Equine granulocytic anaplasmosis in the Czech Republic

P. Jahn, P. Zeman, B. Bezdekova, I. Praskova

Twelve confirmed cases of equine granulocytic anaplasmosis (EGA) and five additional suspected cases, showing a compatible clinical history and specific IgG titres of 1280 or above, were recorded in the Czech Republic during the period 2002 to 2008. The diagnosis was based on clinical signs, the detection of Anaplasma phagocytophilum morulae in neutrophils in blood smears, serology and molecular methods. Pyrexia (39.8 to 41.3°C), depression, partial or total anorexia, limb oedema and icterus were the most frequently observed clinical abnormalities. Haematological examination revealed thrombocytopenia in all the horses, and mild anaemia and leucopenia in five of them. Several horses showed high titres of specific antibodies immediately after onset of the disease, suggesting that they had previously been exposed to A. phagocytophilum. Genotyping of the A. phagocytophilum strains distinguished two genetic variants, with divergence in the sequence of the ank gene of the bacterium, circulating in the Czech Republic.

Materials and methods

All horses admitted to the clinic between the years 2002 and 2008 were initially included in the study. The geographical area served by the clinic covers the whole of the Czech Republic, although animals from the most western regions are admitted less frequently. On admission to the clinic, all horses underwent a standard clinical examination, and the owners were interviewed about the horses’ history.

In horses with a history of pyrexia, depression and anorexia, a jugular blood sample was drawn on the day of admission and divided into aliquots for routine haematological examination, A. phagocytophilum serology anduffy coat preparation. A diagnosis of EGA was based on compatible clinical signs and the detection of A. phagocytophilum morulae in the cytoplasm of at least three neutrophils in blood smears stained with the commercial staining kit Hemacolor (Merck). Depending on the opportunity, one or more successive blood samples were taken while the horse was hospitalised and/or convalescing at the owner’s stable. Paired samples of sera for serological examination and buffy coats for DNA isolation were stored frozen at –80°C until processed in the laboratory.

Sera were analysed for the presence of IgG antibodies to A. phagocytophilum using the immunofluorescence method. Tested sera were titrated to endpoint with a commercial goat anti-horse IgG conjugate (Kirkegaard & Perry Laboratories). A laboratory culture of A. phagocytophilum (NTN-1 strain) grown in HL-60 cells and fixed on microscope slides was used as the antigen. Indirect fluorescent antibody (IFA) tests were performed in the usual way and evaluated at x1000 magnification under a fluorescence microscope.

Selected positive sera were analysed in detail using the Western blotting method. Membrane-bound proteins of A. phagocytophilum, derived from the above laboratory culture, were reacted with the horses’ serum samples, followed by incubation with protein G-horseradish peroxidase conjugate (ICN Biomedicals). Bound sera were visualised with chemiluminescence using a commercial detection kit (Lumi-GLO; Kirkegaard & Perry Laboratories) and a radiographic film (Foma).

DNA from the buffy coat samples was isolated using a DNeasy Tissue extraction kit (Qiagen), and then used as a template for nested PCR amplification of a 546 bp segment of the 16S rRNA gene of A. phagocytophilum (Massung and others 1998). The PCR products were analysed in the conventional manner using agarose gel electrophoresis and ethidium bromide staining. Genotyping of equine A. phagocy-
anaplasmosis. Acute blood smear stained with Hemacolor (Merck).

Results

From the total of 8446 horses treated at the university clinic in the period from 2002 to 2008, 12 (0.14 per cent) animals (two in 2002, one in 2003, three in 2004, four in 2005, one in 2006, one in 2007, and none in 2008) were diagnosed as suffering from EGA. Another five probable cases, showing a compatible clinical history and elevated specific IgG titres (≥1280), were examined at the clinic but too late after the onset of disease (12 days), which precluded a conclusive diagnosis; these cases were discounted from the study. Three of the affected horses were admitted at the end of May, six in June and three in October. One stallion, four geldings and seven mares were affected; the breeds affected were warmblood (five horses), thoroughbred (three), Arabian (two), paint horse (one), and standardbred (one). Three of the horses were three to four years of age and nine of them were between five and 11 years old. Seven of the affected horses resided in South Moravia, three in North Moravia and two in Central Bohemia, suggesting that EGA is distributed throughout the Czech Republic.

The clinical signs included pyrexia of 39.8°C to 41.3°C (11 cases), depression (10 horses), partial or total anorexia (eight horses), limb oedema (six horses), ataxia (four horses), icterus (nine horses) and trembling (one horse). An increased heart rate (46 to 80 bpm) was found in five horses. Oedema of the testes, polyuria/polydipsia, stiffness and reluctance to move were observed in the stallion. In two horses, the clinical signs had been interpreted by their owners as mild colic; however, signs of colic were not observed in these horses either on admission or during hospitalisation at the clinic. The duration of clinical signs before admission to the clinic was estimated to have been one to five days.

