DETECTION OF *BRUCELLA* SPECIES BY DNA PROBE HYBRIDIZATION ASSAY

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SUMMARY

A DNA probe hybridization assay was applied to detect Brucella DNA sequences by hybridization of non-radioactive probes to Brucella DNA dotted on nylon membranes . The assay was conducted using 2 probes prepared from genomic DNA of B. abortus and B. melitensis reference strains. Hybridization studies with the B. abortus and B. melitensis probes have shown them to be specific only with DNA preparations from 5 B. abortus and 2 B. melitensis reference strains and local isolates, respectively. No hybridization signal was detected with DNA from 2 gram-negative bacteria showing serological cross-reactivity with Brucella species. Sensitivity experiment has demonstrated the ability of the 2 probes to detect at least 1 pg of Brucella DNA. The diagnostic usefulness of of the hybridization assay for the detection of Brucella species in 60 field samples was evaluated. The hybridization assay showed a sensitivity of 89% and specificity of 88% compared to bacteriological culture procedures. It could be concluded that probe hybridization assay using non-radioactive probes may provide a valuable additional diagnostic tool for the rapid diagnosis of Brucella species.

INTRODUCTION

Brucellosis is an economically important zoonotic disease affecting many animal species and humans in many areas of the world (World Health Organization, 1986). It is caused by gram-negative bacteria of genus *Brucella*. The genus *Brucella* comprises six species: *B. abortus*, *B. melitensis*, *B. suis*, *B.ovis*, *B. canis and B. neotomae*. Bovine brucellosis and brucellosis of small ruminants is caused primarily by *B. abortus* and *B. melitensis,* respectively. The disease is characterized by a marked decrease in reproductive efficiency owing to abortion, clinical disease, infertility and diminished levels of milk production (Corbel,1997).

Diagnosis of brucellosis has traditionally relied on the detection of circulating antibodies followed by the bacteriological isolation of living

organisms (Alton et al., 1988). It is known that serological methods are not always sensitive or specific (Perry and Bundle, 1990 and Diaz-Aparicio et al., 1994). Moreover, they have been reported to cross-react with antigens other than those from *Brucella* species (Corbel et al., 1984 and Alton et al., 1988). Microbiological isolation and identification are the most reliable methods of diagnosing brucellosis (Greenlee et al., 1994 and Corbel, 1997). Bacteriological methods have the advantage of detecting the organism directly and thus limit the possibility of false positive reactions. However, these procedures are time-consuming due to the fastidiousness of the organism, and represent great risk of infection for laboratory diagnosticians. Because of these difficulties, the development of new procedures for the detection of *Brucella* species is of great practical importance.

Nucleic acid based detection methods are very promising tools for the diagnosis of brucellosis (Bricker, 2002). Recently, several studies have been reported on the utilization of probe hybridization assays for many *Brucella* species (Fayazi et al., 2002; Newby et al., 2003 and Rajashekara et al., 2004). In Egypt, few preliminary studies were carried out on the use of DNA probe assay as a detection method using isolates of *Brucella* species (Gabal et al., 1994 and Hosein et al., 1996). This method has not been used for the direct detection of *Brucella* species in clinical or field samples from naturally infected animals. The aim of the present study was to apply the hybridization assay using non-radioactive probes for detection of *Brucella* species and assess its usefulness in the diagnosis of animal brucellosis.

MATERIALS AND METHODS

Bacterial strains:

The *Brucella* reference strains and local *Brucella* field isolates used in the present study are listed in Table (1). *Brucella* local field isolates were identified and typed by colonial morphology, Gram staining, biochemical and antigenic characteristics according to the procedures described by Alton et al. (1988). *Escherichia coli* O: 111 and *Yersinia enterocolitica* O: 9 were used as negative controls and selected because of their reported cross-reactivity with *Brucella* species in serological tests.

Extraction of genomic DNA.

