MYCOPLASMA HAEMOLAMAE INFECTION IN AN ALPACA DETECTED BY BLOOD SMEAR EXAMINATION AND 16S rDNA PCR AND DENATURING GRADIENT GEL ELECTROPHORESIS

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HAEMATOLOGY:

CBC data is reported in Table 1. On blood smear examination, numerous small basophilic staining coccoid structures consistent with Mycoplasma haemolamae were noted heavily clustered on many erythrocytes and freely in the background. Erythrocyte features included moderate anisocytosis, mild polychromasia and moderate rubricytosis (about 20 nRBC /100WBC were counted with mostly metarubricytes and fewer polychromatophilic rubricytes). The reticulocyte percentage was calculated to be 1% and the absolute reticulocyte concentration was about 40,000/•L on a new methylene blue stained slide. This was interpreted as a moderate anaemia with mild regeneration. Anaemia was likely due to haemolysis secondary to the presence of M. haemolamae infection. Low numbers of neutrophils exhibited mild toxic changes with cytoplasmic basophilia and Döhle bodies. Mild toxic change is likely due to acute inflammation. Molecular testing to confirm M. haemolamae infection was recommended.

FOLLOW-UP AND TREATMENT:

Treatment for M. haemolamae infection was started with oxytetracycline. On day 11 of hospitalization and after 3 days of treatment with oxytetracycline, the anaemia had improved (HCT 20.9%; reference range 22.7-46.7% and haemoglobin 9.5 g/dL; reference range 10.9-18.9 g/dL). No coccoid structures consistent with Mycoplasma haemolamae were noted on the blood smear. Rare nRBC's and rare polychromasia was noted. A limited biochemical profile was performed and contained the following parameters albumin, potassium, chloride, calcium, phosphorus, urea and creatinine. Mild hypoalbuminaemia

(albumin 25.7 g/L; reference interval 27.0-51.0 g/L) and mild hypernatremia (Sodium 160.50 mmol/L; reference range 140.0-159.0 g/L) were revealed. Patriot was discharged on day 18 of hospitalization and monitored over the next 4 months with a series of CBCs and molecular testing.

He continued to have a mild anaemia 2 months post discharge (Table 1). He was clinically stable. Four months post discharge, Patriot received a Bluetongue vaccination (Bovilis BTV8 Intervet) and the following morning he was depressed, lethargic, with elevated temperature (39.2 °Celsius; reference range 38-39) and pale mucous membranes. On haematology anaemia had persisted. There was also a mild leukocytosis (32.3 x10e9/L; reference interval 4.9-22.3 x 10e9/L) with a mild mature neutrophilia (30.7x10e9/L; reference interval 1.0-16.6 x10e9/L). Mycoplasma haemolamae was not noted on the blood smear (Table 1). He was treated with flunixin and ceftiofur by the referring veterinarian and his clinical signs improved though he did not recover completely.

Table 1: Serial haematology parameter, blood smear examination and molecular testing results.*Haematological reference intervals are from ISIS 2006.

Haematology Values	Laboratory Reference Interval*	Day 6 Hosp	Day 11 Hosp	2 mo post discharge	4 mo post discharge	Units
WBC	4.9-22.3	13.8	17.6	17.4	32.3	x10•/L
Neutrophils	1.0-16.6	12.3	15.1	13.1	30.7	x10•/L
Lymphocytes	0.10-6.39	0.83	0.65	2.28	0.97	x10•/L
Monocytes	0-1.51	0.14	1.17	2.01	0.32	X10•/L
RBC	3.52-19.16	4.22	6.33	6.11	6.69	x 10^{12}/L
HCT	22.7-46.7	13.7	20.9	20.6	18.8	%
HGB	10.9-18.9	6.2	9.5	9.23	8.4	g/dL
MCV	15.6-59.6	32.4	33.1	33.6	28.2	fL
MCHC	32.0-67.2	45.5	45.2	44.9	44.8	g/dL
PLT	27-1392	242	522	456	910	x 10•/L
Blood Smear for M. haemolame		Positive	Negative	Negative	Negative	
PCR and DGGE for M. haemolamae		Positive	Negative	Positive	Positive	

