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EPIDEMIOLOGICAL STUDIES ON JOHNE'S DISEASE IN CATTLE USING THE CULTURE AND PCR TECHNIQUES

(With 2 Tables and 2 Figures)

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دراسات وبائية عن مرض اليونز في الماشية باستخدام طريقتي البلمرة والزرع مع دراسة حساسيتهما في الكشف عن ميكروب السل الكاذب

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لمعرفة مدي إنتشار مرض السل الكاذب بين قطعان الماشية تم فحص قطيعين من الأبقار الفيريزيان بالنمسا بأجمالي ٧٢ حيوان بإستخدام طريقه الزرع التقليدية و طريقه البلمره. أثبتت النتائج المتحصل عليها أن معدل إنتشار المرض وصلت إلي ١٢,٥% بأستخدام هذه الطريقة في حين لم تتعدى نصف هذه النسبة تقريبا في حاله إستخدام طريقه الزرع التقليدية (٦,٩%). أثبتت النتائج كذلك أن حساسية طريقه البلمرة وصلت إلي ١٠٠% تقريبا في حين لم تتعدى ٥٥,٦ % للطريقة التقليديه. من ناحيه اخري فان خصوصية الطريقتان للكشف عن الميكروب المسبب للمرض كانت ١٠٠% حيث لم نجد أية نتائج إيجابية عند فحص ٣٣ عينه جمعت من حيوانات معروف سلفا خلوها من المرض. هذه النتائج تؤكد أن إختبار البلمرة أكثر حساسية بالإضافة إلي سرعة إجراؤه مما يؤكد أنه الاختبار الأمثل في تشخيص المرض والذي يعتبر الخطوة الأولى في التخلص من هذا المرض وغيره. كما يوصي الباحثان بضرورة إعادة الاختبار بصفة دوريه للتأكد من عدم إصابة الحيوانات.

SUMMARY

The prevalence rate of Johne's disease in two herds of cattle in Austria was determined using the classical (culture) and a recent technique (PCR). PCR technique based on a 314 bp segment was conducted by using primers complementary to the *IS*900, insertion sequence specific for *M. paratuberculosis*. The obtained results revealed that the sensitivity

of PCR technique was more or less twice that of cultural technique as the positive results obtained by the first technique were 12.5% while the second technique gave only 6.9% positive results. Sensitivity of the PCR technique in detection of *M. paratuberculosis* was 100% while it was only 55.6 % for the conventional culture. No positive results were obtained by either test when applied on 33 faecal samples from animals known to be free from Johne's disease, indicating that both are 100% specific. The PCR as compared with the classical culture technique is the simpler, faster and more sensitive with 100% specificity in detection of Mycobacteria.

Key words: *Paratuberculosis, Johne's disease, faecal-Culture, PCR, sensitivity, specificity.*

INTRODUCTION

Johne's disease is a continuing enigma for cattle producers and veterinarians. It is a chronic granulomatous intestinal disease of ruminants causing chronic or intermittant diarrhoea and weight loss leading to emaciation and eventually death (Chiodini et al., 1984). The disease is caused by an acid fast organism named *M. paratuberculosis*. It is generally accepted that cattle become infected when calves ingest faeces contaminated with the organism (Merkal, 1984, and Whitlock et al., 1986). The organisms multiply in the intestinal lamina propria, enter the intestinal lumen, and are shed in the faeces. The clinical signs are detected months to years after faecal shedding begins, when the animal becomes in an advanced stage of infection. Johne's disease causes considerable economic losses in the dairy industry. The disease causes infertility, long intercalving intervals, extensive culling with reduction of the milk production (Buerglet & Duncan, 1978). It was reported that, Johne's disease infects about 2.9% of dairy and 0.8% of beef cattle population in the United states (Merkal et al., 1987). The national economic impact has been estimated to be as high as $ 1.5 billion annually (Jones, 1989).

Attempts to control the disease are severely hampered by lack of rapid and reliable tests to detect the infected animals. Diagnosis of the disease by faecal culture was the most reliable method (Merkal et al., 1968). The current procedures are laborious and primary isolation of the causative agent from faeces takes 6-16 weeks (Chiodini et al., 1984, and Barclay et al., 1985).

DNA probe would offer many advantages in diagnosis of Johne's disease. The polymerase chain reaction allows sensitive detection of DNA of the organism under test (Saiki et al., 1985). The specificity of this technique has been achieved by using primers complementary to a specific target nucleic acid sequence. It was noted that an oligonucleotide probe derived from *IS*900, a stable insertion element present in multiple copies (15-20) per *M. paratuberculosis* genome is the most specific one for this organism (McFadden et al., 1987; Green et al., 1989; Vary et al., 1990, and Moss et al., 1992).

This work was conducted to study the prevalence rate of Johne's disease using the PCR technique as a recent and rapid technique. However, the sensitivity and specificity of the technique was compared with the results of the conventional culture method.

MATERIALS and METHODS

Samples:

Seventy two faecal specimens were collected from two cattle herds. The first one is the Veterinary University herd, Vienna, Austria, containing 35 frisian cattle. The previous routine examination (cultural and serologically) indicated that 33 animals were free from the disease and the two other animals were suspected to be infected). The second herd is a private one with a history of paratuberculosis, with 37 frisian cattle. A specimen was collected directly from the rectum of each animal under complete aseptic conditions and transported without delay to the laboratory. Sterile glassware and instruments were used in all processing steps. The specimens were stored at -20 °C if the investigation was delayed for some time.

