats in southern Europe by Torkan *et al.* (2014) [19].

The occurrence of *Candidatus Mycoplasma haemominutum* was found to be 13 per cent. Petry *et al.* (2020) [10], Salim *et al.* (2020) [9], Demkin and Kazakov (2021) [6] and Malangmei *et al.* (2021) [8] also recorded similar findings. Global prevalence studies of haemotropic mycoplasmas revealed the highest prevalence for *Candidatus Mycoplasma haemominutum* when compared with other two species viz, *Mycoplasma haemofelis* and *Candidatus Mycoplasma turicensis*. A study conducted by Jenkins *et al.* (2013) [14] reported a prevalence of 31 per cent of haemotropic mycoplasmosis among domestic cats in Newzeland using a real-time PCR assay with twenty five per cent of cases caused by *Candidatus Mycoplasma haemominutum*, 7.5 per cent of cases were due to *Mycoplasma haemofelis* and 4.5 per cent *Candidatus Mycoplasma turicensis*. Maher *et al.* (2010) [13] reported 20.6 per cent prevalence of haemoplasma among the domestic cats in Greece with higher occurrence of *Candidatus*

Mycoplasma haemominutum than *Mycoplasma haemofelis*. Higher prevalences of *Candidatus Mycoplasma haemominutum* than the present study was reported by Ghazisaeedi *et al.* (2014) [4] and Do *et al.* (2020) with prevalences of 63% and 83% respectively.

(2014) [5] found the evidence of horizontal transmission of *Candidatus M. haemominutum* by direct contact between cats by aggressive interactions or vectors, but not did not record any horizontal transmission of *M. haemofelis*. This may be the reason for occurrence of higher proportion of *Candidatus Mycoplasma haemominutum* than *M. haemofelis* in the present study also.

Hence, it is concluded that Polymerase chain reaction was known to be more sensitive technique for diagnosis than cytological examination for haemoplasma detection as it has got the potential to differentiate the species of mycoplasmas and assays were based on amplification of segments of the haemoplasma 16S rRNA gene (Ghazisaeedi *et al.*, 2014) [4].

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