1 Application of PCR and Reverse Line Blot Hybridization to Detect Arthropod
2 Transmitted Haemopathogens in Horses Presented at a Referral Veterinary Facility in
3 New Jersey USA
4
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7
8 Abstract
9 Equine granulocytic ehrlichiosis is a tick-borne zoonotic disease caused by the
10 intracellular bacterium *Anaplasma phagocytophilum.* This disease has been well
11 documented in California , USA and sporadic cases have been recorded in New Jersey
12 and other states. Equine piroplasmosis is a reportable disease in the United States with
13 the most recent outbreak reported in Florida in 2008. In order to detect these diseases in
14 an area of low prevalence, two PCRs followed by a reverse line blot (RLB) hybridization
15 process were performed on DNA extracted from blood of 133 horses presented at a large
16 equine referral clinic in New Jersey. One PCR amplified the 16S rRNA gene for
17 *Anaplasma/ Ehrlichia* and gene and the other the 18S rRNA gene for *Theileria/Babesia* .
18 Amplified products were applied to a membrane to which oligonucleotide probes for
19 equine granulocytic ehrlichiosis (*Anaplasma phagocytophilum*) and equine
20 piroplasmosis (*Theileria equi* and *Babesia caballi*) were covalently attached. Two horses
21 were positive for *Anaplasma phagocytophilum* .This is the first published record of *A*
22 *phagocytophilum* DNA to be detected in horses in the United States using the reverse line
23 blot hybridization process. This technique is useful for large scale epidemiological

24 screening and detection of several tick-transmitted haemopathogens simultaneously in

25 animals with vague clinical signs of disease.

26 Keywords: horses, *Anaplasma phagocytophilum*, HGE, zoonotic diseases, New Jersey

27 Introduction

28 Granulocytic ehrlichias are obligate intracellular gram negative bacteria that are
29 transmitted by ticks. Equine granulocytic ehrlichiosis was first described by Gribble in
30 1969 based on cases presented at the University of California, Davis Veterinary teaching
31 hospital from as far back as 1961[1]. Since then equine granulocytic ehrlichiosis has been
32 reported in several states including Colorado[2], Florida,[3], Connecticut[4] , Massachusetts[5] ,
33 Minnesota, Wisconsin[6] and New Jersey[7]. It has been reported in several other countries
34 including Mexico, Spain[8], France[9], the Netherlands[10] , Sweden [11],Czech Republic[12] and
35 Israel[13]. In the United States, *Ixodes scapularis* is the vector in north eastern upper and
36 mid western United States and *I pacificus* is thought to be the chief vector in California[1]
37 [14].The agent of human granulocytic ehrlichiosis (HGE) and granulocytic ehrlichiosis of
38 horses formerly *Ehrlichia equi* have been reclassified as *Anaplasma phagocytophilum*
39 based on similarities in the *groESL* gene and 16S rRNA gene sequences[15]. Clinical signs
40 in horses are often non-specific and include fever, lethargy, anorexia, depression,
41 petechiation , limb oedema, icterus, ataxia and reluctance to move [16 17]. Diagnosis of
42 equine ehrlichiosis is based on clinical signs and finding the characteristic inclusion
43 bodies in the cytoplasm of neutrophils or eosinophils of affected horses. Other
44 haematological findings in clinically affected horses include mild anaemia, leukopaenia
45 and thrombocytopaenia [7]. At the onset of clinical signs or within a few weeks after
46 infection, antibodies to *A phagocytophilum* may be detected serologically. However, due
47 to persisting convalescent antibody titres it is difficult to interpret a positive serological
48 result [11]. Serological confirmation of ehrlichiosis may be made if an increase in antibody
49 titres is observed in acute and convalescent sera [18]. The infection in the acute stages may

