Recovery of Brucella melitensis from Artificially Infected Dromedaries

Johnson B¹, Kinne J¹, Jose Sh¹, Pfeffer M², Shanmugaraja R¹, Pandarakandy Sh¹, Ali F¹, Maio E¹, Caveney MR¹, Sollner NK¹ and Wernery U¹*¹
¹Central Veterinary Research Laboratory, Dubai, United Arab Emirates
²Institute for Animal Hygiene, University Leipzig, Germany
*Corresponding author: Wernery U, Central Veterinary Research Laboratory, Dubai, United Arab Emirates

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Abstract

Fourteen serological positive dromedaries intratracheally and intranasally infected with Brucella melitensis were euthanased 12 months after infection. A full necropsy was performed on all 14 dromedaries and 43 different organ samples from each dromedary tested for the presence of the pathogen using 2 selective Brucella media and 3 culture techniques as well as RT PCR.

From a total of 43 different organs 21 (49%) were culture negative and 22 (51%) positive. The pathogen resided mainly in body lymph nodes. The highest culture result was achieved when the enrichment method was used. However, it is proposed, to use all 3 culture methods (direct, concentrated, enriched) as few samples were also negative in the enrichment method but positive in the other methods. BHI is the optimal agar because the Brucella colonies are easier to identify than on Farrell’s agar. RT PCR is not sensitive enough to identify Brucella directly from organ samples, as the pathogen concentration is very low. Culture is still the “gold standard” for the diagnosis of brucellosis.

Keywords: Brucella melitensis; Dromedaries; Samples; Isolation

Introduction

Dromedary brucellosis is widespread in camel rearing countries and is preliminary caused by Brucella melitensis. The pathogen has been mainly isolated from aborted fœtuses, milk, hygromas [1], but rarely from dromedary organs of infected dams [2]. In connection with a serological investigation performed at CVRL, Dubai [3,4], we had the opportunity to culture the pathogen from a great number of organ tissues from 14 dromedaries infected with B. melitensis. The results of these investigations are presented here.

Materials and Methods

Fourteen non pregnant female dromedaries (Animal ID C1-C14) of different age (14-23 years) were intratracheally and intranasally infected with a B. melitensis strain, referred to as EM2 [3] belonging to the genetic group East Mediterranean (former African group). This strain was genotyped with multiple-locus variable number tandem repeat (MLVA) [5]. It was previously isolated from a dromedary placenta. The purpose of this infection experiment was the evaluation of serological tests for the in vivo diagnosis of dromedary brucellosis. All 14 infected dromedaries became serologically as well as blood culture positive and were euthanased 12 months after infection. A full necropsy was performed on all camels and 43 different organ samples were taken from each dromedary. Each panel of samples included different lymphoid tissues, internal organs, neuronal tissues (brain and spinal cord), joint fluids of both tarsal and carpal joints, and all four udder cisterns. Each tissue sample was tested for the presence of B. melitensis, using 3 methods; the direct, the concentrated and the enrichment culture methods explained hereafter.

Two types of selective Brucella agars were used

Farrell’s media (Brucella medium base CM0169, Oxoid, supplemented with filtered horse serum SHS100, E and O Laboratories, UK and Brucella selective supplement SR0083A, Oxoid) and Brain-Heart-Infusion agar (Brain Heart Infusion CM 1135, Oxoid, with 1% bacteriological agar and supplemented with filtered horse serum SHS100, E and O Laboratories, UK and Brucella selective supplement, SR0083A, Oxoid).

Direct culture method

The cut surface of organs and lymph nodes was streaked on the surface of the selective Brucella agar plates and 0.1ml of joint fluids was cultured by spread plate method on the Brucella selective agars mentioned above.

Concentration culture method

Organs and lymph nodes were finely minced and transferred into a sterile filter bag (Bag Page, Inter Science. France). 30ml of sterile PBS was added to it, then blended and homogenized in a Lab Blender Mixer (Inter Science, France) for 6 minutes at high speed. The filtrate was decanted into sterile 50ml Falcon tubes and centrifuged at 3000g for 30min. The supernatant was discarded and 0.1ml of the sediment was streaked on the selective Brucella agar plates.

Enrichment culture method

A 1.0ml aliquot of the sediment which was used for the concentration culture method described above was transferred into 7ml of Trypticase Soy Broth (Merck 1.05459.0500) with Brucella selective supplement SR0083A (Oxoid) in a Greiner tube for incubation at 37°C for 6 days. After direct culture, the remaining joint fluid was also enriched in Trypticase Soy Broth with Brucella supplements.