DNA was isolated as described by Fekete et al. (1992). Briefly, bacterial cells from 10 ml of culture were collected by centrifugation at 10,000 rpm for 15 minutes at 4°C. After washing in phosphate buffer saline, the cellular pellets were resuspended in lysis buffer (10 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl [pH 7.6]), Ribonuclease A was added to a final concentration of 50 µg/ml and the mixture was incubated for 1 hour at 50°C. Sodium dodecyl sulfate was added to a final concentration of 0.5% and protinase K was added to a final concentration of 500 µg/ml. The reaction mixture was incubated for 2 hours at 50°C and was extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). The aqueous phase was extracted with

chloroform-isoamyl alcohol (24:1), from which the aqueous phase was retained. DNA was precipitated with 100% cold ethanol after addition of sodium acetate to a final concentration of 0.3 M and collected by centrifugation. After washing with 70% ethanol, the DNA pellet was dried before being resuspended in 25 µl of sterile ultrapure water. The DNA concentration was measured by spectrophotometry at 260 nm. DNA preparations were stored at -20°C until further use.

Probe preparation and dot blot hybriddization:

Whole genomic DNAs from *B. abortus* 544 and *B. melitensis* 16 M reference strains were used as molecular probes. Dot blots of DNA were made using positively-charged nylon membranes (Sigma Co., USA). DNA dotting on membrane, probe labelling, hybridization and the detection procedures were performed using non-radioactive DIG-labelling and detection kit (Roche Diagnostics, USA) according to the supplier's instructions. Briefly, blots (membranes) were air dried and baked for 1 hour at 80°C. The membranes were prehybridized for 4 hours at 68°C in hybridization solution (5%SSC, 0.1% (w/v) N-laurylsarcosine, 0.02% (w/v) SDS and 1% (w/v) blocking reagent). After an overnight hybridization at 68°C with DIG-labelled probe, membranes were washed twice with 2X SSC; 0.1% (w/v) for 5 minutes at room temperature and then twice with 0.1X SSC;0. 1% (w/v) for 15 minutes at 68°C. After washing, membranes were air dried and subsequently subjected for detection of the bound probe.

Sensitivity of *Brucella* detection by hybridization probes:

The sensitivity of hybridization assay was determined by using purified genomic DNA from *B. abortus* 544 and *B. melitensis* 16 M reference strains. DNA stock from each strain was ten-fold serially diluted in sterile ultrapure water (from 1 ng to 10 fg) and aliquots of 25 µl from each dilution were analyzed by dot blot hybridization as described above.

Processing, testing and bacteriological examination of field samples:

A total of 60 milk, placental tissues and supramammary lymph node samples were collected from serologically-positive cattle, buffalo, sheep, goat and camels by the standard Rose Bengal and tube agglutination tests for brucellosis. Processing of field samples for DNA extraction was done according to the method previousely descibed (Fekete et al., 1992). Probe hybridization was performed as described above for bacterial strains. For bacteriological testing, tissue homogenates or fluid samples were streaked on plates of brucella agar selective media. Seeded plates were incubated in 10% CO_2 atmosphere for at least 3 days. Suspected *Brucella* colonies were picked up, purified and identified according to the standard procedures described by Alton et al. (1988). The sensitivity and specificity of the hybidization assay as a diagnostic test were calculated as described by Ryan (1991). The sensitivity of the hybidization assay was calculated by dividing the number of samples that tested positive by the hybidization assay by the number of samples found positive by culture. The specificity of the hybidization assay

was calculated by dividing the number of samples that tested negative by hybidization assay by the number of samples found negative by culture.

RESULTS

Hybridization of *Brucella* probes with *Brucella* DNA from reference strains and local isolates:

The results of dot blot hybridization of probes prepared from *B. abortus* 544 and *B. melitensis* 16M reference strains with DNAs from tested *Brucella* reference strains and local isolates are given in Fig.(1). The *B. abortus* probe reacted positively with DNAs from *B. abortus* 544 and S19 reference strains and *B. abortus* biovar 1 local isolates tested (Fig.1A). Similarly, the *B. melitensis* probe hybridized to DNAs from *B. melitensis* 16M reference strain and the 3 tested local *B. melitensis* biovar 3 isolates (Fig.1B). On the other hand, no hybridization signal was detected for DNAs from *E. coli* O: 111 and *Y. enterocolitica* O: 9.

