# *Evaluation of serological responses in horses challenged with* Burkholderia pseudomallei *using current diagnostic tests for glanders*

Ulrich Wernery[1*] Marina Rodriguez Caveney[1], Renate Wernery[1], Rekha Raghavan[1], Karine Laroucau[2], Ginu Syriac[1], Shruti Miriam Thomas[1], Jeeba John[1], Marina Joseph[1], Shantymol Jose[1], Sunitha Joseph[1] and Patrick Woo[3]

[1]*Central Veterinary Research Laboratory, United Arab Emirates.*
[2]*European Union Reference Laboratory for Equine Diseases, Anses, Laboratory for Animal Health, Bacterial Zoonosis Unit, Maisons-Alfort, France.*
[3]*Department of Microbiology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong.*

[*]*Corresponding author at: Central Veterinary Research Laboratory, PO Box 597, Dubai, United Arab Emirates Tel.: +971 4 337 5165, e-mail: cvrl@cvrl.ae.*

*Veterinaria Italiana* 2019, **55** (3), 261-267. doi: 10.12834/VetIt.1701.9026.2
Accepted: 19.04.2019 | Available on line: 30.09.2019

**Keywords**
Haematology, Inoculum, Melioidosis, Serology, Subcutaneous, *Burkholderia pseudomallei*.

**Summary**
Six horses were challenged experimentally with a strain of *Burkholderia pseudomallei* isolated from a fatal case of the infection in a dromedary camel years earlier in the Emirate of Dubai. Three horses were inoculated subcutaneously and in 3 the bacterium was administered by the oral route. Four of the horses became serologically positive based on reactions to one or more of the OIE described tests for glanders. *B. pseudomallei* was re-isolated from the 4 serological positive horses. Only one of the subcutaneously infected horses, developed fever for 3 days. The white blood cell values and the neutrophil counts were also elevated. The study confirmed that existing serological test for diagnosing glanders cannot differentiate between glanders and melioidosis in horses.

## *Positività alle prove sierologiche ufficiali per Morva in cavalli infettati con* Burkholderia pseudomallei

**Parole chiave**
Ematologia, Inoculo, Melioidosi, Sierologia, Sottocutaneo, *Burkholderia pseudomallei*.

**Riassunto**
In questo studio stati infettati sei cavalli con un ceppo di *Burkholderia pseudomallei* isolato da un dromedario infetto deceduto nell'Emirato di Dubai. Il batterio è stato inoculato per via sottocutanea in tre cavalli e somministrato per via orale negli altri tre. Quattro cavalli, da cui è stato isolato nuovamente l'agente patogeno, sono risultati sierologicamente positivi ai test OIE per la morva. Solo uno dei cavalli infettati per via sottocutanea ha avuto un'alterazione febbrile della durata di 3 giorni. I loro valori di globuli bianchi e di neutrofili erano considerevolmente elevati. Lo studio ha confermato che nei cavalli il test sierologico per la morva non è sufficiente a differenziare la diagnosi di morva da quella di melioidosi.

## *Introduction*

Melioidosis is an infectious disease which resembles glanders. It is caused by *Burkholderia* (*B.*) *pseudomallei* a Gram-negative bacillus which share over 99% genetic homology with *B. mallei*, the etiologic agent of glanders (Rainbow *et al.* 2002). Melioidosis is a disease common to both, animals and people. It is associated with the development of suppurative or caseous lesions in infected organ. *B. pseudomallei*, is a motile, Gram-negative, facultative anaerobic bacillus which grows readily on routine diagnostic media, but preferably on Ashdown's medium on which it develops a distinctive colonial morphology (Markey *et al.* 2013). *B. pseudomallei* is usually found in soil and water and may affect a wide range of animal species (Lefèvre 2010). In terms of global distribution, melioidosis is predominantly associated

