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Case Report

Burkholderia mallei infection in a horse imported from Brazil

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Summary

A horse imported from Brazil developed a respiratory illness 2 weeks after arrival in Germany. After an initial but inefficient treatment glanders was diagnosed based on serological and molecular biological findings. The present case highlights the potential risk of an importation of glanders in free areas. The fact that veterinarians in countries where glanders has been eradicated for decades are not familiar with the clinical symptoms of the disease, can favour the entry of the disease. In order to prevent the spread of glanders, the sanctions of the veterinary authorities in such cases of the infection are of utmost importance.

Introduction

Glanders, caused by *Burkholderia mallei*, is a highly contagious disease in equids that is notifiable to the World Organisation of Animal Health (OIE). Germany has been free from glanders for the last 50 years, as have many other European and non-European countries. Based on clinical, serological and molecular biological findings glanders was diagnosed in a horse imported from Brazil to Germany.

This report presents a rare case of glanders in Europe and recalls the omnipresent potential risk of an importation of this notifiable disease in glanders-free areas.

Case details

History

An 8-year-old mare of the Crioulo breed was imported to Germany in 2006 from a stable located in the area of Sao Paulo, Brazil. The International Animal Health Certificate documented that the horse had not stayed in the states of Pernambuco and Alagoas, where glanders occurred in the past, during the last 2 years. It was also documented in the certificate that glanders had not occurred during the last 6 months in the area of Sao Paulo, where the horse was kept. Furthermore, the certificate confirmed that the prescribed complement fixation test (CFT) was negative for glanders at a serum dilution of 1 in 10. About 3 weeks before exportation to Germany, the horse was sent to a stable in Rio de Janeiro. During veterinary inspection at Frankfurt airport, no clinical signs of illness were registered and the horse was transported to the stable of destination, where it was kept isolated from other horses.

Diagnostic procedure and case management

Physical examination

Two weeks after arrival of the horse at the destination stable, the local veterinarian registered respiratory signs of illness with a body temperature of 38.7°C. Nasal discharge, stellate scars and purulent crusts on the mucous membranes of the nose, tachypnoea and increased respiratory sounds were identified. Furthermore, thickening of the metatarsal and metacarpal regions, and purulent crusts on the skin on these parts of the legs were detected. At that point, the veterinarian thought that this could be the result of bandaging the legs during the flight. The treatment consisted of an i.m. injection of procaine benzylpenicillin (2.8 mg), benzathine benzylpenicillin (1.8 mg), dihydrostreptomycin (6.2 mg), procaine hydrochloride (480 mg) and oral administration of dembrexin hydrochloride (125 mg), b.i.d. The lesions on the legs were bandaged using a rivanol and tetracycline

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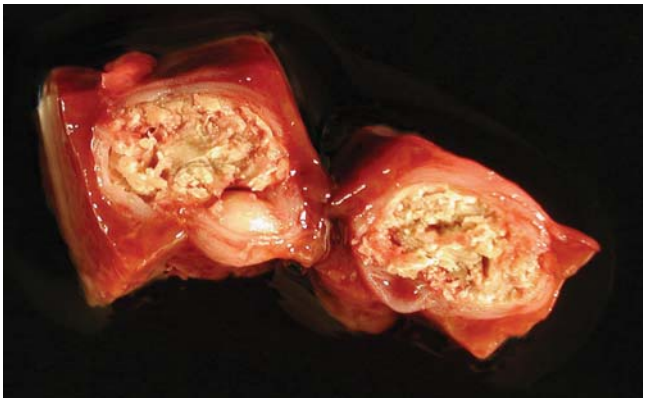

Fig 1: Large white nodule in the lung with dry, white to yellow cut surface containing onion-skin layered grey material.

hydrochloride solution. The bacteriological examination of nasal swabs resulted in the isolation of *Escherichia coli, Streptococcus equi* ssp. *zooepidemicus* and *Klebsiella pneumoniae*. In view of the results of the bacterial sensitivity tests, the antibiotic treatment was changed to cefquinome sulphate (675 mg/day) for 5 days, followed by sulphadimethoxine (10.4 mg) and trimetoprim (2 mg) b.i.d. for 10 days.