Sensitivity of *Brucella* Probes:

To determine the sensitivity of *Brucella* DNA detection by hybridization probes, a 10-fold dilution series of purified genomic DNAs from *B. abortus* 544 and *B. melitensis* 16M reference strains was prepared and subjected to hybridization to *B. abortus and B. melitensis* probes, respectively . Fig.(2) shows the results dot blot hybidization of the 2 probes with different *Brucella* genomic DNA dilutions tested. The lowest quantity of DNA detected on the membrane was 1 pg. No detectable difference was observed for *B. abortus* and *B. melitensis* probes regarding the detection limit (Panels A and B).

Probe testing and bacteriological examination of field samples;

To assess the usefulness of the hybridization assay in the detection of *Brucella* DNA in field samples from infected animals, a total of 60 milk, placental tissues, lymph node tissue samples, collected from serologically-positive cattle, buffalo, sheep, goat and camels, were tested by dot blot hybidization assay and culture procedures. A two-by-two comparison of results of the probe assay to those of culture procedures are shown in Table (2). Of 34 culture-positive samples, 31 were positive by the probe assay . Of 26 culture-negative samples, 3 gave a positive result by the probe assay . The sensitivity of the probe assay was 89 % compared with culture techniques and its specificity was 88 %. The bacteriological testing of the 31 field samples found positive on probe testing revealed the isolation and identification of 21 *B. abotus* biovar 1 and 10 *B.melitensis* biovar 3, respectively (Table 3). As shown in Fig.3, *Brucella* genomic DNA was detected by hybidization assay , using the *B.abortus* probe, in cattle milk, buffalo milk, cattle placental tissue, cattle and camel lymph node tissue samples (Panel A). On the other hand, the *B. melitensis* probe detected *Brucella* DNA in cattle, buffalo, sheep, goat and camel milk and in camel lymph node tissue samples (Panel B). All DNA preparations form *Brucella*-free field samples (milk, placental and lymph node

tissues) included in the hybridization assay did not react with either of the used *Brucella* probes.

DISSCUSION

Although eradication programs have been adopted, brucellosis remains a global problem with severe economic consequenes. Control of brucellosis in animals relies mainly on veterinary sanitation measures focused on the reduction or eradication of this disease in farm animals (Refai, 2002). A critical tool to the success of these measures is unquestionably a rapid and accurate diagnosis of the disease. Therefore, there is an demanding need for sensitive, specific and fast diagnostic proceures for Brucellsis in livestock. Therefore, the aim of the present study was to apply the probe assay for detection of *Brucella* species and to assess its utility in the diagnosis of brucellosis in animals.

In the present study, a dot blot hybridization assay , using non-radioactive *Brucella* whole geomic DNA as molecular probes, was applied to detect *Brucella*-specific target DNA sequences. The use of non-radioactive digoxigenin as a label reduces the hazard involved in the use of isotopes, as well as the need for specialized equipment and training required in radioisotope handling (Lebacq, 1990).

Using whole genomic probes can cause problems with cross reactivity (Hopper et al., 1989). However, De Ley et al. (1987) have shown that evolutionary neigborhood of genus *Brucella* is sparsely populated. Consequently, there was no difficulties in using whole genomic *Brucella* DNA probes.

Purified genomic DNAs (10 ng/ 25 µl-assay mixture) from each of the tested *Brucella* reference strains and local isolates were evaluated as targets. Whole genomic DNA probes from *B. abortus* and *B. melitensis* hybridized to DNAs from reference strains and local isolates of *B. abortus* biovar 1 , and DNAs from *B. melitensis* biovar 1 reference strain and *B. melitensis* biovar 3 local isolate, respectively. The absence of cross hybridization of *Brucella* probes when used with genomic DNAs extracted from *E. coli* O: 111 and *Y. enterocolitica* O: 9 that are repeatedly reported to serologically cross-react with *Brucella* species (Alton et al., 1988) represents an evidence of their specificity for detecting *Brucella* species sequences.

In this study, a sensitivity test was conducted to determine the limit of detection of the probe hybridization assay. Decreasing concentrations of purified genomic *Brucella* reference strain DNAs were subjected to probe testing. There was no difference in the threshold sensitivities of the 2 *Brucella* probes used with the tested diluted DNA samples from *B. abortus* 544 and *melitensis* 16M reference strains (1pg of DNA). Lebacq (1990) reported equal general threshold of hybridization assays by using non-radioactive probes. However, a lower level of sensitivity (1 ng of *Brucella* DNA) was observed by Hopper et. (1989).