with tropical and subtropical areas of Thailand, Vietnam and India, but also with North Australia. For many years, it was considered a tropical disease of warm and humid countries (Limmathutotsakul *et al.* 2015). Since 1970 however, melioidosis has also been reported in countries with a temperate climate such as parts of Africa, southern Australia, Latin America and Europe. On occasion, *B. pseudomallei* has been introduced into new environments in which it may cause sporadic disease as was the case with regard to the outbreak in the zoological gardens in Paris in 1975 (Nouvel *et al.* 1976) and in Brazil in 1982 (Galimand *et al.* 1982). Shipment of contaminated soil, water and infected animals (Ryan *et al.* 2018) could potentially give rise to melioidosis outbreaks anywhere, including countries in which glanders occurs. On this basis, melioidosis could be considered a re-emerging disease. While serological tests such as the complement fixation, indirect haemagglutination, ELISAs and other assays are effective herd surveillance tools, they do not differentiate between melioidosis and glanders infected horses. False-positive reactions for glanders may occur in areas where melioidosis is endemic since available serological tests may detect antibodies that cross-react with those of *B. mallei*. In the case of melioidosis, no serological tests have yet been validated for use in veterinary medicine (OIE 2018).

An experimental study of *B. pseudomallei* infection in horses was undertaken in Dubai, United Arab Emirates, with a triple aims, the first of which was to investigate the clinical signs and pathological lesions of melioidosis in this species. The second objective was to raise positive equine sera to *B. pseudomallei* for the purpose of developing serological tests for the diagnosis of the disease in horses and thirdly, to investigate if the sera of infected horses cross-react in currently available diagnostic tests for glanders. The results of the third objective is reported herein.

## Material and methods

### Design study

The experiment lasted 2 months. The duration of the study was based on the development of clinical signs and health deterioration, which are detailed in the 'Outcome of the study' section.

### Horses

Six retired horses each above 25 years of age and of different gender, all with a degree of intercurrent bone and ligament diseases were selected for this experiment. They were kept isolated in individual horse boxes in a desert area. Two experienced equine grooms were responsible for the horses which were fed timothy hay *ad libitum* and 2 kg of grain mixture twice a day in the morning and afternoon. All horses had access to fresh water via automatic drinkers in their boxes. The front and rear of the barn were kept closed by big doors which as a precautionary method, were covered with an insect proof-netting outside each door. The entire barn was air-conditioned with no open windows. Grooms and researchers entered through a separate side door, which led to a separate room. Furthermore, the entrance to this room was secured by an insect proof netting and a mat containing disinfectant. This room was used by 2 grooms and 3 researchers to change into disposable gowns and rubber boots; each person put on three layers of gloves one of which (middle one) had shoulder protection (Veterinary gloves, Henry Schein, USA). Besides wearing a Particulate Respirator N95 mask (3M-8210, Mexico) and goggles, all 5 persons also wore an anti-fog, protection-face shield (VWR, USA).

### Inoculum

The source of the strain of *B. pseudomallei* used for this study was a dromedary camel that died of the infection in 1997 (Wernery *et al.* 1997). The strain was grown on Ashdown's medium. The inoculum had a concentration of 6.6 x $10^7$ cfu/ml. Three of the horses were injected with 2 ml of the inoculum subcutaneously on the left side of the neck (Figure 1). The remaining 3 horses were fed a piece of bread that had been injected with 5 ml of the same inoculum.

### Samples

Blood was collected for serological tests, which are discussed in results section and temperature was monitored daily. Blood was withdrawn from the jugular vein until horses were euthanased (please see

