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BACTERIOLOGY (N BOREL, SECTION EDITOR)

The Resurrection of Glanders in a new Epidemiological Scenario: A Beneficiary of "Global Change"

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Abstract
Purpose of Review Glanders in horses is a re-emerging zoonotic disease. This review summarizes the existing literature and focusses on the current epidemiological situation, new diagnostic procedures, therapeutic aspects, and measures for the eradication and control of glanders. Personal observations from the field of activity of the OIE reference laboratory for glanders are included.
Recent Findings Successful eradication and control of glanders is based on the diagnostic value of various diagnostic assays. Shortcomings in the prescribed methods regarding specificity and sensitivity impair test interpretation in the laboratory and may obstruct supervising authorities. Serological assays based on purified or recombinant antigens will be available in future, after successful validation.
Summary Successful eradication and control of glanders can only be achieved by combining highly sensitive and specific testing methods with effective culling strategies. Close cooperation between authorities and owners, as well as the strict compliance to biosafety approaches in animal holdings are essential.

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Introduction

Glanders is caused by the Gram negative, non-motile, intracellular bacterium *Burkholderia (B.) mallei* [1••]. It evolved from the Southeast Asian soil bacterium *B. pseudomallei* by continuous genome reduction and large-scale insertion driven re-arrangement events during its adaption to its natural host and reservoir, the horse [2, 3]. Glanders is also a rare, but often fatal disease in humans [4]. The underlying mechanisms of pathogenicity are poorly understood, but modulation of the immune response signalling pathways and of various virulence factors are believed to result in the evasion of intracellular killing and persistent infection [5]. An outbreak of equine glanders is notifiable to the EU and OIE (Table 1). The notification will lead to trade restrictions for the affected equid population and severe economic losses in the affected countries in the long run.

The Disease in Different Hosts

Burkholderia mallei can invade its host through the mucous membranes, the gastrointestinal tract, and the integument. The incubation time has always been a matter of intensive debate (6 days to several months); however, clinical signs in experimental infection may develop within 3 days. The initial low fever, which is the first sign of infection, will often go unnoticed. Poor hygiene, crowding, and stressful conditions such as transport or adverse climate, favour the spread of *B. mallei* and the onset of clinical disease [1••, 6••, 7, 8]. The disease is acute (days to weeks) in donkeys and chronic (months to

Table 1 Glanders – disease timeline 2005-2014 [20••]

2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
Brazil	Brazil Eritrea India	Brazil India Iran Mongolia Russia	Brazil India Iran Mongolia	Brazil India Iran Kuwait Mongolia Myanmar	Bahrain Brazil Eritrea India Iran Kuwait Mongolia Myanmar Pakistan	Afghanistan Bahrain Brazil India Iran Lebanon Myanmar Pakistan	Afghanistan Brazil India Iran Pakistan	Brazil India Iran Pakistan Russia	Brazil Germany* India Iran Iraq

* one notified case, unknown source of infection

years) in horses [9]. However, new data indicate that a chronic course is also possible in donkeys [9]. Mules can show symptoms of acute as well as chronic disease. Carnivores [10] and camels are also susceptible to glanders and develop clinical disease [11]. Glanders is characterised by ulcerating nodular lesions of the skin (also known as farcy), on mucous membranes (nasal form) or on inner organs such as the lungs (pulmonary form), liver, and spleen. All different forms can coincide. Generalised symptoms include fever, malaise, depression, cough, anorexia, weight loss, and quick loss of stamina [1••, 6••, 12]. Unilateral nasal discharge and enlargement of tributary lymph nodes are frequently observed during clinical episodes. In acute cases progression of nasal symptoms, respiratory failure (bronchopneumonia) and septicaemia result in the death of the patient. The clinical picture of glanders in horses can be confused with melioidosis caused by *B. pseudomallei*. In endemic areas glanders should always be considered when tying-up syndrome is observed [13•]. In chronic glanders episodes of worsening with successive improvement of the body condition may be seen [1••, 12, 14••]. Chronic cases may recover clinically, but remain carriers for life since the bacterium can "retreat" into its niche for long periods of time and infectivity returns if the host's immune system is weakened. Thus, intermittent excretion of bacteria may occur at any time. These pre-symptomatic or carrier animals are the source of infection for the healthy equine populations and are central to spreading of disease [1••, 6••, 7, 15•, 16]. Fatal infections in persons dealing with solipeds have been observed [17•]; however, transmission from solipeds to humans appears to be rare, even in cases of close contact. The reasons for this observation are unclear and further investigations are required.