In order to assess the usefulness of the probe hybridization as a diagnostic method, a variety of field samples were evaluated for the detection of *Brucella* species. The 2 *Brucella* probes reacted positively with DNAs from milk, placental and lymph node tissues samples collected from different animal species. On the other hand, DNA preparations form *Brucella*-free field samples included in the hybridization assay failed to serve as targets for the used *Brucella* probes and all gave negative results. This indicates the specificity of probe hybridization for the selective detection of *Brucella* species in samples from infected animals.

The sensitivity and specificity of the dot blot hybridization as a diagnostic test were assessed. When the probe testing was applied to the field samples, its sensitivity with respect to bacterial culture was 89% (of 34 culture-positive samples, 3 were -positive). Many factors could account for false-negative results in the samples that were culture-positive. Samples with *Brucella* organisms in numbers below the detection limit, or inefficient DNA extraction in some samples may account for such results. Morever, freezing of samples prior to hybridization testing may result in damage to bacterial cells allowing penetration of endonucleases and subsequent degradation of target DNA. The probe assay showed a specificity of 88 % (of 26 culture-negative samples, only 3 gave a positive result by DNA probe testing). A false-negative bacteriological result can be caused by massive contamination of the samples, by inhibition of some *Brucella* species in the selective media (Blasco, 1992), or by a viability loss before culturing. In all these circumstances DNA can still be detected by the probe assay .

Beside its sensitivity and specificity, the blot hybridization assay described in the present study has several advantages over the current microbiological diagnostic methods for *Brucella* species. This method facilitates the simultaneous screening of large number of samples, reducing the amount of required sample testing materials. A major advantage is the speed with which the probe assay can be performed, where results could be obtained within less than 24 hours. This is much less time than that required to obtain results by conventional detection methods (at least several days or even weeks). Moreover, the probe assay eliminates the hazards of handling the infectious organism in the laboratory. This is significant because *Brucella* is a serious human pathogen.

The results recorded in the present study have demonstrated the reliability, high sensitivity and specificity of the hybridization assay for use in the detection of *Brucella* DNA and its utility in detecting the presence of the *Brucella* species in field samples from naturally infected animals. These data may form a strong basis for the development of new alternative diagnostic methods for brucellosis in animals.

It is clearly important not only to detect but also to identify the species of *Brucella* implicated in natural infection (Bricker, 2002 and Fayazi et al., 2003). With comparative genome sequence analysis among *Brucella* species (Halling et al., 2005), development of DNA hybridization-based methods that can be used to easily differentiate between *Brucella* species and biovars could be possible. This would be of significance for epidemiological studies and implantation of sound control measures for brucellosis in animal livestock.

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Table 1: *Brucella* strains and isolates used in this study.

Species and biovars	Strain	Source	Origin
B. abortus 1	544	Reference strain	Central Veterinary Laboratory, Weybridge, UK
B. abortus 1	S19	Reference vaccine strain	
B. abortus 1		Local Field Isolate (cow milk)	Animal Reproduction Research Institute Giza, Egypt
B. abortus 1		Local Field Isolate (buffalo milk)	
B. abortus 1		Local Field Isolate (cow uterus)	
B. melitensis 1	16M	Reference strain	Central Veterinary Laboratory, Weybridge, UK
B. melitensis 3		Local Field Isolate (sheep lymph node)	Animal Reproduction Research Institute Giza, Egypt

Table 2: Comparison of the results of probe testing and culture assay with field samples.

Test result	Probe-positive	Probe-negative	Total
Culture-positive	31*	3	34
Culture-negative	3	23**	26
Total	34*	26**	60

* Sensitivity of the probe assay was calculated to be 89% compared to culture assay.
** Specificity of the probe assay was calculated to be 88% compared to culture assay.

Table 3: Brucella identification in 31 field samples found positive on probe assay.