50 also be confirmed using autogenous cell cultures. Acute infections can also be
51 demonstrated using the polymerase chain reaction techniques (PCR) , to amplify a region
52 of the 16S rRNA gene [19] or *groESL* gene [15].
53 The piroplasms, *Theileria equi,* formerly *Babesia equi* and *B caballi* parasitize the red
54 blood cells of equids and are also transmitted by Ixodes ticks. Affected horses may be
55 asymptomatic or have acute fever, anaemia, dyspnoea, and sudden death may occur. *T*
56 *equi* infections may result in abortion and neonatal death [20]. Transplacental transmission
57 from chronically infected horses has been demonstrated[21]. Currently there are restrictions
58 on the international movement of horses that are serologically positive as infected
59 animals are thought to be infected for life at a level at which the piroplasms can be
60 transmitted once the vector is present [22]. According to Mehlhorn and Schein, *T. equi* is
61 present in all areas of Africa, the Middle East , Asia , South, Central and North America,
62 in all coastal countries of the Mediterranean and all States of the former Soviet Union [23].*T*
63 *equi* has been eradicated from the United States and Australia[24] and Japan where the
64 disease is considered to be nonendemic. These countries' disease free status is
65 continually at risk via the importation of horses[25] and a recent outbreak on a Florida farm
66 has implicated an imported horse as the source of infection with subsequent transmission
67 to other animals iatrogenically via used needles and syringes [26]. As clinical signs of tick
68 transmitted diseases are non-specific and it may be difficult to observe organisms in
69 blood smears, it is often difficult to arrive at a definitive diagnosis especially where these
70 diseases are at a very low prevalence. Serological tests for *A phagocytophilum* and *B*
71 *caballi* or *T equi* cannot differentiate between current and past infection. Due to the non-
72 specific nature of clinical signs of these tick transmitted diseases, they may be under

73 diagnosed in areas where the prevalence is low[10]. *Anaplasma/ Ehrlichia* spp and
74 *Babesia/Theileria* spp DNA in blood of horses may be simultaneously detected using the
75 reverse line blot hybridization technique. This method has been applied to detect *A*
76 *phagocytophilum* DNA extracted from horse blood in the Netherlands[10] and *B. caballi*
77 and *T equi* DNA extracted from blood of Spanish horses[27].
78 Blood samples from horses with fever and non-specific clinical signs submitted to the
79 diagnostic laboratory of an equine referral centre in New Jersey were screened for
80 *Theileria/Babesia* DNA and *Anaplasma/ Ehrlichia* DNA using PCR and the reverse line
81 blot hybridization in order to determine the presence of these tick-borne haemopathogens
82 in an area of low endemnicity for *A phagocytophilum* and zero prevalence for
83 *Babesia/Theileria* genera. As these pathogens are both transmitted by Ixodes ticks, a
84 method by which these pathogens may be detected simultaneously is valuable for arriving
85 at a definitive diagnosis of disease and also for large scale epidemiological screening in
86 areas where one of the diseases is endemic. *A phagocytophilum* is also an important tick
87 transmitted zoonotic disease, hence its detection in horses may serve as an indicator of
88 the human risk associated with acquiring the infection according to the geographic
89 location and potential for contact with the tick vector.
90
91 Materials and Methods
92 EDTA blood was collected from 133 out of a possible 1342 cases admitted to an equine
93 referral centre in New Jersey between May and December 2007. Horses with fever or
94 those with non-specific clinical signs were included in the study. Blood smears were
95 Geimsa stained and examined under light microscopy. Complete blood counts were

96 performed using an automated haemocytometer (ABX Pentra 60, ABX Diagnostics,
97 Irvine CA). The remaining sample was shipped to the University of Georgia , Department
98 of Infectious Disease where DNA was extracted from each sample using the DNeasy
99 blood and tissue kit (Qiagen Sciences Maryland, USA) according to the manufactures
100 instructions. Extracted DNA was then stored at -20˚ C until analysed by PCR and
101 Reverse Line Blot hybridization.

102 PCR

103 Two separate PCR reactions were performed on each sample. PCR for the 16S rRNA
104 gene for *Anaplasma*/*Ehrlichia* was conducted using primers as described by Bekker et
105 al[28] and for the 18S rRNA for *Theileria*/ *Babesia* using primers previously described [29]
106 with the following adaptations : PCR reactions for *Anaplasma*/*Ehrlichia* or
107 *Theileria*/*Babesia* were performed using Sigma RedTaq Readymix (Sigma, St Louis MO,
108 USA)in a 50 µl reaction volume according to the manufacturers instructions. Briefly a 50
109 µl reaction volume consisted of 5 µl of extracted DNA , 25 µl of RedTaq(Sigma St
110 Louis, MO,USA) , 25 pmol forward and 25 reverse primers (Sigma, St. Louis MO,
111 USA) and PCR grade water. Positive control DNA for *A phagocytophilum* was supplied
112 by Prof. John Madigan , UC Davis. Positive control *T equi* DNA was obtained from a
113 Trinidadian horse, from which amplified products from 18S rRNA gene sequences were
114 identical to *T. equi* DNA sequences on gene bank. PCR grade water (Sigma, St. Louis
115 MO, USA) was used as a negative control. The reactions were performed in a Techne
116 Flexigen thermal cycler(Techne, Cambridge, UK) as follows. There was an initial
117 denaturing step of one cycle at10 minutes at 94 ˚ C, followed by 40 cycles of 20 sec 94 ºC
118 30 sec at 47ºC and 30 sec at 72ºC and a final hold at 4 ºC. 10 µl of amplified products

