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# Serodiagnosis of glanders with reference to endemic- and non-endemic settings

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**Iahtasham Khan**

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| Dekan: | Univ.-Prof. Dr. Leo Brunnberg |
| Erster Gutachter: | Prof. Dr. Lothar H. Wieler |
| Zweiter Gutachter: | Prof. Dr. Heinrich Neubauer |
| Dritter Gutachter: | Prof. Dr. Heidrun Gehlen |

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**Abbreviations**

| | |
|---|---|
| AP | Alkaline Phosphatase |
| AGID | Agar (ose) gel immunodiffusion |
| ATCC | American type culture collection |
| *B.* | *Burkholderia* |
| BALB/c | albino, laboratory-bred strain of the house mouse |
| BC | Before Christ |
| BCIP | Bromo-chloride-indolphosphate |
| cELISA | Competitive Enzyme Linked Immunosorbent Assay |
| °C | Grad Celsius |
| C8-HSL | Octanoyle-homoserine lactone |
| C10-HSL | *N*-decanoyl-homoserine lactone |
| CIDC | Central Veterinary Institute of Wageningen UR, The Netherlands |
| CIE | Counterimmunoelectrophoresis |
| CMS | Department of Clinical Medicine and Surgery, Faisalabad, Pakistan |
| CPS | Capsular Polysaccharide |
| CFD | Complement Fixation Diluent |
| CFT | Complement Fixation Test |
| DMF | Dimethylformamide |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme Linked Immunosorbent Assay |
| FAO | Food and Agriculture Organization |
| Fig. | Figure |
| FLI | Friedrich-Loeffler-Institut, Germany |
| G | Gram(s) |
| h | Hour(s) |
| H2O | Water |

| | |
|---|---|
| HA | Haemagglutination Assay |
| HS | Haemolytic System |
| IATA | International Air Transport Association |
| IB | Immunoblotting |
| IFA | Immunofluroscent assay |
| IgG | Immunoglobulin G |
| IHA | Indirect Haemagglutination Assay |
| IL | Interleukin |
| K | Kappa |
| KDa | Kilo Dalton(s) |
| LPS | Lipopolysaccharide |
| mAbs | Monoclonal antibodies |
| mCIE | modified Counter-Immuno-Electrophoresis |
| mg | Milligram |
| $MgCl_2$ | Magnesium chloride |
| MIC | Minimum Inhibitory Concentration |
| Min | Minute(s) |
| MLST | Multilocus Sequence Typing |
| mM | Millimolar |
| mL | Millilitre(s) |
| MLVA | Multiple Loci VNTR Analysis |
| MNGCs | Multinucleated giant cells |
| µm | Micrometer(s) |
| µl | Microliter(s) |
| µg | Microgram(s) |
| NaCl | Natriumchlorid |
| NBT | Nitroblue-Tetrazoliumchloride |
| OD | Optical density |

| | |
|---|---|
| OIE | Office International Des Epizooties/ World Organisation for Animal Health |
| *P.* | *Pseudomonas* |
| PBS | Phosphate Buffer Saline |
| PCR | Polymerase Chain Reaction |
| PFGE | Pulse Field Gel Electrophoresis |
| RBT | Rose Bengal Test |
| RD | Remount Depot, Equine Rearing Establishment, Sargodha, Pakistan |
| RFLP | Restriction Fragment Length Polymorphism |
| RPM | Revolutions Per Minute |
| RNA | Ribonucleic acid |
| rRNA | Ribosomal ribonucleic acid |
| RVFC | Remount Veterinary and Farms Corps, Sargodha, Pakistan |
| spp. | Species |
| SDS-PAGE | Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis |
| Tab. | Table |
| TLR | Toll-like receptor |
| T2SS | Type II secretion system |
| T3SS | Type 3 secretion system |
| T6SS | Type 6 secretion system |
| U | Units |
| UAF | University of Agriculture Faisalabad, Pakistan |
| USDA | United States Department of Agriculture, Ames, Iowa, USA |
| V | Volt |
| VNTR | Variable Number Tandem Repeats |
| WB | Western Blot |

## 1 Introduction

The causative agent of glanders in solipeds is *Burkholderia mallei (B. mallei)* which is a Gram-negative, nonmotile, intracellular bacterium. *B. mallei* is an obligate mammalian pathogen (Neubauer *et al.,* 2005). Infection in equids provokes different syndromes depending on the route of entry. Glanders is characterized by nodules and ulcerations in the upper respiratory tract and the lungs. The skin form is known as farcy. In case of acute infection severe febrile condition is seen (Schlater, 1992). Glanders presents mostly in chronic form in horses and is acute in donkeys and mules. The disease is highly contagious (Mandell *et al.,* 1995). Glanders in horses was described already in ancient times by Greek and Roman writers e.g. Aristotle, 350 BC as "malignant" or "bad disease" (Henning, 1956; Steele, 1980). The agent also infects occasionally humans in close contact to infected animals with a high case fatality rate (Mandell *et al.,* 1995; Neubauer *et al.,* 1997). *B. mallei* has been used as an agent of biological warfare against humans and animals during World War I and II (Lehavi *et al.,* 2002). Although test and slaughter policy in solipeds has been successfully applied to eradicate glanders from the United States and Western Europe, it is still endemic in Africa, the Middle East, Central and South America and parts of Asia (Neubauer *et al.,* 2005). Since 1999 a number of glanders outbreaks in horses have been registered in Pakistan, predominantly in the area of the Punjab. Solipeds including horses, mules and donkeys were the affected animals. Cutaneous, pulmonary and septic clinical forms with rare neurologic signs were seen in these animals (Lopez *et al*., 2003). Almost 90% of infection exists as nonclinical or latent form (Neubauer *et al*., 2005).

Eradication programs have been launched accordingly and attempts have been made to comparatively evaluate various tests for serological diagnosis e.g. the Rose Bengal Test (RBT) (Naureen *et al*., 2007). In Pakistan, low indemnity paid to the owner and lack of stringent implementation of the Glanders and Farcy Act (1899) limit the owners' preparedness to destroy glanderous animals (Muhammad *et al*., 1998). Economics constraints contribute to the persistence of glanders in this region because modern methods for control of this disease (monitoring and euthanasia) are not viable options (Hornstra *et al*., 2009). Disease can easily spread through drinking water troughs and commonly used harness contaminated by nonclinical or latent infected horses or latent carriers with crowding conditions and stress (Marek and Manninger, 1945). Horses and mules can be asymptomatic carriers and many hosts can play a role to maintain *B. mallei* (Al-Ani *et al*., 1987). Communal stables and drinking water troughs are available throughout the Pakistani Punjab main districts including Sargodha, Faisalabad, Lahore etc. and *B. mallei* has been isolated from these water troughs recently (Hornstra *et al*., 2009). To our best knowledge, no study was conducted to assess the seroprevalence of glanders in Punjab, Pakistan.

The complement fixation test (CFT) and mallein test, were the means of diagnostic choice in former eradication programs worldwide when the prevalence of glanders was considerably high (Marek and Manninger, 1945). CFT is the preferred diagnostic tool because it has the ability to detect clinically, inapparent carriers and chronically infected horses which are responsible to spread glanders to healthy equine populations and cause new outbreaks (Mark and Manninger, 1945). Historically, the CFT remained also the OIE (Office International Des Epizooties, Paris, France) mandatory test for international trade of equines although a high number of sera is reported to be diagnosed false positive (Marek and Manninger, 1945; Wernery *et al*., 2004, 2005). Cross reactions have also been reported between *B. mallei* and *Streptococcus equi* (etiologic agent of strangles), resulting in false positive reactions (Al-Ani, 1993). Old, pregnant and debilitated animals showed false negative results in CFT. Use of crude whole-cell preparations in CFT caused false positive results due to genus-specific cross-reacting antigens. Demands have been changed now and need for a technique which can reduce false-positive (and false-negative) results is highly indispensible (Neubauer *et al*., 2005). According to the principles of validation of diagnostic assays (OIE, 2008), improvement of test quality always includes the evaluation of new analytical test substances e.g. new antigens in the CFT. Hence, the accuracy of the CFT is believed to be particularly as high as 90–99%. Anti-*B. mallei* antibodies are detected within one week after infection and the test remains positive also in chronic cases (Steele, 1980; Sprague *et al.,* 2009). Low specificity and sensitivity of CFT and ELISA is associated with the use of crude preparations of whole cell test antigens (Neubauer *et al*., 2005). Maiden attempt was made by Sakamoto (1929) towards using new and specific antigen; he used an extract of *B. mallei* cultures to diminish the rate of questionable positives in CFT. He observed 1% of false-positive reactors readily found in the horse population. Interestingly, he was able to improve specificity and sensitivity of the precipitin test when compared to CFT and pathology, using a mixture of soluble carbohydrate antigen which could be supposed to be a mixture of capsular polysaccharides (CPS) and mainly lipopolysaccharides (LPS) (Neubauer *et al*., 2005). Validation of commercially available CFT products presumably containing preparations of *B. mallei* museum strains in a current outbreak situation is missing.

Based on the previously mentioned facts, the present study was designed to:

1. Determine the seroprevalence of glanders in apparently healthy equids in various districts of the Punjab, Pakistan.
2. Comparatively evaluate two commercially available CFT antigens: (c. c. pro and CIDC) using sera from glanders free (Germany), potentially exposed and glanderous equids (Pakistan).
3. Comparatively evaluate three commercially available antigens: (c. c. pro, CIDC and USDA) using sera from glanders free (Germany) and glanderous/immunised animals.

# 2 Literature

## 2.1 Characterisation of *Burkholderia mallei*

### 2.1.1 History and taxonomy

Glanders is known since the horse was domesticated. In 1882, the German military physician Friedrich Loeffler isolated the causative agent of equine glanders after culturing it from a glanderous horse's liver and spleen in Berlin, Germany (Wilkinson, 1981; Neubauer *et al*., 1997). *B. mallei* has also been used as biological weapon. During World War I, German agents are believed to have intentionally infected a large number of Russian horses and mules on the Eastern Front. During World War II, the organism was again used by Japanese forces as a biological weapon (Dance, 2005). *B. mallei* has been classified as a Category B bioterrorism agent by the Centre for Disease Control and Prevention, Atlanta, USA (CDC) on the basis of its potential for dissemination, its zoonotic nature and the need for enhanced diagnostic capacity and surveillance (CDC, 2000; Rotz *et al*, 2002). The genus *Burkholderia* consists of more than 40 different species, which occupy a wide array of ecological niches (Vandamme *et al.,* 2007). The genus *Burkholderia* species found in soil, water, rhizophore, animals and humans (Stoyanova1 *et al*., 2007). Several species are source of important diseases in humans, plants and animals. Other species play a vital role in bioremediation. From a strict taxonomic point of view, *B. mallei* should be considered as subspecies of *B. pseudomallei*, *B. thailandensis* and *B. oklahomensis* are genetically distinct and considered separate species (Godoy *et al*., 2003; Glass *et al*., 2006). The organisms were classified into several genera including *Bacillus*, *Corynebacterium*, *Mycobacterium*, *Loefflerella*, *Pfeifferella*, *Malleomyces*, *Actinobacillus*, and *Pseudomonas* (Steele, 1979). The current genus *Burkholderia* was proposed in 1992 on the basis of 16S ribosomal ribonucleic acid (rRNA) sequence data, DNA-DNA homology values, cellular lipid and fatty acid composition, and phenotypic characteristics (Yabuuchi *et al*., 1992).

### 2.1.2 Microbiological characteristics

*B. mallei* is a Gram-negative, nonmotile, nonsporulating and facultative intracellular obligate mammalian pathogen. No report about its growth in soil or water is available although it can remain viable at room temperature. Outside the body, it is susceptible to heat, sunlight and common disinfectants (Van Der and Bishop, 2004; Nicoletti, 2007). It ranges in size from 0.3 to 0.8 μm in width and 2 to 5 μm in length and can only be weakly stained with simple stains.

Miller *et al*. (1947) described the presence of a capsule in *B. mallei,* although they did not succeed to prove its presence. Nearly 50 years take a Russian scientist, Popov, proved its existence and predominant role in as a virulence factor (Popov *et al*., 1991). Gold standard method of diagnosis of glanders is isolation and identification of *B. mallei* from pure culture (Blue *et al*., 1998). It has to be stressed that characteristic properties of the infectious agent may undergo changes during infection resulting in hindrance to use classical methods of microbiological diagnosis (Piven, 1997). *B. mallei* can be grown on routine culture media including nutrient, blood, and MacConkey agars. Viscid, smooth and creamy colonies of *B. mallei* can be obtained after 48 h at 37°C (98.6°F) on routine culture media. In the presence of nitrogen the organism can grow aerobic and facultatively anaerobic (Pitt, 1998; OIE, 2008). On sterile potato slices growth appears as shiny, moist, yellowish tinged transparent film after 3 days of incubation. The growth becomes brown to chocolate along with same colour sediment on prolonged incubation (Davies, 1955; Naureen, 2006).

The organism produces smooth 'S' type colonies on culture medium when directly cultured from samples which transform to rough 'R' type (or variant) on sub-cultivation. The former type is regarded as the original form of *B. mallei* (Mochida, 1939). It was found that the 'R' variant is less virulent than the 'S' type, or even non-pathogenic in its filamentous form. However, mallein produced from both types (S and R) had the same potency (Mochida, 1939). The formation of different variants is believed to be caused by the disruption of the lipolysaccharide (LPS) producing operon resulting in avirulence. Opinion differs concerning the ability of the organism to grow on MacConkey's agar. Buxton and Frasser, 1977 found that their *B. mallei* isolates did not grow on MacConkey's agar while Songer and Post (2005) stated that their *B. mallei* strains grow on this agar, but the colonies were non-lactose fermenter. *B. mallei* can selectively be differentiated by its lack of motility, the ability to ferment a characteristic subset of carbohydrates and its colony morphology (Steele, 1980; Blue *et al.,* 1998). In acute cases in man, diagnosis was sometimes successful from blood culture (Minett, 1959). Isolation of the organism from equine blood culture has also been described (Muhammad *et al.,* 1998). Glycerol enrichment enhances growth. In contaminated samples, supplementation of media with substances that inhibit the growth of Gram-positive organisms (e.g. crystal violet, proflavine) has proven to be useful, as has pre-treatment with penicillin (1000 units/ml for 3 hs at 37°C) (Minett, 1959). Xie *et al.* (1980) developed a selective medium comprised of polymyxin E (1000 units), bacitracin (250 units), and actidione (0.25 mg) incorporated into nutrient agar (100 ml) containing glycerine (4%), donkey or horse serum (10%), and ovine haemoglobin or trypton agar (0.1%). After 3–4 days of intraperitoneal inoculation of male guinea pigs with cultures of *B. mallei* peritonitis and orchitis, the "Strauss reaction", can be seen. The sensitivity (Se) of this technique is only 20% and at least five animals must be inoculated. However, re-isolation of *B. mallei* from testes of

infected animals is proving the specificity of this response (Neubauer *et al.,* 1997; Scholz *et al.,* 2006; OIE, 2008).

### 2.1.3 Antibiotic resistance

Species of *Burkholderia* showed resistance against antibiotics by adopting different mechanisms including enzymatic modification, alterations in drug targets and limited permeability (Burns, 2007). *B. mallei* is resistant to a wide range of antibiotics including ß-lactam antibiotics, macrolides and aminoglycosides (Dance *et al.*, 1989; Heine *et al*., 2001). Most *B. mallei* strains showed resistance to amoxicillin, ampicillin, penicillin G, bacitracin, chloromycetin, carbenicillin, oxacillin, cephalothin, cephalexin, cefotetan, cefuroxime, cefazolin, ceftriaxone, metronidazole, and polymyxin B (Kovalev, 1971; Neubauer *et al.,* 1997; CDC, 2000). Most recent studies showed that ~ 94% of the *B. mallei* isolates in Pakistan were susceptible to both, enrofloxacin and ofloxacin, and all were intrinsically resistant to ampicillin ($MIC_{90}$ ≥128). Some isolates (17%) showed resistance against amoxicillin, cephradine, cefuroxime, norfloxacin, ceftizoxime and ceftriaxone (Naureen *et al.,* 2010).

### 2.1.4 *Burkholderia mallei* antigens

Bacterial pathogens exhibit different surface antigens which are potent virulence factors and play a pivotal role to escape from the host immune response or to attach to the host cell surface. *B. mallei* is antigenically heterogeneous (Pitt *et al.,* 1990; Goldberg, 2007). *B. mallei* and *B. pseudomallei* exhibit two polysaccharides: Capsular polysaccharides (CPS) and LPS. *B. mallei* and *B. pseudomallei* produce two virulent kinds of CPS (CPS-1 and CPS-2) which are structurally characterized only in *B. pseudomallei* (Knirel *et al.,* 1992; Reckseidler *et al.,* 2001). Cross reactivity between *B. mallei* and *B. pseudomallei* CPS was expected due to > 90% resemblance of the CPS gene locus (Kim *et al.,* 2005). It has been suggested that this polysaccharide contributes to the antiphagocytic effect by reducing the level of C3b binding to the bacterial surface (Reckseidler-Zenteno *et al*., 2005). In *B. mallei* subtractive hybridization strategy identified the genes of the capsular polysaccharide locus. Mutation of these genes result in lack of reactivity of these mutants with antisera raised against *B. pseudomallei* that proved their role in CPS production (DeShazer *et al*., 2001). Relatively little is known about the lipid A-core structure of *B. mallei* and *B. pseudomallei* (Kawahara *et al*., 1992). The LPS O-antigen polysaccharides from *B. pseudomallei* and *B. mallei* LPS have been structurally characterized (Knirel *et a*l., 1992; Burtnick *et al*., 2002). The LPS of both

species is a disaccharide which differs from one another at the location of O-actyl substitutions on the talose residue. Polyclonal LPS specific antisera show cross-reactions between *B. mallei* LPS and that of *B. pseudomallei* (DeShazer *et al*., 2001). None of six *B. mallei* isolates reacted with a monoclonal antiboby (mAb) specific for *B. pseudomallei* LPS (Anuntagool and Sirisinha, 2002). On the other hand, mAb 3D11 is specific for the LPS of *B. mallei* isolates (Anuntagool and Sirisinha, 2002). *Burkholderia mallei* strain specific mAbs have also been described that effectively distinguish 80% of the different *B. mallei* strains tested. *Burkholderia pseudomallei*-specific mAbs appear to react with a distinctive antigen present only in strain ATCC 23343 (Feng *et al*., 2006). Khrapova *et al*. (1995) evaluated the use of mAbs for gel immunodiffusion and agglutination tests as a basis of new-generation preparations for fluorescent antibody assays and indirect haemagglutination tests. Use of *B. mallei* specific anti-LPS mAb 3D11 or use of O-PS in glanders serodiagnosis might reduce the number of false-positive results and would further refine sensitivity and specificity (Neubauer et al., 2005). Sprague *et al*. (2009) used *B. mallei* Dubai 7 and the mAb 3D11 (Biotrend, Köln, Germany) in a competitive Enzyme Linked Immunosorbent Assay (cELISA) and found a higher specificity (98.5%) than with the inhibitory ELISA (iELISA; 85.5%). Immunization of mice with LPS of *B. pseudomallei* induced the production of IgM and IgG3 response and partial protection against *B. mallei* (Nelson *et al*., 2004). The genes required for the synthesis of *B. mallei* O-antigen have been identified (Burtnick *et al*., 2002). The O-antigen has unique structural features that once identified could be used to develop a diagnostic test that could differentiate between *B. mallei* and *B. pseudomallei.*

### 2.1.5 Virulence factors

Studies of *B. mallei* virulence, the associated host response, and candidate vaccine development have been reviewed (Whitlock *et al*., 2007). A capsular polysaccharide was identified in *B. mallei* using subtractive hybridization technique (DeShazer *et al.,* 2001). *B. mallei* capsule mutants proved avirulent in hamsters, mice and the horse (DeShazer *et al.,* 1998; Lopez *et al.,* 2003). It has been demonstrated that capsular polysaccharide is an indispensable virulence factor in hamsters and mice (DeShazer *et al.,* 2001). Passive immunization studies in mice showed that anti-capsule antibodies are protective against lethal bacterial challenge (Jones *et al.,* 1997). The capsule, a type 3 secretion system (T3SS) and type 6 secretion system (T6SS) play the pivotal role in pathogenesis. Intracellular survival of *B. mallei* involves T3SS-dependent escape of bacteria into the host cytoplasm and BimA-dependent actin-based motility. Formation of multinucleated giant cells (MNGCs) enables the pathogens to escape the host immune responses, to replicate and persist in host cells (Galyov *et al.,* 2010). In the host cell environment free iron is limited. Making use of micro-array

analysis the effects of growth in different iron concentrations on the regulation of gene expression in *B. mallei* were examined (Tuanyok *et al.,* 2005). It was noted that *B. mallei* is able to survive in host cells by using alternative metabolic pathways for energy production. *B. mallei* virulence associated genes regulated by a complex quorum-sensing system are still under investigation (Ulrich *et al.,* 2004). *B. mallei* encodes two *luxI* homologs that produce *N*-octanoyle-homoserine lactone (C8-HSL), (*N*-decanoyl-homoserine lactone) C10-HSL, 3-hydroxy-C8-HSL, and 3-hydroxy-C10-HSL and four *luxR* homologs. The signal for the BmaR3-BmaI3 quorum-sensing system is 3-hydroxy-C8-HSL (Duerkop *et al.*, 2008). The *B. mallei* O-antigen is responsible for the resistance against macrophage killing (Wikraiphat *et al.,* 2009) and gives way of survival in serum by inhibiting killing by the alternative pathway of complement (DeShazer *et al.,* 1998). *B. mallei* LPS O-antigen is lacking an O-actyl at group 4′ position of the talose residue. After introduction of mutations in the *B. mallei* animal-pathogen-like T3SS, the interaction between T3SS and pathogenicity of ATCC (American type culture collection) 23344 in vivo was analysed (Ulrich and DeShazer, 2004). Induced mutation explained that T3SS was required for complete virulence of *B. mallei* in the BALB/c (albino, laboratory-bred strain) mouse model. The T6SS gene cluster1 (T6SS-1; BMAA0744-0730) is induced inside murine macrophages showing that it plays a role in the intracellular survival of the pathogen (Burtnick *et al.,* 2010). Regarding a type II secretion system (T2SS), *B. mallei* secrets the T2SS proteins only poorly. Two T2SS genes have mutations and these findings may explain this 'secretion phenotype' (Nierman *et al.,* 2004). Toxins i.e. extra-cellular enzymes which disrupt host cellular functions include pyocyanin, lecithenase, collagenase, plasmocoagulase, and lipase (Narbutovich *et al.,* 2005; Songer and Post, 2005). Pyocyanin interferes with the terminal electron transfer, whereas, lecithenase, collagenase, and lipase are lytic in their action.

