Molecular study on *Pasteurella multocida* and *Mannheimia granulomatis* from Kenyan Camels (*Camelus dromedarius*)

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Abstract

Background: Outbreaks of a Haemorrhagic Septicaemia (HS) like disease causing large mortalities in camels (*Camelus dromedarius*) in Asia and in Africa have been reported since 1890. Yet the aetiology of this condition remains elusive. This study is the first to apply state of the art molecular methods to shed light on the nasopharyngeal carrier state of *Pasteurellaceae* in camels. The study focused on HS causing *Pasteurella multocida* capsular types B and E. Other *Pasteurellaceae*, implicated in common respiratory infections of animals, were also investigated.

Methods: In 2007 and 2008, 388 nasopharyngeal swabs were collected at 12 locations in North Kenya from 246 clinically healthy camels in 81 herds that had been affected by HS-like disease. Swabs were used to cultivate bacteria on blood agar and to extract DNA for subsequent PCR analysis targeting *P. multocida* and *Mannheimia*-specific gene sequences.

Results: Forty-five samples were positive for *P. multocida* genes *kmt* and *psl* and for the *P. multocida* Haemorrhagic Septicaemia (HS) specific sequences KTSP61/KTT72 but lacked HS-associated capsular type B and E genes *capB* and *capE*. This indicates circulation of HS strains in camels that lack established capsular types. Sequence analysis of the partial 16S rRNA gene identified 17 nasal swab isolates as 99% identical with *Mannheimia granulomatis*, demonstrating a hitherto unrecognised active carrier state for *M. granulomatis* or a closely related *Mannheimia* sp. in camels.

Conclusions: The findings of this study provide evidence for the presence of acapsular *P. multocida* or of hitherto unknown capsular types of *P. multocida* in camels, closely related to *P. multocida* strains causing HS in bovines. Further isolations and molecular studies of camelid *P. multocida* from healthy carriers and from HS-like disease in camels are necessary to provide conclusive answers. This paper is the first report on the isolation of *M. granulomatis* or a closely related new *Mannheimia* species from camelds.

Keywords: *Pasteurellaceae*, *Pasteurella multocida*, *Mannheimia*, Camels, *Camelus dromedarius*, Haemorrhagic Septicaemia

Background

*Pasteurella multocida* capsular types B and E are the specific cause of seasonal outbreaks of Haemorrhagic Septicaemia (HS) in tropical cattle and buffaloes [1–3]. The veterinary literature provides a long record of an HS-like disease causing significant mortality in camels [4–8]. Yet the aetiology of HS in camels remains elusive [1, 3, 9]. Previous reports on HS-like disease in camels often failed to isolate the pathogen or provided only limited information on phenotypical characteristics of the isolates [9]. Attempts to infect camels with highly virulent *Pasteurella multocida* capsular type B strains produced only very mild clinical symptoms that resolved completely within 3 days [9].

A respiratory disease in Ethiopian camels caused by *Pasteurella (Mannheimia) haemolytica* has been described by Bekele [10]. This pilot study is the first of its' kind to use state of the art molecular methods for investigating *Pasteurellaceae* in camels.
Methods
In 2007 and 2008, 388 nasopharyngeal swabs were collected at 12 locations in North Kenya from 246 clinically healthy camels in 81 herds that reportedly been affected by outbreaks of HS-like disease. Flocculated swabs (FLOQSwabs®) were used for DNA extraction while swabs in Amies transport medium (Sterilin®) were used for standard bacteriological investigation. Bacteriological examination and extraction of DNA was carried out at a laboratory in Nairobi (Analabs Ltd.). DNA eluates from 341 swabs and 19 isolated Pasteurella-like cultures were transferred to the Institute for Microbiology and Epidemiology at Freie Universität Berlin (IMT/FUB) in Germany. At the IMT/FUB, DNA was extracted from 19 culture isolates, two of which did not belong to the Pasteurellaceae based on PCR results. Seventeen isolates were subsequently selected for biochemical testing to differentiate M. granulomatis from other Mannheimia spp., according to Ewers et al. (2004) [11]. Three hundred five samples with positive reaction in the 16S rRNA gene PCR, as well as, heat denatured DNA from bacterial cultures were investigated for the presence of P. multocida- and M. haemolytica-specific DNA sequences. For P. multocida the specific sequences tested were kmt, psl, and KTSP61/KTT72. Samples that proofed positive in one of these PCRs were further tested for capsular genes capA, capB, capD, capE, and capF, and for virulence-associated genes toxA, ptfA, pfhA oma87, ompH, hgbA, hgbB, exbB/tonB, lbpA, nanB, and nanH. To detect pathogenic Mannheimia spp., we screened for the leukotoxin (lktA) and the outer membrane protein (pomA) genes. All PCRs, except for the P. multocida capsular gene PCR, which was performed as a multiplex PCR, were conducted as single PCRs according to previously published protocols [12–18].