Haematological examination revealed mild anaemia (packed cell volume 0.26 to 0.29 l/l, reference range 0.30 to 0.42 l/l) in five horses and thrombocytopenia in all of them (6 x 10^9 to 61 x 10^9 platelets/l, reference range 100 x 10^9 to 250 x 10^9 platelets/l). Leucopenia (3.1 x 10^9 to 4.5 x 10^9 cells/l, reference range 6 x 10^9 to 10 x 10^9 cells/l) with neutropenia and lymphopenia were observed in five horses. All clinically affected horses had A. phagocytophilum morulae detectable in 5 to 10 per cent of neutrophils in peripheral blood smears during the acute phase of infection (Fig 1).

The serological response to the infection in individual horses is summarised in Table 1. Antibody levels and their dynamics varied considerably among the horses. The most typical response was an at least two-fold increase in IgG titres (seven horses); however, some horses showed no significant increase (three horses) or even a decrease (two horses) in antibody levels by the end of the first month of convalescence. The latter horses were distinguished by high IgG titres (5120 to 20,480) present as early as on the day of admission, one to five days after the onset of disease. Fig 2 illustrates the specific profile of reactive antibodies revealed by Western blot. Immunoblots of all tested sera showed antibodies that reacted with the immunodominant MSP (~44 kDa) and several other A. phagocytophilum proteins. During the course of convalescence, additional major antigen bands became apparent on immunoblots, but subsequent follow-up indicated a slow return towards the initial band pattern (Fig 2, horse 1). Some sera with high initial IgG titres showed a particularly strong reaction to multiple antigens as early as on the day of admission, with very few bands becoming more readily apparent by the end of the first month of convalescence (Fig 2, horse 2).

The PCR test was positive in all 10 horses that were examined. Genotyping of the A. phagocytophilum strains (from nine horses) revealed that the horses were infected with two distinct genetic variants of the bacterium, which shared the same 16S rRNA, groL, msp4 sequences but diverged in the sequence of the ank gene; the variants are therefore referred to as ank variant 1 (GenBank/EMBL/ DDBJ accession number EU389555) and ank variant 2 (GenBank/EMBL/DDBJ) accession number EU839565) below. The ank vari- ant 1 was detected in horses 1, 3 and 4, whereas ank variant 2 was detected in horses 2, 5, 6, 9, 10 and 11 (the strain from horse 12 is yet to be sequenced). There was no apparent sex, breed or age predisposition or geographical distribution for either variant. Although the ank variant 2 appeared to be associated with, on balance, more pronounced clinical signs (Fig 5), the differences were not statistically significant, and the stallion, which was infected with ank variant 1, also experienced a serious clinical course. The most striking difference demonstrated was between the degrees of protective immunity in horses infected with either one of the two variants. All three horses infected with ank variant 1 had low anti-A. phagocytophilum IgG titres, not exceeding 1280 at the start of the disease, whereas four of six horses infected with ank variant 2 exhibited titres ranging between 2560 and 20,480; the difference fell somewhat short of statistical significance (one sided P=0.091, mid P=0.045, Fisher’s exact test).

Nine horses were treated with oxytetracycline at a dose of 5 to 10 mg/kg once a day for five to seven days. This treatment was followed by a rapid improvement of their health status within 12 hours. Three horses had been treated with penicillin before admission to the clinic; they were not switched to oxytetracycline but recovered spontaneously. The clinical signs in these horses persisted for three to 14 days until recovery.

Discussion

EGA is regarded as an emerging infection; in the past 25 years, cases of this disease have increasingly been diagnosed throughout Europe. In the Czech Republic, the first

