DOI : 10.5958/2277-8934.2015.00026.0 *Vol 22 No 2, p 163-168*

PRODUCTION OF A CASEOUS LYMPHADENITIS VACCINE FOR DROMEDARIES*

Berlin M.[1], M. Joseph[2], S. Jose[2], R. Raghavan[2], G. Syriac[2], N. Paily[2] and U. Wernery[2]
[1]Beuth Hochschule für Technik Berlin-University of Applied Sciences, Berlin, Germany
[2]Central Veterinary Research Laboratory, P. O. Box 597, Dubai, UAE

ABSTRACT

CLA is one of the most important bacterial infectious diseases of dromedaries and generally in livestock. For the development of CLA, the main antigen of the pathogen is its exotoxin phospholipase D (PLD) which is produced by all *C. pseudotuberculosis* strains. Therefore, PLD is essential for the production of a vaccine. The highest amount of PLD is already found in the Brain Heart Infusion Broth supernatant after 24 hours incubation at 37°C. The mixture of two CLA biotypes (ovine/caprine; equine/bovine) containing 750 µg PLD gave a complete protection against a challenge doses containing 4.0×10^3 cfu/ml of an ovine/caprine biotype. Seroconversion was tested with a newly established indirect ELISA using PLD as antigen and protein A as conjugate.

Key words: *Corynebacterium pseudotuberculosis*, dromedary, vaccine

Caseous Lymphadenitis (CLA) or pseudotuberculosis caused *by Corynebacterium (C.) pseudotuberculosis* is a widespread bacterial disease in Old World camels (OWC). It also affects sheep and goats worldwide and produces an ulcerative lymphangitis in cattle. The organism has also been isolated from abscesses of New World camels (NWC) (Wernery *et al,* 2014). CLA is characterised by the abscessation of one or more superficial lymph nodes but may also cause pneumonia, hepatitis, nephritis, mastitis, arthritis, orchitis and meningitis through lymphogenous and haematogenous distribution of the pathogen from the primary site to these internal organs and tissues. The disease is endemic in horses in California and is named "Pigeon Fever". It causes abscesses that lead horses chest to swell (Promed, 2007).

C. pseudotuberculosis is a short, irregular ovoid, Gram positive coccoid rod. The facultative intracellular bacterium multiplies in infected phagocytes and gets disseminated via lymph or blood to secondary sites where it causes abscesses. The virulence of *C. psuedotuberculosis* is attributed to the major exotoxin, phospholipase D (PLD), which increases vascular permeability and also facilitates dissemination of the pathogen into lymph nodes where it inhibits chemotaxis and death of neutrophils as well as inactivation of complement (Markey *et al,* 2013). Two biotypes are known, ovine/caprine and equine/bovine. It has recently been shown that strains isolated from dromedaries in the UAE belong to both biotypes using the nitrate reduction test (Berlin, 2015).

CLA can affect entire dromedary herds and has caused severe eradication problems in NWCs in Europe (Wernery *et al,* 2014). CLA is very difficult to treat although many antibiotics are sensitive towards this pathogen.

We report here about the production of a CLA toxin vaccine in dromedaries.

Materials and Methods

Selection of strains

Over the last 11 years CVRL has isolated 73 *C. pseudotuberculosis* strains from dromedaries. These strains are tested with the nitrate reduction test and it was found that 49% (36) being nirate negative belonged to the ovine/caprine biotype and 51% (37) to equine/bovine biotype which are nitrate positive. For CLA vaccine production for dromedaries, one of each biotype was therefore chosen: MB4356 (Shaheen) ovine/caprine, MB4280 (Corina) equine/bovine, since it is known that the genes of the ovine strains are more conserved than the genes of the equine strains

* This is the abstract of M. Berlin's Bachelor Thesis with the title: *Corynebacterium pseudotuberculosis* in camels-epidemiology and phases of vaccine production (2015). Beuth Hochschule, Berlin, University of Applied Sciences.