## 2.2 Glanders

### 2.2.1 History

Glanders is one of the oldest known equine diseases. Aristotle first described glanders in horses around in 350 BC and named it "malleus", (Latin for "hammer"). Disease symptoms were reported by Hippocrates approx in 425 BC. Another word root may be the Greek word "melis" meaning "honey" (Howe *et al*., 1971; Groves and Harrington, 1994; Marr and Malloy, 1996). Glanders was suggested as the cause of 6$^{th}$ plague of Egypt as mentioned in the Bible (Marr and Malloy, 1996). Apsyrtus described glanders with considerable accuracy and recognized its contagiousness. A short time before the fall of the Western Roman Empire, the Greek veterinarian Vegetius Ranatus who lived in the time of Theodosius (381 AD), distinguished seven different varieties of the disease, and two of these viz., '*malleus humidus*'

and '*malleus farciminosis'* have respectively been identified with glanders and farcy. He also recognized the contagious properties of discharge of the external ulcers (Huidekoper, 1903; M´Fadyean, 1904).

Humans, goats, dogs, cats, rabbits, camels and some carnivores living in the vicinity of infected equids or had contacted to infected carcasses have been naturally infected (Loeffler, 1886; Kovalov, 1971). The first report about human glanders was published in the beginning of penultimate century in different European countries (Neubauer *et al*., 1997). Glanders caused huge economic losses in Europe till World War I. The disease was not studied by a scientific approach until the early part of 19$^{th}$ century. Due to serious problems with glanders in French cavalry horses, King Louis XV laid the foundation of the first veterinary school, Lyon, France in the mid- 18$^{th}$ century. Due to the zoonotic nature of disease, many early researchers get infected and died of glanders (Wilikinson, 1981).

### 2.2.2 Transmission

Prior to *B. mallei* discovery, Solleysel, between 1667 and 1682 expressed the view that glanders could be transmitted through the air. Viborg (1979) supposed the presence of "*B. mallei"* in nasal secretions and pustules and ulcers discharge of infected animals. Caspard de Saunier in 1734 stated that the disease is transmitted directly or indirectly through contaminated harnesses, water troughs, and feed mangers. On the basis of results of pathogenesis studies carried out during the late 19$^{th}$ and early 20$^{th}$ century it became clear, that glanders is readily transmissible by ingestion or inoculation in equines and other animals (Nocard, 1986; Schütz, 1898; M'Fadyean, 1904; O'Leary, 1908; Dudgeon and Symonds, 1918). Stomach feeding of *B. mallei* caused a rise in temperature (41°C) in horses after 24 h which remained high during observation period of 25 days. Common water troughs are potential source of infection and animals become infected by drinking water contaminated with nasal secretions (Kinsley, 1911). A high number of *B. mallei* bacteria are present in nasal secretions and skin exudates of glanderous equids that can contaminate fomites and spread the disease (Gregory and Wagg, 2007). *B. mallei* can enter into the body through breached skin, mucous membranes and by aerosol inhalation. Whitlamsmith (1898) documented transmission of glanders from affected (mallein positive but clinically normal) horse to mallein negative (healthy) animals. The author believed that transmission occurred by inhalation or ingestion of dried particles of nasal discharge. Among equids, the disease spreads intensified under stress causing factors including over-crowding and poor hygiene. Carnivores can catch the disease by ingestion of glanderous meat (Huidekoper, 1903; Hart, 1916; Battelli *et al.*, 1973; Alibasoglu *et al.*, 1986). Humans can contract the disease from clinically affected animals (Ward, 1879; Dixson, 1882; Stewart, 1904; Herold and Erickson,

1938; Selvi and Paykoc, 1949), during post-mortem (Pospisil, 2000; Wittig, 2006), and during handling of morbid tissues and cultures in laboratory (Gaiger, 1913; Hunter, 1920; Howe and Miller, 1947; Alibasoglu *et al.*, 1986; Srinivasan *et al.*, 2001). Human to human transmission has also been reported (Pospisil, 2000).

### 2.2.3 Pathogenesis

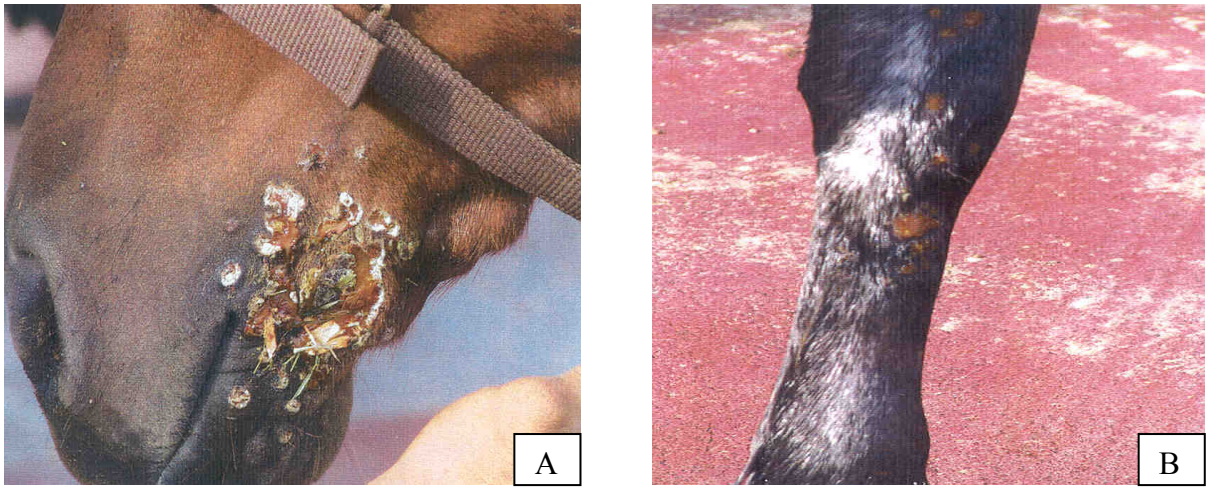
*B. mallei* is believed to find its gate of entry through mucous membranes and the integuments of the host. Bacteria penetrate the mucosa and travel via lymph vessels and finally reach lymphatic sentinels. *B. mallei* proliferates in these lymph nodes and takes its way to the blood stream and spreads to the visceral organs haematogenously. Thus, lymphatic and blood circulatory systems play a vital role in the formation of musculoskeletal lesions (Jubb *et al*., 1993; Steele, 1979; Fritz *et al*., 1999; Pitt and Dance, 2005). Degree of virulence (chronic and acute) of *B. mallei* strains has been studied in different animal models (Miller *et al*, 1948). Its chronicity is facilitated by the presence of a cytoplasmic membrane, three layered cell wall and a capsule which are helpful to escape from host immune response and which are impermeable against some antibiotics (Dance *et al.*, 1989). The incubation period depends on route of entry and virulence of the strain of *B. mallei*, ranging from a few days to several weeks (Groves and Harrington, 1994). Host predilection sites include lungs, upper respiratory tract and lymphatics. *B. mallei* endotoxin causes lymphangitis, lymphadenitis, tissue reaction of smooth muscle and sloughing of mucous membranes (Kovalev, 1971). Cutaneous glanders is characterized by superficial or deep subcutaneous nodules which may ulcerate and discharge pus. Pulmonary glanders is characterized by encapsulated nodules that might develop central calcification (Al-Ani, 2007).

### 2.2.4 Clinical signs

Early infection clinical signs of glanders in equids are thirst, fever, shivering, drooping of the head, tachycardia, tachypnea, weight loss, rough hair coat, indolence, prostration, and reluctance to move (Schlater,1992). In the nasal form of glanders, rupture of the nasal nodules leads to hemorrhagic or mucopurulent nasal discharge. External naries are swollen and crusted (Van Der Lugt and Bishop, 2004; Nicoletti, 2007; Al-Ani, 2007). Pulmonary glanders can remain subclinical but clinical form leads to depression, anorexia, malaise, catarrhal bronchopneumonia, coughing, and fever (40°C [104°F]). In more advanced stages, dyspnea and rales may be observed. Cutaneous glanders develops superficial or deep subcutaneous abscesses with or without ulceration which lead to palpable lymphangitis (farcy pipes) and

lymphadenitis (farcy buds). Sometimes nodules may develop in internal organs including the liver, spleen, and testes (Acha and Szyfres 1994; Muhammad, 1998; Waag and DeShazer, 2004; Al-Aini, 2007). Cutaneous lesions of glanders are shown around mouth and fetlock joint of horses (Figure 1). Glanders manifestations in man may be variable, six forms of infections have been reported (Domma, 1953). Wernery *et al*. (2011) confirmed natural infection of dromedaries with glanders and observed clinical signs including profuse bilateral nasal discharge, fever, emaciation and fatigue.

**Figure1: Cutaneous lesions of glanders: around mouth (A) and on fetlock joint (B) of glanderous horse**



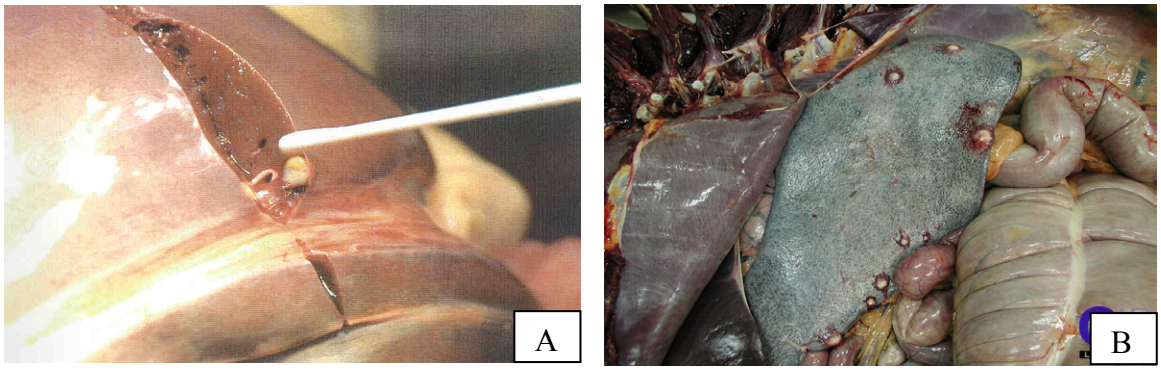

Figures source: Dr. U. Wernery, Central Veterinary Research Institute, Dubai, UAE

### 2.2.5 Pathology and histopathology

Variable post-mortem lesions of glanders were observed in equids including granulomas and ulcers in a variety of tissues, nodules and fulminating ulcers were found on mucous membranes of nasal passages (nasal form of glanders), larynx, and upper lip. In pulmonary glanders lungs may show nodular foci underneath the pleura while diffuse miliary granulomatous nodules with a caseonecrotic centre. Edema of lungs lobes and severe bronchopneumonia is observed in acute glanders. Visible nodules were found in liver and spleen of glanderous animals (Figure 2; Wernery *et al*., 2004). Cervical, mandibular and mediastinal regions show lymphadenitis that will lead to ulceration with sebaceous contents. Liver, spleen and testis may show lymphadenitis (Zubaidy and Al-Ani, 1978; Arun *et al.,*

1999; Lopez *et al.,* 2003; Van Der Lugt and Bishop, 2004; Al-Ani, 2007). Histopathologic lesions in equids may include granulomatosis or pyogranulomatosis lesions in lungs with inflammatory elements including macrophages, epithelioid cells, small foci of haemorrhage, edema and interlobular and intra-alveolar fibrin. Vasculitis and thrombosis of nasal blood vessels with neutrophilic granulocyte infiltration was observed. Neutrophilic granulocytes and macrophages infiltration in ulcerated submucosa was observed as well. Nodules are encapsulated and necrogranulomatous with inflammatory cells, including neutrophils, lymphocytes and macrophages (Miller *et al.,* 1948; Popov *et al.,* 1991; Mota *et al.,* 2010). Bazargani *et al*. (1996) found muscle abscesses during postmortem of glanderous racehorses in Tehran, Iran. Those authors did not find any visible lesion in the lungs and spleen. However, such nodules were reported from glanderous horses during a recent outbreak in Dubai, UAE (Wernery *et al*., 2005). It may now be speculated that the Iranian *B. mallei* strains were less virulent or the disease was diagnosed earlier.

**Figure 2: Nodules were found in the glanderous equid`s liver (A) and spleen (B) after post-mortem**




Figures source: Dr. U. Wernery, Central Veterinary Research Institute, Dubai, UAE

### 2.2.6 Immune response

Currently no vaccine is available against glanders for both humans and animals. Vaccine candidate formulations were made by desiccation of *B. mallei* bacteria or by treating bacteria with glycerine or urea (Mohler and Eichhorn, 1914; Kovalev, 1971). A mixed kind of immune response has been observed with both, T helper (Th) 1 and 2 cells, and associated cytokines. IgG1 subclass antibodies were observed in BALB/c mice during *B. mallei* infection

(Amemiya *et al*., 2002). In another study, a heat shock protein (hsp) of the *Escherichia coli* homologue for large 60 KD hsp (GroEL), was found to be more immunoreactive than the *E. coli* homologue for large 70 KD hsp (DnaK) in sera from a patient and mice previously infected with *B. mallei* (Amemiya *et al*., 2007). Combined use of interleukin (IL)-12 and nonviable *B. mallei* induced better immune response rather than use of killed *B. mallei* alone (Whitlock *et al*., 2007). Attempts to transfer passive immunity with hyperimmune sera remained unsuccessful although passive transfer of specific-mAb against *B. mallei* conferred early protection (Kovalev, 1971; Vyshelesskii, 1974; Trevino *et al*., 2006). Studies on *B. mallei* live attenuated and heat killed vaccines in mice showed only partial host protection. These studies might be helpful for the future development of subunit or whole cell vaccines (Ulrich *et al*., 2005; Whitlock *et al*., 2007). BALB/c mice having experimental *B. mallei* intrapertoneal injection died with chronic glanders after 70-day post-infection. Spleen get infiltrated with the neutrophils and macrophages 5 h post-infection and an influx of activated macrophages, neutrophils and T cells followed by 24 h post-infection. In early *B. mallei* infection, Gr-$1^{+}$ (antigen) cells are important to control the infection while T cells (nitric oxide) are important during the later stages to combating the infection (Rowland *et al*, 2010).

Antibodies appear within a week during *B. mallei* infection and infected animals quickly attain the maximum peak of titers. These will stay for weeks and decline slowly (M'Fadyean, 1896).

### 2.2.7 Epidemiology

The horse is **the** natural reservoir of *B. mallei.* Among solipeds, horses show chronic while donkeys show acute form of glanders. Carnivores become infected by eating infected meat (Minett, 1959; Neubauer *et al*., 2005). Glanders is considered a re-emerging disease and is still endemic in North Africa, Middle East, South America, and Asia (Wittig, 2006; Dvorak and Spickler, 2008). Many outbreaks were reported during the last decade from China, Russia and Mongolia (Zhang and Lu,1983), Turkey (Alibasoglu *et al* .,1986), India (Krishna *et al.,* 1992), Iran (Bazargani *et al,*.1996), Iraq (Al-Ani *et al*., 1998), Pakistan (Muhammad *et al*.,1998), Brazil (Mota *et al*., 2000; Elschner *et al.,* 2011). From 1980 to 2007 focal outbreaks either reported to OIE or published in scientific journals indicate the disease is prevalent in developing countries like Pakistan (Muhammad *et al.,* 1998; Saqib *et al.,* 2003; Naureen *et al.*, 2007). As far as could be ascertained, the first document on occurrence of glanders in Pakistan and India was published in 1877 (Anonymous, 1877). This report suggests that glanders entered into this region by British cavalry regiments during the Bengal Presidency. Only few studies have been undertaken on the prevalence of the disease in the 2 draft-equine populated areas of Punjab, Pakistan. A half percent incidence of glanders was noted in 1977

in Faisalabad metropolis (Nasreen, 1977). No human disease was reported. Equine glanders was reported to OIE from many countries before 1996 (OIE Report, 2003) (Table 1). Humans contract disease by contact of infected horse or their exudates (Neubauer *et al*., 1997). In 2001 the first human case was reported in English language medical literature since 1949 (Srinivasan *et al.,* 2001).

**Table1: Equine glanders and its eradication.**

| Country or territory | Year of eredication | Country or territory | Year of eredication |
|---|---|---|---|
| Australia | 1891 | Moldavia | 1975 |
| Austria | 1952 | Namibia | 1925 |
| Bulgaria | 1954 | The Netherlands | 1957 |
| Canada | 1938 | Norway | 1889 |
| Croatia | 1959 | Poland | 1957 |
| Denmark | 1928 | Portugal | 1952 |
| Egypt | 1928 | Romania | 1960 |
| Estonia | 1945 | Serbia and Montenegro | 1959 |
| Finland | 1943 | Slovakia | 1954 |
| Republic of Macedonia | 1957 | South Africa | 1945 |
| France | 1965 | Spain | 1956 |
| Georgia | 1960 | Sudan | 1989 |
| Germany | 1955 | Sweden | 1943 |
| Greece | 1965 | Switzerland | 1937 |
| Hungry | 1956 | Taipei China | 1950 |
| India | 1988 | Great Britain | 1928 |
| Ireland | 1920 | Northern Ireland | 1910 |
| Israel | 1951 | United States of America | 1942 |
| Japan | 1935 | Zimbabwe | 1911 |

### 2.2.8 Present situation of occurrence

Glanders can be considered a re-emerging disease as the number of outbreaks in solipeds and even other animals e.g. zoo carnivores (tigers and lions) and camels is steadily increasing in the last two decades (Alibasoglu *et al.,* 1986; Wittig *et al.,* 2006; Wernery *et al.,* 2011; ProMED, 2011). Some recent outbreaks have been reported to OIE (Table 2).

