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LABORTATORY DIAGNOSIS OF RUMINANTS BRUCELLAE BY AMOS PCR

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ABSTRACT

Four hundred twenty two blood, molk, tissue specimens and stomach content of aborted foeti collected from different localities at Sharkia Governorates were examined for detection of brucellae by PCR, 5 out of 110 blood samples proved to be positive, 8 out of 90 milk samples were also positive 7 out of 220 tissus specimens (4 from lymph nodes, 2 from spleen and one from stomach content of aborted foetus). Proved to harboue brucellae

INTRODUCTION

The AMOS PCR assay is based on the insertion of the genetic element insertion sequence (IS 711) at a unique chromosomal losus of brucella Species. The AMOS PCOR assay is a multiplex primer assay that was used of five-primer cocktail one primers annels to the IS 711 element, while each of the other four primer hybridized to one of the brucella species at a locus near by and at variable distanecs outside the element. Highly sensitive and specitic diagnostic test for brucella based on PCR.***(Fekete et al,1990)*** a suitable reaction conditions and oligonucl-eotide primers for the detecion of B. melitensis and B. abortus by PCR and the technique proved be applicable in the diagnosis of brucellosis ***(Bailey et al, 1992).*** PCR proved to be faster than bacteriological tests and more accurate than imminologic methods because it directly detected the presence of the organism ***(Fekete et al, 1992 (Ouahrani-Bettache et al, 1993),*** Isolated an IS element of *B.* ovis and named is6501. PCR assay comprises fine oligonucleotide primers which can identify selected biovars four species are Brucella. Individual biovars within species species are not differentiated. The assay can identify three biovats (1. 2 and 4) of *B. abortus.* All biovar of *B. melitensis,* biovar 1 of *B.suis* and all biovar of *B.ovis.****(Bricker and halling 1994)*.** The specificity and high sensitivity of the PCR may provide a valuable tool for the diagnosis of brucellosis.

(Romero et al, 1995). The specificities of PCR ELISA were 100%. When testing milk samples form beucella, free cattle. A PCR positive sample was negative by ELISA and 7 ELISA-poaitive Were PCR negative. The specificities of both tests, were 100% while sensitity was 98%. ***(Romero et al, 1995).*** The possibitity of using polymerase chain reation (PCR) for the laboratory diagnosis of brucellosis with representative regional collection of 44 brucella strains. ***(Balakhonov et al, 1996).***

All species of genus brucella contain several copies (between10- 40) of an insertion sequence appears of IS 711. The position of copies of this insertion sequence appears to differ in each species and this can be used to discriminate between them. The obtained patterns reflect the position of the insertion sequence in genome-this method can be used to different species, strains, within a species***. (Ouahrani-Bettache et al, 1996).*** AMOS PCR evaluated to determine two hundreds thirty one isolates and tested them by the conventional tests and brucella AMOS PCR. ***(Ewalt and Bricker 2000).***

This study was detect one the efficacy of PCR in the laboratory of Brucella in ruminants bred in Sharkia Governorate.

MATERIALS & METHODS

Three ml aliquots blood samples from each animal, creamy sediment mixure of milk from dairy animal and specimens of tissue were obtained form slaughtered animals as well as two stomach content of aborted foeti were examined as shown in table (1).

Blood samples: ***(leal-Kleve zas et al,)***

Four hundreds microliers of blood samples were taken and centrifuged as 4000×g for 3 min., the cell pellets were resuspended HCO_3, 100 m MEDTA (PH 7.4). Mixed and centrifuged at 4000 ×g for 3 min.treatment with erythros lysis solution was repeated until the leukocyt e pellets lost all reddish colouring. 10 ul of proteinase k (20 mg /ml) were added to the sample and mixed thoroughly and incubated for 30 min. at 50°C. 400 ul of 5 asturated phenol was mixed and centrifuged ar 8000×g for 5 min, the aqueous layer was transferred to a fresh tube and equal volume of chloroform- isoamyl alcohol was added and centrifuged at 8000 xg for 5min. The aqueous layer was transferred to a fresh tube and 7.5 M

ammonium acetate was added, the two volume of 95% ethanol was added and centrifuged at 8000 xg for 5 min. The pellets were rinsed with 20 ul of TE buffer (10 mM) Tris HCL (PH. 80),1 m Mdisodium EDTA).

Milk samples:-

Frozen milk was thawed at room temperature and 400 ul from fatty layer was mixed with 400 ul of lysis solution (2%) Triton ×-100,1% sodium dodecyl sulfate, 100 m M NACL, 10m Tris-HCL (PH. 8.0) and 10 ul of proteinase K (20 mg 1ml) were added and incubated for 30 min at 50°C then proceed as in blood samples.