119 were subjected to gel electrophoresis at 90V for 30 mins in 1.5 % agarose gel prestained
120 with ethidium bromide. Amplified products of approximately 400 bp were visualized
121 under uv light using Syngene Multigenius bioimaging system (Syngene Cambridge ,
122 UK).
123 Reverse Line Blot Hybridization.
124 The membrane (Pal Cooperation, Pensacola Fl. USA) containing oligonucleotide probes
125 listed in Table 1 was prepared in a miniboter (Immunetics, Cambridge Massachusetts,
126 USA) as previously described [30]. The reverse line blot hybridization was performed as
127 described previously[29].
128 Results
129 Clinical data for the horses sampled are summarized in table 2 and 3. Out of the 133
130 horses tested, two (1.5 %) were positive for *A*. *phagocytophilum* via RLB (Figure 1) .
131 The case history for each *A phagocytophilum* positive horse is presented below. DNA of
132 *Theileria* or *Babesia* spp was not detected in any of the samples by PCR and RLB.
133 Case 1: A 5 year old quarterhorse gelding was referred to the clinic in December 2007
134 with a history of fever (rectal temperature 40.5 ˚ C) of unknown origin for about one
135 week, with lethargy , mild, cough and hind limb oedema. On presentation, he appeared
136 bright and alert, with normal body temperature, respiration and heart rate. The right distal
137 hind limb was oedematous. The complete blood count indicated a mild anaemia with
138 microcytotic RBCs and monocytosis (Table 4). The serum biochemistry profile indicated
139 a hypocalcaemia and hyperkalaemia. The horse was hospitalized and treated daily with
140 oxytetracycline (7mg/kg IV bid) for 3 days and his hind limbs were bandaged. Following
141 treatment, his temperature remained normal , the hind limb oedema resolved and the

142 horse was discharged with instructions to monitor manure production and body

143 temperature twice daily.

144 Case 2: A 5 year old thoroughbred gelding used for foxhunting was referred in November

145 2007 for evaluation of choke. The horse appeared alert and responsive , normothermic

146 with a normal heart rate and an elevated respiratory rate and a capillary refill time of 2

147 secs. A complete blood count revealed mild anaemia and leukopaenia. All panels of the

148 serum biochemistry profile were within normal limits. Endoscopic examination

149 (Olympus Medical Systems, Japan) revealed the presence of a feed bolus extending from

150 the proximal oesophagous to the thoracic inlet. Choke was confirmed, the animal treated

151 for 48hrs with a combination of oxytocin, buscopan (Boeringer Ingelheim Vetmedica Inc

152 St. Louis, MO),potassium penicillin (22000IU,IV q 6hrs), gentomycin (6.6 mg/kg IV)

153 and crystalloid fluid therapy (Plasmalyte®, Baxter, Deerfield IL). The choke resolved

154 and the horse was discharged on ceftiofur sodium (Pfizer Animal Health, NY, NY)

155 (3mg/kg IM for 5days) with instructions to monitor its progress.

156 Both horses are reported to have recovered fully and doing well.

157 Discussion

158 *A phagocytophilum* has a very wide host range including man ,wild life and domestic

159 animals [31 32]. Previous studies on the epidemiology of granulocytic ehrlichiosis in the

160 United States have focussed on dogs ,wildlife and ticks [33 34] and human infections[35].

161 Equine granulocytic ehrlichiosis was at first thought to be endemic only in California ,

162 but it is now known to have a much wider geographic range in the United States,

163 occurring in areas where the tick vector is present[19]. Clinical signs of acute infection with

164 *A phagocytophilum* were seen in case 1. That is , fever, lethargy, anorexia , oedema and