**Table 2: Recent outbreaks of glanders.**

| Country | Year | Reference |
|---|---|---|
| Pakistan | From 1999 to 2007 | Naureen *et al*., 2007; Hornstra *et al.,* 2009 |
| India | from 2006 up to now | Malik *et al.,* 2009 |
| United Arab Emirates | 2004 | Wernery 2004, 05; Scholz, *et al.,* 2006 |
| Brazil | 2000 up to now | Mota *et al.,* 2000; Elschner *et al*., 2009; Khan *et al.,* 2011 |
| Turkey | 1986 upto 2008 | Alibasoglu *et al*., 1986; Akcay, 2008; OIE, 2010 |
| Bahrain | 2010, 2011 | WAHID, 2010a; Wernery *et al*., 2011 |
| Kuwait | 2010 | WAHID, 2010b |
| Iran | 2011 | Promed, 2011 |
| Lebanon | 2011 | WAHID, 2011a |
| Afghanistan | 2011 | WAHID, 2011b |

**Figure 3: Map of outbreaks of equine glanders during last two decades.**

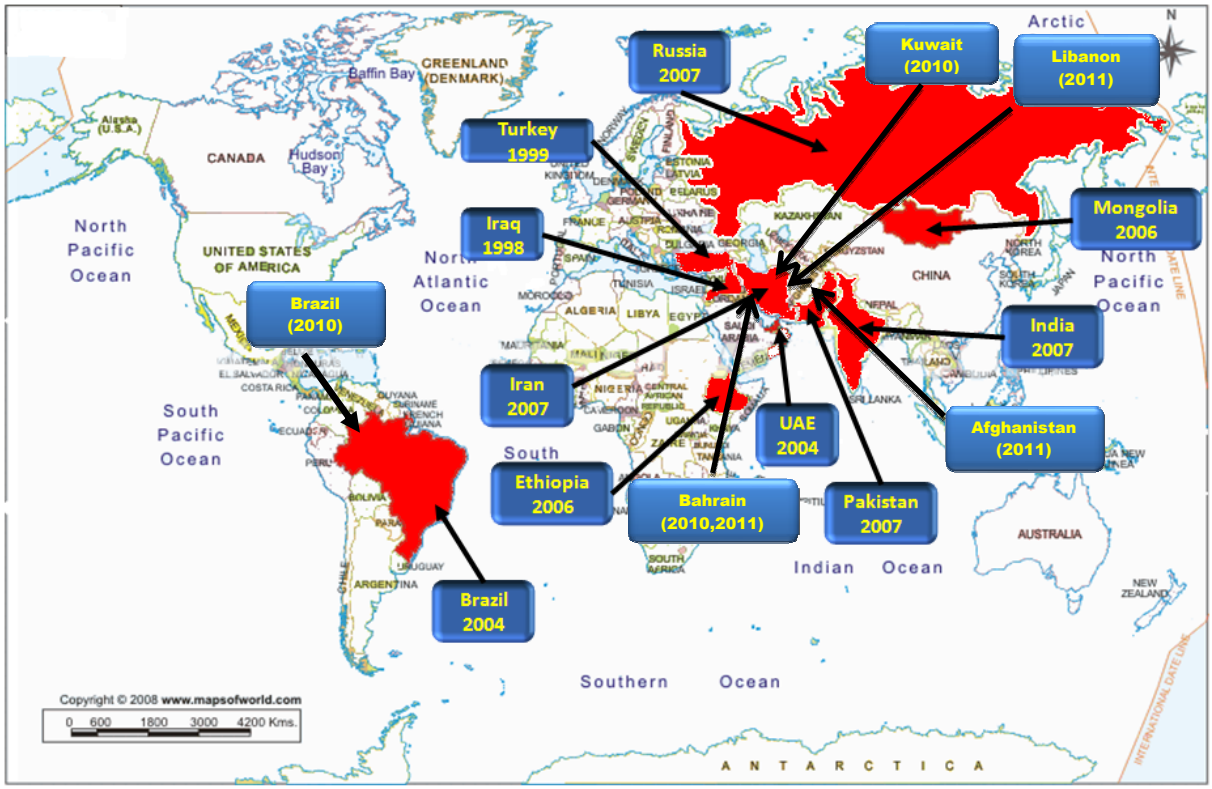



Figure source: Dr. M. Saqib, Department of Clinical Medicine and Surgery, University of Agriculture, Pakistan

As I collected the sera for this study from three countries: Pakistan, Brazil and Germany, I have briefly encompassed the current situation of glanders in these countries as follows:

### 2.2.8.1 Glanders in Pakistan

Amazing pittance (equivalent of only $ 1.1 in Pakistan at present) as compensation to the owner from destruction of suspected animals and lack of stringent enforcement of glanders control laws as well as government's concern with other more grave socioeconomic issues do not create an environment to implement "the test and slaughter glanders policy" in a true letter and spirit. As a salvage measure, equine owners sell their glanderous animals at reduced price that leads to spread of disease in new areas. It also triggers to investigate the role of therapy as an alternative to animals' destruction (Muhammad *et al*., 1998). Sporadic cases of

glanders have been reported from 1999 to 2007 from various areas (Sargodha, Faisalabad, and Lahore) of Punjab, Pakistan. MLST results indicate that the Punjab *B. mallei* isolates are more closely related to each other than to the known sequenced strains because the average pair wise distance (APD) among Punjab isolates is twice lower than the APD calculated for the other 2 situations investigated (Hornstra *et al.,* 2009). In 2006, glanders was confirmed in a dozen of horses stabled at the Lahore Polo Club located in the Racecourse Park in GOR (golf and riding), Punjab, Pakistan. Horses with positive mallein test were not destroyed. Instead, the horses were shifted to the Veterinary Hospital, University of Agriculture in Faisalabad, Pakistan. That was the contravention to the government's Glanders and Farcy Act, which states clearly that horses infected with glanders have to be killed (Khan, 2005). Half a percent incidence of glanders was reported in 1977 (Nasreen, 1977), 1 percent in 1981 (Vaid *et al.,* 1981), while a report of 1983 (Bashir, 1983) documented a prevalence of 1.45 and 16.7 percent in horses and mules, respectively. These reports indicate that glanders is endemic in this region. However, no action has as yet been taken by the concerned authorities to stamp out the disease. Prevalence of this zoonotic disease should be cause of concern to public health authorities, veterinary, and medical communities.

#### 2.2.8.2 Glanders in Brazil

In Brazil, the disease is limited to certain areas, to some north-eastern states of the country, where it is endemic and, consequently, notified to the OIE in the six-monthly reports as present in a zone of the country. The disease has not been recorded in other regions of the country since 2008, when it was detected in the State of Sao Paulo (OIE, 2010). Clinical, pathological, epidemiological, serological and most reliable isolation of *B. mallei* from foci of glanderous equids proved existence of glanders in the "Zona de Mata" in the states of Pernambuco and Alagoas (Mota *et al*., 2000). Another Brazilian worker studied the molecular performance and phenotypic characterization of *B. mallei* isolated from clinical and serological positive horses belonging to the Metropolitan District of Recife and Zona de Mata of Pernambuco and Alagoas (Silva *et al*., 2009). In 2010, glanders was also diagnosed in donkeys in the state of Pernambuco, Brazil (Mota *et al*., 2010). Very recently glanders was reported in horse from Sobradinho. Sobradinho is a municipality located in the state of Bahia. Infected horses were not responding to antibiotics. Horse serum was positive in CFT and the horses were inconclusive in mallein test made after 45 days (WAHID, 2010).

#### 2.2.8.3 Glanders in Germany

Glanders has been eradicated from Germany and no case has been reported since 1955 (Elschner *et al*., 2011). Equids were taken as reference negative control for the current studies.

### 2.2.9 Importance of glanders as a disease notifiable to OIE

Due to its high degree of infectivity, incapacitation of infected animals and high economic losses glanders is a disease notifiable to OIE. In laboratories, *B. mallei* is highly infectious for human when it is aerosolized (Rosebury and Kabat, 1947; Neubauer *et al*., 1997). Glanders caused high mortality and morbidity in untreated cases. In the septicemic form, the case fatality rate recorded up to 95% in 3 weeks or higher in untreated cases and more than 50% if treated. Mortality rate in the pulmonary form is 90–95% if untreated, and 40% if treated. In its chronic form, the case fatality rate is 50% even in treated cases (Available at http://www.cfsph.iastate.edu/Factsheets/pdfs/glanders.pdf). This character makes glanders a notifiable disease to local authorities in many countries worldwide.

### 2.2.10 Economic importance of equids

Equids including donkeys, mules and horses have multiple economic effects. According to Food and Agriculture Organization (FAO) Census 2008, there were approximately 59,000,000 horses in the world, with around 33,500,000 in the America, 13,800,000 in Asia and 6,300,000 in Europe and smaller portions in Africa and Oceania (FAO, 2009). The American Horse Council estimates that horse-related activities have a direct impact on the economy of the United States of over $39 billion, and when indirect spending is considered, the impact is over $102 billion (American Horse Council, 2006). A major part of horse racing's economic importance lies in the gambling associated with it (Campbell, 2008). Approximately 100 million horses, donkeys and mules are still used as draught animals for agriculture and transportation in less developed countries including Pakistan (Brown, 2006). Equids have been used extensively during warfare. The first archaeological evidence of horses used in warfare dates to between 4000 to 3000 BC (Newby *et al.,* 1999). During warfare donkeys have been used throughout history for transportation of supplies, pulling wagons and as riding animals. During the Soviet war in Afghanistan, the United States used large numbers of mules to carry weapons and supplies over Afghanistan's rugged terrain to the mujahideen

(Bearden and Milt, 2003). Horse meat is used as food source for human and carnivorous animals' consumption in many countries (USDA, 2008). Donkeys are also used for many tasks including farm tillage, threshing, raising water, milling etc. Some donkeys are used to sire mules, as companions for horses, to guard sheep and raised for milk and meat purposes (Starkey and Starkey, 1997).

## 2.3 Laboratory diagnosis of glanders

### 2.3.1 *B. mallei* isolation

To avoid contamination infectious material for *B. mallei* isolation should be taken from unopened glanderous lesions aseptically. On blood agar, *B. mallei* produced small non-haemolytic, slimy, light greyish-yellow colonies after 48 hs which changed to yellowish-brown after 72 hs. Growth on potato slices is similar to that of light yellow honey pigmentation (Muhammad *et al*., 1998). After a few days on glycerol agar, there is a confluent slightly cream coloured growth that is smooth, moist, and viscid. With constant incubation, the growth thickens and becomes dark brown and rough (OIE, 2008). On plain nutrient agar, the growth is much less luxuriant, and growth is poor on gelatin (Steele, 1980). Pitt (1998) also stated that *B. mallei* grows below optimal well on nutrient agar and forms smooth, grey translucent colonies (0.5–1 mm in diameter) in 18 hours at 37 °C. Due to variations in characteristics, fresh isolates should be used for identification (OIE, 2008). For phenotypic identification several markers can be used e.g. that indole is not produced. Commercially available test kits (e.g. API (Analytical Profile Index) system, BioMerieux or the Biolog, Hayward, California) can be used for identification of organisms of the *Pseudomonas* group (OIE, 2008). However, ready-to-use commercial kits e.g. the API 20 NE (bioMérieux, Hazelwood, Mo.) and RapID NF (Remel, Lenexa, Kans.) misidentified *B. mallei* as non-virulent bacteria (Glass and Popovic, 2005).

### 2.3.2 Antigen, protein and polysaccharide detection

Development of monoclonal antibodies (mAbs) that could effectively differentiate between *B. mallei* and *B. pseudomallei* is urgently needed. Various attempts have been made so far to develop mAbs that could recognize as many different strains and/or clinical isolates of these two pathogens but the cross-reactivity between them due to close antigenic relationship is the main constraint. *B. mallei* LPS showed cross-reactivity with polyclonal antisera raised against *B. pseudomallei* while *B. mallei* antisera showed no cross-reactivity with mAbs specific for *B.*

*pseudomallei* O-polysaccahrides (O-PS) (Pitt *et al.,* 1992; Burtnick *et al.,* 2002). It was also observed that *B. mallei* LPS does not react with a monoclonal antibody (Pp-PS-W) specific for *B. pseudomallei* O polysaccharide (O-PS) (Mary *et al*., 2001). Another recent study was made to develop *B. mallei*-specific mAbs that could effectively distinguish 80% of the different *B. mallei* strains tested, and all the *B. pseudomallei*-specific mAbs appeared to react with a distinctive antigen present only in the ATCC 23343 strain, but not in any other strains of *B. pseudomallei* tested (Feng *et al.,* 2006). Khrapova *et al.* (1995) evaluated the effectiveness of using mAbs in the gel immunodiffusion and agglutination tests as a basis of new-generation preparations for fluorescent antibody assay and indirect haemagglutination test to detect pathogenic pseudomonads. Development of monoclonal antibody reagents specific for *B. mallei* epitops suggests more specific ELISAs in the future that will help to resolve the problem of false positive and false negative CFT results for horses traded. No test has been validated so far (OIE, 2008). Agglutination test with hyperimmune antisera raised in rabbit is commonly used for serotyping of bacteria but its limitation to differentiate *B. mallei* from *B. pseudomallei* was elaborated by Cravitz and Miller, 1950. Direct immunofluorescence using hyperimmune serum raised against *B. pseudomallei* proved to be specific for members of the *pseudomallei* group rather one species alone (Moody *et al*., 1956; Neubauer *et al*., 1997). Sandwich ELISA was developed for rapid antigen detection of *B. pseudomallei*. The assay was based on the use of specific mAbs to capture antigen in clinical specimen culture positive for *B. pseudomallei*. Sensitivity and specificity of the assay were 75 and 98%, respectively (Anuntagool *et al*., 1996).

### 2.3.3 Serological diagnosis

Various serological tests including CFT, IHA, Counter Immuno Electrophoresis (CIE), Agar-gel immunodiffusion (AGID), Dot ELISA, cELISA, RBT, indirect fluorescent test (IFT), immunoblotting (IB) are used. CFT is the only OIE approved test for international trade of equids and glanders eradication programs in the past. Although its limitations to yield false positives are well known, false negative test results and natural anticomplementary activity in the sera of some equids can not be ignored. RBT has been validated in Russia only and was recently (2007) comparatively evaluated in Pakistan. In agglutination test cross agglutination with *B. pseudomallei* was observed (Hagebock *et al*., 1993; Neubauer *et al.,* 1997; Neubauer *et al.,* 2005; Naureen *et al.,* 2007). AGID needs large amounts of antibodies (a titer ≥ 1:128) to produce detectable precipitin lines (Al-Ani and Jerry, 2007). Rapid multiplex immunofluroscent assay (IFA) which was used to detect antibodies against *B. pseudomallei* showed cross reactivity with the genetically related species, *B. mallei* and *B. thailandensis* (Hirotoshi *et al.,* 2007). Sensitivity of conventional ELISA was found equal to that of

fluorescent antibody test (FAT). Dot ELISA is rather more sensitive than CFT, IHAT and CIE as it could detect antibodies much earlier than these three tests. However, results of the dot ELISA may not be reliable up to 6 weeks after mallein injection. ELISAs do not seem to be in practical use (Verma *et al.,* 1990; Joyce *et al.,* 1993; Acillo, 1998; Sprague *et al.,* 2009). There is general agreement that CFT is superior to other serological tests in diagnosis of glanders (Sen *et al*., 1968; Al-Ani *et al.,* 1993). Immunoblot was evaluated for glanders serodiagnosis. Elschner *et al.* (2011) validated western blot (WB) assay for the diagnosis of glanders. The authors tested 305 sera which were randomly selected out of 2,282 sera collected from various geographical areas of Germany. The authors tested also 205 sera collected from glanderous and /or animals immunised against *B. mallei*. WB showed markedly higher diagnostic specificity and sensitivity (100%). It was suggested to use WB as a confirmatory test for the diagnosis of glanders. Naurren *et al*. (2007) comparatively evaluated Rose Bengal plate agglutination test (RBPT) with malleinisation and other conventional serological tests including indirect haemagglutination test (IHAT), CFT, and modified counter immunoelectrophoresis test (mCIET). All tests except mCIET showed 100% specificity. They determined diagnostic sensitivities of RBT, IHAT, CFT, mCIET, and mallein test using sera of culture-positive equids to be 90.0, 97.1, 91.4, 81.4, and 75.7%, respectively. On comparing glandered and glanders free equids, the highest agreement (0.987) was found between RBT and CFT followed by RBT and IHAT (0.940), RBT and mallein test (0.871), and RBT and mCIET (0.852). RBT was suggested to be supplementary easy to use field test for the diagnosis of glanders. Parthasarathy *et al.* (2006) developed a microarray technology by immobilizing *B. mallei* and *B. pseudomallei* polysaccharides. This technique has an upper hand over the current serodiagnostics in detecting successfully *B. mallei* and *B. pseudomallei* serum (human and animals) antibodies. It was the first serological test in which well characterized (capsular polysaccharide and O-antigen saccharides) were used in the microarray to avoid false positives. Jana *et al.* (1982) described their technique of preparation of the antigen used in serological tests including IHA and CFT. They used three *B. mallei* strains (Bucarest-2 obtained from Pasture Institute, Paris and two Indian strains isolated from army equines) for the diagnosis of glanders in equines by counter-immuno-electrophoresis (CIE), CFT and IHA. They tested 155 horse and 57 mule sera with clinical and culture positive test results, in contact and apparently normal animals. CIE proved to be rapid and a more sensitive test as compared to CFT and IHA. Sprague *et al.* (2009) determined the sensitivity and specificity of CFT, an indirect (i) and a competitive (c) ELISA using formalin-fixed *B. mallei* whole cell antigen and a well characterized mouse monoclonal antibody for serodiagnosis of glanders. They determined that CFT is still a very reliable serodiagnostic test for horse populations with low glanders prevalence. The cELISA has a high sensitivity and specificity corresponding to CFT. They mentioned that automatisation, ability to use non-complement sera and use of sera of various host species are the additional benefits of

cELISA. Katz *et al.* (2000) developed a competitive enzyme-linked immunoassay (cELISA) for the serodiagnosis of four pathogens, *Babesia equi* and *Babesia caballi* (piroplasmosis), *Trypanosoma equiperdum* (dourine), and *Burkholderia mallei* (glanders) in horses. They calculated apparent test specificities for *B. equi, B. caballi, T. equiperdum,* and *B. mallei* cELISAs to be 99.2%, 99.5%, 98.9%, and 98.9%, respectively. Additionally, they determined kappa (K) values of agreement between CFT and the cELISA for the serodiagnosis of *B. equi, B. caballi, T. equiperdum,* and *B. mallei* infections in experimentally exposed horses to be 76 and 0.55, 89 and 0.78, 97 and 0.95, and 70 and 0.44%, respectively. They determined that the cELISA was more reproducible, objective, and a convenient approach for piroplasmosis, dourine, and glanders serodiagnosis than the CFT as it resolved the problem of haemolysed or anticomplementary sera. Verma *et al.* (1990) developed a dot enzyme linked immunosorbent assay (dot ELISA) for the diagnosis of equine glanders. *B. mallei* antigens (Zagreb, Bogor and 1163 India) were bound to nitrocellulose coated on a dipstick. The reaction was amplified by an avid-biotin system with biotinlayted anti-horse IgG and horse peroxidase-avidin. They found the sensitivity to be highest for the Dot ELISA with additional benefits including easy and rapid performance. The sensitivity was not affected by anticomplementary sera and infection was detected early. Hagebook *et al.* (1993) reported that intrapalpebral mallein test can induce the production of antibodies against *B. mallei* which leads to positive CFT result. Similarly, they stressed on the unreliability of dot ELISA results to detect glanders infection up to 6 weeks post mallein injection. Al-Ani *et al* (1993) evaluated microplate ELISA for the diagnosis of glanders using sera from 122 horses which were divided into three groups on the basis of clinical examination, bacteriological isolation and mallein test i.e. group I (22 glanderous horses), group II (73 potentially exposed horses) and group III (negative control). They determined infected horses had ELISA titers of 1571$\pm$ 41.3(OD 0.84$\pm$0.04) while potentially exposed horses had ELISA titers of 224 $\pm$ 23 (OD 0.73$\pm$0.05), control horses had titers <50 (OD < 0.1). Phung *et al.* (1995) used purified specific glycolipid antigen (GL) of *B. pseudomallei* in ELISA. They detected IgG in 49 out of 50 human melioidosis sera. IgG was also detected in 2 out of 185 (Japanese) and 16 out of 181 (Vietnamese) control sera, respectively. Finally, they calculated sensitivity to be 98.0% and specificity to be 98.9% and 91.1% in the Japanese and Vietnamese sera, respectively. Al-Ani *et al.* (1989) successfully used agar-gel immunodiffusion (AGID) test for the diagnosis of glanders. They concluded that AGID test is a rapid, inexpensive and accurate in clinical cases of glanders but the only disadvantage is the need of large amount of antibodies (titers of $\geq$ 1:128) to produce a visible precipitin line. Katz *et al.* (1999) developed a rapid array-immunoblotting on a single nitrocellulose strip to diagnose dourine, piroplasmosis and glanders at the same time. Antigens used in the immunoblot method were identical to those used in the CFT. They stated that immunoblotting offers an alternative method to CFT suspicious and anticomplementary sera. They further mentioned that immunoblotting is a rapid and convenient single

confirmatory test method for piroplasmosis, dourine and glanders serodiagnosis. Cravitz and Miller (1950) mentioned that agglutination test is one of the oldest serological tests for the diagnosis of glanders in suspected animals. Sera from non-infected humans and animals can show titers of 1:500. The test would be considered as positive if the suspected sera would have titers of 1:1,000 or above. Gangulee *et al.* (1966) in India and Zhang and Lu (1983) in China successfully used IHA for the serodiagnosis of glanders in suspected animals and they estimated its sensitivity comparable to that of the CFT. The Chinese workers immunized 18 horses with killed *B. mallei* strains. IHA detected 89% reactors while CFT detected 99%. They observed 14.5% of 76 mallein positive tested animals had diagnostic titers, and that 12% were positive in CFT ($\geq$1:10). They suggested the combined use of both tests for a higher sensitivity. Sen *et al* (1968) comparatively evaluated different serological tests. They concluded that the use of agar gel precipitation test using *B. mallei* China 5 was not suitable for the diagnosis of glanders as it detected only 3 out of 10 glanders confirmed ponies whereas either the haemagglutination test, CFT or conglutinin complement absorption test was useful for the detection of glanders with the following diagnostic titers: 1:640, 1:10 and 1:10, respectively. Ma *et al*. (1986) developed an indirect fluorescent antibody assay. This technique could differentiate between *B. mallei* antigens and other cross-reactive antigens which was not possible with CFT.