Incubation

All streaked plates and inoculated broth tubes were incubated at 37°C in an atmosphere of 5% CO₂ for 6 days. After 6 days incubation, all plates were examined for the growth of typical Brucella colonies.
Table 1: Results of Brucella bacteria culture and PCR from 43 different organ samples of 14 artificially *B. melitensis* infected dromedaries.

| Organ number | Organ samples                      | Number of samples tested | Number of positive samples in RT PCR from original samples | Number of positive samples in culture | Number of negative samples in culture |
|--------------|-----------------------------------|--------------------------|------------------------------------------------------------|--------------------------------------|---------------------------------------|
| 1            | Brain                             | 13                       | 0                                                          | 1 (7.7%)                            | 12 (92.3%)                           |
| 2            | Right submandibular lymph node    | 13                       | 0                                                          | 3 (23%)                             | 10 (77%)                             |
| 3            | Pharyngeal lymph node             | 14                       | 0                                                          | 2 (14.3%)                           | 12 (85.7%)                           |
| 4            | Left pharyngeal lymph node        | 13                       | 0                                                          | 2 (15.4%)                           | 11 (84.6%)                           |
| 5            | Spinal cord                       | 14                       | 0                                                          | 1 (7.1%)                            | 13 (92.9%)                           |
| 6            | Tonsil                            | 13                       | 0                                                          | 0                                   | 13 (100%)                            |
| 7            | Trachea                           | 12                       | 0                                                          | 0                                   | 12 (100%)                            |
| 8            | Prescapular lymph node dorsales   | 14                       | 0                                                          | 7 (50%)                             | 7 (50%)                              |
| 9            | Prescapular lymph node ventrales  | 13                       | 0                                                          | 5 (38.5%)                           | 8 (61.5%)                            |
| 10           | Left Lung                         | 14                       | 0                                                          | 1 (7.1%)                            | 13 (92.9%)                           |
| 11           | Right Lung                        | 13                       | 0                                                          | 2 (15.4%)                           | 11 (84.6%)                           |
| 12           | Lymph node mediastinales medii    | 11                       | 0                                                          | 1 (9.09%)                           | 10 (90.9%)                           |
| 13           | Liver                             | 14                       | 0                                                          | 0                                   | 14 (100%)                            |
| 14           | Spleen                            | 14                       | 0                                                          | 3 (21.4%)                           | 11 (78.6%)                           |
| 15           | Heart                             | 13                       | 0                                                          | 0                                   | 13 (100%)                            |
| 16           | Lymph node tracheobronchales medii| 13                       | 0                                                          | 2 (15.4%)                           | 11 (84.6%)                           |
| 17           | Lymph node tracheobronchales sinisteri | 12                  | 0                                                          | 1 (8.3%)                            | 11 (91.7%)                           |
| 18           | Lung Lymph node                   | 11                       | 0                                                          | 3 (27.3%)                           | 8 (72.7%)                            |
| 19           | Right kidney                      | 14                       | 0                                                          | 0                                   | 14 (100%)                            |
| 20           | Left kidney                       | 14                       | 0                                                          | 0                                   | 14 (100%)                            |
| 21           | Urinary bladder                   | 13                       | 0                                                          | 0                                   | 13 (100%)                            |
| 22           | Intestine                         | 13                       | 0                                                          | 0                                   | 13 (100%)                            |
| 23           | Lymphocentrum mesentericum craniale| 13                     | 0                                                          | 0                                   | 13 (100%)                            |
| 24           | Lymphocentrum mesentericum caudale| 11                       | 0                                                          | 0                                   | 11 (100%)                            |
| 25           | Small intestinal lymph node       | 12                       | 0                                                          | 0                                   | 12 (100%)                            |
| 26           | Right uterus                      | 13                       | 0                                                          | 0                                   | 13 (100%)                            |
| 27           | Left uterus                       | 13                       | 0                                                          | 0                                   | 13 (100%)                            |
| 28           | Left ovary                        | 12                       | 0                                                          | 0                                   | 12 (100%)                            |
| 29           | Right ovary                       | 11                       | 0                                                          | 0                                   | 11 (100%)                            |
| 30           | Left hind udder                   | 13                       | 0                                                          | 0                                   | 13 (100%)                            |
| 31           | Right hind udder                  | 11                       | 0                                                          | 0                                   | 11 (100%)                            |
| 32           | Left front udder                  | 13                       | 0                                                          | 1 (7.7%)                            | 12 (92.3%)                           |
| 33           | Right front udder                 | 11                       | 0                                                          | 1 (9.09%)                           | 10 (90.9%)                           |
| 34           | Left udder lymph node             | 14                       | 0                                                          | 4 (28.6%)                           | 10 (71.4%)                           |
| 35           | Right udder lymph node            | 11                       | 0                                                          | 2 (18.2%)                           | 9 (81.8%)                            |
| 36           | Left front udder cistern          | 13                       | 0                                                          | 2 (15.4%)                           | 11 (84.6%)                           |
| 37           | Right front udder cistern         | 11                       | 0                                                          | 0                                   | 11 (100%)                            |
| 38           | Left hind udder cistern           | 14                       | 0                                                          | 2 (14.3%)                           | 12 (85.7%)                           |
| 39           | Right hind udder cistern          | 12                       | 0                                                          | 1 (8.3%)                            | 11 (91.7%)                           |
| 40           | Left tarsal joint fluid           | 11                       | 0                                                          | 0                                   | 11 (100%)                            |
| 41           | Right tarsal joint fluid          | 12                       | 1 (8.3%)                                                   | 1 (8.3%)                            | 11 (91.7%)                           |
| 42           | Left carpal joint fluid           | 11                       | 0                                                          | 0                                   | 11 (100%)                            |
| 43           | Right carpal joint fluid          | 11                       | 0                                                          | 0                                   | 11 (100%)                            |
The enrichment broth was well homogenized and then 0.1ml of broth was quadrant streaked on selective Brucella agars. The streaked plates were incubated for another 6 days at 37°C in an atmosphere of 5% CO₂. After 6 days of incubation, the plates were examined for growth of typical Brucella colonies.