SEND REPRINT REQUEST TO U. WERNERY email: cvrl@cvrl.ae

which present a great variability (Soares *et al,* 2013) and may therefore have an influence on the efficacy of a vaccine.

Cultivation of the two C. pseudotuberculosis strains

The strains were kept in the CVRL microbank in beads. The beads were frozen at -80°C and one bead of each strain were rolled over two blood agar plates each which were incubated at 37°C for 48 hours. After the incubation 10 single colonies were picked with a wire loop and suspended in 10ml of Brain Heart Infusion Medium (BHI, Oxoid UK).

The suspension was added to 240ml BHI medium, thoroughly mixed on a shaker followed by incubation at 37°C for 72 hours. For the determination of colony forming unit (cfu/ml), 1ml of each bacterial suspension was removed from the 250ml volume every 24 hours, in total 3 times.

Estimation of colony forming unit per ml

The 1ml bacterial suspension was 10 fold serial diluted until a dilution of 10^{10} in sterile NaCl and 0.1ml of each dilution plated on blood agar plates which were then incubated for 48 hours at 37°C. After the incubation the colonies were counted and cfu/ml calculated.

Purification of culture supernatant

After one, two and three days of incubation the entire bacterial suspension of approximately 250 ml BHI medium was centrifuged at 4500 rpm for 30min to remove the bacterial cells. The supernatants were then sterile filtered through a Millipore Stericups GP vacuum filtration system with a pore size of 0.22µm. To prove the sterility of the filtered supernatants, 0.1ml each of the filtrate was streaked on 5 agar plates which were incubated for 48 hours at 37°C and inspected for any bacterial growth.

PLD estimation in C. pseudotuberculosis culture supernatants

A laboratory method had to be established to test if any PLD was released into the BHI –culture medium during the 72 hour growth of the ovine and equine biotypes and in which quantity. This was done three times after 24, 48 and 72 hours of incubation.

Qualitative method

For the detection of PLD, the qualitative method was established by Berlin (2015) using camel sera in an indirect antibody ELISA with known PLD antibody titres.

Quantitative method

For the quantitative method recombinant-PLD purchased from Hyphen Biomed was used. 100 µg lyophilised PLD were reconstituted in PBS. With this solution, a calibration curve was established and compared with the PLD concentrations obtained in the BHI culture supernatants after incubation of 24, 48 and 72 hours at 37°C (Berlin, 2015).

Preparation of CLA vaccine

Only the sterile 24 hour culture supernatants of both strains MB4356 Shaheen ovine and MB4280 Corina equine were pooled and mixed thoroughly because the 24 hour BHI culture achieved the highest bacterial count (Table 1). To 2ml of the mixed sterile culture supernatant, 1ml of the adjuvant ADVAX (ADVAX HCXL, Australia) was added and vortexed thoroughly for several minutes to guarantee a good homogenisation of the vaccine. The adjuvant ADVAX was found to be suitable for the immunisation of dromedaries (Eckersly *et al,* 2011; Johnson *et al,* 2015).

Five dromedaries were vaccinated twice (booster after 3 weeks) with 3ml of the CLA vaccine subsutaneously at the base of the camel's neck. One dromedary was not vaccinated (No. 3) and used as negative control. As explained earlier, each 1ml of the sterile mixed 24 hour culture supernatants contained 350µg/ml PLD and as 2ml of culture supernatant was used for the vaccine, each dromedary received 700µg PLD.

Experimental dromedaries

Six female dromedaries older than 18 years were selected for the vaccination and challenge infection experiment. They were kept together in one open pen at CVRL, where they received lucerne hay *ad libidum* and 2kg of concentrate daily as well as fresh water.

From all 6 experimental dromedaries blood was collected from the jugular vein before the trial and regularly after vaccination and challenge and tested for antibodies against *C. pseudotuberculosis* PLD. For this purpose an indirect ELISA was established using a commercial ELITEST CLA kit from Hyphen Biomed, France. This test was established for sheep sera using recombinant PLD as antigen and rabbit anti-sheep IgG HRP conjugate (Oreiby *et al,* 2014). The rabbit anti-sheep conjugate was replaced by Protein A (Sigma, P8651, US) for testing CLA antibodies in dromedary sera (Wernery, 2012; Wernery *et al,* 2014).