Ma *et al.* (1987) also detected glanders in suspected equids with ELISA. They determined its sensitivity corresponding to that of the indirect fluorescent antibody test. However, the ELISA can not be used in any diagnostic laboratory everywhere because the need of the expensive photometer (Acillo, 1998).

Keasey *et al.* (2008) developed a microarray assay comprising approximately 70% of the 4066 proteins of *Yersinia pestis* proteome to identify antibody biomarkers. To differentially diagnose plague from infections caused by other bacterial pathogens that may present similar clinical symptoms in the beginning. They showed extensive antibody cross reaction for Gram-negative bacteria including *Burkholderia mallei, Burkholderia cepecia, Burkholderia pseudomallei, Pseudomonas aeruginosa, Salmonella typhimurium, Shigella flexneri* and *Escherichia coli*. Tiyawisutsri *et al.* (2005) used a modified indirect haemagglutination assay normally used to detect antibodies to *B. pseudomallei* to spot cross-reactivity of antibodies against *B. pseudomallei*, *B. mallei*, and *B. thailandensis* antigens. They demonstrated a lack of cross-reactivity between *B. pseudomallei* and *B. thailandensis* but marked cross-reactivity between *B. pseudomallei* and *B. mallei*. They used pooled antigens prepared from the following isolates: (i) *B. pseudomallei* clinical isolates 199a and 207a, obtained from patients with melioidosis in northeast Thailand; (ii) *B. thailandensis* isolates E27, E32, and E256, obtained from soil in northeast Thailand; and (iii) *B. mallei* isolates EY2233 (Faculty of Science, Mahidol University) and ATCC 23344. Gilmore *et al.* (2007) investigated the reactivity of sera from culture-proven human cases of melioidosis from north Queensland

against antigens derived from *Burkholderia pseudomallei*, *B. thailandensis*, and *B. cepacia* using the indirect haemagglutination assay. Cross-reactivity between sera from culture-positive cases of melioidosis and *B. thailandensis* antigens was demonstrated. They used antigen in the IHA which is a polysaccharide component of the slime layer of the organism. They observed reactivity to *B. thailandensis* only at a lower antigen dilution (1:10) than that used with the other *B. pseudomallei* antigens (1:320). This study indicates intrinsic differences in specific antigens between the two species. Thomas *et al.* (1990) evaluated a CFT modified by the addition of porcine serum (CFPS) and an IHA to detect antibodies to *B. pseudomallei* in pigs. They performed these tests along with cultural examinations of 250 pigs. They estimated diagnostic sensitivity and specificity to be 79.3 and 99.5% and 82.8 and 93.2% for the modified CFT and IHA, respectively. When results from the combination of both tests were considered, the values were 86.2 and 92.8%, respectively. Thus, both are recommended for the serodiagnosis of porcine melioidosis. Very recently, a ELISA has been developed in which purified rBimA (recombinant *Burkholderia* intracellular motility A) protein was used as test antigen in ELISA for the serodiagnosis of glanders. rBimA protein did not cross-react with serum of a melioidosis patient and sera of healthy humans (Kumar *et al.,* 2011).

### 2.3.4 Delayed hypersensitivity testing (Mallein test)

Two Russian scientists, Gelman and Kalning first developed the so called "mallein test" in 1891 (Rutherford, 1906). Mallein is a (purified) protein derivative (PPD) of *B. mallei* which is mostly injected in the intrapalpebral region of the lower eyelid of glanders suspected equids. The mallein test is based on a delayed type hypersensitivity reaction. Visible swelling at the injection site and fever 24 to 48 h post mallein injection shows a positive result. The mallein test has its limitations including feeble reaction in unsensitized animals, limited sensitivity and false positive reactions due to cross reactivity with closely related pathogens (Hagebock *et al*., 1993; Al-Ani, 1993; Muhammad *et al*., 1998; OIE, 2004). Most of the mallein reactors manifested no clinical signs of glanders but tested positive in most serodiagnostic tests (Minett, 1959; Verma, 1975; Nagal *et al*., 1995).

### 2.3.5 Molecular diagnosis

The *B. mallei* genome continues to evolve through random IS-mediated recombination events, and differences in gene content may contribute to differences in virulence observed among *B. mallei* strains (Losada *et al.,* 2010).

Because of genetic resemblance between *B. mallei* and *B. pseudomallei*, more specific molecular techniques for the identification of the latter pathogen are highly demanding. Most of the assays developed can not differentiate both pathovars at the same time (Ulrich *et al.,* 2006; Novak *et al.,* 2006). Real Time (RT) - Polymerase Chain Reaction (PCR) assay, PCR-restriction fragment length polymorphism (RFLP), pulsed-field gel electrophoresis (PFGE), 16S rRNA sequencing, variable number tandem repeat polymorphism (VNTR), and multilocus sequence typing (MLST) have been used to specifically identify *B. mallei* (Lew and Desmarchelier, 1993; Sprague *et al*., 2002; Godoy *et al.,* 2003; Scholz *et al*., 2006). These techniques have little field significance because of their sophisticated performance. Very recently polysaccharide microarray technology and DNA microarray-based technology for detection and identification of *B. mallei* and *B. pseudomallei* was explored (Parthasarathy *et al.,* 2006; Schmoock *et al.,* 2009).

#### 2.3.5.1 Conventional PCR and real-time PCR methods

Commercial ready-to-use biochemical kits (API 20 NE and RapID NF) available for *B. mallei* identification show cross reactivity with other nonvirulent bacterial species leading to false-negative results (Inglis *et al*., 1998; Glass and Popovic, 2005). Similarly due to the high antigenic relatedness of *B. mallei* and *B. pseudomallei*, no *B. mallei* specific antibody based antigen test is available (Waag and DeShazer, 2004). Consequently, various methods for the detection of *B. mallei* and *B. pseudomallei* DNA have been developed. The main constraint of detecting pathogenic *Burkholderia* is their low concentration in tissue and biological fluids of the infected host. During the importunate infection, characteristic properties of the infectious agent may undergo changes: posing hindrance to use classical methods of microbiological diagnosis (Piven, 1997). PCR is a highly sensitive, specific, simple and readily available technique. Rapid and accurate diagnosis of *B. mallei* is important for prompt treatment particularly in case of acute glanders. Different attempts have been made to develop PCR and real time-PCR to differentiate between *B. pseudomallei* and *B. mallei* and to specifically diagnose *B. mallei*. Many primers have been designed for detecting sequences of the 16S, 23S rRNA genes and the spacer region of 16–23S rRNA gene (Kunakorn and Markham, 1995; Tyler *et al*., 1995; Bauernfeind *et al*., 1998; Tkachenko, 2003). Development and validation of PCR or real-time PCR is hindered by the fact that *B. mallei* specific primers sets or probes have not yet been standardised (Sprague *et a*l., 2002; Tomaso *et al*., 2004; Tomaso *et al*., 2005). The detection of *B. mallei* by multiplex PCR and real-time PCR does not depend on *B. mallei* specific primers but on the loss of *B. pseudomallei* specific sequences in *B. mallei* (Thibault *et al*., 2004; Lee *et al.*, 2005). Furthermore, none of these PCR assays has been evaluated with clinical samples or its effectiveness has been validated under the real field

conditions. Altukhova *et al*. (2007) used primers based on the flagellar C gene to identify *B. mallei* and *B. pseudomallei* and to diagnose experimental glanders and melioidosis. On the basis of their results, they recommended PCR for both identification of pure cultures and investigation of experimental material. Similarly, Sprague *et al.* (2002) reported heterogeneity in the fliC gene from several *B. pseudomallei* isolates, which barred the differentiation of *B. mallei* from *B. pseudomallei*. RT-PCR assays were developed targeting the *B. pseudomallei* 16S rRNA, fliC, and the ribosomal subunit protein S21 (rpsU) genes, none of which could differentiate *B. mallei* from *B. pseudomallei* (Tomaso *et al*., 2005). Real-time PCR assays have also been developed that target genes of the conserved type 3 secretion system (TTS1, orf11 and orf13) encoded by both *B. mallei* and *B. pseudomallei* (Thibault *et al*., 2004). In the latter investigation, both *B. mallei* and *B. pseudomallei* were distinguished from *B. thailandensis* by amplification of orf13, but the assay was not successful to differentiate *B. mallei* from *B. pseudomallei*. Furthermore, Antonov *et al*. (2004) attempted to target portions of the *B. mallei* and *B. pseudomallei* 23S rRNA to distinguish these species using standard PCR (not a RT-PCR platform) and reported that this highly conserved genomic target is not adequate for distinguishing *B. mallei* from *B. pseudomallei*. An in silico approach was made to screen the *B. mallei* ATCC 23344 and *B. pseudomallei* K96243 genomes to identify alleles distinctive to *B. mallei*. A total of 5 genes were targeted to develop a *B. mallei*-specific PCR assay. Each primer set was tested against an extensive panel of *B. mallei* and *B. pseudomallei* isolates recovered from various geographic, clinical and environmental locations around the world. This study explains that all *B. mallei* isolates contain a conserved bimABm and that the primer pair AT4/AT5 will be extremely useful for the identification of *B. mallei*, and differentiation from *B. pseudomallei*, in the event this agent is used in a biologic attack (Ulrich *et al*., 2006). Scholz *et al.* (2006) developed a simple, rapid, sensitive and specific PCR assay targeting the flagellin P (flip)-IS 407A genomic region of *B. mallei* for specific detection of this organism in pure cultures and evaluated this assay with clinical samples from a recent outbreak of equine glanders. Primers derived from the known flip-IS407A sequence of *B. mallei* American Type Culture Collection (ATCC) $23344^{T}$ allowed the specific amplification of a 989-bp fragment from each of the 20 *B. mallei* strains investigated, whereas other closely related organism tested negative. Bowers *et al.* (2010) developed recently RT-PCR assay, "BurkDiff", which targets single nucleotide polymorphisms (SNP) to detect and differentiate *B. mallei* and *B. pseudomallei*.

#### 2.3.5.2 Pulse Field Gel Electrophoresis (PFGE)

Bacterial typing using PFGE is a readily and commonly used in house molecular technique. Detailed literature regarding typing of *B. pseudomallei* and related pathogens is available

(Koonpaew *et al*., 2000; Godoy *et al.,* 2003). PFGE is also a highly discriminatory tool for *B. mallei*. In a recent study, 17 distinct ribotypes were resolved for 25 isolates of *B. mallei* using two restriction enzymes (Harvey and Minter, 2005). PFGE and multi locus sequence typing (MLST) were used to type 21 *B. mallei* laboratory strains. Information about all known *B. mallei* strains should be gathered and a commonly available database of PFGE banding pattern types might be helpful in case of future outbreaks of glanders in humans and animals (Chantratita *et al.,* 2006). However, there is the need for standardisation of the technique.

#### 2.3.5.3 Mutilocus Sequence Typing (MLST)

A total of 147 isolates of *B. pseudomallei, B. mallei* and *B. thailandensis* was recently characterised by MLST (Godoy *et al*., 2003). MLST showed similar resolution as that of PFGE and identified the clones causing disease in animals, each of which was also associated with disease in humans. Alleles of six of the seven loci investigated in *B. mallei* were also present within *B. pseudomallei* isolates. This finding proved that *B. mallei* is a clone of *B. pseudomallei* and *B. mallei* should not be considered as a separate species on genetic basis. MLST can be performed with purified DNA so there is no need for excessive cultivation of the agent or the keeping of strain collections. Web-based analysis might enhance diagnostics (Godoy *et al.,* 2003). The MLST scheme developed for *B. pseudomallei* is also applicable to *B. mallei* and *B. thailandensis*.

#### 2.3.5.4 Variable Number Tandem Repeat Analysis (VNTR)

MLST is time consuming and lacks discriminatory power of genetically closely related *B. pseudomallei* and *B. mallei* isolates (Godoy *et al.,* 2003). Recently, a PCR-based method using VNTR loci has become a popular method for the molecular typing of pathogens (Liu *et al*., 2006; Svraka *et al.,* 2006). In a very recent study, the analysis of the genetic information of 23 VNTR loci showed that Pakistani *B. mallei* isolates from the Punjab are genetically distinct to isolates from other countries (Hornstra *et al.,* 2009). Although VNTR markers have higher mutation rates in comparison to other genetic markers which make them inappropriate for determining deep evolutionary relatedness, VNTRs are suitable to discriminate between closely related isolates, determine the degree of relatedness among isolates and discern population structures on a spatial scale (Uren *et al.,* 2007; Pearson *et al.,* 2007). This ability is especially important for *B. mallei* because it is a recently emerged clone of *B. pseudomallei* and has been shown to be genetically monomorphic applying typing methods such as MLST (Godoy *et al*., 2003).

## 3 Materials and Methods

### 3.1 Materials

#### 3.1.1 Sera collection

##### 3.1.1.1 Materials used for blood sampling and sera preparation from Pakistani equids

**Table 3: Equipment and consumables for blood sampling and sera preparation.**

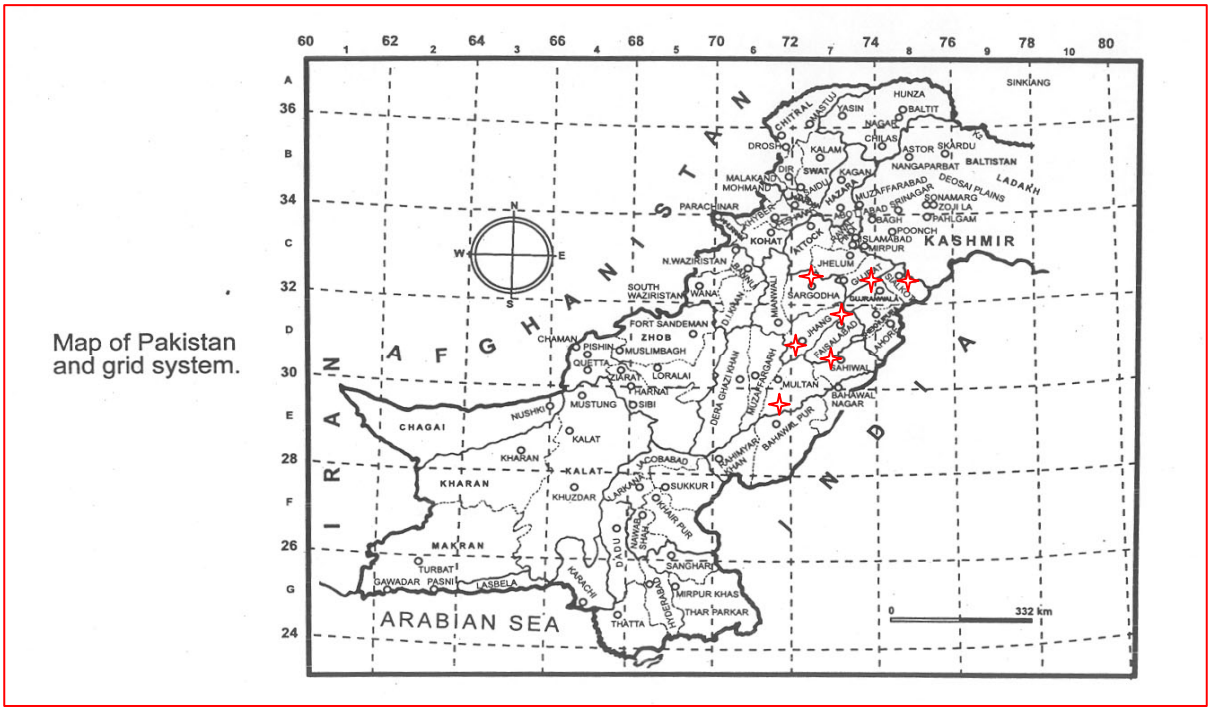
| Name of the equipments /consumable/reagents | Name of manufacturer, if available |
|---|---|
| **Equipment** | |
| Mini Centrifuge Machine | Eppendorf, GmbH, Germany |
| Centrifuge machine | 800D, Jiangsu Zhengji Instruments, China |
| Electric Generator | Loncin, China |
| Ice Box | Biorays Laboratory, Pakistan |
| -20C Freezer | 180-T, Haire, China |
| Medical Emergency kit | Biorays Laboratory, Pakistan |
| Ice Packs | Biorays Lab, Pakistan |
| 500-1000 µl Microdispenser | Eppendorf, Germany |
| 25-100 µl Microdispenser | Eppendorf, Germany |
| 25-100 µl Microdispenser tips | ImuMed, China |
| **Consumables** | |
| Pink Spray 250 ml | Komi Pharma, Korea |
| Surgical Cotton | Imperial Industry, Pakistan |
| 70% Methylated Ethanol | Biorays Laboratory, Pakistan |
| 5 ml Eppendorf tubes | Eppendorf, Germany |
| Cotton Bandages | Kohinoor, Pakistan |
| 3ml Cryo tubes | TPP, Switzerland |
| BD syringes10 ml | Shifa Industry , Pakistan |

| | |
|---|---|
| Blood collection vacutainers with gel Clot activator 17X 75 MM, 6 ml | BIO-VAC, China |
| Gloves | Sigma, Pakistan |
| Masks | Sigma, Pakistan |
| Dry Ice | International World Courier Service, Pakistan |

#### 3.1.1.2 Study area in Pakistan

Pakistan is bordered by Afghanistan and Iran in the west, India in the east and China in the far northeast. In most of the neighboring countries of Pakistan, glanders outbreaks have been reported as mentioned above. So, geographic location of Pakistan is critical with reference to cross boundary animal diseases like glanders. In the present study, both army and civil equine populations of Pakistani Punjab were included (Figure 4). The Punjab has also the highest of equine population among the four provinces (Balochistan, Khyber Pakhtoon Khawa, Sindh and Punjab) of Pakistan (Livestock Economic Survey, 2008–09). To save time, labour and cost, not all districts of Punjab were included in the sampling frame. Only districts with increased equine population and particularly having a previous history of glanders outbreaks were included in the sampling frame (Hornstra *et al.,* 2009). A map of the Punjab province, Pakistan including various districts from where blood samples were collected is shown in Figure 4. A total of 2,013 blood samples were collected. Approximately 8 ml blood was collected aseptically from the jugular vein of donkeys, mules and horses. Blood samples were preserved in an ice box under field condition. Forty four sera were received separately from suspected culture positive glanderous equids brought to Equine Veterinary Hospital, Department of Clinical Medicine and Surgery (CMS), University Agriculture Faisalabad (UAF), Pakistan. Microbiological culture, isolation and identification of *B. mallei* are mentioned in detail by Saqib, 2009. A report of the Livestock Census 2006, Pakistan about the distribution of horses, mules and donkeys in the major districts of Punjab is shown in Table 4.

**Figure 4: Map of Punjab, Pakistan.**




Map of Pakistan showing the locations (red stars) from where blood samples were collected

### 3.1.1.3 Sera preparation and labelling of samples

Each blood sample was immediately transferred from the syringe to 6ml blood vacutainers with gel clot activating factors. Serum was separated after clot formation by the method provided by "Texas Department of State Health Services, Laboratory Service Section: "Serum Separation Protocol for Field Professionals" (Detailed information is available at http://www.dshs.sate.tx.us/lab/CLIAtrainingSlides.pdf). More than 2,013 blood samples were collected. Sera showing haemolysis, bacterial contamination or plaque formation were discarded. Eventually, 1,453 (horses: 183; mules: 873; donkeys: 397) good quality sera (main pool) were available in the end. It is worth mentioning here that 44 sera from culture proven glanderous equids are not included in this 1,453 sera collected from potentially exposed equids. Sera were transferred into cryo tubes. The cryo tubes were labelled with a specific code with permanent marker as explained below:

H/M/12/06/09/312: spp./sex/date/serial number e.g. (H: horse; M: mule; D: Donkey) (F: female; M: male) (date: 12/06/09) (number of relevant questionnaire), respectively. A questionnaire was developed with open and closed questions to get information on descriptive

epidemiology: who (species), when (temporal distribution of equids) and where (spatial distribution of equids). The questionnaires showed that blood was not drawn from pregnant, vaccinated and/or malleinised animals. Blood was harvested from healthy equids although mostly civilian equids kept for draught purpose suffered from emaciation, malnutrition, physical injuries and internal or external parasitism. Some animals suffered from "strangles" in the past. The distribution of sera within the districts and the species is shown in Table 5 and 6. Of 1453, 533 equines were of the civil origin. These 533 equids blood samples were collected from 111 different local sites including communal stables, vegetable markets, animal sales and purchase markets, abattoirs for large and small "Halal" animals and government civil veterinary hospitals (Table 7). All sampling sites showed poor hygienic and sanitary conditions.