Results
The species P. multocida could not be isolated from 312 nasal swabs cultured on Blood Agar. Of the 305 DNA eluates containing sufficient quantities of DNA to undergo molecular characterization, 60 were positive for at least one of the P. multocida species-specific sequences tested by PCR. Forty-five samples gave a positive result for the HS-associated sequence KTSP61/KTT72, known to be present in P. multocida capsular type B strains. Neither capB nor capE gene, encoding HS-associated capsular types B and E, were detected in the sample material. Blast search of 18 KTSP61/KTT72 sequences [19] matched with high scores (98.8–99%) to nucleotide sequences submitted under GenBank accession numbers AF016260 (P. multocida unknown protein 1 gene, partial cds and unknown protein 2 gene, complete cds), AY948545.1 (P. multocida HS-B specific genomic sequence) and AJ421531.1 (P. multocida DNA fragment specific for HS). CapA and capD genes encoding for P. multocida capsular types A and D were identified in five and in one sample, respectively. Screening for adhesin-related genes ptfA and pfhA was positive in 15 and 2 samples, respectively. Outer membrane protein gene ompH was detected in 10, oma87 in 14 samples. While toxA, encoding the dermonecrotoxin, was not found in any sample, iron acquisition-related genes were present as follows: hgbA (n = 12), hgbB (n = 11), exbB/tonB (n = 1). Neuraminidase encoding genes nanB and nanH were present in 9 and 11 samples, respectively.

The biochemical profiles of the 17 Mannheimia spp. isolates characterised phenotypically were inconsistent and differed from the biochemical profile described for M. granulomatis [20], namely Sorbitol: pos.; α-Fucosidase: neg.; β-Galactosidase: variable. Of the 305 eluates tested, none was positive for Mannheimia spp. associated sequences lktA or pomA. According to the 16S rRNA sequence analysis performed on 19 cultures from the nasopharynx of healthy camels, 17 sequences showed closest relatedness (98–99%) to sequences from M. granulomatis, previously classified as P. granulomatis, Bisgaard taxon 20 and P. haemolytica biogroup 3 [21, 22].

Discussion
This study investigated the active (nasopharyngeal) carrier state for Pasteurellaceae in Kenyan camels. Importance of the latent carrier state in the epidemiology of Pasteurellosis was reiterated by Dzifa et al. (2008) [23].

The fact that no P. multocida capsular type B or E specific DNA sequences were identified in this study may indicate the presence of non-capsulated strains or the emergence of a hitherto unknown capsular type of P. multocida in camels. According to the OIE Terrestrial Manual [24] vaccines against Haemorrhagic Septicaemia in cattle and buffaloes must be based on local isolates that represent the prevalent serotype; seed cultures for the production of HS vaccines should contain capsulated organisms. Based on the results of this study the indiscriminate use of vaccines based on HS causing P. multocida isolates from cattle and buffaloes cannot be recommended for the prevention of HS-like disease in camels. This is the first molecular study to confirm the presence of P. multocida capsular types A and D specific DNA sequences in the nasopharynx of healthy Kenyan carrier camels, albeit at low frequency. Both capsular types have been reported in camels previously [25] based on phenotypic characterisation. Failure to culture P. multocida in this study is possibly related to the fastidiousness of the species which does not withstand cold chain transport of several days [26].

The lktA or pomA sequences do not occur regularly in all Mannheimia species or strains [27, 28], hence negative findings do not rule out presence of Mannheimia spp., but lktA-negative M. haemolytica strains are reported to
be less virulent. In this study the most common Pasteurellaceae species cultured from the nasopharynx of healthy carrier camels in North Kenya and identified by 16S rRNA sequence analysis was a *Mannheimia* sp. with 98% to 99% sequence identity to *M. granulomatis*. Only more recently has *M. granulomatis* been recognised as a significant pathogen in domestic and wild ruminants [29–31]. Phenotypic characterization of Pasteurellaceae species is of limited value [32] and published phenotypic characteristics for *M. granulomatis* are based on a limited number of bovine, leprine and deer strains [2, 21, 22, 30]. Hence it is to be expected that biochemical reactions of Kenyan camelid *M. granulomatis* strains differ from those described for *M. granulomatis* under http://www.bacterio.net/mannheimia.html/. Comparison of our results with a previous communication on involvement of *P. (M.) haemolytica* in respiratory disease in Ethiopian camels [10] is also limited, because the methodology used there would not have permitted a differentiation between *M. granulomatis*, *M. (Pasteurella) haemolytica* and other *Mannheimia* spp. The possibility that this respiratory pathogen isolated from Ethiopian camels [10] may in fact have been *M. granulomatis* or a new *Mannheimia* species very closely related to *M. granulomatis* cannot entirely be ruled out.