Tissue samples *(Rambrook et al, 1989):*

Freshly excised tissue was dropped in liquid nitrogen cup and grinded in pestle and morter. 10 volumes of extraction buffer (10 mM Tris- HCL (PH 8.0), 1.0 MEDTA (PH. 8.0), 20 ug/ml pancreatic Rnase and 0.5% SDS) in a beaker., put in 15ml centrifuge tube, incubated for 1 hour at 37°.,10 ul of proteinase k (20 mg/ml) was added and the contenrts were mixed and incubated for 30 min. at 50°C, then proceed as in blood samples.

Determination of DNA concentration:

Was determined by measuring optical density via absorbance measurement at 260 nm and 280 nm. ***(Sam brook et al, 1989).***

Amplifion by PCR: *(leal-kleve zas et al 1995)*

The brucella PCR diagnostic assay primer cocktail composed of Five primers were published by ***(Bricker and Halling 1994)*** and Was performed in a toatal of 50 ul with 100 ng of DNA, 50 pmol of Each primer, 50 mM KCL, 10 Mm Tris-HCL (PH 9.0), 0.1% Triton ×-100, 3m M Mg CL_2, 200 uM (each) DNTPS and 2.5 u Taq. Polymerase. The reaction was conducted in DNA the thermal cycler (Perkin Eimer, syster 2400). At a denaturation temperature 94° for 4 min., this was followed 35 cycles at 94° for 60 sec., 60°C for 60 sec. AND 72°C for 60 sec. and one final extension at 72^0 c for 3 min before stored at 4°C. Eight microliters of the amplified reaining mixture were taken taken and fractionated in a 1.5% agarose gel staining with ethidium bromide comide containg 1×TBE and 100-bp DNA ladder as asize standard, than electrophoreaed at 75 V for 1.5 hours and photographed over Uv light source.

Table (1) Number of samples examined by PCR assay:

Animal Species	No .of Examined Blood samples	No. of Milk samples	No of tissue specimems			No.of stomach content of aborted foeti	Total
			Lymph nodes	spleen	uterus		
Cattle	50	35	48	41	8	-	177
Sheep	24	23	21	20	6	1	95
Goats	29	24	27	25	7	1	118
Camels	7	8	7	7	3	-	32
Total	110	90	103	93	24	2	422

RESULTS & DISCUSSION

One strain amplified 498- bp fragment out of 50 cattle blood samples and identified as *B. abortus*, one strain from 24 sheep blood samples and amplified 731-bp fragments and identified as *B. meliensis and* three strins were amplified a 731- bp fragments out of 29 goats blood samples. While camel samples proved to be brucelle free. As shown in (Table 2) figures 1 &2. Eight samples milk from a total 90 samples proved to be positive be PCR (3 samples of cow's milk, one amplified 498 -bp fragment and two amplifie 731-bp feagments in an incidence of 10 %, two amplifie 731-bp feagments from 23 ewes milk samples in an incidence 8.69, and three amplified 731-bp fragments from 29 gaots milk sampled in an incidence of 10 .34%. (Table 3) Concerning examined tissue specimens by PCR, only one sample out of 97 tissue specimens of cattle (lymph node) was amplified 498-bp fragments with an incdence of 1.03%, one strain was detected from 47 tissue specimens of sheep (lymph rode) and amplified 731-bp fragments with an incidence of 2.13%. In addition to four straina were recovered from 59 tissue specimens of goats (2lymph nodes and 2apleen), all amplified 731-bp fragment and with an incidence of 6.78%.One samole of stomach contents aborted foetus for sheep was amplified 731-bp fragment and proved to be *B. Melitensis as* ahown is table (4).

With a meticulous visiona, the PCR assay is useful technique for Direct detection of brucellae in clinical samples and proved to be Apowerful, sensitive, specific and quick than a culture, reducing The time of diagnosis to one day and providing ability to detect organism even when become non- vialable and there conclusion suported by the findings obtained by ***Bricker and Halling 1995, leal klevezasetal 1995 and 2000, Romero et al 1995 andEwalt and Bricker2000*** who proved that PCR was optimal tool for both screening of herds, testing of

individual animal and obtain adefinitive diagnosis during acute, chronic stage of illness and before antibodies are detectable.

Table (2): Results of PCR on blood samples of examined ruminants for the detection of brycellae.