Prophylactic and Therapeutic Aspects

The main focus of vaccine research has mostly been on *B. pseudomallei*, and only few research groups have made some efforts to develop a vaccine against *B. mallei* [18]. In order to achieve complete protection against the intracellular agent *B. mallei* innate and adaptive immune responses have to be enhanced. Heat-inactivated vaccine candidates including mutants and protein antigen-based subunit vaccines have been developed, but no complete protection could be achieved to date [18, 19]. Vaccine strategies designed to silence negative regulators of the host immune system represent a novel promising way to boost innate immunity [19]. Various efforts have been made to cure glanderous horses [12] using antibiotics. The results are promising but more trials are needed to determine if the treated horses are really free of *B. mallei* or if some bacteria retain the dormant status during treatment without active metabolism (i.e. no uptake of antibiotics). These horses pose a severe risk. Improper use of antibiotics enhances the spread of glanders as demonstrated during several recent outbreaks in India [6••].

Epidemiology

The infected horse is the only known reservoir. In the environment *B. mallei* is susceptible to heat, sunlight, and common disinfectants, but may survive in water for up to 100 days. The agent is transmitted through direct contact or uptake of water or feed contaminated with skin exudates or nasal secretions of affected animals. Commonly used harnesses, grooming tools, but also veterinary equipment or water troughs and feed mangers have often been associated with outbreaks [1••, 6••, 8].

Glanders was endemic throughout the world until the end of the nineteenth century but nearly eradicated after WWII due to the diminishing importance of equids as working animals; the strict application of an internationally accepted testing (malleinisation or CFT) and culling strategy left only few pockets of endemicity in few underdeveloped countries of Asia, Africa, and America [1••]. In the second half of the last century, however, not only did the awareness for glanders diminish, but the knowledge on the clinical presentation, on its epidemiology and on the diagnostic proceedings was also lost; moreover, the necessity for research was simply ignored. This led to an increasing number of outbreaks and re-

introduction of glanders into hitherto disease-free areas. The renaissance of glanders began in the mid-1990s with the globalised trade of infected, but symptom-free pet and racing horses and with the intensive use of mules in agricultural production systems as, for example, in Brazil. To date, Brazil, Pakistan, Iran, and India [20••, 21], as well as an unknown number of countries in Asia, Africa, and America are endemic. Of interest is a recent report from Texan veterinary authorities at the Mexican border, where one of several Mexican stray donkeys was tested positive for glanders indicating the presence of pockets of endemicity on the North American continent [22]. The increasing number of outbreaks continues to pose a major risk for the global horse population. Noteworthy are recent reports from Germany on glanders in an imported horse [7] and a case where the source of infection could not be elucidated [23•]. These cases demonstrate that glanders may occur unnoticed when pre-import tests fail to detect chronic carriers.

The Diagnosis of Glanders: When is a Case Truly a Case?