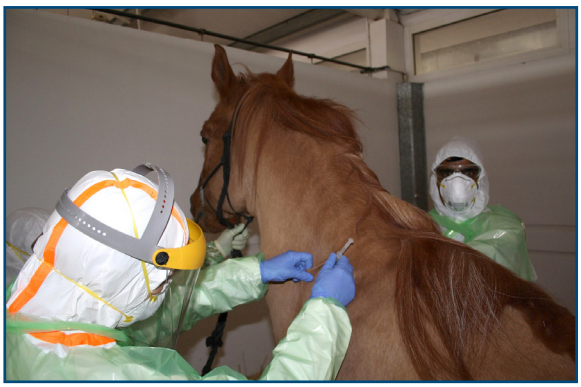

**Figure 1.** *Subcutaneous infection of horse 2 with* Burkholderia pseudomallei resistance.

outcome of the trial in the results section). The blood was allowed to clot and was transported in cool boxes to CVRL where it was centrifuged and serum was frozen and stored at - 20 °C until tested. Additionally, EDTA blood was also regularly withdrawn and blood parameters (RBC, WBC, PCV, haemoglobulin, neutrophils and lymphocytes) were assessed by using the automatic haematology analyser SYSMEX XT 2000i (Kobe, Japan). The reference values for the parameters tested were adapted from the 'Diagnostic cytology and haematology of the horse' (Cowell and Tyler 2002). All horses were regularly monitored twice a day, in the morning and evening, rectal temperatures were recorded and also the haematological values were examined during each visit. Sera were tested using the following serological tests for the diagnosis of glanders. Complement fixation test (CFT) using either two antigens; a commercial antigen from c.c.pro (Oberdorla, Germany) or Dubai-7 (Scholz *et al.* 2006) which was produced according to the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE 2018); two enzyme-linked immuno-sorbent assays (ELISA), an in-house ELISA using Dubai-7 antigen and a commercial ELISA from IDvet, Montpellier, France (Laroucau *et al.* 2014). The fifth serological test used was an immunoblot assay (IB) (Elschner *et al.* 2011). The different serological tests used in this study were carried out in accordance with the detailed methodology described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals of the OIE in 2018, Chapter 2.5.11.

## Results

### Outcome of the trial

Out of the six trial horses, three (1, 2, and 4) injected subcutaneously and one (3) challenged by the oral route, became infected with *B. pseudomallei*.

Horse 1: 30 years of age, had pre-existing chronic arthritis and after subcutaneous infection, it developed fever within 4 days, followed by weight loss, nasal and ocular discharge. Abscessation at the site of injection developed on day 7 post infection (pi). Horse was euthanased on day 13 pi.

Horse 2: 31 years of age. The severity of 'rocked back stance' which was primarily due to previous chronic ailments, increased. After subcutaneous injection, there was severe swelling at the injection site. Mucopurulent discharge from both nostrils and a swelling at the right side of the brisket were observed. The horse was euthanised on day 31 pi.

Horse 3: 26 years of age. Severe swelling of the left chest area followed by weight loss and severe lameness of both front legs was observed after oral infection. The horse was euthanased on day 37 pi.

Horse 4: 28 years of age, it developed slight fever on day 14 pi. following subcutaneous injection. Abscessation at the site of injection was also observed from day 4 pi. and from day 17 pi. the left retropharyngeal lymph node swollened at the size of a golf ball. The animal lost appetite and weight. It was euthanased on day 44 pi.

Horse 5: 23 years of age, with one eye only. It was severely lame for many years after a fracture of the left tibia. The animal developed mild nasal discharge from day 7 to 13 pi and lost weight. It was euthanased on day 51 pi.

Horse 6: 28 years of age, it suffered from severe chronic suspensory ligament injury on the right front leg and was emaciated. After oral infection, it developed mild nasal discharge from day 6 to day 12 pi. The horse was euthanased on day 58 pi.

**Table I.** *Summary of clinical signs and pathological lesions observed in 6 horses infected with* B. pseudomallei.

| Horse ID | Age in years | Route of infection | Clinical signs | Loss of weight | Abscess at injection site | Euthanazed days p.i | Pathology |
|---|---|---|---|---|---|---|---|
| 1 | 30 | Sub cutaneous | Fever (40 °C), nasal and ocular discharge | Yes | Yes, opened | 13 | Pulmonary and renal abscesses; acute hemorrhagic cystitis. No lesions in nasal septum/ conchae |
| 2 | 31 | Sub cutaneous | No fever, mucopurulent nasal discharge | No | Yes, swelling not opened | 31 | Pulmonary hemorrhages; subacute pyogranulative cystitis. No lesions in nasal septum/ conchae |
| 3 | 26 | Oral | No fever, lameness of both front legs swelling at chest, no nasal or oral discharge | Yes | Swelling left chest | 37 | Granulative cystitis. No pulmonary and renal lesions. No lesions in nasal septum/conchae. |
| 4 | 28 | Sub cutaneous | Slight fever (38.6 °C), weight loss, stopped laming, no ocular or nasal discharge | Yes | Yes, opened | 44 | Granulative cystitis. No pulmonary and renal lesions. No lesions in nasal septum/conchae. |
| 5 | 20 | Oral | None | No | No | 51 | No pathological lesions. |
| 6 | 29 | Oral | None | No | No | 58 | No pathological lesions. |

## Other clinical signs

Clear to mucopurulent bilateral nasal and eye discharge was observed in horse 1, less copious discharges were also seen in horses 2 and 3. These clinical signs persisted until days 13 pi, 31 pi and 37 pi, respectively. No clinical signs were observed in horses 5 and 6 and they were euthanased within two months.

