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Special Article - Brucella

Recovery of *Brucella melitensis* from Artificially Infected Dromedaries

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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 fetuses, 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% CO_2 for 6 days. After 6 days incubation, all plates were examined for the growth of typical *Brucella* colonies.

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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 sinistrii	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% CO_2. 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 200µl 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 organims. 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

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		Concentrated		Enrichment	
			Farrell's media	BHI agar media	Farrell's media	BHI agar media	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 medii	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 medii	13	0	1 (7.7%)	1 (7.7%)	0	2 (15.4%)	2 (15.4%)
17	Lymph node tracheobronchales sinistrii	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%)	1 (7.7%)
33	Right front udder	11	0	0	0	1 (9.1%)	0	0
34	Left udder lymph node	14	0	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%)	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	0
41	Right tarsal joint fluid	12	1 (8.3%)	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

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.

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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