It is important to bear in mind that the serological tests used for detecting glanders were tailored to their application in herd diagnosis within control programmes, and their use in individual animals may, therefore, have limitations. In order to obtain reliable results every test must be validated under the conditions of the geographic region it is intended to be used in, e.g. Europe, the Middle East, or South America. Each test will have a different sensitivity and specificity in different geographic regions due to the presence of local cross reacting bacteria, specific immunologic characteristics of the local breed of equids, contact to antibody-inducing local plants, etc. The OIE standards [24••] provide information on which sample types and sample numbers are to be taken and investigated. Special care must be placed on the quality of the serum to be analysed [25•]. Precautions have to be made to collect blood aseptically, to monitor the cold chain during transport and to use a clear identification system for the serum and its corresponding donor. Serological investigations without considering these points have limited informative value [26]. Also, the quality of the test itself and the technique applied can be crucial [25•]. A good example of this is the Complement Fixation Test (CFT). The observed variability in specificity and sensitivity is caused by the various available formulations [27, 28] and differences in test procedures/protocols [14••, 29•]; incubation time and temperature, for example, significantly influence sensitivity and specificity [29•]. Production strains, which have been used for decades have often lost their immunogenic structures, resulting in impaired sensitivity of the test. Antigen should therefore be prepared from *B. mallei* strains circulating in the area of testing. Notwithstanding these limitations, the CFT is the only approved test for the international trade of horses as it has been in use for more than a century and meticulous validation has been done in numerous countries during the last years [14••]. The CFT has proven to be a valuable screening test in the eradication programmes of many countries, but if individual horses are tested it should be expanded to include a confirmatory test in future. Three confirmatory tests may become available soon: A) a Western Blot assay using purified LPS from *B. mallei* strains. This test has been used in the last decade at the OIE reference laboratory in Jena, Germany, and approximately 50 true positive and several hundred true negative sera were tested successfully [30–32]. B) A cELISA making use of a *B. mallei* LPS specific monoclonal antibody (3D11). Different serogroups of *B. mallei* have been described in the past and the loss of single epitopes has been observed [33]. Hence, a test based on a single epitope may be "false" negative in horses infected with such strains. C) A recombinant iELISA making use of a specific epitope which showed promising results for the Indian setting [6••, 34•, 35, 36]. Bearing in mind that these tests have not yet been validated to OIE standards, the OIE has now initiated a project to validate these and several other newly developed tests for their use as confirmatory tests in international trade [37, 38]. Per definition, no serological test will be 100% sensitive or specific, but every test has its unique application within the control of glanders in local settings. Negative CFT results paired with positive Western blot results (or any other positive confirmatory tests) are to be expected. This is caused by the previously described intrinsic limitations of the different test procedures. A major shortcoming of glanders serology is that no approved standard serum is available to validate each new test set up. Prudent regulations have to be made to handle such discrepancies based on scientific knowledge, current epidemiologic situation and validation status of the tests involved. We have to accept that horses with questionable results will have to be culled for the sake of freedom of disease.

Malleinisation, i.e. testing the allergic reaction of patients to crude preparations of *B. mallei* (delayed hypersensitivity test) has even more limitations. Although still in use in some countries [39, 40], it is no longer used for international trade purposes since "false" positives can distort serology results after the application of mallein [33]. In the past it was successfully applied to identify horses with chronic infection or in control programmes without adequate laboratory facilities.

It is often impossible to cultivate *B. mallei* from or demonstrate specific components in a sample due to the nature of the disease. Only few bacteria are found in samples from chronic lesions or exudates, and usually these bacteria are overgrown by environmental bacteria or fungi on solid or liquid media due to the inappropriate application of antibiotics to the patient [6••]. Development of specific selective media has failed because of the fastidious character of *B. mallei*. Consequently,

cultivation has a very low sensitivity. Animal inoculation is believed to have a sensitivity of no more than 20% in chronic cases. Although the immune system of experimental animals is able to hinder the growth of non-pathogenic, environmental bacteria it is still very unlikely that viable *B. mallei* bacteria will be present in the samples taken.

As most lesions caused by glanders are located in the lungs, spleen, liver, and the mucous membranes, samples cannot be obtained from living animals. Necropsy is, therefore, the only option for sampling. Samples should be taken from all lesions or tissues that are conspicuous and may reach the hundreds; they must be investigated in toto. The advice of an experienced pathologist should be sought. An important supporting tool is histopathology. Although the microscopic lesions are not pathognostic, staining with anti-*B. mallei* hyperimmune serum can help to identify those samples which should be subjected to PCR analysis. The morphology of these lesions can be impressive however, the number of bacteria causing these lesions may be very low. None of *B. mallei*-specific monoclonal antibodies available are suitable for the conclusive diagnosis of glanders in tissue sections due to the specific chemistry used in pathology.