As the respiratory signs failed to improve with treatment and the symptoms could not be assigned to one of the more common respiratory infections, the veterinarian suggested that a rare disease, such as glanders, should be considered. He asked for differential diagnostic clarification of glanders and informed the local veterinary authority. The serological examination using the statutory CFT for glanders was ordered.

Serological findings and mallein test

The CFT was performed at the German reference laboratory for glanders according to the manual of the World Organisation of Animal Health (Anon 2007). Malleus-antigen[1] was used for the CFT. Three consecutive serum samples were tested and all sera showed a positive reaction in the CFT. For the mallein test, a purified protein derivative mallein[2] was used for intracutaneous injection of 0.2 ml into the neck. Forty-eight hours after application, this area of the skin was painful and an increase of the thickness of the skin from 3.1 to 8.2 mm was measured.

Pathological findings

Based on the clinical and serological findings, as well as the results of the skin test, and according to Anon (1979), the horse was subjected to euthanasia and a necropsy was performed. Necropsy revealed that the horse was in good general condition. Increased scaling of the skin was noted at the metacarpus and metatarsus of the right front leg and the left hind leg, but not associated with any changes in the subcutis or regional lymph nodes. The upper and lower respiratory tract were examined in detail. The head was split sagittally and the nasal septum removed. No lesions were observed at the nares, conchae, nasal cavity, nasal septum, guttural pouches, nasopharynx, larynx and trachea. The lymphoid tissues of the nose, Waldeyer's ring and larynx were moderately enlarged. In the lung, a 1.5 x 1.5 x 0.5 cm, white nodule was present in the left cranial lobe. Multiple, up to 0.5 cm in diameter large, firm nodules were scattered throughout all lung lobes. They had either a dry, white to yellow cut surface or contained onion-skin layered, grey material (**Fig 1**). Numerous similar nodules were found in the liver (**Fig 2**) and an additional 3 in the spleen. Altered tissue samples were collected for histological and immunohistological examination, for isolation of *Burkholderia mallei* and for the preparation of an inoculum for inoculation of guinea pigs.

Histological and immunohistological examinations

Tissues were fixed in neutral buffered formalin, embedded in paraffin and stained with haematoxylin and eosin. Immunohistochemistry was performed on paraffin sections using a polyclonal anti-*B. mallei*-rabbit serum and a murine monoclonal antibody specific for *B. mallei* (clone 3D11)[3] using overnight incubation at 4°C. The avidin-biotin-peroxidase method[4] was applied according to the manufacturer's instructions. The chromogen used was 3,3´-diaminobenzidine-tetrahydrochloride[5]. Tissue sections were counterstained with Mayer's haematoxylin. As the positive control for the polyclonal antibody, the spleen of a mouse experimentally infected with *B. pseudomallei* was used. For the negative control, the primary antibody was substituted by ascites fluid of nonimmunised Balb/cJ mice[6] or rabbit serum[5].

The histological examination revealed that most nodules were characterised by a massive infiltrate of eosinophils, some lymphocytes and macrophages, a few multinucleated giant cells and foci of calcification. These nodules were interpreted as induced by wandering larvae of helminths, most likely strongyles. A few nodules in the liver showed a distinct morphology with extensive central

Fig 2: Multiple nodules in the liver.

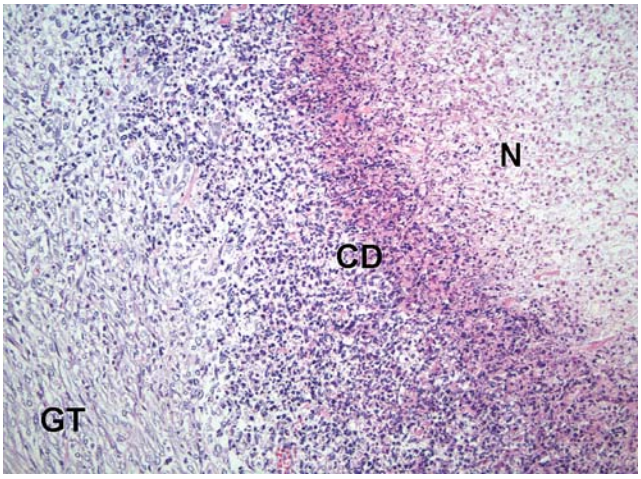

Fig 3: Necrotising panniculitis at the injection site of a guinea pig characterised by extensive necrosis (N), surrounded by cellular debris of leucocytes (CD), and granulation tissue (GT). Haematoxylin eosin, magnification 100x.