PCR

A PCR for the detection of Brucella antigen was only performed on the original samples and not on the concentrated or enriched samples. Briefly, a small piece of tissue about 200mg was placed in an Eppendorf tube containing 2mm glass beads (Sigma, US) and 20ul of proteinase K (20mg/ml concentration from Qiagen protease, Germany) and 200ul ATL buffer (Qiagen, Germany) were added. Sample tube was vortexed thoroughly and incubated at 56°C for 1hr. 300µl buffer AL (Qiagen, Germany) was added and sample tube vortexed for 15secs after which 500µl of the sample was transferred to the MagNA Pure automated extraction platform (Roche Diagnostics Ltd, UK). DNA was extracted using MagNA Pure LV DNA extraction kit according to the manufacturer’s instructions. The DNA was finally eluted with 100µl of Magna pure elution buffer. The PCR was performed according to method described by Probert [6], using ABI 7500 DX machine.

Results

The results of our investigations are shown in Tables 1, 2 and 3

Table 1 summarizes the results of B. melitensis culture and PCR from 43 different dromedary organs of 14 artificially infected dromedaries. From a total of 43 different organs cultured for Brucella bacteria, 21 (49%) were negative in culture and 22 (51%) harbored the bacteria. Following artificial infection with the pathogen, Brucella bacteria resided mainly in body lymph nodes (21%, 9/43), udder tissues and lymph nodes (16%, 7/43) and other tissues (12%, 5/43). An exception was one tarsal joint fluid, from which Brucella bacteria were isolated in numbers too numerous to count. The highest isolation percentage of Brucella bacteria was from prescapular lymph nodes dorsales (50%) followed by udder lymph nodes with 29%, lung lymph nodes with 27% and submandibular lymph nodes with 23%. There was no difference in the isolation frequency in connection with the route of infection.

Table 2 summarizes the results of 3 different culture methods (direct, concentrated, enriched) on 2 different selective agars from organ samples from 14 necropsied dromedaries. In total 43 organ pieces were tested (Table 1) out of which 22 were cultured positive (51%) (Table 2). Samples derived from these positive organs gave sometimes inconsistent culture results, as from some dromedaries the same organ samples were positive and negative in others.

Table 3 shows the results in detail. There were two dromedaries (C6, C14) from which B. melitensis was isolated from only one organ as well as two dromedaries (C2, C5) from which B. melitensis was isolated from 10 organs. Despite repeated culture, from 4 no B. melitensis was isolated.

When using direct method, only 2 (9%, 2/22) organ samples including tarsal joint fluid were positive on Farrell’s agar compared to 6 on BHI agar (27%, 6/22) including the 2 positive organ/tarsal joint s samples from Farrell’s media.