**Table 4: Number of horses, mules and donkeys (study population) in various districts of Punjab, Pakistan (Livestock Census, Pakistan, 2006).**

| District | Horses | Mules | Asses | Total Equine Study Population |
|---|---|---|---|---|
| Faisalabad | 10479 | 6102 | 132214 | 148795 |
| Toba Tek Singh | 2177 | 1863 | 41839 | 45879 |
| Jhang | 15123 | 1310 | 172397 | 188830 |
| Gujranwala | 7213 | 3841 | 66699 | 77753 |
| Sialkot | 5503 | 2238 | 73720 | 81461 |
| Multan | 1783 | 807 | 16901 | 19491 |
| Sargodha | 11720 | 3758 | 130252 | 145730 |
| Total | 53998 | 19919 | 634022 | 707939 |

**Table 5: Spatial distribution of 1,453 blood samples on the basis of various districts, Punjab, Pakistan.**

| District | Horses | Donkeys | Mules | Total |
|---|---|---|---|---|
| Faisalabad | 25 | 77 | 06 | 108 |

| | | | | |
|---|---|---|---|---|
| Toba Tek Singh | 20 | 55 | 04 | 79 |
| Jhang | 20 | 101 | 07 | 128 |
| Gujranwala | 15 | 60 | 0 | 75 |
| Sialkot | 21 | 50 | 0 | 71 |
| Multan | 22 | 50 | 0 | 72 |
| *RVFC,RD, Sargodha | 75 | 01 | 844 | 920 |
| Total | 198 | 394 | 861 | 1,453 |

*Remount Veterinary and Farms Corps (RVFC), Remount Depot (RD), Sargodha, Pakistan

**Table 6: Distribution of 1,453 blood samples on the basis of species.**

| Species | RVFC,RD, Sargodha/ army population | Civil population | Total |
|---|---|---|---|
| Horse | 75 | 108 | 183 |
| Donkeys | 01 | 396 | 397 |
| Mules | 844 | 29 | 873 |
| Total | | | 1,453 |

**Table 7: Distribution of the sampling clusters of civilian equids in six districts of the Punjab, Pakistan.**

| Sampling cites | JHG | TTS | FSD | GUJ | MUL | SKL | Total |
|---|---|---|---|---|---|---|---|
| Vegetable markets | 03 | 02 | 01 | 01 | 02 | 03 | 12 |
| Brick kiln units | 14 | 09 | 04 | 05 | 05 | 06 | 43 |
| Horse show | 01 | 0 | 0 | 0 | 0 | 0 | 01 |
| Slaughter house for | 01 | 01 | 01 | 01 | 0 | 0 | 04 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| small animals | | | | | | | |
| Slaughter house for large animals | 01 | 01 | 01 | 01 | 01 | 0 | 05 |
| Animal health care units | 01 | 02 | 01 | 0 | 0 | 0 | 04 |
| Communal stables | 01 | 02 | 01 | 0 | 0 | 0 | 04 |
| Goods transportation units | 06 | 05 | 06 | 06 | 07 | 06 | 36 |
| Animal sale and purchase markets | 0 | 0 | 02 | 0 | 0 | 0 | 02 |
| Total # of clusters | 28 | 22 | 17 | 14 | 15 | 15 | 111 |

JHG Jhang; TTS Toba Tek Singh; FSD Faisalabad; GUJ Gujranwala; MUL Multan; SKL Sialkot

#### 3.1.1.4 Sera collection from Pakistan

Forty four sera from culture proven (Figure 7) glanderous equids (Figure 6) were obtained from Veterinary Teaching Hospital, University of Agriculture, Faisalabad, Pakistan.

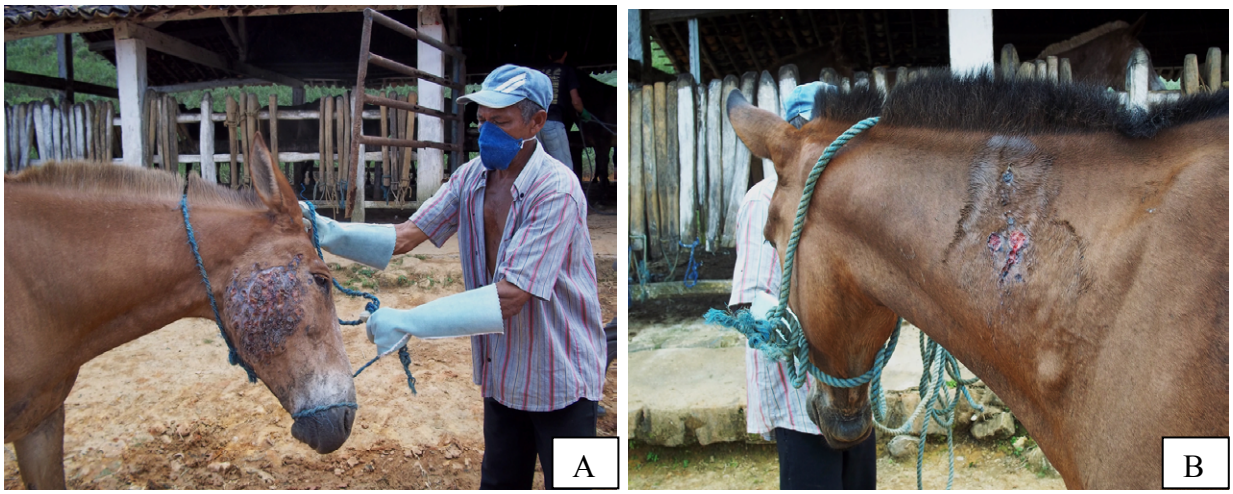
#### 3.1.1.5 Packaging and Shipment

Sera in 3 ml cryo tubes were packed as “Biological Substance Category B” “UN3373” in primary, secondary and tertiary receptacles according to International Dangerous Goods Regulations by International air transport association (IATA) (detailed instructions available at http://www.umdnj.edu/eohssweb/publications/PI650.pdf). All samples were packed in upright position in 2 layers next to each other in four boxes with inner dimensions (L x W x H) 29 x 27x 25 cm. 5 cm space was left on either side for dry ice filling. 4 kg dry ice was used per box. Length of each cryo tube was 2.75 inches/ 7 cm with inner diameter 0.39 inches/ 1 cm. Finally, all sera samples were shipped by air to FLI, Germany.

### 3.1.1.6 Sera collection from Brazil

A total of 113 sera were collected from clinical positive glanderous horses (Figure 5) from various states including Pernambuco, Passira. The sera were supplied by Laboratório Nacional Agropecuário, Ministério da Agricultura, Pecuária e Abastecimento, Rua Dom Manoel De Medeiros, S/N Dois Irmãos Recife Pernambuco 52171-030, Brazil.

**Figure 5: Cutaneous ulcers (A & B) and farcy bud (B) of glanderous Brazilian horse.**




Figures Source: Professor Fernando Leandro dos Santos - Professor of pathology of Domestics Animals - UFRPE/Brazil

### 3.1.1.7 Sera collection from Germany

A total of 200 sera collected from various geographical areas of Germany was investigated.

### 3.1.1.8 Sera collection from immunised rabbits and horses

Immunization of horses and production of hyperimmune sera in rabbits were performed as described in recent publication (Elschner *et al*., 2011). Briefly, horses were immunized with an adjuvanted mixture of crude suspensions of heat killed *B. mallei* strains: Bogor, Mukteswar and Zagreb ($10^9$cfu/ml). Immunizations were done for seven consecutive weeks

with 7 doses ($10^3$ cfu/ml to$10^9$ cfu/ml) of 1.5 ml antigen. Rabbits were immunized with a crude suspension ($10^6$ cfu/ml) of heat inactivated *B. mallei, B. pseudomallei, B. cepacia*, and *Pseudomonas aeruginosa*. The strains used are given in Table 9. Two initial immunizations (days 0 and 3) were made with 10 doses of 0.2 ml ($10^6$ cfu/ml) antigen without adjuvant. Five more immunizations were done with 0.5 ml ($10^6$ cfu/ml) in the ear vein (days 10, 17, 24, 31, and 38). Final sera were obtained 6 weeks post-immunization. Seroconversion in both species was monitored by CFT and IB.

#### 3.1.1.9 Animal ethical issues

Animals' ethical issues pros and cons were taken into account while harvesting the blood samples from donkeys, mules and horses. Guidelines were followed made for the welfare of livestock from which blood is being harvested for commercial and research purposes by the "Animal Advisory Committee, Ministry of Agriculture, Wellington (1996), New Zealand". Detailed information is available at http://www.biosecurity.govt.nz/files/regs/animal-welfare/pubs/blood-harvesting-guidelines.pdf). The animal experiment (immunization of rabbits) was authorized by the government of Thuringia, Germany (registration number 04-106/07). The animal experiment (immunization of horses) was authorized by the government of Thuringia, Germany (registration number 04-105/07).

#### 3.1.1.10 Experimental designs

Following three experimental designs have been used in four different studies:

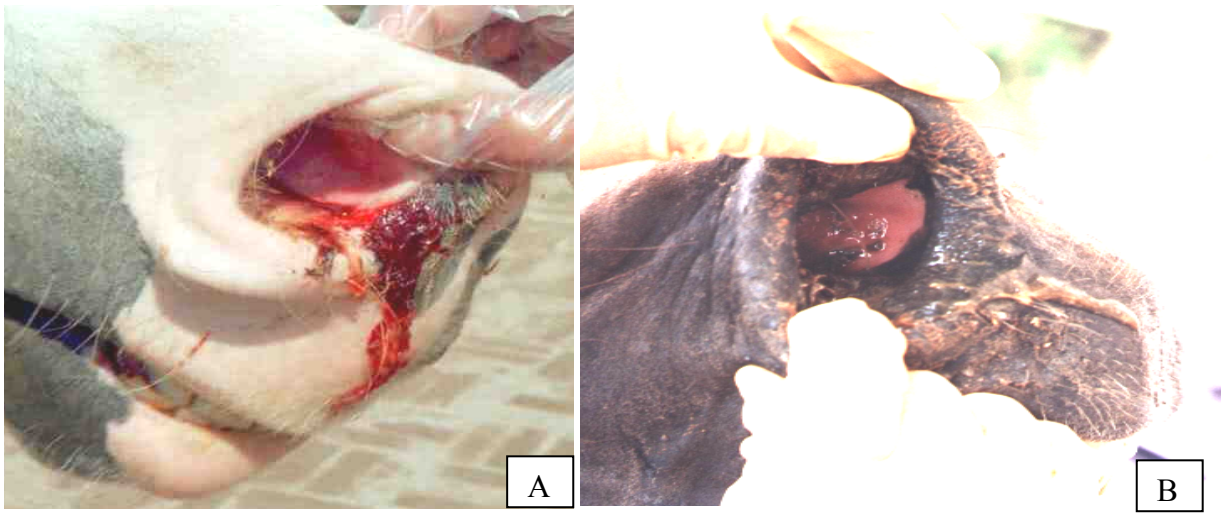
#### 3.1.1.10.A A preliminary prevalence study for six districts of the Punjab, Pakistan

In the first experimental design 533 equids sera were collected from various districts of Punjab, Pakistan to estimate the seroprevalence of glanders. All sera were tested with CFT. Sera with suspicious, false positive and anticomplementary results in CFT were retested with recently published confirmatory IB (Elschner *et al*., 2011).

### 3.1.1.10.B Validation of the CFT antigens of the companies' ccPro and CIDC for use in an South East Asian endemic country, Pakistan

In a second experimental design, a total of 1,678 sera were divided into three groups: group I, II and III. In group I, 25 (horses:19; mules:04; donkeys:02) were obtained from clinical (Figure 6) and culture positive (Figure 7) glanderous equids from the Department of Clinical Medicine and Surgery (CMS), University of Agriculture Faisalabad (UAF), Pakistan. In group II, 1,453 sera samples (horses: 183; mules: 873; donkeys 397) from first experimental design were investigated. In group III, 200 sera (horses: 199; donkey: 01) were randomly collected from different geographical places of Germany. These sera were taken as negative control as glanders has been eradicated from Western Europe and Germany is in the list of glanders free country (Elschner *et al*., 2011). All sera were tested by CFT separately using **two** different commercially available *B. mallei* antigens: **c. c. pro and CIDC**. Sera with suspicious, false positive and anticomplementary CFT results were retested with confirmatory IB. However, all sera of group I (glanderous) and III (non-glanderous) sera were tested with IB for confirmatory serodiagnosis.

**Figure 6: Nasal septum ulcers (B) and nasal bleeding (epistaxis, A) in 2 different Pakistani horses affected with glanders.**




Figures Source: Dr. M. Saqib, Department of Clinical Medicine and Surgery, University of Agriculture, Pakistan

**Figure 7: Confluent and mucoid growth of *Burkholderia mallei* on brain heart infusion agar with 4% glycerol (A), pure culture of *B. mallei* on blood agar plate. Notice non-haemolytic greyish- brown colonies after 72 hours (B).**

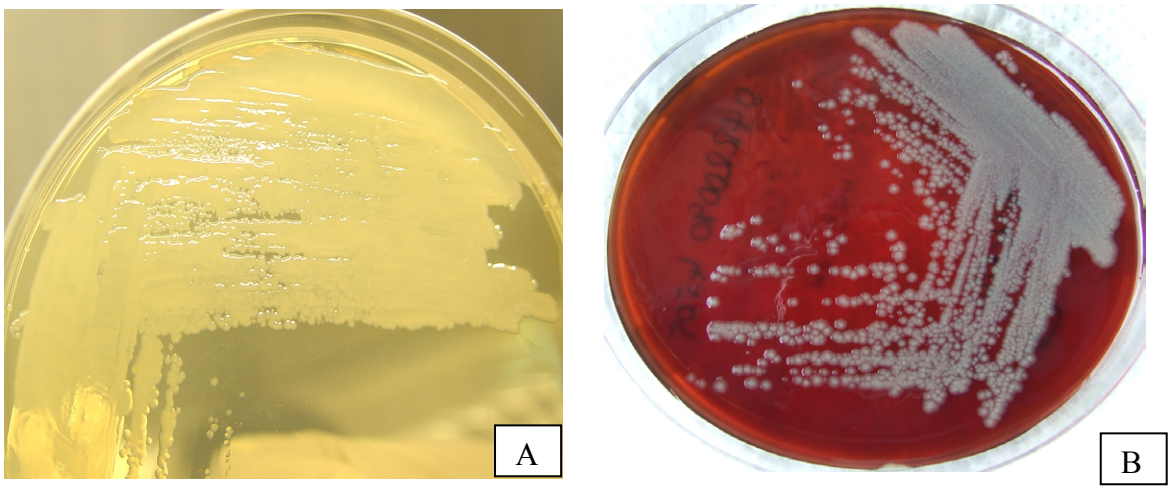



Figure (A) Source: Dr. M. Saqib, Department of Clinical Medicine and Surgery, University of Agriculture, Pakistan

### 3.1.1. 10.C Validation of CFT antigens using positive sera from Asia and South America

A total of 410 sera (horses, 342; donkeys, 07; mules, 30, immunised rabbits, 12; immunised horses, 19) were investigated. The sera were divided into two groups. In group I, 200 sera (horse, 199; donkey, 01) from 3.1.1.10 B experimental design was used. In group II, 210 sera were placed either from culture positive animals (44) or/and equids which were clinically diagnosed glanderous (135) originating from Pakistan (Veterinary Medical Teaching Hospital, University of Agriculture, Faisalabad) and Brazil (Laboratório Nacional Agropecuário, Ministério da Agricultura, Brazil). The 12 sera of immunised rabbits and 19 sera from immunised horses against *B. mallei* were also added to group II. All sera were tested by CFT using **three** *B. mallei* antigens separately: **c. c. pro, CIDC and USDA**. Sera with suspicious and anticomplementary CFT results were retested with confirmatory IB.

### 3.1. 2 Diagnostic antigens

Sera with anticomplementary activity and suspicious results in CFT were retested by Western Blotting. Mixtures of three *B. mallei* (LPS) strains were used as probe antigens: ATCC 23344 (isolated from a human in China in 1944); Zagreb (isolated from a horse in Ex-Yugoslavia) and Mukteswar (isolated from a horse in India) in the 3.1.1.10 A and 3.1.1.10 B experimental designs (Table 8). All bacterial strains were provided by the Federal Institute for Risk Assessment, Berlin, Germany. A mixture of *B. mallei* LPS was used for the detection of antibodies in the test serum. Three *B. mallei* strains were used for the preparation of LPS mixtures: Bogor (originating from a horse in Indonesia); Mukteswar and Zagreb in 3.1.1.10 C experimental design (Table 8).

**Table 8: List of bacterial strains used in this study (antigens preparation and experimental immunisation).**

| Strain | Source | Geographic origin | Species |
|---|---|---|---|
| Mukteswar | Horse | India | *Burkholderia mallei* |
| Bogor | Horse | Indonesia | *Burkholderia mallei* |
| Zagreb | Horse | Yugoslavia | *Burkholderia mallei* |
| ATCC 23343 | Human | Unknown | *Burkholderia pseudomallei* |
| DSM 7288 | Unknown | Unknown | *Burkholderia cepacia* |
| ATCC 9027 | Unknown | Unknown | *Pseudomonas aeruginosa* |

ATCC: American Type Culture Collection, Manassas**,** USA

DSM: German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany

### 3.1.3 Complement fixation test

#### 3.1.3.1 Negative and positive control sera

Following negative and positive control sera were used in CFT (Table 9):

**Table 9: Negative and positive control sera samples used for c.c.pro, CIDC and USDA antigens in CFT.**

| Serum Type | Batch No. | CFT Titer | Manufacturer |
|---|---|---|---|
| Negative control serum | 0502007RR0114 | 0 | Friedrich-Loeffler-Institut (FLI), Jena, Germany |
| Low positive control serum (CIDC) | 04CS1008 | 1:20 | CIDC, Lelystad, The Netherlands |
| High positive control serum (CIDC) | 04CT1202 | 1:50 | CIDC, Lelystad, The Netherlands |
| Positive control serum (c.c.pro) | 040210RR9256 | 1:80 | c. c. pro, GmbH, Oberdorla, Germany |
| Low positive control serum (USDA) | Reagent code 166-L | 1:10 | USDA, Iowa, Ames, USA |
| High positive control serum (USDA) | Reagent code 166-H | 1:80 | USDA, Iowa, Ames, USA |

### 3.1.3.2 CFT-analysis

In the present studies, all sera were tested with the OIE recommended CFT method according to the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Anonymous, 2008) separately using two commercially available c.c.pro (c.c.pro GmbH, Oberdorla, Germany) and CIDC (Central Veterinary Institute of Wageningen UR, The Netherlands) antigens in 3.1.1.10 B experimental design and separately using three commercially available antigens: c. c. pro; CIDC and USDA (United States Department of Agriculture, Ames, Iowa, USA) in 3.1.1.10 C experimental design.

Following commercially available CFT antigens were used in this study:

I. c. c. pro (GmbH, Oberdorla, Germany). This antigen consists of *B. mallei* strains: Zargreb, Mukteshwar and Bogor. This antigen was extracted from *B. mallei* cultures in phosphate buffer saline and the compound contained 0.5% phenol as a conservative. Working dilution in Veronal buffer (VB) is 1:40.

II. CIDC (Central Veterinary Institute of Wageningen UR, The Netherlands) antigen. This antigen also consists of *B. mallei* strains: Zargreb, Mukteshwar and Bogor. Bacteria were heat killed for 3 h in a Koch's steamer at 100 °C and the working dilution in VB is 1:600.

III. USDA (United States Department of Agriculture, National Veterinary Services Laboratory, Ames, Iowa, United States of America). This antigen was prepared by a single Chinese *B. mallei* strain. This strain isolated during World War II was supplied to USDA by the Armed Forces Institute of Pathology (AFIP). This organism was used also to produce mallein which was used for anti-sera production, but the organism is no longer available. Antigens in lyophilized form were reconstituted in distilled water. According to the manufacturer's information about the method of preparation, mallein is the heat-stable product formed in glycerine broth cultures of *B. mallei*. Bacterial cells were killed by heating. The supernatant was decanted, filtered, concentrated by evaporation, and diluted with physiological saline (0.85%) containing 0.5% phenol. The working dilution in VB was 1:100.

#### 3.1.3.3 Reagents, buffers and equipment for CFT

All reagents (veronal buffer, haemolytic system, complement, haemolysin or amboceptor's) except antigen and negative control sera for the complement fixation test were supplied by Virion / Serion (Virion / Serion GmbH, Friedrich-Berguis-Ring 19, Würzburg, Germany).