165 anaemia . There was no indication of tick infestation. The animal also responded to
166 treatment with oxytetracycline. As no morullae were observed in the leukocytes, the
167 clinician could not make a definitive diagnosis of equine ehrlichiosis. Equine ehrlichiosis
168 was an incidental finding in case 2. The animal had been used for hunting thus has had a
169 history of exposure to ticks. Clinical recognition of equine ehrlichiosis may be difficult at
170 first presentation as the severity of the disease varies according to the age of the horse
171 and the duration of illness. It is thought that adult horses over 3 years old may develop
172 more severe and progressive clinical signs compared to younger horses [16]. However
173 older horses , that is those over nine years, appeared to have recovered spontaneously in a
174 study of cases in the Netherlands [10]. In the present study, Case 2 appeared to have
175 recovered spontaneously, and it is not known if the episode of choke was related to
176 infection with *A phagocytophilum*. Lyme disease caused by *Borrelia borgdorferi* is
177 another zoonotic tick transmitted disease that is endemic in North Eastern United States.
178 Clinical signs of borreliosis in horses are similar to equine anaplasmosis and included
179 lethargy, low grade fever and painful joints resulting in reluctance to move[14]. As horses
180 in this study were not tested for *B burgdorferi* DNA, it is not known if this pathogen may
181 have contributed to clinical disease. Other possible differential diagnosis for tick-
182 transmitted infections include equine herpes virus infection, equine infectious anaemia
183 virus, equine arteritis virus and *Leptospira spp*. PCR based methods to detect tick-
184 transmitted haemopathogen DNA in blood of horses are more sensitive than traditional
185 methods based on serology or examination of blood smears for the organism. In detecting
186 antibodies to *A phagocytophilum* via IFA test, serum antibodies were detected between
187 17 to 25 days after challenging two naïve ponies[36]. Another study of 13 horses

188 demonstrated seroconversion titres to *A phagocytophilum* at 19-81 days post infection
189 with a median time of 46 days[19]. PCR followed by RLB for *A phagocytophilum* can
190 therefore be advantageous over serology in detecting acute infections and therefore
191 assisting clinicians in early treatment of cases, hence resulting in a more successful
192 outcome for the equine patient. Although equine piroplsmosis was not detected in the
193 samples in this study, the RLB is advantageous in that it can be used to detect *B caballi*,
194 and *T equi*, using a single PCR for the *Babesia*, *Theileria* genera. Probes for other
195 Ehrlichia/Anaplasma spp of horses can be attached to one membrane hence simultaneous
196 infections with these parasites may be detected. Using the RLB is however not
197 economical for use of single samples. The miniblotter apparatus used in this study was
198 designed for 45 samples. The RLB process is also somewhat tedious as it involves a
199 hybridisation step followed by several washes, and the addition of a conjugate and ECL
200 detection and the use of hyperfilm. Strict attention must be placed on the quality of wash
201 solutions and the temperatures for washing, hybridization and incubation. The quality of
202 the amino-linker used to attach the probes to the membrane is also important in accurate
203 detection of positive DNA[30]. It is important to note that a membrane may be used up to
204 10-15 times hence reducing the overall cost of this technique. Other methods such as
205 multiplex real time PCR may be used to detect mixed infections , however unlike the
206 RLB, multiplex PCR is at present very complicated to standardize. The RLB membrane
207 also contains generic and species specific probes. Hence potentially undescribed
208 *Ehrlichia / Anaplasma spp* or *Theileria/ Babesia spp* can be detected with a positive
209 result at the generic probes. This simultaneous detection of several species of different
210 genera at present is not available using real-time PCR. Using serial dilutions of plasmid

211 clones of the 18SrRNA gene sequences, Nagore et al determined the sensitivity of the

212 RLB assay as 1 gene clone copy compared to PCR which was $10^3 – 10^2$ copies[27].

213 Gubbels, using serial dilutions of infected blood with known parasitaemia determined the

214 RLB sensitivity as 10^{-6} % which corresponds to 3 parasites per µl of blood [30]. The latter

215 technique may be more valid as it takes into account the initial step of the process which

216 includes DNA extraction from blood.

217 Approximately 1342 cases were seen at the clinic during the period May – December ,

218 2007 for various procedures. It is therefore understood that diagnosing tick transmitted

219 haemopathogens of horses in areas of low endemnicity will remain a challenge for the

220 veterinarian, however these diseases should always be in mind when dealing with

221 unrefractory cases or cases of horses with fever. The RLB technique is important for the

222 simultaneous detection of tick transmitted haemopathogens on a moderately large scale

223 and is advantageous in that it may be able to detect previously undescribed

224 *Anaplasma/Ehrlichia* species or *Theileria/ Babesia* species of horses in epidemiological

225 studies on tick transmitted haemopathogens. The RLB would also be advantageous in

226 large scale epidemiological testing for several tick transmitted haemopathogens in an

227 outbreak situation such as the 2008 equine piroplasm outbreak[26] in Florida to quickly

228 obtain a definitive diagnosis in areas where other tick transmitted haemopathogens are

229 known to occur. The RLB may also be applied to determine the vector capacity of ticks

230 present.