Horses subcutaneously infected displayed multiple pyogranulomatous nodules in lungs and kidneys, but did not exhibit conchal ulcers as seen in glanders horses.

## Blood parameters

Infection with *B. pseudomallei* influenced the haematological profile of horse 1 which also had fever (38.8 °C-39.9 °C) for 4 days (from day 3 to day 8 pi). The white blood cell count (WBC) increased from 12 to 38 $10^6$/l and the neutrophil count from 73% to 89%. Haematological values did not change considerably in the remaining infected horses (Figure 2).

## Serology

Blood samples were analyzed for antibodies to *B. mallei* using 5 serological tests, 2 CFTs, 2 indirect ELISAs and one IB. With the exception of the IB test, serological results are summarized in Figure 3. Interpretation of the IB test is based on a qualitative threshold, indicated by a protein band (colorimetric method), not by a numerical value.

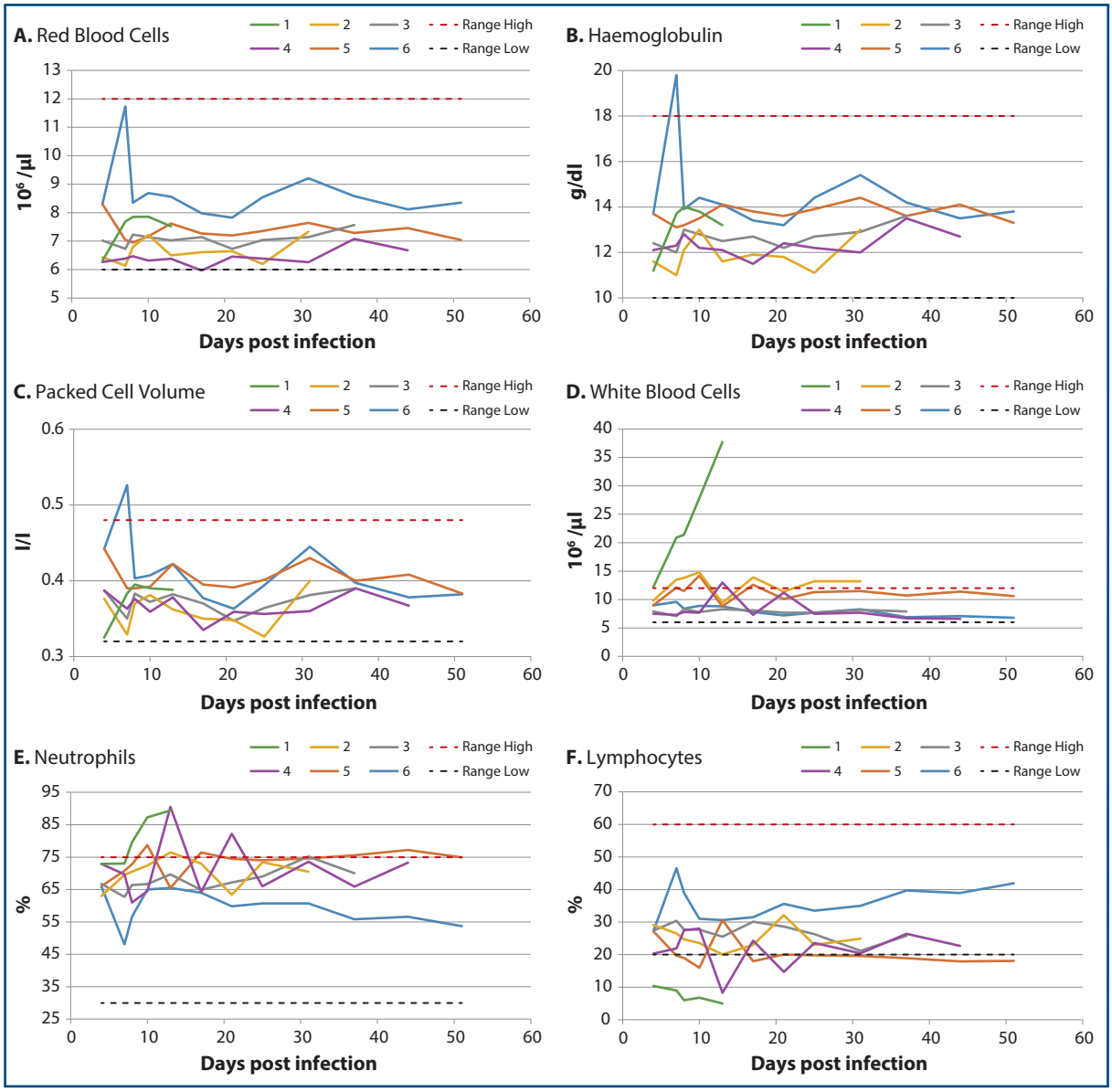


**Figure 2.** *Haematological results of 6 horses before and after infection with* B. pseudomallei.

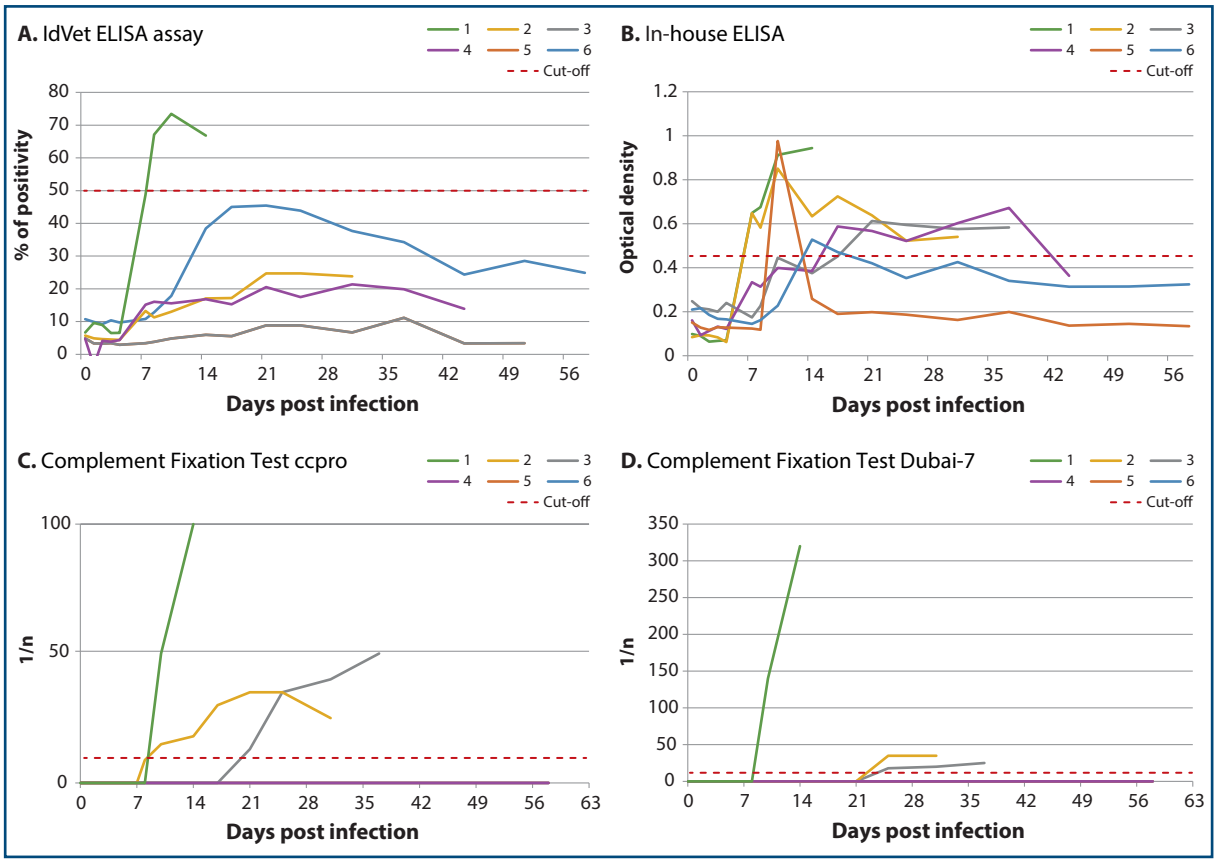


**Figure 3.** *Serological results of 6 horses infected with* B. pseudomallei *using 4 serological tests for diagnosing glanders.*

All subcutaneously infected and one orally infected horses (horse 3) developed antibodies, detected by CFT and / or ELISA.