When the concentration method was used, 11 organ samples were positive on Farrell’s agar (50%, 11/22) and 16 on BHI agar (73%, 16/22). With this method, however, 2 organ samples cultured positive on Farrell’s agar were negative on BHI agar and B. melitensis was only isolated from the right udder cistern on BHI agar with direct method.

There was no isolation difference between Farrell’s and BHI media, when the enrichment method was used. A total of 19 organ samples were B. melitensis positive on both the media (95%, 19/22). Only 3 organ samples were negative using this method, but positive with the BHI concentration method (2x) and BHI direct method (1x).

A total of 282 specimens from 22 positive organ samples from 14 dromedaries were tested. Table 2 shows the summarized results of how many specimens were positive. The highest isolation success was 40 (14.2%) positive specimens with the enrichment technology.

Except for the right tarsal joint of one dromedary with a joint swelling from which numerous B. melitensis colonies were directly isolated, the remaining organ samples contained only 2-4 colonies per agar plate on direct culture. When concentration method was used, the number of B. melitensis per plate increased to between 2-18 colonies and when enriched, B. melitensis was isolated in numbers too numerous to count.

Discussion

Extreme care must be exercised when working with Brucella as humans are highly susceptible to brucellosis and laboratory infections are not rare. Brucella is an intracellular organism with fastidious growth requirements in vitro, which makes its isolation on culture media difficult. Hence, proper culture techniques and right selection of selective media are mandatory for Brucella isolation. For the isolation of Brucella bacteria, liquid specimens like milk or stomach content can be inoculated straight onto selective media, whereas tissue samples should be finely minced and homogenized in a blender and aliquots used for culture as Brucella bacteria are intracellular organisms. Although Brucella melitensis grows well on blood agar, most specimens tested may contain many different bacterial and sometimes fungal species and therefore selective media are required for the isolation of this pathogen. We were fortunate with our investigation, as all 43 organ samples collected from each euthanased dromedary camel were immediately cultured after necropsy.

Our investigations showed that whenever testing a carcass for brucellosis, specimens should be taken from at least 22 different organs mentioned in Table 2, as it is unpredictable which organ may harbor the pathogen.

For the culture of Brucella bacteria from organ samples, we choose Farrell’s and Brain-Heart-Infusion (BHI) agars, which possess the same antibiotics, and antifungal ingredients, which are in the selective supplement SR0083A from Oxoid.

Even though, the selective supplements were the same for BHI and Farrell’s media, it was observed that BHI media was less inhibitory to Brucella bacteria compared to Farrell’s media. The Brucella colonies were easier to identify on BHI media as the colonies appear larger, honey-comb colored and translucent in white light after 6 days incubation. On Farrell’s media the colonies appear small or tiny after 6 days of incubation. On Farrell’s agar the colonies appear small or tiny after 6 days of incubation.
6 days of incubation leading to chances of not detecting them. These differences can only be explained by the different compositions of Farrell’s and BHI media. BHI media contains brain infusion solids and di-sodium phosphate, which are not added to the Farrell’s media. Although there was no isolation difference found between the enrichment technology with Farrell’s and BHI agars, the ideal method for the isolation of *B. melitensis* from dromedary organs is the enrichment method using BHI agar as the *Brucella* bacteria are easier to detect.

Table 2: Isolation of *Brucella melitensis* from 22 positive organs of 14 artificially infected serological positive dromedaries on 2 different agars by 3 different culture methods.