Challenge infection experiment

For the challenge infection of experimental dromedaries of which 5 were vaccinated twice with the CVRL CLA vaccine, the Shaheen ovine strain MB 4356 was chosen and 4.0×10^3 cfu/ml subcutaneously injected at the base of the neck. After the challenge infection all dromedaries were daily examined for any reaction at the injection side and their rectal temperatures recorded. Additionally, blood were regularly withdrawn from the jugular vein and tested for antibodies against PLD and a haemogram performed.

Results

Cultivation of two C. pseudotuberculosis strains

Table 1 shows that both strains reached the highest cell density 24 hours after incubation in BHI broth at 37°C (Table 1).

Table 1. *C. pseudotuberculosis* cfu/ml in relation to incubation time.

Incubation time	24 hours	48 hours	72 hours
MB4356 Shaheen ovine	6.50×10^9	2.35×10^8	3.20×10^7
MB4280 Corina equine	6.15×10^9	2.00×10^8	3.50×10^7

Purification of culture supernatant

The filtered supernatants of BHI broth did not show any bacterial growth after 48 hours incubation at 37°C.

PLD estimation in C. pseudotuberculosis culture supernatant

For the estimation of PLD in culture supernatants, qualitative and quantitative methods were used. For the qualitative method, the ELISA method with known PLD antibody titres detected PLD in culture supernatants and the quantitative method detected 350µg/ml of PLD cultured BHI broth after 24 hours incubation.

Immune status of dromedaries before and after CLA vaccination

Antibody development against PLD was measured before and after vaccination, as well as after challenge infection which is shown in Fig 1.

Result of the challenge infection experiment

After the challenge dose of 4.0×10^3cfu/ml of strain MB4356 Shaheen ovine was given to all 6 dromedaries including the non vaccinated camel No 3, they were adspected every day for any sign of disease. The total white blood cell count (WBC 10^9/L) and the rectal temperatures were recorded over several weeks shown in Figs 2 and 3.

Exactly 7 days after the challenge doses were given, the control camel No 3 developed a swelling at the injection site and an abscess (Fig 4) after 18 days which was lanced and cleaned out (Fig 5). As can be seen from Figs 2 and 3, camel No 3 developed fever and an increased WBC. Despite intensive antimicrobial therapy over 7 days the dromedary had to be euthanised 3 months p.i on human grounds and during necropsy several abscesses were detected in the liver from which *C. pseudotuberculosis* was isolated (Fig 6).

All 5 vaccinated dromedaries remain in good health 6 months p.i. with no sign of any abscess. Their antibody development is shown in Fig 1.

Discussion

Vaccines against CLA for sheep and goats are commercially available, but are often not licensed in countries in which they are not produced. They are not available for camelids. They have, however, been used in experimental vaccination trial in dromedaries. These vaccines which are injected subcutaneously at the base of the neck produced granulomas of different sizes (Wernery, 2012) and therefore these experiments were abandoned. This was the reason why researchers from CVRL embarked on a project to find the most suitable adjuvants for dromedaries (Eckersly *et al,* 2011; Johnson *et al,* 2015).

The commercially available CLA vaccines are formulated from concentrated formalin inactivated *C. pseudotuberculosis* culture supernatants containing PLD. They do not give full protection in small ruminants, but a significant reduction in number of abscesses was observed after vaccination. Several scientists have started research in the production of autogenous vaccines against CLA mainly in NWCs (Beghelli *et al,* 2004; Kobera and Poehle, 2004; Braga, 2007), but also in dromedaries (Wernery, 2012). The researchers tried different approaches. Cell wall proteins as well as toxins of *C. pseudotuberculosis* were used with variable success.