FURTHER MOLECULAR TESTING

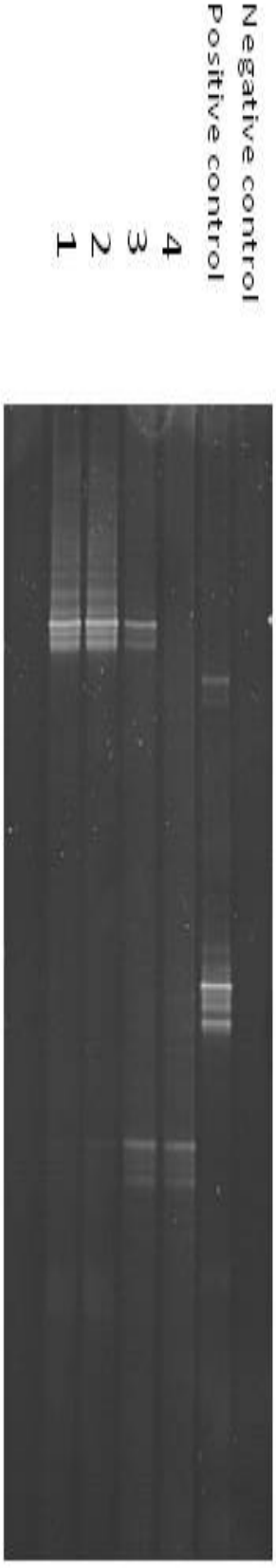
PCR, Denaturing Gel Electrophoresis (DGGE) and sequencing were performed at the VLA from EDTA blood samples from day 6 and 11 of hospitalization and 2 and 4 months post discharge. DNA was extracted from 100uL blood samples using a tissue DNA extraction kit (Sigma). Amplification of the V3 region of the 16S RNA gene was performed according to the method as described previously using universal bacterial primers (GC-341F 5•-CGC CCG CCG CGC GCG GGCGGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG and 534R 5•ATT ACC GCG GCT GCT GG)[1].

DGGE was performed using the Ingeny PhorU 2 x 2 apparatus (GRI Molecular Biology, Essex, UK)[1]. Samples were loaded onto 10% polyacrylamide/bis (37.5; 1) gels with linear denaturing gradients from 30-60% (where 100% is 7 M urea and 40% (v/v) deionized formamide) in 1 x TAE electrophoresis buffer (Severen Biotech Ltd., Worcestershire, UK). Electrophoresis was performed at 10 V at a temperature of 60 •Celsius for 18 h. Gels were stained with SBYR Gold (Cambridge BioScience, Cambridgeshire UK) in 1 x TAE for 30 min at room temperature and visualized under UV illumination. An identical band was seen in three samples (day 6 of hospitalization and 2 and 4 months post discharge) using 16S rRNA PCR and DGGE (Fig 2). The samples from day 6 of hospitalization and 2 months post discharge gave a strong high band on the DGGE whereas a weaker high band and a much lower band were seen in the sample from 4 months post discharge. The sample from 11 days of hospitalization had a much lower fainter band (Fig 2).

The samples from day 6 and day 11 of hospitalization were then sequenced to confirm identity. DNA was amplified from isolates using the 63F and 1387R primers under the conditions described above[2]. PCR products were cleaned up using a Qiagen QIAquick PCR purification kit and products were quantified by running on 2% agarose E-gels with a quantitative E-gel ladder (Invitrogen). 16S rRNA was sequenced in both the forward and reverse direction and sequencing reactions were performed using a DTCS Quick Start sequencing kit (Beckman Coulter) according to the manufacturer's instructions. Samples were ethanol precipitated and run on a Beckman Coulter CEQ8800 Sequencer.

DNA sequencing confirmed that the samples from day 6 of hospitalization and 2 months and 4 months post discharge were M. haemolamae. The DNA sequence had 99% identity to previously reported M. haemolamae 16S rRNA gene (EMBL-EBI accession number AF306346.1). The sample from day 11 of hospitalization was not consistent with M. haemolamae and had a low identity to a variety of Lactococcus, Bacillus and Enterococcus species on sequencing most likely due to contamination.

Figure 2. Denaturing gradient gel electrophoresis of 16S r DNA PCR produce. Lane 1, day 6 of hosp; lane 2, 2 mo post discharge; lane 3, 4 mo post discharge; lane 4, day 11 of hosp; positive control M. bovis.



DISCUSSION

Mycoplasma haemolamae is a wall-less haemotrophic prokaryote previously classified as Eperythrozoon species in the order Rickettsiae as a member of the family Anaplamataceae affecting camelids, but has recently been reclassified as Mycoplasma haemolamae based on its 16S rRNA sequence [3]. Mycoplasma haemolamae is most closely related to Mycoplasma wenyonii, a parasite of cattle. It clusters together on the present phylogenetic tree with other haemoplasmas for which 16S rRNA sequences have been determined [4, 5] . The parasites present as basophilic and coccoid to ring shaped on erythrocytes or in the plasma unattached to erythrocytes [6]. Parasites are 0.4-1.0 •m in diameter causing a slight indentation of the RBC membrane by scanning electron microscopy [6, 7].

Mycoplasma haemolamae was first described in 1990 in the USA [6, 7]. Subsequent reports of M. haemolamae infection in camelids predominantly are from the USA [8, 9] or Canada [10]. There is little data on prevalence studies. Of 1753 llama serum samples tested at 5 institutions across the USA, 208 were positive using serologic antibody titer to the organism formerly known as Eperythrozoon suis (Mycoplasma suis) by an indirect haemagglutination test [6].