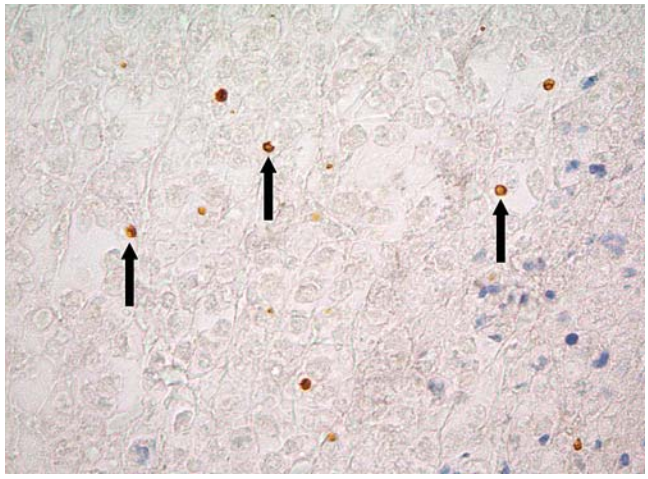

Fig 4: Immunohistological labelling of* B. mallei *antigen (arrows) within the necrotic tissue of the same guinea pig illustrated in Figure 3, avidin-biotin peroxidase complex (ABC) method, monoclonal antibody; haematoxylin counterstain; magnification 400x.

necrosis, ghost cells and calcification surrounded by some lymphocytes, plasma cells and macrophages. Bacterial organisms were not found within any nodule using both Gram and Giemsa staining or immunohistology for *B. mallei*. The skin lesions of the legs were characterised by hyperkeratosis, epidermal hyperplasia, dermal fibrosis and adnexal atrophy. On the basis of these results it was not possible to confirm or to rule out the diagnosis of glanders. At the site of mallein injection, a focal perivascularly accentuated deep dermatitis was present, which was characterised by infiltration of granulocytes, macrophages and lymphocytes.

Strauss reaction as a diagnostic tool

As a diagnostic tool to detect glanders, the Strauss reaction (acute orchitis) in guinea pigs develops after intraperitoneal injection of suspect tissue specimens ensuing the isolation of the agent. Therefore, 5 male guinea pigs were inoculated subcutaneously into the right inguinal fold with a crude suspension of lesions from liver, spleen, lung and lymph nodes. Three days after inoculation one animal died. Seven days after inoculation the other 4 animals were killed, necropsied and samples were collected for histological, immunohistological and microbiological examination. There were no testicular lesions in any of the guinea pigs, but all developed severe diffuse dermal and subcutaneous suppurative to necrotising inflammation with abscess formation at the site of inoculation (**Fig 3**). In 2 animals, the inflammation extended throughout the abdominal wall causing peritonitis and splenomegaly. Myriads of Gram-negative bacteria were identified in histological section at the inoculation site of all animals. Immunohistologically, a specific immunolabelling of *Burkholderia* antigen within the necrotising panniculitis in 2 guinea pigs was observed (**Fig 4**). These results verified the suspicion of glanders as the correct diagnosis.

Antigen detection from bacterial culture and animal tissue by PCR

In accordance with the recommendations of the OIE manual (Anon 2007) for the isolation of *B. mallei*, tissue samples from the horse (liver, lung, spleen, nasal concha, lymph nodes) and from the inoculated guinea pigs (testes, glandula vesicularis, lymph nodes, liver, spleen, abscess material) were transferred to nutrient broth[8] containing 3% glycerol[5] and on calf blood agar plates[8] containing 3% glycerol. After 24 h incubation at 37°C, a subculture of broth was carried out on blood agar plates containing 3% glycerol. After incubation for 72 h at 37°C, suspicious colonies were subcultured and examined by flagellin P gene (*fli*P) -based *B. mallei*-specific real-time polymerase chain reaction (PCR) as described by Tomaso *et al.* (2006). However, no *B. mallei* could be isolated and no bacterial DNA could be detected by PCR.