Horses 5 and 6 which were challenged by oral route remained seronegative throughout the study period.

Horses 1 and 2 which were infected by the subcutaneous route developed a detectable serological response in both, ELISA and IB tests, starting from day 7 pi. Three days later horse 1 was positive to both CFTs, while horse 2 from day 10 pi to ccpro and from day 35 to the Dubai-7 CFT.

## Discussion

The six horses used for this experimental study of *B. pseudomallei* infection were above 25 years of age and all suffered since many years from ligament and bone diseases. In the 10 years preceding the study, they had been kept in a horse retirement enclosure and were to have been euthanased shortly because of age and clinical ailments. However, permission was granted to include them in the *B. pseudomallei* challenge study before euthanasia (see Animal Welfare issue). As it is evident from Figure 3, three subcutaneously (horses 1, 2 and 4) and 1 orally infected horses (horse 3) developed antibodies. CFTs, the IB and the in-house ELISA remained positive for horses 1 and 2 until they were euthanased on days 13 and 31 pi, respectively. On the contrary, horse 2 was negative when tested by the IDvet ELISA. This discrepancy may be explained by the fact that the recently isolated Dubai-7 strain of *B. mallei* is antigenically closer to *B. pseudomallei* whereas the antigen used in the IDvet ELISA was an ATCC strain of the bacterium. Of the 6 horses challenged with *B. pseudomallei*, this commercial ELISA gave positive reactions only on samples collected from horse 1 which developed severe disease. The animal had showed an increasing CFT titer which continued to increase until the end of the study. From horse 1 and horse 2 *B. pseudomallei* was re-isolated. Horse 3, which was orally infected, became CFT, ELISA using Dubai-7 strain of *B. mallei* and IB positive from day 21 pi. Its CFT titre remained low and the IDvet ELISA negative. The animal was euthanased on day 37 pi as in the case of horse 2. Also from this animal *B. pseudomallei* was re-isolated.

Horse 4, which was infected subcutaneously, did not produce CFT and IDvet ELISA antibodies but reacted with the Dubai-7 strain of *B. mallei* ELISA and IB assay from day 21 pi until day 44 pi. As for horse 1, 2, and 3, *B. pseudomallei* was also re-isolated from horse 4. Although due to the exiguous sample size any conclusion should be interpreted with caution,

according to these findings, the IDvet ELISA appears to be more specific than the other serological tests used in this study.

Horses 5 and 6, which were both orally challenged, did not seroconvert until the entire length of the trial. *B. pseudomallei* was not re-isolated from these horses.

Horses which produced antibodies after infection also showed lesions in some organs. The two serologically negative horses, instead, did not have any observable lesions.

This study confirmed that the serological diagnosis of melioidosis is difficult and that glanders and melioidosis cannot be differentiated based on current diagnostic tests for glanders. Although specific assays have been developed to detect antibodies to *B. pseudomallei*, most of them use poorly characterized antigens and are not standardized (Wiersinga *et al.* 2018). A protein microarray that contains 20 recombinants and purified *B. pseudomallei* proteins, provides a standardized test for detection of antibodies in humans (Kohler *et al.* 2016). A similar approach may have the potential to improve the serodiagnosis of this infection in other species including equids in which it has not been investigated yet. It is hoped to use the sera obtained from this study as the basis for further studies that hopefully will lead to a serological test for the diagnosis of melioidosis in equids.

## Statement of animal rights

When reporting experiments on animals, authors should indicate whether the institutional and national guide for the care and use of laboratory animals was followed.