It is known that all *C. pseudotuberculosis* strains isolated so far, possess the PLD gene and also CVRL's isolated 73 strains were no exceptions (Hakimuddin *et al,* 2015). It was mentioned by Afzal *et al* (1996) that *C. pseudotuberculosis* strains which PLD genes were deleted are unable to produce lymph node abscesses. Various vaccination trials had shown that the exotoxin PLD plays an important role in the

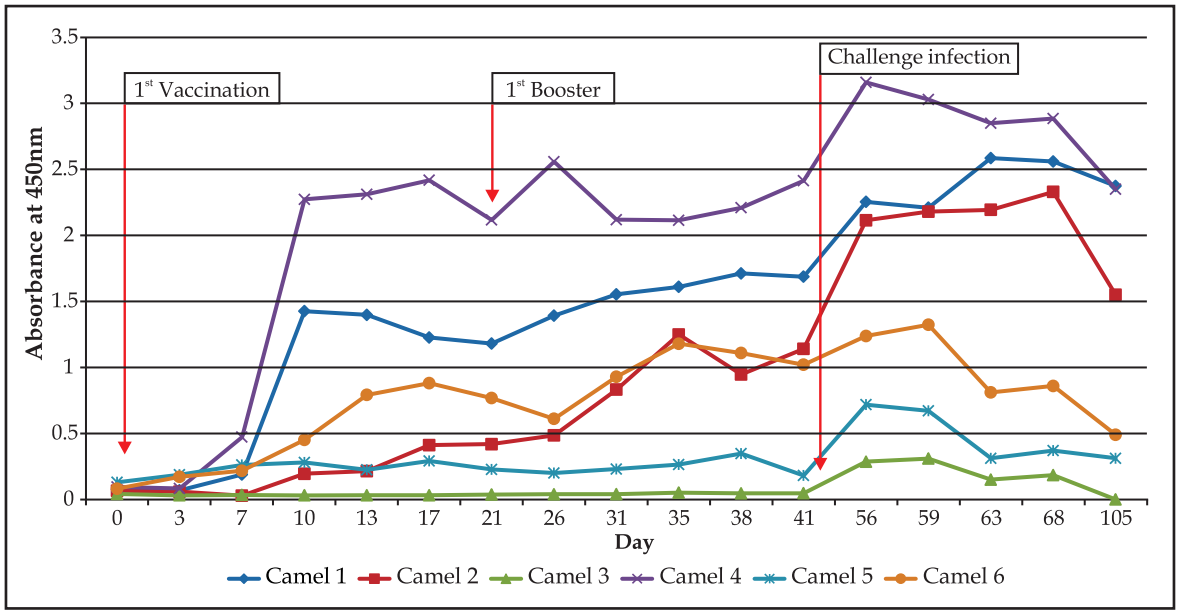

Fig 1. Antibody development against PLD after 2 vaccinations, camel No 3 was not vaccinated, but challenged.