Furthermore, all tissue samples from the horse and the guinea pigs were directly tested by PCR. After grinding the tissue to a fine powder in liquid nitrogen, the DNA was isolated using the DNeasy Blood & Tissue Kit[9] according to the instructions of the manufacturer. Using the *fli*P-based real-time PCR, *B. mallei* DNA was detected in the lung tissue of the horse, but not in any of the other tissues.

Discussion

Glanders is still endemic in the Middle East, Asia, South America and might also be in Africa as recent reports from Turkey, the United Arabic Emirates, Iraq, Iran, India, Pakistan, Mongolia, China and Brazil indicate (Krishna *et al.* 1992; Bazargani *et al.* 1996; Al-Ani *et al.* 1998; Muhammad *et al.* 1998; Arun *et al.* 1999; Mota *et al.* 2000; Wernery *et al.* 2004, 2005).

The diagnosis of *B. mallei* infection still relies on serological proof, i.e. agglutination, CFT and allergic

reaction caused by the intracutaneous application of mallein (Neubauer *et al.* 2005).

Due to the low number of bacteria in infected tissues, abscess material and excreted pus, cultivation on solid or in liquid media is often negative, particularly if samples originate from subclinical or chronic cases (Bongert 1927; Miller *et al.* 1948; Arun *et al.* 1999).

The Strauss reaction (orchitis) is not specific for glanders and has a sensitivity of only 20% (Bongert 1927), although during the outbreak of glanders in Dubai, United Arabic Emirates, the isolation of *B. mallei* using this method was successful (Wernery *et al.* 2004, 2005). Material containing *B. mallei* causes a severe localised peritonitis and orchitis in guinea pigs, and this reaction can only be interpreted as '*B. mallei* - positive' in connection with the re-isolation of the agent (Anon 2007). Because of the lack of pathognomonic clinical signs, the difficult cultivation of *B. mallei* and the low sensitivity of some diagnostic tools, the diagnosis of glanders requires a meaningful combination of data from clinical signs and all the laboratory tests for glanders. It can be stated that the rather nonspecific clinical signs and the low sensitivity of the Strauss reaction, for example, could lead to a wrong diagnosis and might contribute to unintentional introduction of glanders in glanders-free regions.

In the described case, glanders was suspected due to the clinical signs that were nonspecific. Final diagnosis was carried out by means of CFT and skin test. This diagnosis was supported by a positive PCR result for lung tissue of the horse, the positive immunohistological results for sections of subcutis of 2 guinea pigs and characteristic histomorphological findings in sections of liver tissue. The failure to re-isolate the agent could be the consequence of antibiotic treatment of the horse in the run-up to the initial suspicion of glanders. The lack of glanders-positive reaction in the other PCR-tested tissues and the negative immunohistological results in the tissues of the horse underline the difficulty to diagnose glanders. The source of the infection of the horse could not be elucidated.

In conclusion, the present case highlights the omnipresent potential risk of an importation of glanders in free areas in a world of increasing international trade of horses. Nonpathognomonic clinical signs and the fact that veterinarians in countries where glanders has been eradicated for decades are not familiar with its clinical signs and might not immediately suspect the disease, can favour the entry of the disease. This vicious circle might end up in the unnoticed re-entry of glanders (Wittig *et al.* 2006). In order to prevent the spread of glanders, the sanctions of the veterinary authorities in such cases of the infection are of utmost importance.

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Manufacturers' addresses

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[3]Biotrend Co., Cologne, Germany.
[4]Vector Laboratories, Burlingame, California, USA.
[5]Sigma Chemie GmbH, Taufkirchen, Germany.
[6]Biologo Co., Kronshagen, Germany.
[7]Merck Pharma GmbH, Darmstadt, Germany.
[8]Sifin GmbH, Berlin, Germany.
[9]Qiagen GmbH, Hilden, Germany.

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