| Organ Number | Organ samples | Number of organ samples tested | Number of organ samples positive for *Brucella* and their percentage on 2 different *Brucella* selective media by 3 culture methods |
|--------------|---------------|--------------------------------|-------------------------------------------------------------------------------------------------------------------------------------|
|              |               | Direct Farrell’s media BHI agar media | Concentrated Farrell’s media BHI agar media | Enrichment Farrell’s media BHI agar media |
| 1            | Brain         | 13                              | 0 0 0                                                                                                                                | 1 (7.7%) 1 (7.7%) 1 (7.7%) |
| 2            | Right submandibular lymph node | 13 | 0 0 1 (7.7%) | 1 (7.7%) | 3 (23.1%) 3 (23.1%) |
| 3            | Pharyngeal lymph node              | 14 | 0 0 2 (14.3%) | 1 (7.1%) | 1 (7.1%) 1 (7.1%) |
| 4            | Left pharyngeal lymph node        | 13 | 0 0 0 | 2 (15.4%) | 1 (7.7%) 1 (7.7%) |
| 5            | Spinal cord                        | 14 | 0 0 0 | 1 (7.1%) | 1 (7.1%) 1 (7.1%) |
| 8            | Prescapular lymph node dorsales    | 14 | 0 1 (7.1%) | 4 (28.6%) | 3 (21.4%) 7 (50%) 7 (50%) |
| 9            | Prescapular lymph node ventrales   | 13 | 0 0 2 (15.4%) | 4 (30.8%) | 4 (30.8%) 4 (30.8%) |
| 10           | Left Lung                           | 14 | 0 0 0 | 0 | 1 (7.1%) 1 (7.1%) |
| 11           | Right Lung                          | 13 | 0 0 0 | 1 (7.7%) | 2 (15.4%) 2 (15.4%) |
| 12           | Lymph node mediastinales medi        | 11 | 0 0 0 | 0 | 1 (9.1%) 1 (9.1%) |
| 14           | Spleen                              | 14 | 0 0 1 (7.1%) | 0 | 2 (14.3%) 2 (14.3%) |
| 16           | Lymph node tracheobronchales medi        | 13 | 0 1 (7.7%) | 1 (7.7%) | 0 | 2 (15.4%) 2 (15.4%) |
| 17           | Lymph node tracheobronchales sinistri        | 12 | 0 0 0 | 1 (8.3%) | 0 | 0 |
| 18           | Lung lymph node                    | 11 | 0 0 2 (18.2%) | 1 (9.1%) | 3 (27.3%) 3 (27.3%) |
| 32           | Left front udder                    | 13 | 0 1 (7.7%) | 1 (7.7%) | 1 (7.7%) 1 (7.7%) |
| 33           | Right front udder                   | 11 | 0 0 0 | 1 (9.1%) | 0 | 0 |
| 34           | Left udder lymph node               | 14 | 0 0 4 (28.6%) | 3 (21.4%) | 3 (21.4%) |
| 35           | Right udder lymph node              | 11 | 0 0 1 (9.1%) | 2 (18.2%) | 2 (18.2%) 2 (18.2%) |
| 36           | Left front udder cistern            | 13 | 1 (7.7%) | 1 (7.7%) | 1 (7.7%) 2 (15.4%) 2 (15.4%) |
| 38           | Left hind udder cistern             | 14 | 0 0 0 | 0 | 2 (14.3%) 2 (14.3%) |
| 39           | Right hind udder cistern            | 12 | 0 1 (8.3%) | 0 | 0 0 |
| 41           | Right tarsal joint fluid            | 12 | 1 (8.3%) | 1 (8.3%) | 1 (8.3%) 1 (8.3%) 1 (8.3%) |
| Total        |                                    | 282 | 2 (0.7%) | 6 (2.1%) | 17 (6.02%) 26 (9.2%) 40 (14.2%) 40 (14.2%) |

Table 3: Number of dromedaries and organ samples from which *Brucella melitensis* was isolated; organ samples denoted by organ number against each dromedary.

| Number of positive organs | Number of dromedaries | Animal ID | Organs from which *B. melitensis* was isolated (see Table 1 for organ number) |
|--------------------------|-----------------------|-----------|--------------------------------------------------------------------------------|
| 0                        | 4X                    | C3, C10, C11, C12 | Nil |
| 1                        | 2x                    | C6        | 8 |
|                          |                       | C14       | 41 |
| 2                        | 1X                    | C4        | 4, 8 |
| 3                        | 2X                    | C1        | 1, 3, 5 |
|                          |                       | C9        | 8, 9, 34 |
| 5                        | 1X                    | C8        | 2, 9, 11, 14, 39 |
| 6                        | 1x                    | C13       | 8, 9, 16, 34, 36, 38 |
| 7                        | 1X                    | C7        | 8, 9, 10, 14, 18, 34, 35 |
| 10                       | 2X                    | C2        | 2, 8, 9, 14, 18, 32, 33, 35, 36, 38 |
|                          |                       | C5        | 2, 3, 4, 8, 11, 12, 16, 17, 18, 34 |
However, also the enrichment method did not always detect all Brucella-positive organs. Therefore, it has proposed to include all three methods direct, concentration and enrichment methods when Brucella isolation from organ samples is required.

Interestingly, when 282 specimens from 22 positive organs were tested directly with RT PCR, all results were negative except one tarsal joint fluid. From this tarsal joint of one infected dromedary which was severely swollen, numerous B. melitensis colonies were directly isolated. The low concentration of Brucella organisms in original specimens is the reason why all RT PCR were negative. The low sensitivity of Brucella RT PCR has been described previously [7]. RT PCR on concentrated or enriched specimens was not performed as it was obvious that these 2 culture methods alone would identify the positive specimens.

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