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University of Al-Qadisiyah
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Serological, bacteriological and molecular study of aborted cows, buffalos and women infected with *Brucella*

A. A. Najum
Coll. of Vet. Med./ Unive. of Al-Muthana

J.A.AL-Sa'aidi
Coll. of Vet. Med./ Unive. of Al-Qadysia

M.A.Alrodhan
Coll. of Sci./Unive. of Al-Qadysia

Abstract

Abortion is the most obvious manifestation of *Brucella* infection. In this study, 59 aborted buffalos, 91 aborted cows and 150 aborted women were studied. Diagnosis of *Brucella* infection in these abortions was based on clinical, serological and bacteriological studies, then determination of *Brucella* isolates with PCR assay. Serological studies included the use of RB and ELISA tests as screening tests for infection. Results showed that in bovine the RB test shows significant difference between the positive cases in cows and buffalos ($P<0.05$) while in ELISA test there is no significant difference between these two groups. In aborted women there was significant difference in the RB and ELISA tests between the negative and positive women ($P<0.05$).From all positive cases by ELISA test, *Brucella* was isolated from 7 aborted cows, 3 buffalos, and 2 women. *Brucella* isolates were revealed amplification of a 223-bp fragment with B4 and B5 primers except one strain that isolated from blood culture of women .

Introduction

Brucellosis, is a major infectious disease afflicting humans and a wide range of domesticated animals and wildlife. It is known to be a worldwide problem and one of the most important among zoonoses in the Mediterranean region, India, and Central and South America (1). Brucellosis results significant human morbidity (2). Clinical symptoms in human brucellosis include fever, anorexia, polyarthritis, meningitis, pneumonia,endocarditis, and other less common clinical manifestations. Brucellosis in pregnant women carries the risk of spontaneous abortion or intrauterine transmission to infant, especially during early trimesters. Reports from the areas where *Brucella. melitensis* infection is endemic, suggest that there is an increased rate of abortion in asymptomatic pregnant women (3). Although eight biovars of *B. abortus*, the causative agent of bovine brucellosis, have been identified, biovar 1 is most frequently isolated from cattle , in countries where biovar prevalence has been studied (4). Outbreaks of bovine brucellosis are associated with abortion during the last trimester of gestation, and produces weak newborn calves, and infertility in cows and bulls (5).

Materials and Methods

Materials:

1.culture media.

No	Culture media	Company / origin
1	Blood Agar Base	Oxoid / UK
2	*Brucella* Agar	Oxoid / UK
3	MacConky agar	Fluka / India
4	Nitrate Water	Oxoid / UK
5	Peptone water	Oxoid / UK
6	Trypticase Soya Broth	BBL / USA
7	Trypticase Soya Agar	BBL / USA
8	Urea Agar	Difco / USA
9	Gelatin Agar	Difco/ USA
10	Simmon's Citrate	Himedia/India
11	MR – VP Broth	Difco / USA

2. Humen Brucellosis Kits: Provided by NovaTec, Germany
3. Bovine Brucellosis kit: Provided by EUROPEAN VETRINARY LABORATORY; EVL, Netherland.
4. Gram's stain solution and Modified Ziehl – Neelsen stain solution prepared and used as described by Alton *et al* .,(6)
5. Rose Bengal antigen: provided by Omega company, UK.
6. Monospecific antiserum antibrncella abortus: manufactured by Difico, USA. It was provided as a gift from FAO.
7. Monospecific antiserum antibrucella melitensis: manufactured by Difico, USA. It was provided as a gift from FAO.
8. Antibiotic supplements: provided by Himedia, India.
9. Fetal calf serum: Provided by Difico company, USA.
10. Materials used in PCR: Wizard Genomic DNA purification kit, Promega company, USA, GoTaq® Green Master Mix: provided by promega company, USA
11. Primers used for diagnosis: The system used was B4/B5 (Baily)primers system. As advised by Baily *et al*.,(7). And provided by Alpha DNA Company, as described below.