---

**TABLE 1: Results of PCR and serology in 12 horses with equine granulocytic anaplasmosis**

<table>
<thead>
<tr>
<th>Horse</th>
<th>Breed</th>
<th>Age (years)</th>
<th>PCR</th>
<th>Duration of disease</th>
<th>Treatment with oxytetracycline</th>
<th>First titre</th>
<th>Second titre</th>
<th>Interval between samplings (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Arabian</td>
<td>10</td>
<td>+</td>
<td>1</td>
<td>No</td>
<td>80</td>
<td>1280</td>
<td>34</td>
</tr>
<tr>
<td>2</td>
<td>Warmblood</td>
<td>8</td>
<td>1</td>
<td></td>
<td>+</td>
<td>No 2560</td>
<td>5120</td>
<td>27</td>
</tr>
<tr>
<td>3</td>
<td>Thoroughbred</td>
<td>4</td>
<td>4</td>
<td>+</td>
<td>No</td>
<td>3290</td>
<td>10,240</td>
<td>33</td>
</tr>
<tr>
<td>4</td>
<td>Paint horse</td>
<td>5</td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>Yes 1280</td>
<td>5120</td>
<td>36</td>
</tr>
<tr>
<td>5</td>
<td>Arabian</td>
<td>9</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>Yes 10,240</td>
<td>2560</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>Thoroughbred</td>
<td>9</td>
<td>5</td>
<td>+</td>
<td>+</td>
<td>Yes 20,480</td>
<td>20,480</td>
<td>49</td>
</tr>
<tr>
<td>7</td>
<td>Standardbred</td>
<td>11</td>
<td>5</td>
<td>+</td>
<td>Yes</td>
<td>20,480</td>
<td>5120</td>
<td>34</td>
</tr>
<tr>
<td>8</td>
<td>Warmblood</td>
<td>5</td>
<td>5</td>
<td>+</td>
<td>+</td>
<td>Yes 160</td>
<td>5120</td>
<td>36</td>
</tr>
<tr>
<td>9</td>
<td>Warmblood</td>
<td>11</td>
<td>1</td>
<td>+</td>
<td>Yes</td>
<td>3290</td>
<td>2560</td>
<td>28</td>
</tr>
<tr>
<td>10</td>
<td>Warmblood</td>
<td>3</td>
<td>1</td>
<td>NA</td>
<td>NA</td>
<td>Yes 40</td>
<td>5120</td>
<td>6</td>
</tr>
<tr>
<td>11</td>
<td>Warmblood</td>
<td>9</td>
<td>5</td>
<td>NA</td>
<td>Yes</td>
<td>5120</td>
<td>5120</td>
<td>17</td>
</tr>
<tr>
<td>12</td>
<td>Thoroughbred</td>
<td>4</td>
<td>3</td>
<td>+</td>
<td>Yes</td>
<td>20,480</td>
<td>20,480</td>
<td>24</td>
</tr>
</tbody>
</table>

NA Not analysed

---

**FIG 1: Typical Anaplasma phagocytophilum morula detected in a neutrophil of a horse suffering from equine granulocytic anaplasmosis. Acute blood smear stained with Hemacolor (Merck).**

x 1000
two clinical cases were diagnosed in 2002. No cases of EGA could be identified retrospectively in the archives of clinical records at the authors’ clinic in the period from 1925 to 2001.

Most cases in the present study were diagnosed in May and June, and three cases were admitted in October. One case of EGA in France (Bermann and others 2002) was diagnosed in March; cases of EGA in Germany (Büscher and others 1984) and Austria (Fröhlich and Edelhofer 1993) – that is, countries with climatic conditions similar to those in the Czech Republic – were recorded in June. The seasonality of the disease is most likely dependent on tick activity.

The most common clinical sign in this group of horses was pyrexia, which was apparent in 11 of the 12 cases. Pyrexia was reported to be the most constant clinical sign both in natural (Büscher and others 1984, Bermann and others 2002, Butler and others 2008) and experimental infections, and is the first sign to become apparent (Franžén and others 2005). The only afebrile horse in the present study was a five-year-old mare. It is possible that this horse was pyrexic for only a short period, and the owner did not recognise it.

Less frequent clinical signs among these horses were depression (83 per cent of cases), icterus (75 per cent) and anorexia (67 per cent). Butler and others (2008), who described six horses with natural infection, and Franžén and others (2005), who studied six horses with experimental infection, observed depression and anorexia in all cases. Icterus was not reported in the study of Franžén and others (2005), but has been mentioned in several other clinical reports (Gribble 1969, Büscher and others 1984, Fröhlich and Edelhofer 1992, Bermann and others 2002).

Ataxia is an inconsistently occurring clinical sign of EGA; it was observed in four (33 per cent) of the horses in this study. Butler and others (2008) identified it in only one of six naturally infected horses, whereas Franžén and others (2005) observed ataxia in all six experimentally infected horses, occurring on days 2 to 4 after the onset of clinical disease.

Limb oedema was the most delayed clinical sign in all experimentally infected horses in the study of Franžén and others (2005); it occurred in 83 per cent of naturally infected horses in the study of Butler and others (2008), but in only 50 per cent of the horses in the present study. The authors believe that the comparatively low occurrence of limb oedema among the present cases was due to their prompt treatment with oxytetracycline after the diagnosis of EGA had been established. No horse received oxytetracycline in the study of Franžén and others (2005), and only one in the study of Butler and others (2008).