**Conclusions**

This study has documented the carrier state for acapsular *P. multocida* or unknown capsular types of *P. multocida*, closely related to the *P. multocida* strains causing HS in cattle and buffaloes, in healthy camels. At the same time the study found no evidence for the presence of *P. multocida* capsular types B and E or their specific DNA sequences in healthy camels in North Kenya. Further isolations and molecular studies of camelid *P. multocida* from healthy carriers and from HS-like disease in camels are necessary to provide conclusive answers. To our best knowledge this is the first report on the isolation of *M. granulomatis* or a new *Mannheimia* species from camels.

**Abbreviations**

DNA: Deoxyribonucleic acid; HS: Haemorrhagic Septicaemia; IMT/FUB: Institute for Microbiology and Epizootics at Freie Universität Berlin; PCR: Polymerase Chain Reaction; RNA: Ribonucleic acid; rRNA: Ribosomal ribonucleic acid

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**Availability of data and materials**

All data are available in the main text of the manuscript. Materials are available at the Institute of Microbiology and Epizootics, Centre for Infection Medicine, Free University Berlin, Germany.

**Authors’ contributions**

All authors conceived and planned the study. IG and MY performed the sampling, DNA extraction, primary cultures and organized the transfer of samples to Germany. AB and CE designed the laboratory and molecular experiments. All authors contributed on the draft of the manuscript. All authors read and approved the final manuscript.

**Ethics approval**

All animal samples were taken for the purpose of diagnostic of bacterial infectious agents. Informed consent was obtained from the camel owners.

**Competing interests**

The authors declare that they have no competing interests.

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**References**

1. Bain RVS, De Alwis MCL, Carter GR, Gupta BK. Haemorrhagic Septicaemia. Rome: FAO Animal Production and Health Paper 33; 1982. p. 54.
2. Songer JG, Post KW. The Genus Mannheimia and Pasteurella. In: Songer JG, Post KW, editors. Veterinary Microbiology, Bacterial and Fungal Agents of Animal Disease. Chapter 23. USA: Elsevier Inc; 2005. p. 434.
3. Office International Epizootics (OIE). Report of the 2nd Meeting of the OIE ad hoc Group on Disease of Camellid. Paris, 3–5 May 2010.
4. Steel JH. A Manual of the Diseases of the Camel and of his Management and Uses. Madras: Indian Veterinary Manuals III, The Lawrence Asylum Press (printed by GW Taylor); 1900. p. 54–5.
5. Leese AS. The Camel – a treatise on the one-humped camel in health and disease. Stanford, Lincolnshire, UK: Haynes & Son; 1927. p. 270–2.
6. Masia R. Les maladies microbiennes du dromadaire et leur importance en Afrique du Nord. Thèse pour le Doctorat Vétérinaire, L’École Nationale Vétérinaire d’Aloffm, Imprimerie R.Foulon, Paris; 1953. p. 33–34.
7. McGarne JJ, Higgins A. (1986) Infectious Diseases of the Camel, Viruses, Bacteria and Fungi. Pasteurellosis. In: Higgins A, editor. Bailler Tindall, The Camel in Health and Disease; 1986. p. 103.
8. Schwartz HJ, Doli M. The one-humped camel in Eastern Africa - A pictorial guide to disease, health care and management. Berlin, Germany: Verlag Josef Margraf; 1992.
9. Wernery U, Kinne J, Schuster RK. 1.1.8 Pasteurellosis. In: Camelid Infectious Disorders. Paris, France: World Organisation for Animal Health OIE; 2014. p. 58–65.
10. Bekele T. Studies on the respiratory disease ‘sonbobe’ in camels in the eastern lowlands of Ethiopia. Trop Anim Health Prod. 1999;31(6):333–45.
11. Ewers C, Lübke-Becker A, Wieler LH. Mannheimia haemolytica and the pathogenesis of pneumonic pasteurellosis. Berl Munch Tierarztl Wochenschr. 2004;3:497–115.
12. Dougherty SW, Rufflo CG, Adler B. The type 4 fibrinial subunit gene of *Pasteurella multocida*. Vet Microbiol. 2000;72:79–90.
13. Ewers C, Luebbe-Becker A, Bethke A, Kiessling S, Filter M, Wieler LH. Virulence genotype of *Pasteurella multocida* strains isolated from different hosts with various disease status. Vet Microbiol. 2006;114:304–17.
14. Kanten RW, Hansen LM, Hinojosa J, Beber O, Ruehl WW, Hirsh D. Pasteurella multocida produces a protein with homology to the P6 outer membrane protein of *Haemophilus influenzae*. Infect Immun. 1995;63:989–93.
15. Townsend KM, Frost AJ, Lee CW, Papadimitriou JM, Dawkins HJ. Development of PCR assays for species- and type-specific identification of *Pasteurella multocida* isolates. J Clin Microbiol. 1998;36:1096–100.
16. Townsend KM, Hanh TX, O’Boyle D, Wilkie I, Phan TT, Wijewardana TG, Trung NT, Frost AJ. PCR detection and analysis of Pasteurella multocida from the tonsils of slaughtered pigs in Vietnam. Vet Microbiol. 2000;72:69–78.