Primers name	Sequences	Predictive product
B4	5'-TGGCTCGGTTGCCAATATCAA-3'	223- bp region within a gene coding for 31-kDa membrane protein specific to the genus *Brucella*
B5	5'-CGCGCTTGCCTTTCAGGTCTG-3'	

Methods:
1.Samples collection .
a. Cows and Buffalos.
1.Clinical observations: Clinical examination has been performed on aborted cows and fetuses throughout the last few days of gestation period. At parturition, retained placenta, body temperature and bleeding status has been registered .
2. Blood samples:
Hundred and fifty blood samples were (91 from cows and 59 from buffalos) were obtained during the period extended from May, 2010 to December, 2010, at Al-Samawa, Al- Askari, Al-Majied, Al-Khuder, Al – Zurajia and Bani salama regions. serum samples have been obtained for serological assessments using (RB and ELISA)..
3. Aborted fetuses :
Fifteen aborted fetuses, at the first and last stage of pregnancy (9 from aborted cows and 6 from aborted buffalos) were used for brucellosis. The specimens were cultured in duplicated *Brucella* agar plates as described by Alton *et al* (6).
a-Women
1- Clinical signs:
Clinical signs of aborted women were recorded by physician to show pregnancy period, body temperature, sweating, bleeding and abdominal and back pain.
2- Blood samples:
Hundred and fifty blood samples 5ml have been obtained from aborted women aged (14 – 45 years) during the period extended from Jun, 2010 to November, 2010. Two ml of blood samples from each aborted women was injected into prepared sterile trypticase soya broth with 2% sodium citrate as described by Alton *et al* (8).
3. Aborted Placentas:
Hundred and fifteen placenta samples from aborted women have been obtained, at maternity and children hospital of Al-Samawa, after curtag operation (by gynecologists) placenta samples were cultured by two methods after slicing with sterile scissors dipped in ethanol and flamed, first part was purified on selective medium(*brucella* agar) with mixture of antibiotics. They were cultured on duplicate agar plates as described before by Alton *et al*. , (8).
2.Serological assessments were preformed according to OIE, (9).

3.Identification and isolation of *Brucella*: *Brucella* growth was confirmed by bacteriological and biochemical tests as suggested by Alton *et al.* , (8).

4. Molecular Identification by PCR-based assay:

A-DNA purification

The main basic approach used for DNA purification was performed according to instruction manual of Promega (the supplier of the Kit)

B-The PCR amplification process.

-The reaction mixture: Enzymatic amplification of DNA was carried out in a final volume of 25 µl according to the recommendations of manufacture Prepare one of the following reaction mixes on ice: For a 25µl reaction volume:

Component	Volume	Final Conc.
GoTaq® Green Master Mix, 2X	12.5µl	1X
upstream primer, 10µM	0.25–2.5µl	0.1–1.0µM
downstream primer, 10µM	0.25–2.5µl	0.1–1.0µM
DNA template	1–5µl	<250ng
Nuclease-Free Water to	25µl	N.A.

PCR consisted of a preheating at 95ºC for 5 min after this initial denaturation step, the mixture was subjected to 40 amplification cycles as follow:

Loop's steps	Temperature	Time	Number of cycle
denaturation	94 ºC	1 min	40
annealing	55 ºC	1 min	
extension	72 ºC	1 min	
Final extension	72 ºC	7 min	1
Hold	4 ºC	indefinite	1

C-Detection of PCR products by agarose electrophoresis.A horizontal slab gel electrophoresis apparatus was used. Ten µl of amplified products were mixed with 3 µl of loading buffer and analysed by electrophoresis in 2% agarose gel stained with 0.5 µg /ml ethidium bromide, at 100 V for 1hr. in 1 X TBE buffer. Then visualized under UV light using ultraviolet transelumenater, DNA ladder (100-1000) was used and the gel was photographed when necessary by digital camera.

A sample was considered positive for *Brucella* spp. when a specific fragment of 223 bp was detected in the gel Baily *et al*., (7).