With regard to the numerous further diagnostic approaches based on DNA [41–48] or analytical methods such as matrix-assisted laser desorption/ionisation mass spectrometry and Raman spectroscopy [49, 50]: they all depend on the presence of specific DNA or on a *B. mallei* isolate. If the *B. mallei* DNA is destroyed by the chemical composition of the sample or if the sample is taken from a region of the lesion where no *B. mallei* bacteria are present, the test will obviously fail. Clinical validation of molecular diagnostics is missing due to the lack of a statistically significant number of true positive samples. Therefore, for all these direct techniques one rule applies regarding clinical samples: if the test is positive the patient has glanders; if the test is negative it does not imply that the patient does not have glanders, and the proof of freedom of disease is missing. In summary, only if the agent, i.e. the isolate or parts thereof such as DNA, proteins, LPS, are detected, they can be considered evidence that *B. mallei* is or was present in the sample or the animal, respectively. Only highly specialised and certified laboratories working under P3 conditions can carry out the complete diagnostic procedures currently available for glanders.

Glanders Control and Eradication Aspects

Since ancient times, the control of animal diseases was of major importance for human societies to ensure sufficient food supply. Considering the disastrous impact of glanders on horse populations, it is not surprising that Hippocrates and Aristoteles reported on glanders as early as 425 and 350 BC. Special care was taken to guarantee a productive and healthy horse population and the physical fitness of the individual horse was closely monitored as it was the most efficient and valuable weapon in wars up to WWII [51]. The success of the eradication programmes in the USA, Canada, and in Western European countries such as the UK or Germany was based on few principles: a meticulous testing (CFT and/or malleinisation) and culling policy and strict import controls at borders. A key issue for the acceptance of the harsh countermeasures applied was the fair re-imbursement for the culled animals [33]. Because of our involvement in most outbreaks of glanders in the past two decades we have been able to monitor the disease and its epidemiology and found no changes with regard to earlier descriptions: a clinically inapparent carrier is imported to a disease-free equid population and starts to spread the disease. The disease runs undetected for a longer period of time and newly infected but clinically inapparent animals continue to spread the disease [1••]. The success of an eradication programme will, therefore, depend on the ability of the involved authorities to identify ‘healthy’ carriers, to trace back their contacts, and to destroy all infected animals. Because *B. mallei* is not able to establish an environmental reservoir, the outbreak will stop when the last carrier is removed from the population. This explains why it is essential to test every soliped for the existence of “clinically” healthy shedders. It is strongly recommended to maintain serological monitoring in defined intervals for a certain period of time after eradication of disease. Suspicious seropositive cases should be quarantined immediately. All mallein and/or serologically positive animals have to be destroyed. Safe destruction or burial of carcasses, decomposition of manure, and disinfection of premises must be part of the control program. Carcasses of animals affected with glanders must be condemned, and the meat should not be allowed to be used for consumption. Anecdotal reports describing human infection due to consumption of contaminated horse meat exist. Consequently, standard operating procedures for meat hygiene and inspection are imperative in the control of transmission of glanders to humans and carnivores. Further pre-requisites for the efficient control of infectious diseases are: the recording of all holdings in which equines/equids are kept; the registration of every individual animal at birth by marking them, e.g. with a chip. Stock registers on farms and a central database are beneficial (essential) in an outbreak situation. Movement control regulations are advised so that all contacts of an index case can be identified and tested in order to enable the tracing back. Water hygiene is crucial in the control of the spread of *B. mallei* especially in areas where public water troughs are in use. Locations where high numbers of animals from different areas come together need to be controlled meticulously,

i.e. markets, exhibitions, slaughterhouses, collection centres, shows, races, hospitals, etc. Passive surveillance should always be in place, e.g. meat inspection. Data on routine export/import tests and reports of meat inspection should be made available to public authorities. Strict veterinary regulations including serological testing of animals prior to transport can reduce the risk of importation of glanders into free areas. Products of equine origin such as semen, ova, or sera must also be strictly controlled before import. Outbreaks of glanders should immediately be notified to the OIE and to the authorities in the country concerned so that appropriate counter measures for disease surveillance (endemic areas) and eradication (endemic and non-endemic areas) can be implemented. Biosecurity and management practices in resident equine populations are important curtailing factors in preventing disease spread to the local horse population. It is essential to observe the epidemiology of glanders within a geographic context and with simultaneous consideration of the local social or regional customs [1••].