## Acknowledgement

The authors herewith express their gratitude to H. H. Sheikh Mohammed Bin Rashid Al Maktoum, Ruler of Dubai and Vice President of the United Arab Emirates as well as Saeed Al Tayer, Chairman of Dubai Racing Club and Dr. Ali Ridha, Director General of the Central Veterinary Research Laboratory. We thank the following persons who helped us with this project: Brenda Cooke, Heather Copland, Zulfiqar Ali Kiani, Tahir Zaman, grooms: Shyam Singh and Karan Singh.

## References


Cowell R.L. & Tyler R.D. 2002. Diagnostic cytology and haematology of the horse. 2nd Ed. Mosby, St Louis, p. 201.

Elschner M.C., Scholz H.C., Melzer F., Saqib M., Marten P., Rassbach A., Dietzsch M., Schmoock G., de Assis Santana V.L., de Souza M.M., Wernery R., Wernery U. & Neubauer H. 2011. Use of a Western blot technique for the serodiagnosis of glanders. *BMC Vet Res*, **7**, 4.

Galimand M. & Dodin A. 1982. Le point sur la mélioidose dans le monde. *Bull Soc Path Exot*, **75**, 375-383.

Kohler C., Dunachie S.J., Müller E., Kohler A., Jenjaroen K., Teparrukkul P., Baier V., Ehricht R. & Steinmetz I. 2016. Rapid and sensitive multiplex detection of *Burkholderia pseudomallei* - Specific antibodies in melioidosis patients based on a protein microarray approach. *PLoS Negl Trop Dis*, **10**(7): e0004847.

Laroucau K., Bertin C., Roche M., Donnet F., Colaneri Falcão M.D., Madani N., Zientara S., Pourquier P., Santana V.L., Saqib M. & Comtet L. 2014. A new ELISA assay for glanders diagnosis. III Pan-American Congress of Zoonoses, La Plata, 4-6 June 2014.

Lefèvre P.-C., Blancou J., Chermette R. & Uilenberg G. 2010. Infectious and parasitic diseases of livestock. Paris, Lavoisier, 1189-1196.

Limmathurotsakul D., Golding N., Dance D.A.B., Messina J.P., Pigott D.M., Moyes C.L., Rolim D.B., Bertherat E., Day N.P.J., Peacock S.J. & Hay S.I. 2016. Predicted global distribution of *Burkholderia pseudomallei* and burden of melioidosis. *Nat Microbiol*, pii: 15008.2015.

Markey B., Leonard F., Archambault M., Cullinane A. & Maguire D. 2013. Clinical veterinary microbiology. 2nd Ed. Mosby, Elsevier, 275-284.

Nouvel J., Dodin A., Demontory M.C. & Chove M.A.1976. La mélioidose à la ménagerie du Jardin des Plantes de Paris. *Bull Acad Vet*, **49**, 223-230.

Rainbow L., Hart C.A. & Winstanley C. 2002. Distribution of type iii secretion gene clusters in *Burkholderia pseudomallei, B. thailandensis* and *B. mallei. J Med Microbiol*, **51**, 374-384.

Ryan C.W., Bishop K., Blaney D.D., Britton S.J., Cantone F., Egan C., Elrod M.G., Frye C.W., Maxted A.M. & Perkins G. 2018. Public health response to an imported case of canine melioidosis. *Zoonoses Public Health*, **65** (4), 420-424.

Scholz H.C., Joseph M., Tomaso H., Al Dahouk S., Witte A., Kinne J., Hagen R.M., Wernery R., Wernery U. & Neubauer H. 2006. Detection of the reemerging agent *Burkholderia mallei* in a recent outbreak of glanders in the United Arab Emirates by a newly developed fliP-based polymerase chain reaction assay. *Diagn Microbiol Infect Dis*, **54**, 241-247.

Wernery R., Kinne J., Hayden-Evans J. & Ul Haq A. 1997. Melioidosis in a seven-year-old camel, a new disease in the United Arab Emirates (UAE). *J Camel Pract*, **4** (2), 141-143.

Wiersinga W.J., Virk H.S., Torres A.G., Currie B.J., Peacock S.J., Dance D.A.B. & Limmathurotsakul D. 2018. Melioidosis. *Nat Rev Dis Primers*, **4**, 17107.

World Organisation for Animal Health (OIE). 2018. Manual of diagnostic tests and vaccines for terrestrial animals. Chapter 2.5.11 Glanders and mMelioidosis. Paris, OIE.