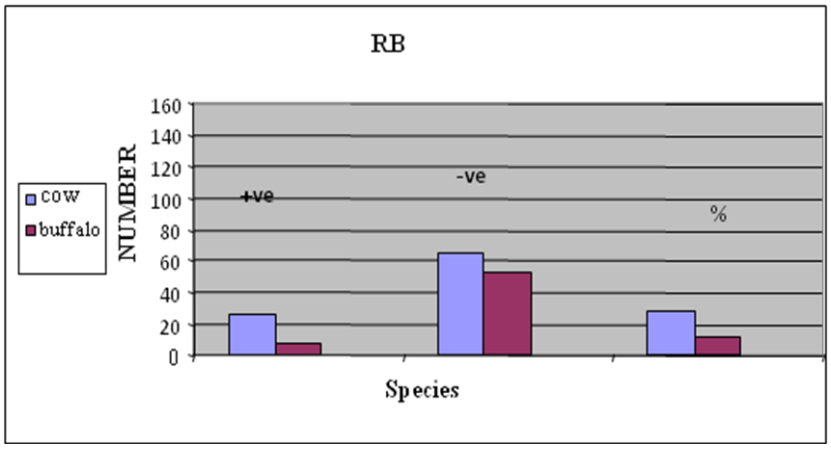
5. Statistical Analysis: All data were analyzed using the statistical package for social science (SPSS) for Windows program on the computer. Chi-square test was used to compare differences between the frequencies. Student *t* test was used to compare mean values between groups.

Results

All positive cases for *Brucella* infection were found in late stage of pregnancy (5 – 9 month), some cases accompanied with abortion with retention of placenta, aborted in buffalos were in herds not sporadically like cows (table 1). Pneumonia and pericarditis were the main complications shown in aborted fetus positive for brucellosis. Placentas were, edematous and opaque with bleeding in some of cases (Figure 1), ELISA, RB tests and clinical signs results are shown in (table1), that RB test results were positive in 33cases (22%) including 26cases

(28.5%) in cows and 7 cases (11.86%) in buffalo. ELISA results were positive in 38cases (25.3%), including 21cases (23%) in cows and 17cases (28.8%) in buffalo. the difference in RB positive cases between groups was significant ($P<0.05$), whereas difference in RB negative cases between cows and buffalos was not significance($P>0.05$) (figure 2). According to ELISA technique, statistical analysis showed insignificant difference ($P>0.05$) between groups(figure 3). *Brucella* organisms first recognized in smears obtained from fetal stomach stained with modified ziehl neelsen stain, which appeared red clumps against a blue background, *Brucella* culture recognized on the basis of colonial morphology which appeared round translucent pale honey color (figure 4) and results of biochemical test shown in (table 2). Out of 91 aborted cows, 7 (13.18%) were positive by culture, whereas out of 59 aborted buffalos, 3(6.77%) were positive for culture (table 1). Out of 15 aborted fetuses from cows and buffalos 4 from aborted cow's fetuses and 2 from buffalo's aborted fetus were positive by culture . Out of 12 uterine fluid swabs 2 from aborted cows and 1 from aborted buffalo were positive by culture. Out of 122 milk samples positive 1 from aborted cows, was positive by culture, all cattle isolates agglutinate with monospecific antisera for A . Clinical signs in women included fever sweating, hypotension, pain, aches and bleeding most women infected with *Brucella* were aborted at first stage of pregnancy except 4 cases were at 2^{nd} – 3^{rd} stage of pregnancy (table 3). ELISA , RB test and clinical signs results shown in (table 3), revealed that RB results were positive in 15 cases (10%). ELISA results were positive in 18 cases(12%), the difference in RB and ELISA positive cases was significant ($P<0.05$) (figure 5 and 6). Out of the 150 aborted women, 2 (1.33%) were positive for culture (table 3) from 18 patient blood samples that positive for ELISA, one isolate were positive by culture. Out of the 150 placenta specimens taken from aborted women, one isolate was obtained by indirect method of culture on *Brucella* selective media that contains vancomycin, colistin, nystatine and nitrafurane antibiotic supplements and results of biochemical test shown in (table 2). Blood isolates agglutinate with monospecific antisera for A, while placenta isolate agglutinate with monospecific antisera for M.The primer pair used in this study succeeded in the amplification of a 223-bp fragment from *Brucella* isolates culture that were studied. meanwhile, the DNA extracted from culture harboring *Brucella*'s DNA, so that they yielded predicted 223-bp fragment, where no fragment was amplified in DNA extracted from *B.abortus* that isolated from blood of aborted women (Figure 7).