Animal Species	No. of examined samples	No. of Positive samples	%	Amplication fragments	Identified strain	No. of negative samples	%
Cattle	50	1	2.00	498-bp	B. abortus	49	98
Sheep	24	1	4.17	731-bp	B.melitensis	23	95.83
Goats	29	3	10.3	731-bp	B.melitensis	26	89.66
Camels	7	-	4	-	-	7	100
Total	110	5	4.55	-	-	105	95.45

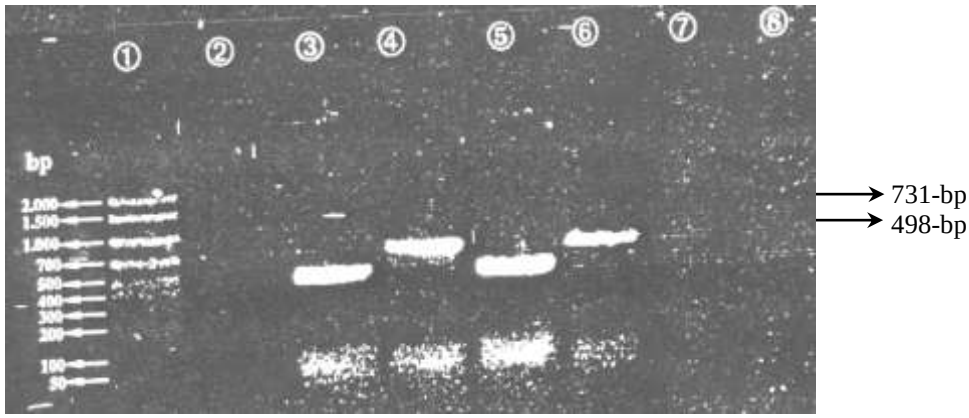
Table(3): Results of PCR on milk samples of examined ruminants for the detection of brucellae .

Animal species	No. of examined samples	No. of Positive samples	%	Amplification fragments	Identified Strain	No. of negative Ssmples	%
Cow	30	3	10.00	498-bp(1)731-	B.abortus B.	27	90.00
Ewes	23	2	0.69	bp(2)	melitensis	21	91.30
Goats	29	3	10.34	731-bp	B. melitensis	26	89.66
She-camels	8	-		731-bp	B. melitensis	8	100.00
				-	-		
Total	90	8	8.89	-	-	82	91.11

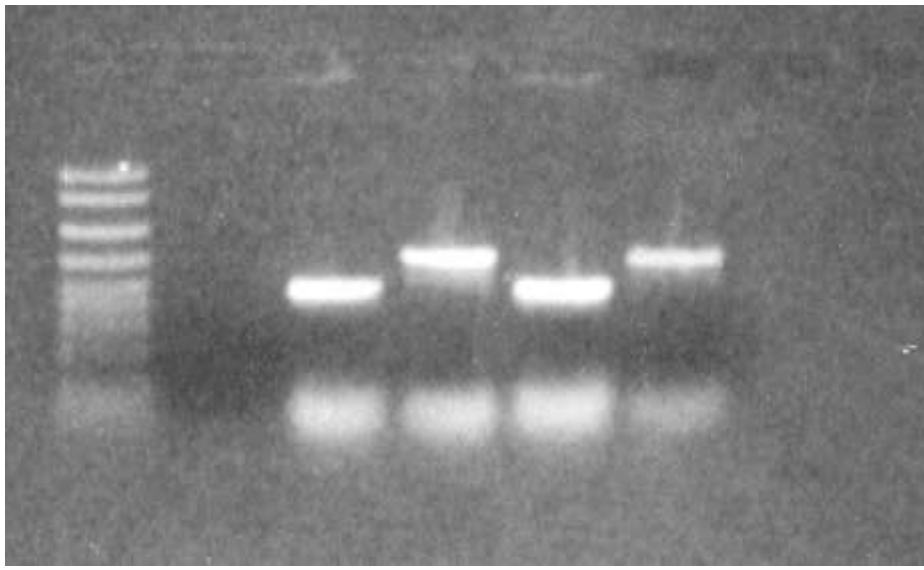
Table (4): Results of PCR on tissue specimens and stomach contents of abortes foeti of ruminants for the detection of bruecellae.

Animal species	No.of examined tissue specimens	No.of stomach content of foeti	No. of positive samples	Amolification fragments	Identified strain
Cattle	97	-	1	798-bp	B. abortus
Sheep	47	1	2	731-bp	B. melitensis
Goats	59	1	4	731-bp	B. melitensis
Camels	17	-	-		
Total	220	2	7	-	-

Detection of Brucella in a Cattle and Sheep blood samples By Multiplex AMOSPCR assay



Figure(1): Lane 1:100 bp as a size atandard, Lane 2: negative control (buffer containing no template), Lane 3: positive control (Brucella abortus 544), Lane4:positive conteol (Brucella melitensis 16M), Lane 5:Amplitied 498-bp fragment (cattle blood Sample), Lane 6:Amplified 731-bp fragment (sheep blood sa mple), Lanes 7,8: negative .