Field sample	Animal species	Typing of *Brucella* isolates		Total
		***B.abortus* biovar 1**	***B.melitensis* biovar 3**	
Milk	Cattle	9	2	11
	Buffalo	1	1	2
	Sheep	0	1	1
	Goat	0	1	1
	Camel	0	1	1
Placental tissue	Cattle	2	0	2
Lymph node tissue	Cattle	9	0	9
	Camel	0	4	4
Total		21	10	31

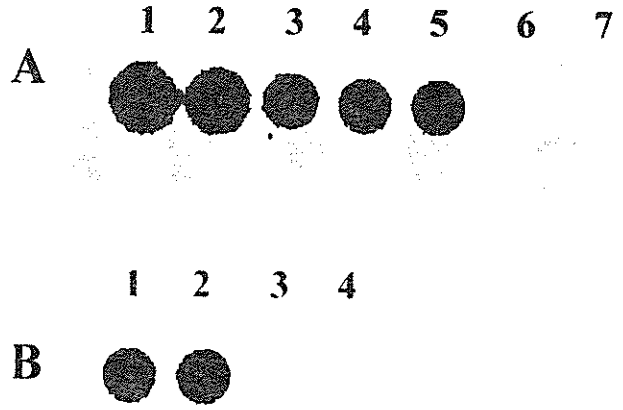

Fig. 1. Specificity of dot blot hybridization for detection of *Brucella* DNA using the *B. abortus* (A) and *B. melitensis* (B) probes, respectively. A) The letter-number coordinates are as follows: A1: *B.abortus* 544; A2: *B.abortus* S19; A3-A5: *B. abortus* biovar 1 isolates from cow milk, buffalo milk, and cow uterus, respectively; A6: *E. coli* O: 111;A7: *Y.enterocolitica* O: 9. B) The letter-number coordinates are as follows: B1: *B.melitensis* 16M; B2: *B. melitensis* biovar 3 isolate from sheep lymph node; B3: *E. coli* O: 111; B4: *Y.enterocolitica* O: 9.

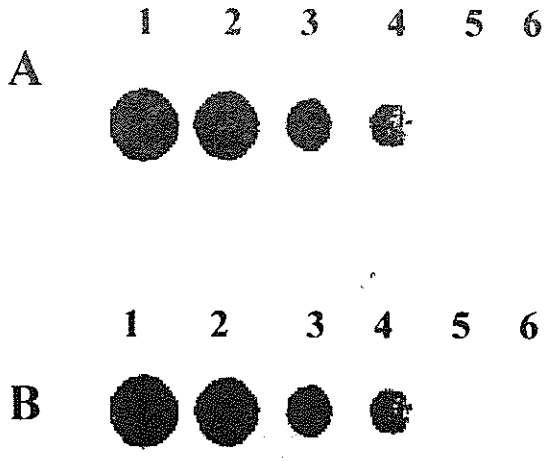

Fig. 2. Sensitivity of dot blot hybridization using the *B. abortus* and *B. melitensis* probes for the detection of DNA from *B. abortus* 544 (A) and *B. melitensis* 16M (B) reference strains, respectively. Tenfold serial dilutions (from 1 ng to 10 fg) of purified genomic DNA of each strain were analyzed by the appropriate probe. A1-A6 and B1-B6 are serial DNA concentrations of *B. abortus* 544 and *B. melitensis* 16M, respectively.