Fig. (1): Aborted cow fetus due to brucellosis, with edema, opaque and bleeding of placenta



*(+ve= positive, -ve = negative, %= percentages)
Fig. (2): illustrate the positive, negative and percentages of RB results for cows and buffalos

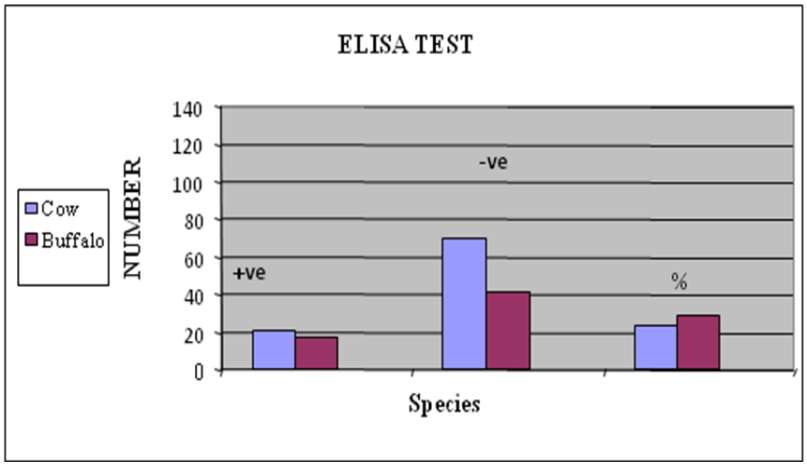


Fig. (3): illustrate the positive, negative and percentages of ELISA results for cows and buffalos.

Fig. (4): Colonial morphology of *Brucella* on blood agar, showing round, translucent, with pearly appearance colonies.

Table (1). History, clinical signs, pregnancy period, Samples and isolation, RB and ELISA results of aborted cows, buffalos and their fetuses.

No.	History & clinical signs	Pregnancy period	Samples & isolation of agent	R.B	ELISA
1	Aborted before 2hrs with edema. of placenta	7 Months	Fetal stomach (+)	++	0.362
2	Pneumonia of Fetus.	8 Months	Fetal stomach (+)	++	0.409
3	Aborted before 6hrs with opaque placenta edema.	6 Months	Fetal stomach (+)	++	0.469
4	Aborted before 12hrs.	9 Months	Uterine fluid (+)	+++	0.772
5	Aborted before 24hrs with retained placenta.	9 Months	Uterine fluid (-)	+++	0.666
6	Aborted before 24hrs with Opaque placenta	8 Months	Uterine fluid (+)	++++	1.037
7	Aborted before 5hrs with pneumonia of Fetus	7 Months	Fetal stomach & Fetal organ (+)	+++	1.00
8	Aborted before 48hrs	7 Months	Uterine fluid (+)	++++	1.08
9	Aborted before 72hrs	6 Months	Uterine fluid (-)	+++	0.846
10	Aborted before 14 d.	5 Months	Milk (-)	+++	1.00
11	Aborted before 23 days.	8 Months	Milk (+)	++	0.676
12	Abortion pneumonia of Fetus with opaque of placenta	9 Months	Fetal stomach & Fetal organ (+)	+++	0.650
13	Retained of placenta	9 Months	Fetal stomach (+)	++	0.570
14	Opaque of placenta	8 Months	Placenta &uterine fluid(-)	+++	0.840
15	Aborted before 40 d with retained placenta	6 Months	Milk (-)	++	1.026
16	Aborted before 60 d. (Abortion also occurred in all herds before 5 months at late pregnancy)	9 Months	Milk (-)	_	1.016

Table (2) Results of biochemical test on *Brucella* isolates obtained from aborted cows, buffalos and women .

No.	Type of isolates	oxidase	catalase	Vp-Mr	Citrate utilization	Indol production	Nitrate reduction	Gelatinase
Bovin								
1	*B.abortuss*	+	+	-	-	-	+	-
2	*B.abortus*	+	+	-	-	-	+	-
3	*B.abortus*	+	+	-	-	-	+	-
4	*B.abortus*	+	+	-	-	-	+	-
5	*B.abortus*	+	+	-	-	-	+	-
6	*B.abortus*	+	+	-	-	-	+	-
7	*B.abortus*	+	+	-	-	-	+	-
8	*B.abortus*	+	+	-	-	-	+	-
9	*B.abortus*	+	+	-	-	-	+	-
10	*B.abortus*	+	+	-	-	-	+	-
Women								
1	*B.abortus*	-	+	-	-	-	+	-
2	*B.melitensis*	+	+	-	-	-	+	-