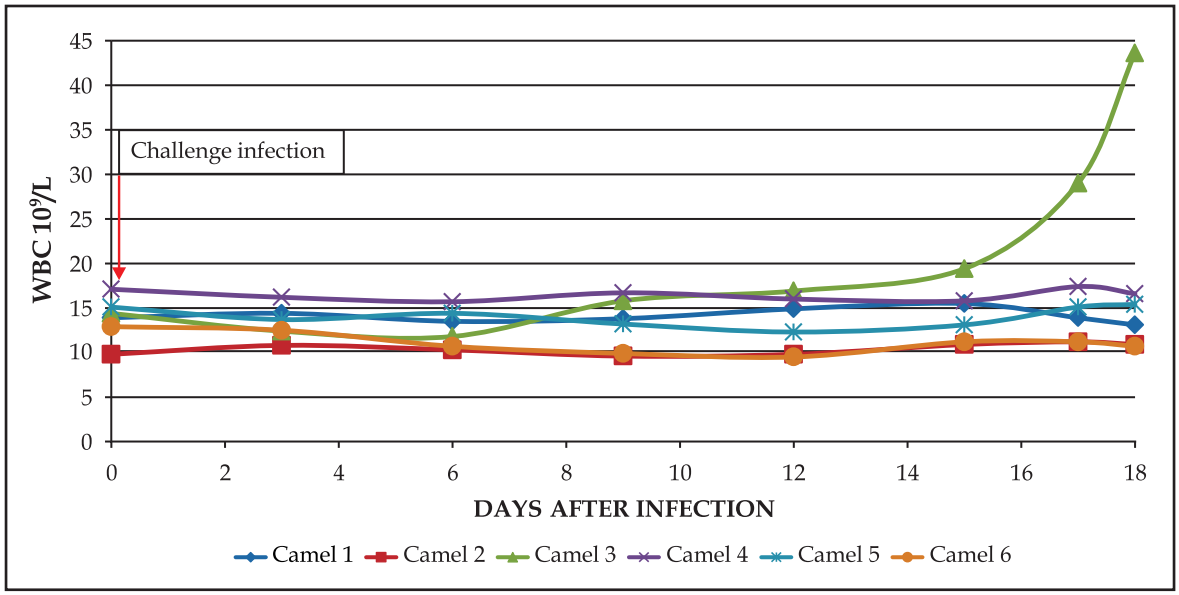

Fig 2. Total white blood cell count of CLA experimental dromedaries, camel No 3 was not vaccinated, but challenged.

development of the disease indicating the need to block PLD by vaccination.

No abscesses developed in an experimental trial with alpacas when they were given a CLA vaccine containing high level of toxin (PLD-500µg/ml; Braga, 2007). In our study we used the 24 hour BHI sterile culture supernatant which contained 350µg/ml PLD resulting in a final dosis of 750µg/per vaccine shot per dromedary. This dosis was sufficient to protect 5 dromedaries with variable CLA ELISA antibody titers from a challenge infection when vaccinated twice. The culture supernatants containing PLD were not treated with formalin since Berlin (2015) found out that formalin-treated PLD showed a significant lower ELISA- antibody binding capacity than non-formalin treated PLD. Additionally, we could show that mice which were intraperitoneally inoculated with sterile non-formalin treated BHI culture supernatants showed no side effects.

In several experiments we showed that a prolonged incubation period to 48 or even 72 hours of the *C. pseudotuberculosis* BHI culture did not increase the PLD level, most probably because the amount of bacteria decreased when further incubated.