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354

Table 1 5' - 3' sequences of oligonucleotide probes used in this study

Oligonucleotide probe	5'- 3' Sequence	Reference
Theileria equi	TCTGCTGTTTCGTTGACTG	This paper
Anaplasma platys	GTCGTAGCTTGCTATGATA	[28]
Ehrlichia canis	TCTGGCTATAGGAAATTGTTA	[37]
Babesia/Theileria genera	TAATGGTTAATAGGARCRGTTG	[30]
HGE	GCTATAAAGAATAGTTAGTGG	[28]
Anaplasma/Ehrlichia genera	GGGGGAAAGATTTATCGCTA	[37]
Anaplasma phagocytophilum	TTGCTRTRARGAATARTTAGTGG	[28]
Babesia caballi - like	CGGGTTATTGACTTCGCTTTTTCTT	[27]
Babesia canis vogeli	GTTCGAGTTTGCCATTCGTTT	This paper
Babesia canis canis	TGACGGTTTGACCATTTGG	This paper
Babesia gibsoni	ACTCGGCTACTTGCCTTG	This paper

Babesia canis rosi	CGGTTTGTTGCCTTTGTG	38

355

Table 4 Complete blood count for 2 horses RLB positive for Anaplasma phagocytophilum

		Rylie		Mcgregor		Reference range	
		10^3/mm3	%	10^3/mm3	%	10^3/mm3	%
WBC		10.6		5.8		5.5 - 12.5	
NEU		7.29	68.9	68.3	3.95	2.0 - 8.0	30.0 - 75.0
Lymphocytes		1.6	15.1	16.6	0.96	1.0 - 5.0	25.0-50.0
Monocytes		1.54	14.6	14.6	0.85	0.1 - 1.0	1.0 - 8.0
Eosinophils		0.11	1	0.1	0.01	0.0 - 0.4	1.0-10.0
Basophils		0.04	0.4	0.4	0.02	0.0 - 0.2	1.0 - 3.0
RBC		7.02		5.75		6.5 - 12.6	
HGB	g/dl	11.5		10.3		11.0 -19.0	
HCT			30.7	28.8		32.0 - 52.0	
MCV	um^3	44		50		80.0 - 100.0	
MCH	pg	16.4		17.9		26.0 - 34.0	

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MCHC	g/dl	37.5	35.8	31.0 - 35.0

Table 2 Clinical presentation of sample population

	No. (%)
Not recorded	74(55.6)
ataxia	1(0.8)
colic	1(0.8)
colic, weight loss	1(0.8)
fever	14(10.5)
fever , diarrhoea	9(6.8)
Fever of unknown origin	1(0.8)
fever, anorexia	1(0.8)
fever, colic	6(4.5)
fever, cough	1(0.8)
fever, distress	1(0.8)
fever, jugular vein thrombosis	1(0.8)
fever, lameness	5(3.8)
fever, lethargy	1(0.8)
fever, nasal discharge	1(0.8)
fever, neck pain	1(0.8)
fever, vasculitis	1(0.8)
fever, weight loss	3(2.3)
lameness	5(3.8)
lethargy	1(0.8)
lethargy, diarrhea	1(0.8)
limb deformity, high white cell count	1(0.8)
muscle fasciculations	1(0.8)
upper respiratory noise	1(0.8)

Table 3 Breed and sex of sample population

No. (%) of breeds in the study

	No. (%)
Not recorded	74 (55.6)
Appaloosa	1(0.8)
Arab	6 (4.5)
Belgian	1(0.8)
Grade quarter horse	1(0.8)
Haflinger	1(0.8)
miniature horse	1(0.8)
Paint	1(0.8)
pony	1(0.8)
Quarterhorse	3 (2.5)
Selle Francais	2 (1.5)
Standardbred	13(9.8)
Thoroughbred	23 (17.3)
Warmblood	5(3.8)

Sex distribution of population No. (%)

	No.(%)
Not recorded	74(55.6)
colt	18(13.5)
filly	10(7.5)
gelding	19(14.3)
mare	11(8.3)
stallion	1(0.8)

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