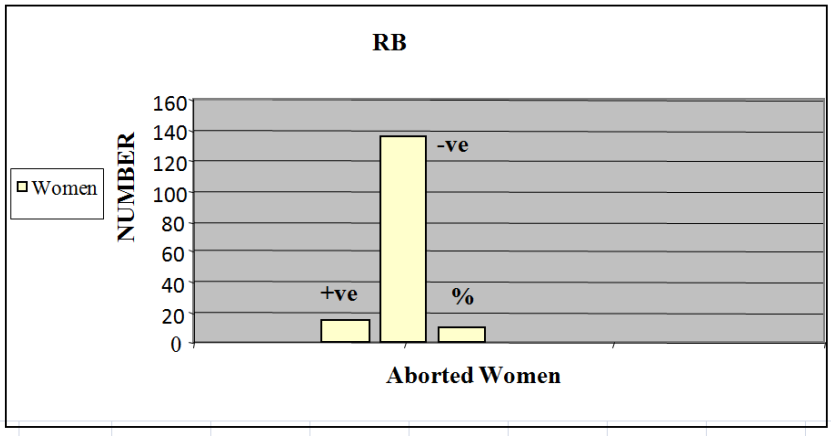


Fig. (5): illustrate the positive, negative and percentages of RB results for women.

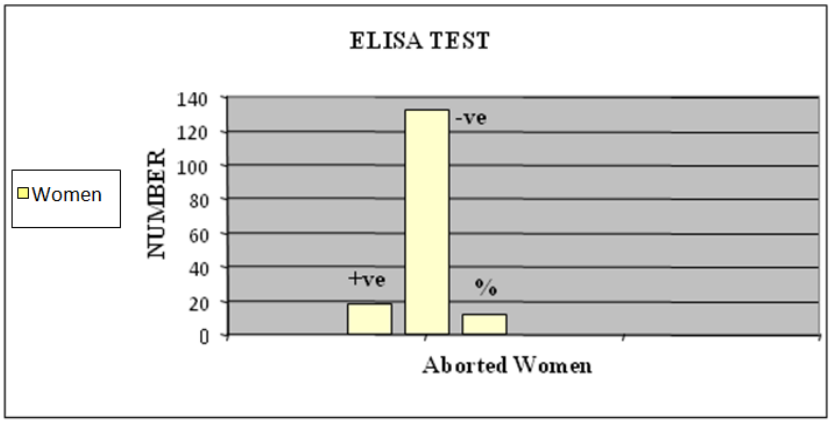


Fig. (6): illustrate the positive, negative and percentages of ELISA results for women.

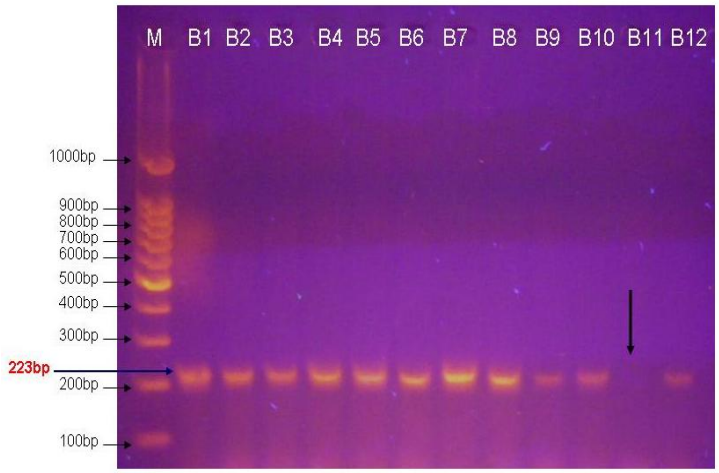


Figure (7): Agarose gel electrophoresis for PCR products of *Brucella* isolates,where 223bp PCR products appear as positive results

Table (3) Clinical signs, pregnancy period, samples and isolation, RB and ELISA results of aborted women.