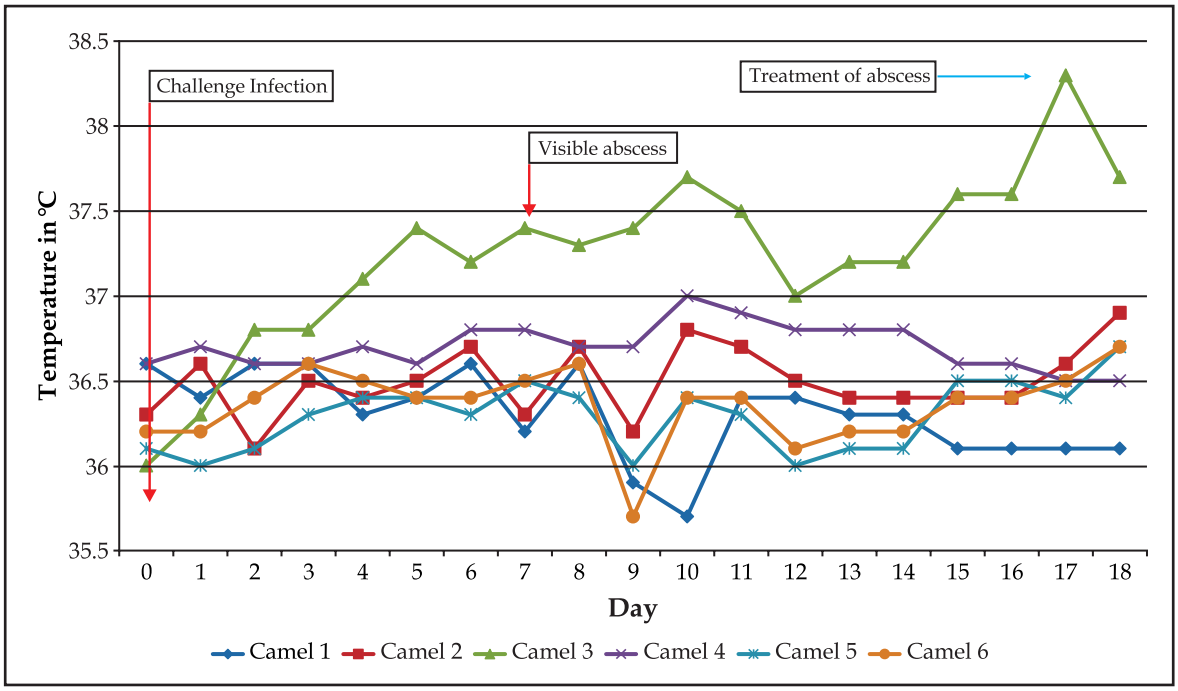

Fig 3. Rectal temperatures of experimental dromedaries, camel No 3 was not vaccinated, challenged.

Fig 4. Swelling in the unvaccinated camel No 3 seven days after s.c, challenge infection with 4.0×10^3 cfu/ml of *C. pseudotuberculosis* Strain MB4356.

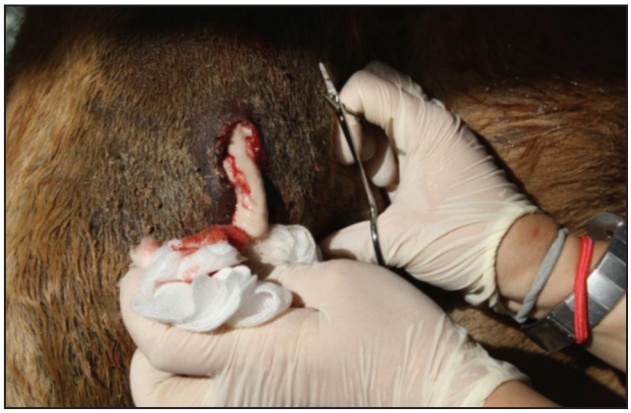

Fig 5. CLA abscess lanced 18 days p.i.

Fig 6. Abscess formation in liver of control dromedary No 3 euthanised 3 months p.i.

After challenge with a *C. pseudotuberculosis* dose of 4.0×10^3 cfu/ml, only the non vaccinated dromedary No 3 developed severe abscessation at the injection site. The development of abscess formation started exactly after 7 days and despite intensive antibiotic treatment and daily cleaning of the multiple abscesses, the dromedary had to be euthanised on human grounds 3 months later. During necropsy several abscesses were found in the liver.

In his experiment with alpacas, Braga (2007) used a challenge dose which was much higher with 1.0×10^6 cfu than ours. It is difficult to decide which is the right challenge dose but we believe that our dose is more realistic. The vaccinated and challenged

5 dromedaries did not develop any changes in their haematology profile, did not develop fever or any swelling at the injection site. They seroconverted and produced different levels of antibodies. After 6 months all 5 camels remained healthy. Afzal *et al* (1996) reported an incubation period of 40 days after artificial infection of dromadaries with CLA pathogen.

In contrary, the non vaccinated challenge dromedary No 3 developed severe CLA with high temperature, high total white blood cell count (WBC) and increased neutrophils. Although these values improved drastically after treatment, the dromedary had to be euthanised due to poor prognosis. It is also interesting to note that the antibody level remained low despite the artificial infection. This may have two reasons; the animal was treated when first abscesses appeared and secondly the animal's immune system may have been suppressed.

Many different antigen and conjugate preparation have been used in ELISA tests for the serological diagnosis of CLA such as cell wall antigens, whole cell extracts, PLD, culture supernatants and recombinant exotoxins. However, most tests detect antibodies raised against the exotoxin PLD (31-31.5 k Da) which is very immunogenic (Pépin and Paton, 2010). For our serological investigations before and after vaccination as well after the challenge infection we used with good results a commercial CLA kit with protein A as conjugate.