Reagents, buffers and equipment used in CFT are mentioned in Table 10.

**Table 10: Reagents, buffers and equipment used in CFT**

| Name of the reagents, buffers and equipment | Name of manufacturer, if available/ formulation |
|---|---|
| Phosphate buffer Saline (PBS), pH 7.4 | 12mM $Na_2HPO_4$ x 2 $H_2O$ (2,136 g)<br>12 mM $NaH_2PO_4$ x $H_2O$ (1,656 g)<br>0,145 M NaCl (8,5 g) |
| Orbital shaker | Polymax 1040, Heidolph Instruments GmbH, Schwabach, Germany) |
| Centrifuge Labofuge 400 | Heraeus, Germany |

| | |
|---|---|
| Pipettes (0,5 ul-10 ml) | Eppendorf Research |
| Water bath GFL | Labortechnik GmbH, Germany |
| Water bath P-DIG | Medingen, Germany |
| Microtiter plates type COS96fb | Corning, USA |

### 3.1.4 WB-analysis

**Table 11: Equipment, consumables, buffers, chemicals and reference sera used in WB.**

| Equipment | Name of manufacturer, if available/formulation |
|---|---|
| Automatic pipettes (0.5ul -10 ml) | Eppendorf, Germany |
| XCell SureLock™ Mini-Cell | Invitrogen, Germany |
| XCell II™ Blot Module | Invitrogen, Germany |
| Novex® blot module | Invitrogen, Germany |
| Microliter syringe 0–250 ul | Hamilton, Germany |
| Microliter syringe 0–25 ul | Hamilton, Germany |
| Tweezers (plastics) | - |
| Orbital shaker | Heidolph Instruments GmbH, Germany |
| Power-pack 200 (30 Volts, 200 volts) | BIO-RAD, Germany |
| **Consumables** | |
| Mini-Incubation trays | BIO-RAD, Germany |
| NuPAGE-Gels 4–12% Bis-Tris,10 Well, 1mm | Invitrogen, Germany |
| NuPAGE-Gels 4–12% Bis-Tris Zoom TM Gel,1mm | Invitrogen, Germany |
| Nitrocellulose membrane filter paper sandwich, 0,45um average pore size | Invitrogen, Germany |
| Silver Staining kit Silver Xpress® | Invitrogen, Germany |

| | |
|---|---|
| Frame for drying gels | Novel experiment technology, Germany |
| Protein™ Standard Kaleidoscope | BIO-RAD, Germany |
| **Buffers** | |
| NuPAGE® Transfer Buffer (20X) | Invitrogen, Germany |
| NuPAGE® MOPS-SDS-Running Buffer (20X) | Invitrogen, Germany |
| Blocking solution ,125 ml<br>Stored at –20 °C | Candor Bioscience, GmbH, Germany |
| Low Cross™ Buffer, 125 ml | Candor Bioscience, GmbH, Germany |
| Washing buffer (10x stock solution) 500 ml | Candor Bioscience GmbH, Germany |
| Sample buffer Laemmli 2x | Sigma, Germany |
| AP-Buffer<br>pH 9,5 ± 0,2:100ml<br>100 mM; NaCl (0,58g)<br>100mM Tris (1,2g)<br>5mM $MgCl_2$ x $6H_2O$(101,65mg) | Sigma, Germany |
| PBS-buffer; pH:7.0<br>12mM $Na_2HPO_4$ x 2 $H_2O$ (2,136 g)<br>12 mM $NaH_2PO_4$ x $H_2O$ (1,656 g)<br>0,145 M NaCl (8,5 g) | Sigma, Germany |
| **Chemicals** | |
| Anti-horse immunoglobulin G (IgG)-alkaline Phosphatse (AP) (Rabbit) | Sigma, Germany |
| Methanol , 100% | Sigma, Germany |
| Nitroblau-Tetrazoliumchloride (NBT) (Analytical grade), 1g<br>0,5g NBT in 10 ml 70% | Sigma , Germany |

| | |
|---|---|
| dimethylformamide(DMF) solution | |
| Bromo-chloride-indolphosphate (BCIP)<br>0, 5 g BCIP (Toulidin salt) in 10 ml DMF (100%)<br>Stored at -20 °C | Sigma, Germany |
| NBT-BCIP Staining solution<br>10 ml AP-buffer<br>66 µl NBT<br>33 µl BCIP | Sigma, Germany |
| 37% formaldehyde | Sigma, Germany |
| Glycerin, ≥ 86% | Roth, Germany |
| N, N-Dimethylformamid | Roth, Germany |
| Acetic acid, 100% | VWR Prolabo BDH, Germany |
| 0.9% NaCl-solution | |
| **Reference sera** | |
| Positive control serum (Horse) | FLI, Jena, Germany (FLI No 08RR2072) |
| Negative control serum (Horse) | FLI, Jena, Germany (FLI No 07RR0114) |

### 3.2 Methods

#### 3.2.1 CFT Principle

Antigen/antibody complexes can be measured by their ability to fix complement because an antigen/antibody complex will "consume" complement if it is present, whereas free antigens or unbound antibodies do not. Tests for antigen/antibody complexes that rely on the consumption of complement are termed complement fixation test and are used to quantitate antigen/antibody reactions. This test will only work in the presence of complement fixing antibodies in the test serum. The reaction will not be visible and a further indicator or haemolytic system (HS) is used. The HS comprises of sheep RBCs which have been

sensitized by addition of anti-sheep erythrocyte antibodies, grown in rabbits (amboceptor), forming an immune complex of antibodies and erythrocytes. Not "consumed" complement will destroy the HS resulting in haemolysis. If the complement is consumed by the antibodies, the HS will be visible as a sediment of erythrocytes.

#### 3.2.1.1 CFT procedure

Sera were diluted 1/5 in VB saline containing 0.1% gelatin. Diluted sera were inactivated for 30 minutes at 58–60°C in a water bath. Inactivation temperature for horses was set at 58°C for 30 minutes while it rose to 63°C for 30 minutes for sera of mules and donkeys or non-equids. Twofold dilutions of the sera were prepared in 96-well round-bottom microtitre plates. First of all, 25 µl of VB was added in all the rows of 96 wells plate except the 2$^{nd}$ row. Inactivated test sera were mixed well on shaker before pipetting into wells. 25 µl of diluted inactivated test serum was pipetted in the wells of the first, second and third rows. Two fold serial dilutions were made starting from 3$^{rd}$ row till end, and 25 µl of resulting mixture were discarded from last row. 25 µl of antigen of working dilution was added to each row except in the first row. 25 µl of complement, diluted to the number of units required, were added to each well. Control wells containing diluent only, complement and diluent and antigen, complement and diluent, were set up to contain 75 µl in each well. Positive and negative serum control wells were also maintained. The plate was shaken 60 sec. on a shaker before incubation. In case of **cold CFT**, plates were covered and incubated at 4°C for 16–20 hrs. In case of **warm CFT**, plates were incubated at 37°C for one hour only. Haemolytic system (HS) was prepared by mixing amboceptor working dilution 1:1 with 1% erythrocyte suspension. One should bear in mind that the sedimented erythrocytes have to be mixed carefully and gently to create the homogenous mixture. HS system was incubated for 30 minutes at 37°C in a water bath. The plates from the first day were also prewormed at 37°C for 30 min. in an incubator to keep both the haemolytic system and plates at the same temperature. 50 µl of the freshly prepared HS was added into each well and shaken carefully. Plates were moist incubated at 37°C for 15–30 min. Incubation was stopped when the complement control with 2 and 1 units showed complete haemolysis. The plate was centrifuged for 5 min at 2,000 rpm. The absence of anticomplementary activity was checked for each serum in the first row. The first row of 96 wells plate was taken as serum control and no diluted antigen was added in this row. A sample that produced 100% haemolysis at the 1:5 dilution was negative, 25–75% haemolysis was suspicious, and no haemolysis (100% fixation) was considered positive.

### 3.2.2 WB assay principle

The WB (alternatively known as immunoblot) is an analytical technique used to detect specific antigens (for example protein) in a given sample of biological homogenates or extracts. It uses gel electrophoresis to separate native or denatured proteins by their weight (denaturing conditions) or by the 3-D structure of the protein (native/ non-denaturing conditions). The proteins are then transferred by a buffer system using their electric charge to a nitrocellulose membrane. They are detected using antibodies specific to the target protein. These antibodies are linked to an enzyme and this enzyme precipitates a non soluble strain onto a membrane, precursor stain is added to the incubation buffer. Antigen preparations for the WB assay and test procedure of immunoblot were followed with little modification in 3.1.1.10 A and 3.1.1.10 B experimental designs and used as such in 3.1.1.10 C experimental design as described by Elschner *et al.* (2011).

#### 3.2.2.1 Antigen preparation for the WB assay

*B. mallei* strains ATCC 23344, Zagreb and Mukteswar were used for experimental designs 3.1.1.10 A and 3.1.1.10 B. *B. mallei* strains Bogor, Zagreb and Mukteswar were used for experimental design 3.1.1.10 C. *B. mallei* strains (Table 7) were grown on blood agar plates over night at 37°C under aerobic conditions. For LPS purification, a 10 µl loop full of bacterial colony material was re-suspended thoroughly in 6 ml saline (0.9% NaCl, pH 7.4) up to a density comparable to McFarland scale 4.0. A volume of 3 ml of 37% formaldehyde was added to achieve a final formaldehyde concentration of 12.3%. The suspension was rigorously vortexed and then washed by shaking at 15 rpm using an orbital shaker (Polymax 1040, Heidolph Instruments GmbH, Schwabach, Germany) over night at room temperature. Controls for sterility confirmed inactivation of bacteria. Cells were pelletized by centrifugation at 3.500 x g for 15 min and the supernatant was discarded. Soluble proteins were removed by 3 repeated centrifugation and washing steps with 9 ml phosphate buffered saline (PBS, pH 7.4) shaking at 15 rpm for 10 min. The LPS-containing pellet was re-suspended in 9 ml PBS. Two further formalin extraction steps by adding 1 volume of 37% formaldehyde into 2 volumes re-suspended cells in PBS were applied. By 3 repeating washing steps with PBS most proteins were removed. After the last washing step the pellet was re-suspended in PBS. The purity of the LPS-preparation was analyzed by SDS-PAGE and silver staining (Figure 9). For SDS-PAGE several dilutions from the prepared LPS containing cell suspension (3 ml) in Laemmli Buffer (Sigma, Munich, Germany) were loaded on a precast denaturing 4–12% polyacrylamide gradient gel (Invitrogen, Karlsruhe, Germany)

and separated at a constant voltage of 200V for 50 min using the Xcell SureLock™ MiniCell (Invitrogen) (Figure 9, lane a). Aliquots were ready to use for WB analysis.

Immunoblot assay was performed as described previously (Elschner *et al.,* 2011). Briefly, partly purified lipopolysaccharide (LPS) antigen was prepared from the three virulent *B. mallei* strains Bogor, Zagreb and Mukteswar. Mixture ratio of these *B. mallei* strains was adjusted as described (Elschner *et al.,* 2011). A mixture of all antigens was separated on a precast preparative 4–12% polyacrylamide gradient gel (Invitrogen, Karlsruhe, Germany) and subsequently transferred to a 0.45 µm nitrocellulose membrane (Invitrogen) using the Novex® Blot Module (Invitrogen). The membrane was blocked overnight in Blocking Solution (Candor Bioscience, Weißensberg, Germany), washed and cut into strips of 3mm and stored at –20°C. Strips were incubated with equine or rabbit sera in a 1:50 dilution in Low Cross Buffer (Candor Bioscience) for 1.5 h at room temperature followed by three washing steps in Washing Buffer (Candor Bioscience) 20 min each.

#### 3.2.2.2 Immunoblotting (IB)

The strips were then incubated for 1.5 h at room temperature in Low Cross Buffer containing alkaline phosphatase-conjugated rabbit anti-horse-IgG or goat anti-rabbit-IgG (Sigma, Munich, Germany, 1:5,000). After three additional washing steps, the strips were stained with NBT-BCIP® solution (Sigma). The immunostaining was stopped by washing with distilled water after 10 minutes. The IB was scored positive if the banding pattern of the *B. mallei* LPS ladder lying within the region of 20 to 60 Kilo Dalton (kDa) was clearly visible, scored suspicious if within the region of 20 to 60 kDa a clouded precipitation was seen, and scored negative if no reactions were seen in the area between 20 and 60 kDa (Elschner *et al.,* 2011).

### 3.2.3 Statistical analysis

Glanders is considered as a rare disease even in endemic countries and expected prevalence can be considered <0.1% in random sample collections. Calculations however were only done based on the numbers of positives and negatives in the collections. Diagnostic specificity and sensitivity were calculated using standard formulae (Martin, 1977). In experimental design 3.1.1.10 C, McNemar test was used to compare sensitivities of CFT antigens by using software version 17.0 (SPSS).

Kappa (K) statistics was calculated as described by Viera and Garret, 2005. To compare the sensitivity, specificity and overall agreement between the various tests, the statistical formulae given by Thrusfield (1995) were used as described below:

| | | **Gold standard test** | | **Total** |
|---|---|---|---|---|
| | | Positive | Negative | |
| Test to be compared | Positive | a | b | a+b |
| | Negative | c | d | c+d |
| | Total | a+c | b+d | a+c+ b+d = N |

The notations used above are:

a = Number of samples positive to both conventional and the gold standard tests

b = Number of samples positive to conventional but negative to the gold standard test

c = Number of samples negative to conventional but positive to the gold standard test

d = Number of samples negative to both conventional and the gold standard tests

a + b + c + d = Total number of samples (N)

Definitions and formulae of the indices used for comparing the different assays are described as follows:

**Sensitivity:** It is the capacity of the test to detect diseased animals, when compared with the gold standard test (a/a+c x 100).

**Specificity:** It is the capacity of the test to detect non-diseased animals, when compared with the gold standard test (d/b+d x 100).

Overall agreement: Is the proportional similarity of the results of both tests

(a+d/N x 100).

In case there were no gold standard for a particular condition and it is necessary to evaluate the diagnosis by the level of agreement between different tests, this assumes that agreement between test is evidence of validity, whereas disagreement suggests that the test are not

reliable. The Kappa test can be used to measure the level of agreement beyond that which may be obtained by chance. The kappa statistic ranges within -1 and +1.

Observed agreement = (a+d) / (a+b+c+d).

This is compared to the expected agreement, which would be obtained by

chance, which is given by the formula: [{(a+b)/n} x {(a+c)/n}] + [{(c+d)/n} x {(b+d)/n}].

Kappa is agreement greater than expected by chance divided by potential excess (OA - EA)/ (1-EA). It should be noted that the Kappa value gives no indication which of the tests is better and that a good agreement may indicate that both test are equally good or equally bad (Viera and Garret, 2005). Kappa is evaluated on the basis of following calculated "K" values:

**Evaluation of Kappa statistic (Thrusfield, 1995)**

| **Kappa Value** | **Evaluation** |
|---|---|
| >0.81 | Almost perfect agreement |
| 0.61–0.80 | Substantial |
| 0.41–0.60 | Moderate agreement |
| 0.21–0.40 | Fair agreement |
| 0.01–.020 | Slight agreement |
| 0.00 | Poor agreement |

## 4 Results

### 4. A On the current situation of glanders in various districts of the Pakistani Punjab

Blood samples were collected from 111 different local sites including communal stables, vegetable markets, animal sale and purchase markets, abattoirs for large and small "Halal" animals and government civil veterinary hospitals (Table 7). All sampling sites showed poor hygienic and sanitary conditions. The animals at the sales and purchase markets came from different cities and surrounding areas and most of the animals had clinically manifest health problems and were in need of medication. The majority of the draught equids were emaciated, suffering from parasites and displayed poor health condition. The animals showed poor body scoring, malnourishment, and suffered from different kinds of skin injuries due to overloading, dehydration, parasitism, and diarrhoea (Figure 8). Most of the animal owners complained about past strangles infections. Five hundred and thirty three blood samples (Table 2) were collected from non-clinical glanderous (Muhammad *et al*, 1998; Radostits *et al*, 2000) horses, mules and donkeys kept to draw carts for the transportation of people, bricks, and different goods. Samples were also collected from horses in the Jhang district kept for horse shows and from clinically healthy horses from the existing strangles hospital. The sex distribution of the horses (113 male/10 female), donkeys (349 male/44 female), and mules (16 male/1 female) ranged from nine months to 16 years (median: 4 years). None of the equids sampled had a history of previous malleinisation. None of the sera tested positive in the Western blot. Depending on the antigen used in the CFT (ccPro/CIDC/both antigens) 7/8/8 horse sera, 58/8/14 donkey sera, and 4/2/0 mule sera reacted false-positive (Table 12). The diagnostic specificity of the ccPro antigen based CFT was 84.71%, and of the CIDC antigen based CFT 92.72%.

**Figure 8: Sampling from draught donkey**


**Table 12: Geographic distribution and false positive CFT results of 533 equine blood samples on the basis of animal species in various districts of the Punjab, Pakistan.**

| Total number of animals tested vs. number of false positive CFT results | | | | |
|---|---|---|---|---|
| | Horses | Donkeys | Mules | Total |
| | ccPro/CIDC/ | ccPro/CIDC/ | ccPro/CIDC/ | ccPro/CIDC/ |
| District | both antigens | both antigens | both antigens | both antigens |
| Faisalabad | 25 (1/4/2) | 77 (3/1/0) | 06 (3/2/0) | 108 (7/7/2) |
| Toba Tek Singh | 20 (5/0/0) | 55 (15/1/4) | 04 (1/0/0) | 79 (21/1/4) |
| Jhang | 20 (0/3/5) | 101 (9/1/4) | 07 (0/0/0) | 128 (9/4/9) |
| Gujranwala | 15 (0/0/1) | 60 (10/2/2) | 0 | 75 (10/2/3) |
| Sialkot | 21 (0/0/0) | 50 (8/2/0) | 0 | 71 (8/2/0) |
| Multan | 22 (2/1/0) | 50 (13/1/4) | 0 | 72 (15/2/4) |
| Total | 123 (8/8/8) | 393 (58/8/14) | 17 (4/2/0) | 533 (70/18/22) |

## 4.B Performance of complement fixation test and confirmatory immunoblot as two-cascade testing approach for serodiagnosis of glanders in an endemic region of South East Asia

A total of 1,678 serum samples were comparatively analysed by two CFT antigens and IB was used as gold standard method. All results are given in detail in Table 12.

In group I all of 25 IB positive sera from culture positive equids were tested positive with both CFT antigens resulting in 100% sensitivity, respectively.

In group II of 1,453 potentially exposed animals 15 samples were tested positive and 319 sera were tested suspicious by use of c.c.pro antigen. Using CIDC antigen 44 samples were tested positive and 266 samples were tested suspicious. In 171 cases out of the total number of 473 positive or suspicious results, both antigens resulted in agreeing results. All 473 CFT positive or suspicious samples were tested negative with IB. Nine hundred and two sera were negative with both antigens. The remaining 78 sera showing anticomplementary activity (ACA) with both antigens were tested negative with IB, and were excluded from all calculations. Considering the CFT positive and IB negative samples as “false positive” samples in calculation, the c.c.pro antigen showed lower specificity (75.71%) than the CIDC antigen (77.45%) in group II.

In group III consisting of 200 true negative animals originating from a non-endemic area, ten and twelve samples were tested positive or suspicious using the c.c.pro or CIDC antigen, respectively. Eight samples showed ACA and were excluded from calculations. All positive, suspicious and anticomplementary sera of group II were tested negative with IB. Considering the CFT positive or suspicious and IB negative samples as “false positive” the c.c.pro antigen showed a higher specificity (94.79%) than the CIDC antigen (93.75%) in group III.

On comparing glanderous and non-glanderous animals, slightly higher agreement (0.79) was found between CFT using c.c.pro antigen and IB followed by CFT using CIDC antigen and IB (0.76). However both, 0.79 and 0.76 values rank as good agreement. A prevalence of glanders less than 0.1% is assumed.