Glanders is a transboundary disease and, therefore, all countries in a region should harmonise their programmes in order to contain the spreading of infection. However, in the majority of cases upon introduction of the pathogen, failure to comply with the OIE disease specific recommendations is observed [15•]. Territorial states like India face imminent problems in their attempts to control glanders due to unidentified pockets of disease, attempts to treat the animals using antibiotics, the selling of infected equids, and movement across borders of administrative districts [6••, 52]. The lack of sound compensation policies for culling cause severe opposition from the mostly very poor animal owners who, therefore, prefer to sell their animals beforehand [6••, 42, 52]. Turkey initiated a nationwide eradication program on glanders between 2000 and 2001 and reported the successful eradication of glanders [53], but no detailed follow-up report is available to this day. Moreover, it is not known to what extent the wars in Iraq and Syria, both bordering on Turkey, have had an impact on glanders control. With the increasing number of *B. mallei* genomes available [54, 55] molecular epidemiology is a promising technique to speed up the time needed for eradication and to control reimport effectively. Molecular typing procedures based on VNTR in *B. mallei* genomes are able to elucidate infection chains and show the global distribution of *B. mallei* clusters [56, 57•].

The OIE is currently implementing aspects of biosafety and biosecurity in the newly established "high-health, high-performance horses" disease risk mitigation strategy [58], mainly focused on the harmonisation of conditions for international movement of competition horses. A further effective countermeasure would be the implementation of a (mandatory) post-arrival quarantine and re-testing of all imported horses. The animals should remain in quarantine until testing is completed but at least for the duration of 14 days to allow a period of observation during which any latent disease may become active as a consequence of the stress imposed by travel [59].

New developments have to be "incorporated" in the current strategy of glanders control: A) Melioidosis caused by *B. pseudomallei* is a very successful emerging disease with temporary reservoirs in horse holdings; it infects horses efficiently. It also infects humans, and 50% of cases are fatal even if treated in time. It has emerged in areas endemic for glanders in Brazil and has caused human disease and death [60]. No promising concepts to control or eradicate this saprophyte currently exist. B) The availability of effective antibiotics may be an option in glanders control under certain circumstances: Although regulations call for culling of diseased animals, certain situations, e.g. wildlife conservation, highly valuable breeding stock, could benefit from effective treatment schemes, and post-exposure prophylaxis [12]. C) There is an obvious need for cheap and sensitive pen-side tests for remote areas in developing countries. Such techniques are available, but have not yet been validated for glanders. D) The finding of cases of glanders in so far free countries forces us to reconsider the concept of global transport using CFT. We also need new risk assessments for not endemic countries with a history of intensive trade with endemic countries.

Conclusion

Glanders has always posed a special challenge to public veterinary health. The disease cannot be described as a clinical entity, and it cannot be diagnosed based on the presentation of clinical signs alone. Chronic infected patients displaying no clinical signs or only unspecific lesions (e.g., scars on mucous membranes or the skin) and without anti-*B. mallei* antibodies circulating in their bloodstream pose a diagnostic problem. They will spread the disease accidentally and maintain the infectious cycle as reservoirs. In our experience, well-kept, but infected pet and sport horses represent this phenotype. Disease is noticed most often in these horses when the immune system is compromised after exposure to stressful situations, e.g. travel. Despite these problems experienced clinicians regularly identify infected horses. Saqib et al., 2012 [12] described clinical severity scores based on a set of clinical findings often present in glanderous horses.

Compliance with Ethics Standards

Conflict of Interest Mandy C. Elschner, Heinrich Neubauer and Lisa D. Sprague declare they have no competing interests.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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