No.	Clinical signs	Gestation period (weeks)	Bacterial isolation		RB	ELISA
			Blood	Placenta		
1	Fever , sweating abdominal pain	7	-	-	++	20.18
2	High fever , pain bleeding aches	10	-	-	++	15.41
3	Pain on back, fever , bleeding and sweating	8	+	-	+++	24.1
4	Fever ,pain bleeding aches	10	-	-	++	10.23
5	Fever ,abdominal , pain , bleeding	32	-	-	+	13.04
6	Bleeding , pain, headache, fever	25	-	-	++	22.37
7	Sweating , pain on back , aches, bleeding ,fever	17	-	-	+	16
8	Pain , fever, bleeding	22	-	-	+++	22.44
9	Pain , fever , bleeding , hypotension	9	-	-	++	18.02
10	Fever , generalized aches, pain	7	-	-	++	22.37
11	High fever, bleeding, pain, sweating, aches	11	-	+	++++	25.11
12	Fever, bleeding pain on the back	8	-	-	+++	21.58
13	Pain, sweating, fever, hypotension	10	-	-	+++	20.09
14	Fever, bleeding, joint, pain	7	-	-	++++	30.74
15	Fever, pain on the back, and abdomen, bleeding	9	-	-	+++	22.05

Discussion

Results of clinical sings of aborted cows and buffalos were like results of many researches who explained that abortion in cattle due to brucellosis occurred at late stage of pregnancy and may result in the birth of nonviable calves and retained placentas (Anderson *et al* ., (10) The results showed that RB gave positive result in aborted cows but were negative for ELISA and bacterial isolation, also it gave a negative result in buffalo aborted before 2 month or more. Corbel, (11)suggested that infection with *Yersinia enterocolitica* O:9, *E.coli* O:157, O 116, *Pseudomonas maltophilia, Vibrio cholerae* O:1, *Salmonella spp and Francisella tularensis* are likely to cause cross-reactions in serological tests with smooth *Brucella* antigens and give false positive for RB. ELISA is effective in detecting all immunoglobuline (antibodies) classes and sub-classes important in diagnosis (8) and appears to be the most sensitive serological test (12). *Brucella* strains isolated from cattle were obtained from aborted fetuses and vaginal discharge and were compared to that isolated from milk samples, the number of *Brucella* organisms in milk and colostrum samples was lower than that in abortion material, fetus stomach, fetal fluids and membranes, also milk samples is highly contaminated (9) . Our study showed that most aborted women positive for brucellosis were aborted in first stage of pregnancy This results is agreement with Young, (13). Three aborted women from the tested group were negative to brucellosis by using the RB test, while they were positive by using the ELISA test. Although RB test is known to have many false positive and false negative results, but generally it is simple, rapid and can be used as an excellent screening method for infection. One isolate that obtained from blood culture of aborted women and one isolate was obtained from placenta by indirect methods, this results confirmed that mentioned by (14) were isolated *Brucella* from placenta of aborted women . Results of PCR were the same as that obtained by Baily et al., (7) who used PCR amplification contained a single pair of oligonucleotide primers designed to amplify a 223 bp product and demonstrated that the assay was sensitive and specific for *B.melitensis* and *B.abortus*.

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دراسة مصلية، جرثومية وجزيئية للأبقار والجاموس والنساء المجهضة المصابة بالبروسيلا

الخلاصة

تم دراسة 59 جاموسة مجهضة و91 بقرة مجهضة 150امراة مجهضة . شخصت الإصابة بالبروسيلا بالاعتماد على الفحوصات السريرية والمصلية والجرثومية وأكدت العزلات باستخدام تقنية تفاعل سلسلة البلمـــرة ، الفحوصـــات المصلية تضمنت استخدام فحصي الروزبنكال والاليزا للتحري عن الإصابة بالمرض . أظهرت النتائج الخاصة بالأبقار والجاموس بان فحص الروزبنكال أعطى فروقات معنوية بين الأبقار والجاموس ، بينما لم يظهر فحـــص الاليـــزا أي فروقات معنوية بين المجاميع. أما النساء المجهضات فلم توجد أي فروقات معنوية بين فحصي الروزبنكال والاليز. من كل الحالات المرضية الموجبة لفحص الاليزا قد تم عزل جراثيم البروسيلا من 7ابقار مجهضة و3 جاموسة مجهضـــة و2 نساء مجهضات على التوالي وان تقنية تفاعل سلسلة البلمرة قد نجحت بتشخيص 11 من عزلات البروسيلا باستخدام البادىءب4وب5 ماعدا العزلة المعزولة من دم النساء المجهضات.