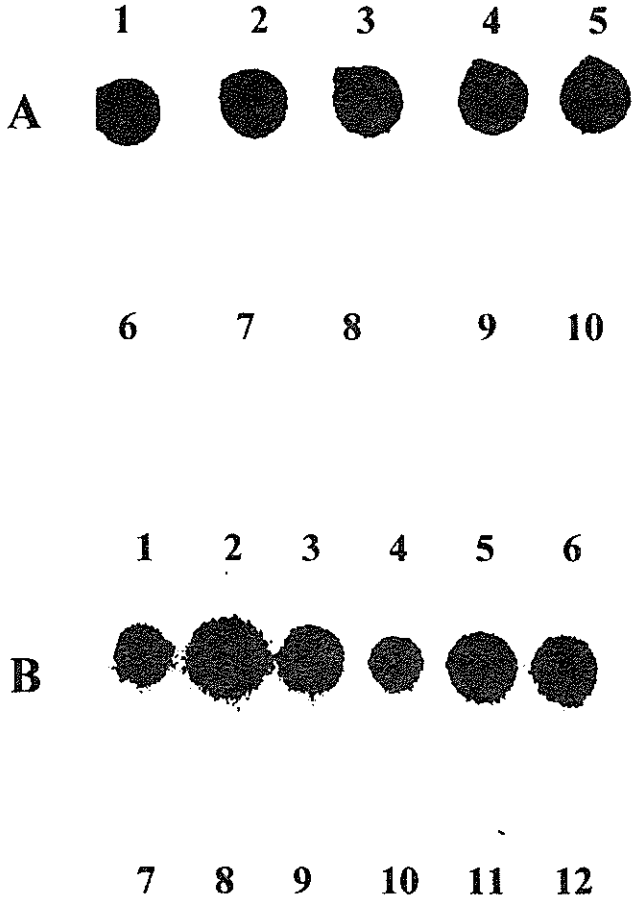

Fig. 3. Representative dot blot hybridization of different types of tested field samples by using the *B.abortus* (A) and *B. Melitensis* (B) probes, respectively. A) The letter-number coordinates are as follows: A1: cattle milk; A2:buffalo milk; A3: cattle placental tissue; A4: cattle lymph node tissue; A5: camel lymph node tissue. [Negative controls (A6-A10: *Brucella* -free cattle milk, buffalo milk, cattle placental tissue, cattle lymph node tissue and camel lymph node tissue samples, respectively)]. B) The letter-number coordinates are as follows: B1-B6 : cattle, buffalo, sheep , goat , camel milk , and camel lymph node tissue samples, respectively. B7-B12: Negative controls (*Brucella*-free cattle milk, buffalo milk, sheep milk , goat milk , camel milk, and camel lymph node tissue samples, respectively).

الملخص العربي

الكشف عن ميكروبات البروسيلا
باستخدام اختبار مجس تهجين الحمض النووي

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استخدم اختبارالتهجين مع مجس الحمض النووي غير المشع في الكشف عن الحمض النووي المميز لميكروب البروسيلا وذلك باستخدام مجسين محضرين من عترة مرجعية لكلا من البر وسيلا ابورتس والبر وسيلا ميليتنسس . تم اختبار ثلاث عترات مرجعية من البر وسيلا ابورتس والبر وسيلا ميليتنسس بالإضافة إلي أربع عترات بروسيلا معزولة محليا وأعطت جميعا نتائج إيجابية للكشف عن الحمض النووي الخاص بميكروب البروسيلا. ولم يعط الاختبار أي نتائج إيجابية بالنسبة لنوعين آخرين تم اختبارهما من البكتيريا سالبة الجرام والمعروفة بحدوث تفاعلات غير نوعية مع ميكروبات البروسيلا سيرولوجيا . وقد تم تحديد مدي حساسية الكشف بواسطة اختبار التهجين باستخدام تركيزات مختلفة من الحمض النووي الديوكسي ريبوزي المستخلص من عترات قياسية لكلا من البروسيلا ابورتس والبروسيلا ميليتنسس حيث تم الكشف عن حتى تركيز واحد بيكوجرام من الحمض النووي بواسطة هذا الاختبار .

ولتحديد القدرة (الكفاءة) التشخيصية لاختبار مجس التهجين للكشف عن الحمض النووي الخاص بميكروبات البر وسيلا في العينات الحقلية من الحيوانات المصابة بالمرض ، تم اختبار عدد ستون عينة (لبن, انسجة مشيمية, غدد ليمفاوية فوق الضرع) أخذت من ابقار وجاموس و أغنام و ماعز و جمال ايجابية للبروسيلا بالأختبارات السيرولوجية و ذلك باجراء كل من اختبارالتهجين مع مجس الحمض النووي والفحص البكتيريولوجي. وطبقا لنتائج كل من الأختبارين فقد تم حساب كل من حساسية وتخصص الاختبار بنسبة 89% ، 88% مقارنة بالفحص البكتيريولوجي علي الترتيب .

وقد أمكن الاستنتاج بأمكانية استخدام اختبار التهجين بإستخدام مجس الحمض النووي غير المشع كاختبار ذو فائدة قيمة في التشخيص السريع للإصابة بميكروب البروسيلا في حيوانات المزرعة لما يتميز به الأختبار من الكشف بحساسية عالية و بتخصص والقدرة علي الكشف عن الحمض النووي المميز للميكروب في العينات الحقلية من الحيوانات المصابة به و بذلك يمثل أضافة لطرق التشخيص المعملي المستخدمة حاليا للكشف عن الإصابة بالمرض .