References

Afzal M, Sakir M and Majid Hussain (1996). *Corynebacterium tuberculosis* infection and lymphadenitis (Toloa or Mala) in the camel. Tropical Animal Health and Production 28:158-162.

Beghelli D, D' Alterio GL, Severi G, Moscati L, Pezzotti G, Foglini A, Battistacci L, Cagiola M and Ayala-Vargas C (2004). Evaluation of the immune response to vaccination against *C. pseudotuberculosis* in an alpaca herd in Italy. Preliminary results. In 4th European Symposium in South American Camelids/ DECAMA European Seminar, Goettingen, Germany, 7-9. October.

Berlin M (2015). *Corynebacterium pseudotuberculosis* bei Kamelen-Epidemiologie und Phasen der Impfstoffherstellung. Bachelor of Science Thesis (B.Sc.). Beuthhochschule für Technik Berlin-University of Applied Science pp 1-43.

Braga WU (2007). Protection in alpacas against *Corynebacterium pseudotuberculosis* using different bacterial components. Veterinary Microbiology 119(2-4):297-303.

Eckersley AM, Petrovsky N, Kinne J, Wernery R and Wernery U (2011). Improving the dromedary antibody response: The hunt for the ideal camel adjuvant. Journal of Camel Practice and Research 18(1):35-46

Hakimuddin F, Ismail M, Abidi F, Wernery U and Khazanehdari K (2015). Sequence differences in *Corynebacterium pseudotuberculosis* isolated from caprine and camel species. Contribution No174 ID Poster 174/ P03:18 Advanced Molecular Diagnostics for Biomarker Discovery, Event Proc. JSBN9783000488054, Technical University of Munich, 23-27.3.15. pp 53.

Johnson B, Dietrich F, Petrovsky N, Kinne J, Wernery R and Wernery U (2015). Characterisation of adjuvants for use in dromedary immunisation. Journal of Camel Practice and Research 22(1):33-48.

Kobera R and Poehle D (2004). Case reports in South American Camelids in Germany. In: Proceedings of 4th European Symposium on SACs and DECAMA Europ. Seminar, 7-9.10.2004, Giessen, Germany 151.

Markey B, Leonard F, Archambault M, Cullinane A and Maguire D (2013). Clinical Veterinary Microbiology. Mosby, Elsevier. pp 135-145.

Oreiby AF, Hegazy YM, Osman SA, Ghanem YM and Al-Gaabary MH (2014). Caseous lymphadenitis in small ruminants in Egypt. Clinical, epidemiological and prophylactic aspects. Tierärztl. Praxis Grosstiere 5:271-277.

Pépin M and Paton MW (2010). Caseous lymphadenitis in sheep and goats. In: Infectious and Parasitic Diseases of Lifestock. Eds. Lefe`vre P-C, Blancou J, Chermette R and Uilenberg G (2010). Lavoisier. pp 1151-1163.

Promed (2007). Pigeon fever, equine USA (Oregon). Available at: www.oregonlive.com/newsflash/regional/index.ssf?/base/news-21/119269254119530.xmls@storylist=orlocal.

Soares SC, Artur S, Eva T, Jochen B, Rommel R, Adriana C, Amjad A, Anderson RS, Anne CP, Carlos D, Eudes GVB , Fernanda AD, Flávia A, Flávia SR, Karina KFN, Luís CG, Sintia A, Syed SH, Syeda MB, Ulisses PP, Vinicius ACA, Maria PCS , Anderson M, Andreas T and Vasco A (2013). The pan-genome of the animal pathogen *Corynebacterium pseudotuberculosis* reveals differences in genome plasticity between the biovar ovis and equi strains. Public Library of Science 8(1):e 53 p18.

Wernery U (2012). Caseous lymphadenitis (pseudotuberculosis) in camelids. Journal of Camel Practice and Research 19(1):21-27.

Wernery U, Kinne J and Schuster RK (2014). Infectious Disorders of Camelids, OIE Book. pp 163-181.