**Table 13: CFT and IB results from sera of group I (sera from culture positive equids from Punjab, Pakistan), group II (potentially glanders exposed equids originating from Punjab, Pakistan, and group III (negative sera from non-endemic area from Germany).**

| Sample group | No of samples | CFT[1] (c.c.pro[2]) | CFT (CIDC[3]) | IB[4] |
|---|---|---|---|---|
| Group I (n = 25) | 25 | positive | positive | positive |
| Group II (n = 1,453) | 163 | positive/suspicious | negative | negative |
| | 139 | negative | positive/suspicious | negative |
| | 171 | positive/suspicious | positive/suspicious | negative |
| | 902 | negative | negative | not tested |
| | 78 | anticomplementary | anticomplementary | negative |
| Group III (n = 200) | 5 | positive | negative | negative |
| | 7 | negative | positive | negative |
| | 5 | positive | positive | negative |
| | 175 | negative | negative | not tested |
| | 8 | anticomplementary | anticomplementary | negative |

[1]CFT: Complement Fixation Test

[2]c.c.pro: CFT antigen from c.c.pro GmbH, Oberdorla, DE

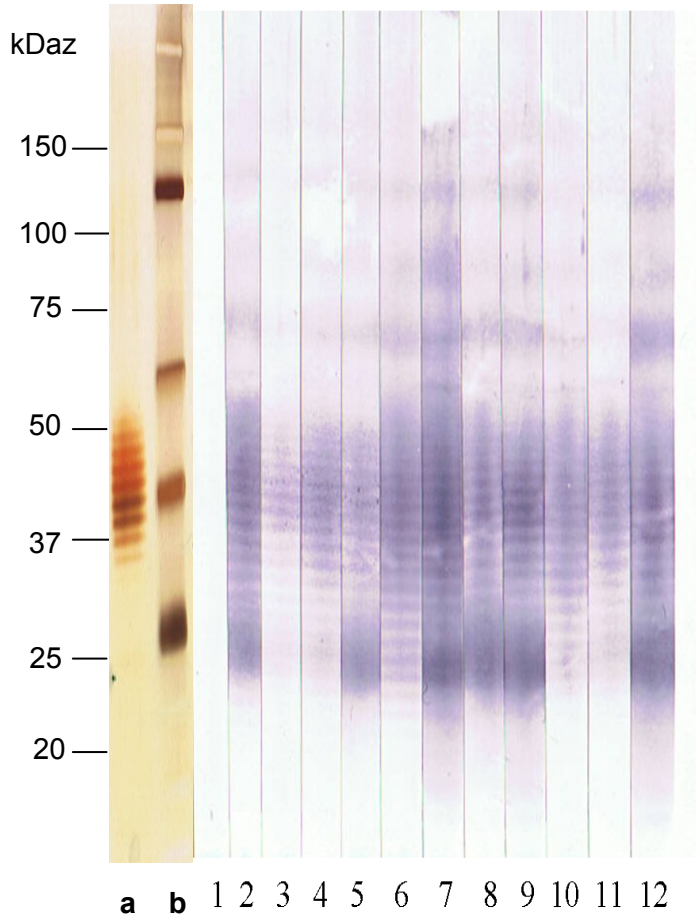
[3]CIDC: CFT antigen from Central Veterinary Institute of Wageningen UR, Lelystad, NL

[4]IB: Immunoblot

## 4.C Comparative evaluation of three commercially available complement fixation test antigens for the diagnosis of glanders

Group I and II sera were comparatively evaluated with three CFT antigens. IB was used as gold standard. The CFT and IB results are summarised in Table 13. All sera of group I tested negative with IB. The c.c.pro antigen tested 193 (96.50%) sera negative and seven (3.5%) sera positive while the CIDC antigen tested 195 (97.50%) sera negative and five (2.5%) sera positive. All 200 (100%) sera tested negative with the USDA antigen. In group II (210) 208 sera tested positive with the IB but two sera (one originating from a Brazilian horse and another from a Pakistani mule, both clinically diagnosed) proved to be negative. These two sera were excluded from all kind of data analysis. The c.c.pro antigen tested 163 (77.61%) animals positive and three (1.42%) animals negative. Forty-four (20.95%) sera showed anticomplementary effect. All c.c.pro-positive sera (163) were tested positive with IB but one serum sample tested negative with c.c.pro was IB-positive. CIDC antigen tested 166 (79.04%) sera positive. Again the same 44 sera showed anticomplementary effect. Of 166 CIDC-positive sera, IB tested 164 positive and two negative. USDA antigen tested 102 (48.57%) sera positive and 64 (30.47%) sera negative. The same 44 sera showed anticomplementary effect. All 102 USDA-positive sera tested positive with IB. Of 64 USDA-negative sera, 62 tested positive with IB and two sera tested negative with IB. All 44 sera showing anticomplementary activity tested positive with IB. These 44 anticomplementary sera were excluded for the calculation of specificity, sensitivity, positive and negative predictive values. IB identified all 200 true negative (group I) and 44 culture-positive (group II) sera correctly with specificity and sensitivity of 100%. The highest specificity was calculated for the USDA antigen (100%) followed by the CIDC (97.50%) and c.c.pro antigen (96.50%).The highest sensitivity was calculated for the CIDC antigen (100%) followed by the c.c.pro antigen (99.39%). The USDA antigen showed substantially less ($P<0.05$) sensitivity (62.19%). The highest positive predictive value was shown by the USDA antigen (100 %) followed by the CIDC antigen (97.04%) and c.c.pro antigen (95.88%). The highest negative predictive value was shown by the CIDC antigen (100%) followed by the c.c.pro antigen (99.48%). The USDA antigen showed a considerably lower negative predictive value (76.33%). Kappa statistic values of agreement were calculated separately between the three CFT antigens and IB testing serum samples from group I and group II animals. Almost perfect agreement (0.96) was found between CFT using either c.c.pro or CIDC antigen and IB. However, substantial agreement (0.64) was found between CFT using USDA antigen and IB.

**Figure 9: Immunoblot analysis of sera from culture positive equids from Pakistan.**




Sliver stain of SDS-PAGE gel (a b), mixture of Bogor, Mukteswar and Zagreb *B. mallei* strains (lane a), mixture of LPS of *B. mallei* strains protein standard (lane b)

Lane 1: negative control; lane 2: positive control; lane 3-12 sera of acutely infected culture positive glanderous horses from Pakistan

**Table 14: Results of the CFT (c.c.pro, CIDC and USDA) and IB testing with sera from the glanders-free (group I) and glanderous and/or immunised animals (group II).**

| Group | Origin | Number (nature) of sera | Species | | | ACA sera | | | | CFT(c.c.pro) | | CFT (CIDC) | | CFT (USDA) | | IB | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | [a]H | [b]D | [c]M | R | H | D | M | R | + | - | + | - | + | - | + | - |
| I | Germany | 200 (true negative) | 199 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | $7^a/0^b/0^c$ | $192^a/1^b/0^c$ | $5^a/0^b/0^c$ | $194^a/1^b/0^c$ | 0 | $199^a/1^b/0^c$ | 0 | 200 |
| II | Pakistan | 44 (culture positive) | 26 | 3 | 15 | 0 | 6 | 1 | 11 | 0 | $19^a/2^b/4^c$ | $1^a/0^b/0^c$ | $20^a/2^b/4^c$ | 0 | $12^a/1^b/2^c$ | $8^a/1^b/2^c$ | 44 | 0 |
| | Pakistan | 22 (clinical positive) | 4 | 3 | *1+14 | 0 | 4 | 3 | 11 | 0 | $0^a/0^b/3^c$ | $0^a/0^b/*1^c$ | $0^a/0^b/*1+3^c$ | 0 | $0^a/0^b/2^c$ | $0^a/0^b/*1+1^c$ | 21 | *1 |
| | Brazil | 113 (clinical positive) | *1+112 | 0 | 0 | 0 | 8 | 0 | 0 | 0 | $104^a/0^b/0^c$ | $*1^a/0^b/0^c$ | $*1+104^a/0^b/0^c$ | 0 | $54^a/0^b/0^c$ | $*1+50^a/0^b/0^c$ | 112 | *1 |
| | In-house | 12 (immunised) | 0 | 0 | 0 | 12 | 0 | 0 | 0 | 0 | 12 | 0 | 12 | 0 | 12 | 0 | 12 | 0 |
| | In-house | 19 (immunised) | 19 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 19 | 0 | 19 | 0 | 19 | 0 | 19 | 0 |
| **Total** | | 410 | 361 | 7 | 30 | 12 | 18 | 4 | 22 | 0 | 170 | 196 | 171 | 195 | 102 | 264 | 208 | 202 |

[a]H Horse, [b]D Donkey, [c]M Mule, R Rabbit, ACA anticomplementary activity

CFT Complement fixation test, CFT (c.c.pro) CFT using c.c.pro antigen, CFT (CIDC) CFT using CIDC antigen,

CFT (USDA) CFT using USDA antigen, IB Immunoblot, + Positive result, - Negative result

*Misdiagnosed as clinically positive by the field veterinarian and therefore excluded from data analysis

## 5. Discussion and Conclusions

Antibodies appear within a week during *B. mallei* infection and infected animals quickly attain the maximum peak of titers. These will stay for weeks and decline slowly (M'Fadyean, 1896). About 90% of mules and horses produce serum antibodies against *B. mallei* post-infection (Verma, 1975; Al-Ani, 1993). In an endemic area like Pakistan, early detection of disease would be helpful for controling glanders by adopting the test and slaughter policy. The usefulness of diagnostic tests depends upon their sensitivity and specificity, accuracy, predictive values and field applicability (Sackett *et al*., 1991). In comparison with other serological tests used for the diagnosis of glanders, CFT is still the OIE prescribed test for international trade of equids (OIE, 2008; Sprague *et al*., 2009). The CFT is considered by many workers to be a highly sensitive test for the diagnosis of glanders (Cravitz and Miller, 1950; Verma, 1980), although it may fail to detect many confirmed cases (Shumilov, 1974). However, both IHA test and CFT are based on crude-whole cell preparations or extracts of the bacteria. Therefore, the potential for the false-positives can not be ruled out (Parthasarathy *et al.,* 2006). There is another highly specific alternative test method available now: Western or immunoblot (IB) for sera which show anticomplementary or suspicious reactions in CFT (Hagebock *et al*., 1997; Elschner *et al.,* 2011). Although IB is a complex and laborious technique it was used already to diagnose equine piroplasmosis, dourine and glanders in 1999 in a prospective study (Katz *et al*., 1999). IB of 6 *B. mallei* strains demonstrated variations in their exopolysaccharides (Anantagool and Sirisinah, 2002). These variations may have an impact on the virulence of the *B. mallei* strain but also on the sensitivity of serological test (Verma, 1975; Verma *et al.,* 1994). IB has recently been evaluated for the diagnosis of glanders and it showed 100% specificity and sensitivity. Therefore, CFT false-positives, suspicious and anticomplementary sera in the current studies were retested with IB.

### 5.A Seroprevalence of glanders in Pakistani Punjab

A recent glanders outbreak in Kabul, Afghanistan, was attributed to illegally imported horses from Pakistan (WAHID, 2011b). This suspicion was based on a cascade of outbreaks in civilian equines in the Pakistani Punjab between 1999 and 2007 which led to the isolation and characterisation of several *B. mallei* strains. The authors speculated on the existence of specific *B. mallei* lineages (genotypes) in the Punjab and that the disease might be spread via communal stables and water troughs (Hornstra *et al*., 2009). Hence, a serum collection was carried out in the districts Multan and Jhang as well as in the Faisalabad, Gujranwala (southeast of Lahore) and Sialkot districts; the latter bordering with India which is also

endemic for glanders. The present study included the civilian working equid population as it is believed that these animals, which are kept under very poor hygienic conditions and traded without control, are the main reservoir for glanders. Sampling was carried out at locations characterised by high animal density (close contact and crowding stress), by a high frequency of movement (multiplicity of animal origin) and physical stress (high working burden and temperature); moreover, since these animals are most likely to use communal water troughs and communal stables, a regular exposure to *B. mallei* can be expected (Hornstra *et al*., 2009). Based on a literature study we assumed that 'healthy' carriers, i.e. subclinical infected animals might also play a most important role for persistence and propagation of disease (Pritchard, 1995). In peace time, i.e. when no outbreak is evident, these animals should at least be detected by serology. Surprisingly, no clinical sign of chronic disease that is scars as marks of local infection or acute glanders or anti *B. mallei* antibodies were observed.

Our study shows that glanders is currently not detectable in several Punjab districts including Faisalabad and Gujranwala known for past outbreaks. It can therefore be assumed that glanders re-emerged in form of local epidemics on introduction of glanderous equines into naive populations. We believe that only small local foci of glanders exist in remote areas of the Punjab from which glanders occasionally spills over into the equine population. This assumption is supported by the finding that a glanders outbreak occurred in a Lahore Polo Club back in 2005 after the introduction of two presumably asymptomatic horses brought from a Sargodha district farm to participate in competitions (Khan, 2005; Hornstra *et al*, 2009). In times of peace, common stables and their surroundings do not appear to be an active reservoir for glanders. *B. mallei* is cleared from wet surfaces or slurry within 100 days in temperate climates (Loeffler and Schütz, 1882). I believe that *B. mallei* is inactivated within few days or even hours by the dry heat and harsh sunlight during the Pakistani summer. Thus, the role of public water troughs and stables for the spread of *B. mallei* may only be of seasonal importance. Another explanation for the absence of glanderous animals in our investigation is that most diseased animals may succumb to glanders during the stressful summer season.

A total of 110 (20.6%) sera tested positive/suspicious with both CFT procedures. These false-positive CFT results were attributed to cross-reactions with other bacteria for example *Streptococcus equi* (Misra and Arora, 1990; Naureen *et al*., 2007). A high prevalence of anti *S. equi* antibodies might be the reason for the observed cross-reactions in our study populations. Others have reported that strangles is endemic in Pakistani army horses (Ijaz *et al*., 2010) and in the civilian equine population of Sargodha and Lahore (Ijaz *et al*., 2011). They reported that the highest (2.6%) incidence of strangles was seen from the end of January to the beginning of May, thus shortly before or at the beginning of the sampling period in our study. Two other Pakistani working groups reported that 32.2% of the mules younger than 2 years and 35.4% of the mules older than 2 years in Pakistan and 54% of the foals in the

Punjab contracted strangles (Ashraf, 2000; Manzoor *et al*., 2008). However, melioidosis can not be ruled out as differential diagnosis. No data on the prevalence of *Burkholderia pseudomallei,* the causing agent of melioidosis, in the Pakistani Punjab, which shows 98% genetic resemblance with *B. mallei*, are available.

Twenty three (4.31%) serum samples predominately from mules showed anticomplimentary activity. The reason for this phenomenon is not clear but sporadic reports describing that mule and donkey sera are more prone to anticomplimentary activity exist (Naureen *et al*., 2007). Diagnostic specificity for both *B. mallei* CFT antigens partly depends on origin (endemic or non-endemic) of animals as it has been observed in past studies. Higher diagnostic specificities were observed testing animals from non-endemic areas (Elschner *et al*, 2011; Khan *et al*., 2011) and lower for endemic areas (Khan *et al*., 2012). In current study, diagnostic specificity of both CFT antigens agrees with this observation.

Our study has shown that it may be very difficult to control glanders in a country like Pakistan. Due to the lack of effective vaccines, the only successful control option for glanders is testing and subsequent culling of positives. The use of local strains can significantly improve the sensitivity of the tests for glanders detection (Sprague *et al*., 2009). To prove our preliminary data, a bigger seroprevalence study among the equines of the Pakistani Punjab, using CFT and Western blot with composite mixtures of local *B. mallei* strains, is highly recommended. Remote regions should be in the focus of this surveillance to identify the pockets of endemicity. Although serological testing will lead to the destruction of some healthy animals due to the occurrence of false positives using the recommended screening test CFT, it is the only way to stop the establishment of *B. mallei* in niches and the transfer from animal to humans (Sprague *et al*., 2009). Testing must be continued even though the disease has seemingly retreated; countermeasures have to be set into action to avoid reintroduction of disease by 'healthy' carriers. It must be stressed that it is impossible to demonstrate absence of disease in every equine tested. An element of risk of spreading the disease still remains even if all precautions have been strictly followed. Public veterinary health actions have to be flanked by a wise re-imbursement policy to receive full acceptance by the animal owners. It is of eminent importance to bear special social or regional customs in mind to gain acceptance of the countermeasures. Finally, all equids of the country must be registered at birth and must be made available for testing. Safe destruction of carcasses, decomposition of manure and disinfection of premises are measures to round up the control program. Last but not least, public health services must monitor human glanders infections in parallel and make medication available to the affected persons.

## 5.B & C Comparative evaluation of two and three CFT antigens

Glanders is a disease of solipeds notifiable to the OIE. Early identification and control of disease is crucial to avoid bans on international trade on equids and considerable losses for the local economy (Sprague *et al*., 2009). Thus, highly specific and sensitive sero-tests are needed for early identification. It is commonly accepted to use the CFT for local outbreak investigation. CFT is also the only OIE recommended serodiagnostic tool for glanders diagnosis in international trade (Neubauer *et al*., 2005). However, it is well known, that CFT has considerable shortcomings i.e. indirect testing for specific antibodies via the complement system, missing standardisation of test materials e.g. erythrocytes or antigen and last but not least a noticeable number of false-positive and -negative results. On the other hand, false-negative CFT results cause spread of glanders to healthy equine population in glanders free areas. Consequently, it is indispensible to develop or improve the serological test that has a high specificity to overcome unreliable predictive values (Sprague *et al*., 2009). A prerequisite to develop such a test is the evaluation of the performance of the currently commercially available CFT antigen preparations in equid populations of various endemic and non-endemic regions. In our preliminary study, specificity and sensitivity of two commercially available CFT antigens obtained from c. c. pro, Germany and CIDC, The Netherlands were separately evaluated by testing sera from clinically and culture positive equids from Pakistan (group I), 1,453 sera samples (horses: 183; mules: 873; donkeys: 397) were randomly collected from apparently healthy but potentially exposed equids of Punjab, Pakistan (group II) and 200 serum samples from horses were analysed which were randomly collected from different geographical areas of Germany (group III). In the latter progressing study, sensitivity and specificity of three commercially available CFT antigens obtained from c. c. pro, Germany, CIDC, The Netherlands and USDA, Ames, Iowa, USA were separately evaluated by testing sera from 410 sera whose detail is mentioned in Table 12.

In case of the experimental design 3.1.1.10. B, the use of CIDC or c.c.pro antigen resulted in specificities of 77.45% or 75.71% for sera from endemic area and 93.75% or 94.79% for sera from non-endemic areas, respectively. The specificity showed considerable differences in analysing samples from endemic and non-endemic areas. I assume that adequate performance of both CFTs in groups II and III is partly caused by the identical composition of formulations based on the same *B. mallei* isolates (Bogor, Mukteswar and Zagreb). During comparative evaluation of three commercial available antigens, c. c. pro, CIDC and USDA showed, 96.5%, 97.5% and 100% specificity, respectively. In a very recent study, the diagnostic specificity of CFT using c. c. pro antigen was determined to be 94.5% which is almost same in case of experimental design 3.1.1.10. B and less than in experimental design 3.1.1.10. C (Elschner *et al*., 2011). It could be due to operator made errors. The diagnostic specificity of

c. c. pro and CIDC antigen was found to be less than in the past study in which the c. c. pro antigen and antigen prepared from a newly isolated strain (Dubai 7) showed 100% specificity testing a sera collection from Middle Eastern countries (Sprague *et al*., 2009). Similarly, 100% specificity was found for a home made sonicated CFT antigen preparation of three *B. mallei* strains PRL-4, PRL-7 (recent isolates from Pakistan) and China 5/ATCC10399 (isolated from China) (Naureen *et al*., 2007). The specificity of the c. c. pro and CIDC antigens found in this study is in the expected range for CFT in accordance with many publications in the past. They reflect the true situation in the field. It can be supposed that the populations investigated in recent publications (Naureen *et al*., 2007; Sprague *et al*., 2009) do not represent the working equine population in the field but is biased by selection e.g. race or Arabian horses. Another reason of higher specificity (100%) of CFT could be the use of local isolates as CFT antigens (PRL-4 and PRL-7) to test Pakistani equids sera and Dubai7 to test sera from Middle East origin. However, USDA antigen made from a Chinese *B. mallei* strain showed absolute agreement of specificity as mentioned in both previous studies (Naureen *et al*., 2007; Sprague *et al*., 2009). The reason why no true positive sera were found from Pakistani potentially exposed equids (1,453) may be the random sampling irrespective to the consideration of glanderous animals. The collection should represent a typical population of equids of that area. No selection bias should be present. An intention of our sampling was also to gain as much truly negative sera from Pakistan as possible to enable a reliable calculation of specificity. While testing the Pakistani sera (1,453), false-positive CFT results with c. c. pro (24.29%) and CIDC (22.54%) antigen may be attributed to cross-reactions to other bacteria (Piven *et al*., 2005). However, the need for an additional confirmatory test like the IB is obvious. For the testing of suspicious and anticomplementary sera, we used the IB technique as used successfully in the past studies (Katz *et al*., 1999; Elschner *et al.,* 2011). In experimental design 3.1.1.10.B results, 100% sensitivity was calculated for (c. c. pro and CIDC) antigens. While comparing three CFT antigens (c. c. pro, CIDC and USDA) highest sensitivity was found for CFT using CIDC antigen (100%) followed by c. c. pro antigen (99.39%). However, the USDA antigen showed significantly ($P<0.05$) less sensitivity (62.19%). In another recent study diagnostic sensitivity of c. c. pro antigen in CFT was determined to be 100% which is almost the same as in our study (Elschner *et al*., 2011). One possible reason for our finding of the unsatisfactory sensitivity of USDA antigen is that it is made from a museum strain of human origin which might has lost antigenicity e.g. by destruction or blockage of various epitopes during subcultivation (Neubauer *et al*., 2005). On the other hand, antibodies against local "serogroups" of *B. mallei,* which were described in the historic literature only, may not react with an antigen of other "serogroups". This would especially be a problem if the test antigen is made from a single strain like in the case of the USDA antigen. The assumption that addition of antigens prepared from local and recently isolated strains to the CFT antigen can improve sensitivity is supported by findings of recent