Figurd(1): Original ultraviolet photograph stained with Ethidium bromide on 1.5% agarose ge1.

Detection of Brucella melitensis in three blood samples of goats by Multiplex AMOS PCR assay

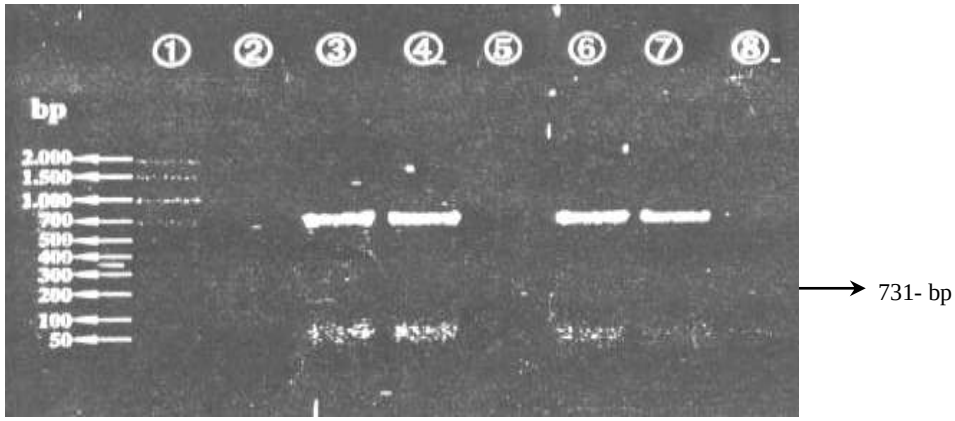


Figure (2): Lane 1:100bp ladder as a size standard, Lane 2: negative control (buffer containing no templaet), Lane 3: positive control (Brucella melitensis 16M), Lane4: Amplified 731-bp fragment, Lanes 5,8: negative, Lanes 6,7: Amplified 731-bp Fragments, products of less than 100-bp were presumed to be primer-dimer coplexes

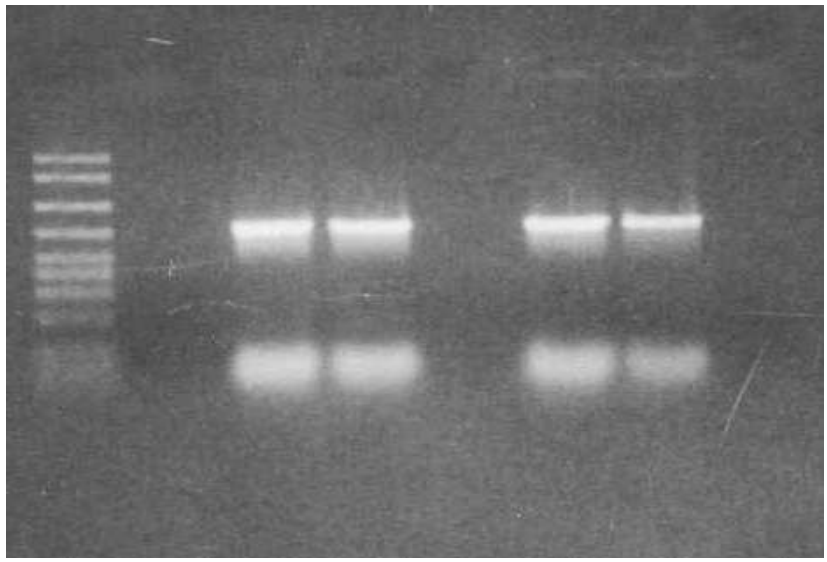


Figure (2): Original ultraviolet photograph staine d with Ethidium bromide on 1.5% agarose ge 1

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التشخيص المعملى لمرض البروسيلا في المجترات
بطريقة تفاعل سلسلة أنزيم عديد البلمرة أموس

د . محمد السيد عنـان **د . يوسف عبد الجليل أحمد**

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(422) عينة دم، لبن، عينات أنسجة ، مكونات معدة لجنين مجهض جمعت من مناطق مختلفة ومتفرقة بمحافظة الشرقية قد فحصت لتشخيص مرض البروسيلا بواسطة سلسلة أنزيم عديد البلمرة أموس وأظهرت الدراسة (5) من (110) عينة دم كانت إيجابية ، (8) من (90) عينة لبن كانت إيجابية،(7) من (220) عينة من الأنسجة" 4 من الغدد اللمفاوية، واثنين من الطحال وواحدة من مكونات معدة لجنين مجهن" كانت إيجابية .