Animals may present acutely in recumbency and weak, or may demonstrate chronic illness including weight loss and anorexia [6, 7]. Mycoplasma haemolamae has been reported in the presence of chronic inflammatory and infectious conditions in young camelids and was most likely an opportunistic infection in these cases [7-9]. Patriot was lethargic and had signs of acute inflammation, with mild neutrophilic toxic change on day 6 of hospitalization (when he was positive for M. haemolamae). The inflammation may have been due to his oedematous scrotum. It is possible that his infection with M. haemolamae was an opportunistic infection but no definitive conclusions were made. At 4 months post discharge he had a mature neutrophilia which was likely due to inflammation or a glucocorticoid response and he was depressed following vaccination and again he was positive for M. haemolamae. Patriot was also positive for M. haemolamae at 2 months post discharge when he was clinically stable. It has previously been reported that despite an intense immune response and even with antibiotic treatment, camelids infected with

M. haemolamae probably remain chronic carriers after clinical signs have resolved[11]. Patriot had recurrent parasitemia over a 4.5 month period but re-infection could not be completely ruled out. In this case Patriot only had a negative PCR while being treated with oxytetracycline.

Clinical manifestation of haemotrophic mycoplasma infection can vary from subclinical infection to life-threatening haemolytic anaemia [12]. Animals who are found to be Mycoplasma haemolamae positive have been reported to present with mild to moderate regenerative or non-regenerative anaemia [6, 7]. In our case the alpaca had chronic weight loss and moderate regenerative anaemia on hospitalization. The mechanism by which Mycoplasma species cause haemolytic anaemia is thought to involve an immune-mediated process in response to bacterial attachment to and deformation of the outer surface of red blood cells [5, 12]. As an example, acutely ill pigs with Mycoplasma suis infection induce the production of IgM cold agglutinins against RBCs thus leading to an immune-mediated haemolytic anaemia[12]. Further work is needed to characterise the mechanism of haemolytic anaemia in alpacas with M. haemolamae.

Hypoglycemia in association with Mycoplasma infection has been observed in many species including camelids[6], lambs[13] and pigs[14]. The proposed mechanism of hypoglycaemia is that glucose utilization by the haemoparasites exceeds gluconeogenesis. It is unclear if the hypoglycaemia in these cases was solely due to massive erythrocyte parasitism, or if other aetiologies including anorexia or bacterial sepsis were contributing. Unfortunately, in this case glucose was not measured.

Haemoplasmas have not been cultured in vitro and hence diagnosis has been difficult historically. Previously diagnosis of M. haemolamae has been based on blood smear examination and indirect IHA for detection of antibodies to Mycoplasma suis [6]but more recently a PCR method has been described [3]. Mycoplasmas tend to fall off erythrocytes easily, the bacteremia is cyclical and at times there are only low numbers of organisms present. Specificity of blood smear examination is also decreased by the possibility that stain precipitate and other erythrocyte features (such as Howell-Jolly bodies) can be mistaken for organisms. PCR was found to be a more sensitive test than blood smear for M. haemolamae [11]. Accordingly, in this case, the samples at 2

months and 4 months post discharge were negative on blood smear but positive on PCR and DGGE confirming that molecular testing is more sensitive than blood smear examination for diagnosis of M. haemolamae infection.

The use of DGGE for the detection and differentiation of Mycoplasma species has been previously described [1, 15]. DGGE can theoretically detect single base mutations in DNA [16, 17]. The method is based on the prevention of migration of DNA fragments following strand separation caused by chemical denaturants. DGGE enables the rapid and specific identification of Mycoplasma species. This is the first known report of an investigation into the suitability of DGGE to detect M. haemolamae DNA from blood samples. 16S rRNA sequencing was carried out to confirm the identity of the bands found on DGGE.

As universal primers were used, the approach described here will not be limited to species that have already been characterized; novel and unknown species will also be detected. The disadvantage of this is that bacteria other than members of the Mollicutes will also generate a band on the DGGE gel. The sample from 11 days of hospitalization gave a low band on DGGE and on sequencing was a vague match to a number of Lactococcus, Bacillus and Enterococcus species most likely due to contamination.

The mode of transmission is not known but biting or blood sucking vectors such as ticks are highly suspected [8, 10]. A case of possible in utero transmission in a 4 day old cria has been reported, however transmammary or postnatal blood inoculation during the birthing process could not be ruled out [10]. Further studies need to be done to elucidate the mode of transmission.

In conclusion, to the author's knowledge, this is the first case report of M. haemolamae in the UK. This is also the first time PCR and DGGE combined analysis was applied to M. haemolamae. It was demonstrated that this was a rapid specific test for M. haemolamae. These results suggest that parasitemia is not cleared by the standard tetracycline regime used and alpacas become chronic carriers after infection with haemoplasmas.

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