studies (Naureen *et al*, 2007; Sprague *et al*, 2009). A higher sensitivity (85.24%) of an in-house RBPT using local *Brucella melitensis* isolates instead of the commercially available antigen (78.59%) was also seen for the diagnosis of brucellosis in goats (Shahaza *et al*, 2009). C. c. pro antigen had a sensitivity of only 80% when testing sera from Dubai, UAE. However, when using a preparation of the local *B. mallei* strain Dubai7 as test antigen the sensitivity increased to 100% (Sprague *et al*., 2009). A decrease of sensitivity may also result from the complement fixation technique itself. Naureen *et al*. (2007) determined a sensitivity of 97.1% for the IHAT using a composite mixture of antigens prepared from three *B. mallei* strains (PRL-4, PRL-7 and China 5). In contrast the sensitivity for CFT using the same composite mixture of 3 antigens was only 91.4%. Another reason for a decrease in CFT sensitivity may be the test protocol used or the diagnostic reagents applied i.e. complement or blood cells. According to "validation of an assay for the diagnosis of infectious diseases" (OIE, 2010), there are various inherent factors which interfere with the analytical performance of an assay including instrumentation, operator error, reagent choice (both chemical and biological), calibration, accuracy , pH and ionicity of buffers and diluents, error introduced by detection of closely related analytes etc. To minimise these inherent errors, a ready-to-use complement and haemolytic system was used. This could be one possible reason to achieve higher sensitivity and reproducibility of CFT using c. c. pro and CIDC antigen than in past studies (Naureen *et al*., 2007; Sprague *et al*., 2009; OIE, 2010). Another possibility to achieve a higher diagnostic sensitivity is the use of cold CFT instead of warm CFT (Burgess and Norris, 1982). Furthermore, the OIE recommended protocol for CFT was used in these current studies while the Pakistani workers (Naureen *et al*., 2007) adopted a CFT procedure described by Verma *et al*., 1990. Interestingly, c. c. pro and CIDC antigens which were commercially prepared by a composite mixture of the same three *B. mallei s*trains (Bogor, Zagreb and Mukteswar) showed equal sensitivity (100%) during their comparative evaluation and almost equal senstivities (100% and 99.39%). The influence of these factors on the test quality may not be underestimated accordingly. The composition of these CFT antigens also take into account the idea of local circulating strains: The Bogor strain originated from Indonesia is believed to represent strains from Southeast Asian countries. The Mukteswar strain originating from India should represent the *B. mallei* lineage from Asian countries e.g. Pakistan. The Zagreb strain isolated from Ex-Yugoslavia was included to represent strains formerly circulating in European countries e.g. Germany. One possible reason to achieve higher Kappa agreement (0.98) between CFT and RBT in the past study could be the use of more local isolates of *B. mallei* in both serological tests to test local sera collections (Naureen *et al*., 2007). In study 3.1.1.10 B, most sera (1,453 = 86.59%) are from Pakistan. Therefore, Mukteswar and ATCC 23344 (China 7) *B. mallei* strains might be useful as local isolates to indentify the sera from South Asian countries. Hundred percent diagnostic specificity and sensitivity of IB in our studies showed absolute agreement with the recent study on the use of

Western blotting for the diagnosis of glanders (Elschner *et al.,* 2011). From the above mentioned CFT studies from different authors, it has to be concluded that antigens produced from more heterogeneous and local (in-house) *B. mallei* strains might have a positive effect on the diagnostic specificity and/or sensitivity of CFT. In our study, almost perfect agreement between CFTs (c.c. pro and CIDC) and IB showed that IB could be used as a confirmatory serological test which has the additional ability to test the anticomplementary sera. Regarding study 3.1.1.10 C, our data do not support the speculation that adding of newly isolated local *B. mallei* strains can enhance the sensitivity of CFT for the diagnosis of glanders considerably. Another finding of this study also supports our conclusion: the composite mixture of *B. mallei* strains (Bogor, Mukteswar and Zagreb) used in IB as probe antigen had 100% sensitivity. Thus, differences in sensitivity of CFT and IB may result from different antibody classes or types being reactive in both tests. It is known that some pregnant and old equids show false-negative results in CFT (Neubauer *et al*., 2005). I can not exclude age or pregnancy to have caused false negative results for our panel of sera as well. Interestingly, samples from one "clinical" positive Pakistani mule and other from one "clinical" positive Brazilian horse (Table 13) which were tested negative in IB but may have been simply be misplaced in group II of design 3.1.1.10. C. Especially these animals may pose problems in an eradication campaign being glanders free. Forty four (20.85%) sera of group II animals showed anticomplementary activity with c.c. pro, CIDC and USDA antigen in experimental design 3.1.1.10. C. All 44 sera had to be excluded to calculate specificity and sensitivity. Interestingly, 18 of these sera were taken from horses which have been culture positive. Considering only this group of sera (n = 44) for calculating sensitivity, the sensitivity would drop dramatically when compared to that of IB. Thus, exclusion of anticomplementary sera from calculation of sensitivity and specificity has always made the CFT appearing "better" than it actually is. Use of IB as confirmatory serodiagnostic test in current studies is justifiable. Use of IB technique used to overcome the anticomplementary effect of sera was already proposed by Katz *et al.*, 1999. He could not prove his proposal on a significant number of sera as a decade ago when glanders was a rare disease worldwide (Naureen *et al*., 2007). I now have proven his assumption in sera collections from glanders free (Germany) and endemic areas in Asia (Pakistan) and South America (Brazil). Especially in remote areas where repetition of sampling would pose a major problem, the use of CFT is clearly limited. New rapid handheld (penside) tests have to be developed for these areas. Zhang and Lu (1983) recommended simultaneous application of two tests (IHA and CFT) to increase the detection rate of glanders. Limitations of one test are compensated by the other test. Using the sera samples from non-glanderous and glanderous animals, kappa statistics agreement was determined between IB and the CFTs (c. c. pro, CIDC and USDA). In experimental design 3.1.1.10.B, almost good kappa agreement between CFT and IB were found (0.79 and 0.76). While in experimental design 3.1.1.10.C, almost perfect agreement (0.96) was found between

IB and CFT using either c. c. pro or CIDC antigen. Significantly less agreement was found between IB and CFT using the USDA antigen (0.64). Thes findings again stress the fact that IB could be used as a confirmatory serological test. The use of the best test antigen in glanders control has to be considered very carefully. Furthermore, as crude antigen was used in all commercial preparations, there is need to evaluate CFT using purified antigens to avoid false-positive CFT results as observed to test Pakistani sera. In conclusion, combined use of IB and CFT is highly suggestive to increase the detection rate of glanders in the future. As no concise data can be obtained from Pakistan and Brazil, any estimate on prevalence (0.1%) remains speculative. The predictive values calculated in our study are based solely on our results from our sera panel and were included in the current study to make our data more comparable to previous publications in the field.

## 6. Summary


### Serodiagnosis of glanders with reference to endemic- and non-endemic settings

Glanders is a highly infectious and zoonotic disease of solipeds caused by *Burkholderia mallei*. Progressive loss of efficiency and fatal outcome resulted in massive economic losses which forced veterinary authorities throughout the world to implement disease control measures; these included mass testing using the complement fixation test and/or malleinisation, and the culling of positives. This led to the eradication of glanders from Western Europe and North America in the 1950s. However, in the last decade, the number of outbreaks in Asia and South America increased steadily and glanders regained the status of a re-emerging transboundary disease. Pakistan has been an endemic country for the past 120 years but concise data on the presence of disease are not available.

A total of 533 serum samples were collected from draught equines, a suspected risk group for glanders, from various districts of the Pakistani Punjab. The complement fixation test (CFT) and the highly sensitive Western blot technique were used for serodiagnosis. No positive animal (horse, mule, and donkey) was found. Glanders seems to be restricted to remote, sporadic pockets of endemicity and may cause outbreaks after being introduced into naive populations by (asymptomatic) shedders.

Various serological tests were used for the diagnosis of glanders in the past but still complement fixation test (CFT) is the internationally prescribed test for trading equines. A new immunoblot (IB) technique has recently been introduced to overcome the well known shortcomings of CFT i. e. a considerable number of false positive and negative results and anticomplementary effect of sera. The objective of this study was the comparative evaluation of two glanders CFT antigens commercially available at Central Veterinary Institute of Wageningen UR, Lelystad, The Netherland (CIDC) and at c.c.pro GmbH, Oberdorla, Germany (c.c.pro) in an glanders endemic area regarding specificity and sensitivity. A total of 1,678 serum samples from the endemic region (Province Punjab, Pakistan) and a non-endemic area (Germany) were analysed. All sera tested positive or suspicious with CFT were analysed by the confirmatory IB to exclude CFT false positive results. Both CFT antigens showed 100% sensitivity. The use of CIDC or c.c.pro antigen resulted in specificities of 77.45% or 75.71% for sera from endemic area and 93.75% or 94.79% for sera from non-endemic areas, respectively. The results demonstrate the different performances of identical tests in different epidemiologically settings. Based on these results, the combined use of CFT and IB is highly suggestive for the serodiagnosis of glanders. Good agreement was calculated between CFT (using either c.c.pro or CIDC antigen) and IB.

In response to third objective of comparatively evaluation of three commercially available antigens: (c. c. pro, CIDC and USDA) using sera from glanders free (Germany) and glanderous/immunised animals, the sensitivity and specificity of three commercially available complement fixation test (CFT) antigens from c.c.pro (c.c.pro), Central Veterinary Institute of Wageningen UR (CIDC) and the United States Department of Agriculture (USDA) were comparatively evaluated by testing 410 sera collected from glanders-endemic and non-endemic areas (200 true negative randomly collected sera and 210 sera collected from experimentally immunised animals (12 rabbits, 19 horses), clinical-positive (135) and culture-positive (44) horses, donkeys and mules). Immunoblotting (IB) was used as gold standard test. Highest sensitivity was shown for the CIDC antigen (100%) followed by the c.c.pro antigen (99.39%). However, the USDA antigen showed substantially less ($P<0.05$) sensitivity (62.19%). Highest specificity was found for the USDA antigen (100%) followed by the CIDC (97.5%) and c.c.pro antigen (96.5%). Positive and negative predictive values for each antigen were calculated to be: 95.88 and 99.48 (c.c.pro), 97.04 and 100 (CIDC), 100 and 76.33% (USDA). Almost perfect agreement (0.96) was found between CFT using either c.c.pro or CIDC and IB. Due to almost perfect agreement (0.96), CFT using c.c.pro or CIDC antigen can be combined with IB to increase the detection rate of glanders among infected animals.

## 7. Zusammenfassung

### Serologische Diagnostik von Rotz unter Berücksichtigung endemischer und nicht-endemischer Tierseuchensituationen

Rotz ist eine anzeigepflichtige, hochkontagiöse Infektionskrankheit der Einhufer und wird durch das Gram-negative, unbewegliche Bakterium *Burkholderia (B.) mallei* verursacht. Der Ausbruch und die Anzeige der Erkrankung führen durch Handelssperren zu erheblichen ökonomischen Verlusten für die betroffenen Länder. Deshalb forcieren die Veterinärbehörden weltweit Überwachungsuntersuchungen mittels Komplementbindungsreaktion (KBR) und Malleintest. Durch eine strikt durchgeführte Ausmerzung positiver Tiere, konnte Rotz in Westeuropa und Nordamerika in den 50iger Jahren ausgerottet werden. Durch eine steigende Anzahl von Rotzerkrankungen in den letzten 10 Jahren in Südamerika und in Asien wird Rotz jedoch als „re-emerging" Tierseuche eingestuft.

Für die serologische Diagnostik von Rotz sind verschiedene Testverfahren beschrieben. Die KBR ist jedoch die für internationale Handelsuntersuchungen vorgeschriebene Methode. In der Diagnostik mit der KBR bereiten insbesondere falsch positive Ergebnisse oder Proben mit antikomplementären Eigenschaften immer wieder Probleme. Deshalb kam in der vorliegenden Arbeit zusätzlich zur KBR ein Immunoblot-Verfahren (IB) zur Anwendung. Pakistan gilt seit 120 Jahren als endemisch für Rotz. Präzise Daten über die gegenwärtige Situation sind jedoch nicht verfügbar.

Deshalb wurden in einer vorläufigen Prävalenzstudie 533 Serumproben von Zugtieren (Pferde, Esel, Maultiere), einer potentiell verdächtigen Risikogruppe, in verschiedenen Bezirken der Provinz Punjab in Pakistan gesammelt und mit der KBR und einem hochsensitiven IB untersucht. Es wurden keine positiven Tiere nachgewiesen. Es kann geschlussfolgert werden, dass Rotz scheinbar nur in begrenzten, weiter abgelegenen Gebieten vorkommt und durch asymptomatische Ausscheider nur sporadisch in erregerfreie Populationen eingebracht wird.

In einer weiteren Studie der vorliegenden Arbeit kamen ebenfalls die KBR und der IB zur Anwendung wobei insbesondere die Nutzung verschiedener diagnostischer KBR-Antigene unter Beachtung verschiedener endemischer Situationen bewertet wurde. Gegenstand dieser Studie war die vergleichende Evaluierung zweier Rotz-KBR-Antigene, kommerziell erhältlich bei Central Veterinary Institute in Wageningen UR, Lelystad, Niederlande (CIDC) und c.c.pro GmbH, Oberdorla, Deutschland (c.c.pro) hinsichtlich ihrer Spezifität und Sensitivität in einer Rotz-endemischen Region Pakistans. Insgesamt wurden 1678 Serumproben (davon 25 positive Proben von Tieren bei denen die Infektion durch die Isolierung des Erregers bestätigt wurde) aus der Provinz Punjab (Pakistan) und einer nicht endemischen Region (Deutschland) getestet. Alle KBR-positiven und -verdächtig reagierenden Proben wurden mittels IB untersucht, um falsch positive KBR-Ergebnisse auszuschließen. Beide KBR-Antigene zeigten eine Sensitivität

von 100%. Die KBR unter Einsatz des CIDC- oder c.c.cpro-Antigens zeigte Spezifitäten von 77,45% und 93,75% oder 75,71% und 94,79% bei Anwendung auf Proben aus der endemischen oder nicht-endemischen Region. Die Ergebnisse zeigen, dass die Spezifität identischer serologischer diagnostischerverfahren, angewendet in Regionen mit unterschiedlicher epidemiologischer Lage erheblich schwanken kann.

**Für eine dritte Studie** wurden drei kommerziell erhältliche KBR-Antigene zur Rotzdiagnostik, das c.c.pro- und CIDC-KBR-Antigen und das KBR-Antigen des United States Department of Agriculture (USDA), vergleichend eingesetzt. Das c.c.pro-Antigen und CIDC-Antigen bestehen aus einer Mischung dreier *B. mallei* Isolaten (Bogor, Zagreb und Mukteswar), wohingegen das USDA-Antigen aus nur einem *B. mallei* Isolat (Chinese) hergestellt wurde. Insgesamt wurden 410 Seren, gesammelt von 200 Pferden aus Deutschland (Gruppe I, negativ) und 210 Serumproben (Gruppe 2, positiv) getestet. Die Seren der Gruppe I galten als negativ, weil Rotz in Deutschland seit mehr als 50 Jahren ausgerottet ist. Die positive Gruppe II setzt sich zusammen aus 44 Tieren aus Pakistan, bei denen die Infektion durch die Isolierung des Erregers bestätigt wurde, aus 135 klinisch krank beurteilten Tieren aus Pakistan und Brasilien, 12 Seren stammten von einem immunisierten Kaninchen und 19 Seren von einem immunisierten Pferd. Die Durchführung der KBR erfolgte gemäß den Vorgaben des OIE - "Manual of Diagnostic Tests and Vaccines for Terrestrial Animals" unter Nutzung des kommerziell verfügbaren Komplement- und hämolytischen Systems der Firma Virion/Serion. Das c.c.pro- und CIDC-Antigen wurde mit dem Serum für 18h bei 4°C (Kältebindung) und das USDA-Antigen für 1h bei 37°C (Wärmebindung) inkubiert. Alle Proben wurden auch im IB analysiert. Die höchste Sensitivität erreichte das CIDC-Antigen (100%) gefolgt durch das c.c.pro-Antigen (99,39%). Das USDA-Antigen zeigte eine deutlich geringere ($P<0,05$) Sensitivität (62,19%). Die höchste Spezifität konnte mit dem USDA-Antigen (100%) erzielt werden, gefolgt vom CIDC-Antigen (97,50%) und c.c.pro-Antigen (96,50%). Dabei zeigten die KBR mit dem c.c.pro- oder CIDC-Antigen zusammen mit dem IB die höchste Übereinstimmung (0,96). Ausgehend von diesen Ergebnissen wird für die serologische Diagnostik von Rotz die Kombination aus KBR mit dem c.c.pro- oder CIDC-Antigen und dem IB empfohlen.

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## 9. List of Publications

1. Title: Comparative evaluation of three commercially available complement fixation test antigens for the diagnosis of glanders

Authors: Iahtasham Khan, Lothar H. Wieler, Falk Melzer, Mayada Gwida, Vania L de Assis Santana, Marcilia MA de Souza, Muhammad Saqib, Mandy C. Elschner and Heinrich Neubauer

Journal: Veterinary Record 2011 (DOI 10.1136/vr.d5410)

2. Title: Performance of complement fixation test and confirmatory immunoblot as two-cascade testing approach for serodiagnosis of glanders in an endemic region of South East Asia

Authors: Iahtasham Khan, Mandy C. Elschner, Falk Melzer, Mayada Gwida, Lothar H. Wieler, Riasat Ali , Muhammad Saqib, Heinrich Neubauer

Journal: Berliner und Münchener Tierärztlich Wochenschrift (DOI 10.2376/0005-9366-125-117)

3. Title: On the current situation of glanders in various districts of the Pakistani Punjab

Authors: Iahtasham Khan, Lothar H. Wieler, Mahboob Ahmad Butt, Mandy C. Elschner, Ashiq Hussain Cheema, Lisa D. Sprague and Heinrich Neubauer

Journal of Equine Veterinary Science (DOI 10.1016/j.jevs.2012.03.006)

4. Title: Comparative evaluation of commercially available antigens for the diagnosis of glanders in Pakistan

Authors: I. Khan, H. Neubauer, L. H. Wieler, M. Saqib and M. Elschner

Conference: Deutsche Veterinärmedizinische Gesellschaft (DVG) Fachgruppentagung 22. bis 24. Juni 2010, Jena, Deutschland (ISBN 978-3-941703-70-4)

5. Title: Seroepidemiology of glanders in various districts of Punjab, Pakistan

Authors: Iahtasham Khan, Heinrich Neubauer, Lothar H. Wieler, Mayada Gwida, Falk Melzer, Ashiq Hussain Cheema, Mandy C. Elschner and Henrich Neuabuer

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*DEDICATION*

*TO*

*MY BELOVED MOTHER SHAMIM AKHTER,*

*MY BETTER HALF SOBIA*

*AND TO MY KIDS*

*AIMAL AND RAWAN*

## 11. Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen inAnspruch genommen habe.

Der Inhalt der vorliegenden Arbeit wurde bereits in folgenden Publikationen

veröffentlicht oder zur Veröffentlichung eingereicht:

1. Comparative evaluation of three commercially available complement fixation test antigens for the diagnosis of glanders

   Iahtasham Khan, Lothar H. Wieler, Falk Melzer, Mayada Gwida, Vania L de Assis Santana, Marcilia MA de Souza, Muhammad Saqib, Mandy C. Elschner and Heinrich Neubauer

   Veterinary Record, 2011 (DOI 10.1136/vr.d5410).

2. Performance of complement fixation test and confirmatory immunoblot as two-cascade testing approach for serodiagnosis of glanders in an endemic region of South East Asia

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   Berliner und Münchener Tierärztlich Wochenschrift, 2012 (DOI 10.2376/0005-9366-125-117).

3. On the current situation of glanders in the various districts of Pakistani Punjab.

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   Journal of Equine Veterinary Science, 2012 (DOI 10.1016/j.jevs.2012.03.006).

4. Effect of temperature on diagnostic sensitivity of complement fixation test for the diagnosis of glanders.

Iahtasham Khan, Lothar H. Wieler, Muhammad Saqib, Falk Melzer, Vania L. de Assis Santana, Heinrich Neubauer r and Mandy C. Elschne

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5. Glanders in animals: A review on epidemiology, clinical presentation, diagnosis and countermeasures

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**Iahtasham Khan**