17. Guenther S, Schierack P, Grobbel M, Lubke-Becker A, Wieler LH, Ewers C. Real-time PCR assay for the detection of species of the genus Mannheimia. J Microbiol Methods. 2008;75:75–80.

18. Townsend KM, Boyle JD, Chung JY, Frost AJ, Adler B. Genetic organization of Pasteurella multocida cap Loci and development of a multiplex capsular PCR typing system. J Clin Microbiol. 2001;39:924–929.

19. http://blast.ncbi.nlm.nih.gov/Blast.cgi (Accessed on 18th March, 2017).

20. http://www.bacterio.cict.fr (Accessed on 18th March, 2017).

21. Angen O, Mutters R, Caugant DA, Olsen JE, Bisgaard M. Taxonomic relationships of the Pasteurella haemolytica complex as evaluated by DNA-DNA hybridizations and 16S rRNA sequencing with proposal of Mannheimia haemolytica gen. nov., comb. nov., Mannheimia granulomatis comb. nov., Mannheimia glucosida sp. nov., Mannheimia ruminalis sp. nov. and Mannheimia vanigeno sp. nov. Int J Syst Bacteriol. 1999;49:673–86.

22. Angen O, Quirie M, Donachie W, Bisgaard M. Investigations on the species specificity of Mannheimia (Pasteurella) haemolytica serotyping. Vet Microbiol. 1999;65:283–90.

23. Dziva F, Muhairwa AP, Bisgaard M, Christensen H. Diagnostic and typing options for investigating diseases associated with Pasteurella multocida. Vet Microbiol. 2008;126:1–22.

24. World Organisation for Animal Health – WOAH/OIE. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (mammals, birds and bees). 2012; 7th edition, Volume 1, Chapter 2.4.12. - Haemorrhagic septicemia, 732–744.

25. Seleim RS, Amal RT, Sahar RM, Nada H, Gobran RA. ELISA and other tests in the diagnosis of Pasteurella multocida infection in camels. Presented at Deutscher Tropentag, International Research on Food Security, Natural Resource Management and Rural Development Georg-August-Universität Göttingen, October 8–10 2003; posted on 'health & life sciences' May 2003 http://www.priory.com/vet/camel.htm.

26. Younan M. Biochemical characterization and identification of capsular antigen of ovine Pasteurella strains of different geographical provenance (Syria / South Germany) and of a collection of bovine Pasteurella haemolytica isolates. Veterinary Faculty - Free University Berlin, 1988.

27. Omaleki L, Browning GF, Barber SR, Allen JL, Srikrumaran S, Markham PF. Sequence diversity, cytotoxicity and antigenic similarities of the leukotoxin of isolates of Mannheimia species from mastitis in domestic sheep. Vet Microbiol. 2014;174:172–9.

28. Shanthalingam S, Goldy A, Bavananthivasam J, Subramaniam R, Batra SA, Kugadas A, Raghavan B, Dassanayake RP, Jennings-Gaines JE, Killion HJ, Edwards WH, Ramsey JM, Anderson NJ, Wolf PL, Mansfield K, Bruning D, Srikrumaran S. PCR assay detects Mannheimia haemolytica in culture-negative pneumonic lung tissues of bighorn sheep (Ovis canadensis) from outbreaks in the western USA, 2009-2010. J Wildl Dis. 2014;50(1):1–10.

29. Riet-Correa F, Ladeira SL, Andrade GB, Carter GR. Lechiguana (focal proliferative fibrogranulomatous panniculitis) in cattle. Vet Res Comm. 2002;24(8):557–72.

30. Blackall PJ, Bisgaard M, Stephens CP. Phenotypic characterisation of Australian sheep and cattle isolates of Mannheimia haemolytica, Mannheimia granulomatis and Mannheimia vanigena. Australian Vet J. 2002;80(1–2):87–91.

31. Bojesen AM, Ladeira SL, Andrade GB, Carter GR. Lechiguana (focal proliferative fibrogranulomatous panniculitis) in cattle. Vet Res Comm. 2002;24(8):557–72.

32. Koendgen S, Leider M, Lankester F, Bethe A, Lubke-Becker A, Fabian H, Leendertz FE. Pasteurella multocida Involved in Respiratory Disease of Wild Chimpanzees. PLoS One. 2011;6(8):e24236.