Previous clinical experience suggests that horses younger than four years of age have a generally less severe course of disease (Gribble 1969, Pusterla and Madigan 2007). However, the most severe clinical signs among the 12 horses described here were observed in a four-year-old thoroughbred stallion that was not treated with oxytetracycline (horse 5). Similarly, Butler and others (2008) observed the most severe clinical course of naturally acquired infection in a four-year-old gelding that became recumbent and had to be euthanased. Schusser and others (2007) described a case of a naturally infected one-year-old filly that developed acute renal failure, pneumonia and laminitis as complications, but recovered after oxytetracycline treatment. In horse 3, the clinical course of the disease was characterised by pyrexia, oedema of the distal limbs and testes, polypusia/polydipsia, stiffness and reluctance to move. The owner noticed increased pulsation of the digital arteries at the beginning of the disease, but laminitis was not confirmed at the clinic. Blood biochemistry in this horse did not reveal azotaemia and, as urinalysis was not performed, it was not possible to assess the degree of renal damage. Oedema of the limbs and testes persisted for 14 days and polypusia/polydipsia was apparent for 18 days; both signs resolved spontaneously. The oedema was probably caused by inflammation of small arteries and veins, which is commonly found in horses with EGA. Mild inflammatory vascular or interstitial lesions have also been recognised in the kidneys, heart and central nervous system of animals examined post mortem during the course of the disease (Gribble 1969).

Anaemia, leucopenia and thrombocytopenia are typically found in cases of EGA and have been mentioned in many clinical reports (Gribble 1969, Butler and others 2008). The mechanism by which haemolysis is affected by EGA, giving rise to pancytopenia, is not fully understood (Pusterla and Madigan 2007).

Antibody levels and their dynamics varied considerably among the 12 horses, and may have been influenced by concurrent antibiotic treatment. Several horses showed remarkably high titres of specific antibodies soon after onset of the disease, and two of them (horses 2 and 5) had titres as high as 2560 and 10,240 on the day after the onset of pyrexia, which may suggest that these horses had previous exposure to the infection. The results of the Western blot indicate that at least in some horses the early immune response was directed against a wide range of A. phagocytophilum proteins, and thus that these horses must have experienced repeated episodes of antigen challenge.
before the clinical disease was first diagnosed in them. Neither IFA or Western blot thus gave reliable information as to whether the anti-bodies were indicative of the current disease or a previous clinical or subclinical infection (Fig 2, horse 0).

Franzén and others (2009) reported the persistence of A phagocytophilum DNA in experimentally infected horses, DNA was intermittently detected for up to 129 days in some individuals and those authors hypothesised that the bacterium might occasionally persist in infected horses. However, in that study the experimental animals exhibited simultaneously a steady decrease in antibody titres (except for one horse that was concurrently affected with pneumonia). The authors can only speculate whether the strong initial anti A phagocytophilum immunity in some of the horses in the present study may have been induced by a latent infection. The authors used PCR to test 11 randomly selected, healthy, co-stabled horses from three farms where EGA was diagnosed along with three former clinically affected horses, once during convalescence, but none tested positive for A phagocytophilum (data not shown). Although this cannot completely exclude a persistent infection in any of the horses, the persistence of elevated antibody levels, periodically boosted by an episode of mild infection, seems a more likely explanation for the serological results. In general, the possibility of chronic EGA and the aetiological role of different genetic variants of A phagocytophilum need further investigation.

It is likely that EGA in horses has been underdiagnosed in the Czech Republic, as well as in other European countries, because the clinical signs in some horses are similar to those caused by infections with other pathogens and some horses recover spontaneously. In the authors’ experience, microscopic examination of blood smears (ideally performed several times, as the proportion of infected neutrophils can vary during the course of infection) is a sensitive and practical diagnostic tool suitable for veterinarians considering infection with A phagocytophilum in the differential diagnosis of horses with pyrexia of unknown origin, and may contribute (in combination with the observation of other clinical signs) to better recognition of the disease.

Acknowledgements

This study was supported by the Ministry of Education, Youth and Sports of the Czech Republic (Grant VZ MSM 6215712403).

References


Equine granulocytic anaplasmosis in the Czech Republic

P. Jahn, P. Zeman, B. Bezdekova and I. Praskova

Veterinary Record 2010 166: 646-649
doi: 10.1136/vr.4852

Updated information and services can be found at:
http://veterinaryrecord.bmj.com/content/166/21/646

These include:

- **References**: This article cites 16 articles, 4 of which you can access for free at:
  http://veterinaryrecord.bmj.com/content/166/21/646#BIBL

- **Email alerting service**: Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/