1- Conventional culture technique:

The faecal specimens were processed for culture using a method similar to that described before (USDA, 1974, and Whipple & Merkal., 1985). Briefly, three grams of the specimen were suspended in 30 ml 0.75% hexadecylpyridinum chloride (HPC, sigma, St. Luois, Mo). The mixture was thoroughly vortexed for 30 min., then filtered through two-play thickness sterile guaz and allowed to stand undisturbed for 24 h at room temperature, permitting the particulate matter to settle. The

supernatant was discarded and the pellet was used for inoculation. A loopful was taken from the pellet and streaked on two slants of Herrold's egg yolk medium, HEYM (Merkal & Curren, 1974), one with mycobactin J (Allied laboratories, Inc., Ames, Iowa, 2 mg/L) and the other without mycobactin J. The used antibiotic coktail were 750 µg/ml cyclohexamide; 50 µg/ml chloramphenicol and 200 U/ml penicillin. The inoculated slants were incubated at 37 °C for up to 16 weeks and examined every two weeks for Mycobacterial growth. Mycobactin-enriched slants showing colonies typical for *M. paratuberculosis* while no growth on the non mycobactin-enriched slants were considered positive if typical acid-fast bacilli were detected by the use of Kinyoun acid-fast staining procedures (Smithwick, 1976, and Chiodini & Van Kruiningen, 1986).

2-Polymerase chain reaction (PCR):-

2.1- Extraction of DNA from faecal specimens:

The procedures were performed as that described by Bleumink-Pluym et al. (1994). For extraction of *M. paratuberculosis*, 0.2 M NaOH was used. The mycobacteria were lysed mechanically by high speed Mini-beadbeater (Biospec products, Bartesvilles, UK, USA) for 3 min. in presence of equal amount of 0.1 and 0.5 mm (1:1) zirconium beads and 6M sodium iodide. After lysing the organisms, the zirconium beads were washed three times with ice-cold Tris wash buffer (20 mM Tris HCl, 1 mM EDTA, 100 mM NaCL) mixed with 96 % ethanol (1:1). The DNA was eluted from the beads in 100µl sterile bi-distilled water at 100 °C for 15 min. then stored at -20 °C to be used in PCR.

2.2-Extraction of DNA from M.paratuberculosis culture:

100 mg (wet weight) of Mycobacterial cells of reference strain (316 F) growing on HEYM was harvested from the surface of the medium and suspended in 500 µl of sterile bi-distilled water. 250 µl of the bacterial suspension were added to a 1.5 ml screw-cap polypropylene tube to which 750 µl of 6M sodium iodide solution and 100 µg of 0.1 and 0.5 mm (1:1) zirconium beads were added and the procedures were conducted as described before.

2.3-Amplification procedures:

Enzymatic amplification of DNA was performed by standard PCR technique described previously (Saiki, 1989). Two oligonuclutide primers within the *IS*900 insertion sequence, designated primer MP3 (5'-CTG GCT ACC AAA CTC CCG A-3') and MP4 (5'- GAA CTC AGC GCC CAG GAT-3') were used for the PCR resulting in a 314-bp PCR fragment (Green et al., 1989, and Bauerfeind et al., 1996). The PCR mixture of 25 µl (in Micro- ampl. reaction tubes with cap) contained 1x amplification buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin; 1mM $MgCl_2$; 200 µM (each) dATP, dGTP, dCTP and dTTP; 40 pmol (each) primer (MP3 & MP4); 0.75 U of Ampil*Taq* Gold (Perkin-Elmer, Cetus, Norwak, Conn); 2 µl of DNA extract and Aqua bi-dist. ad 25µl. PCR was performed on a programmable thermal cycler (GeneAmp PCR system 2400, Perkin Elmer, Cetus) for 12 min (at 95 °C for enzyme activation, one cycle), 35 cycles of denaturation (15 sec. at 94 °C); Primer annealing 15 sec. at 61 °C) and primer extension (1 min. at 72 °C) and one cycle at 72 °C for 7 min.

In order to prevent cross contamination, all pre- and post-PCR procedures were performed in separate rooms with different equipments. The master mix was prepared and handled within a safety cabinet and distributed into 0.2 ml micro-amplification tubes with caps (23 µl/tube). The micro-tubes containing the PCR-mixture were transferred to the sample-processing laboratory where 2 µl of the DNA extract were added and the reaction mixes were then shortly cenrifuged before amplification.

2.4- Detection of the amplified DNA:- After amplification, 3 µl of the loading buffer (0.25% bromophenol blue; 0.25% xylene cyanol FF; 15% Ficoll type 4000) were added to each micro-tube, thoroughly mixed then 10 µl was subjected to electrophoresis at 100 V (5V/cm) for one hour in 2 % agarose gel in Tris-Borate-EDTA buffer (0.089 M Tris-borate; 0.089 M boric acid; 0.002 M EDTA) stained by ethidium bromide (0.5 µg/ml). Under these condition, the 314 bp product of PCR was clearely separated. The separated fragments were visualized on an UV transilluminator at wavelength 320 nm (see Fig. 2).

RESULTS

The obtained results are summerized in the following tables and figures:-

Table 1: Prevalence rate of Johne's disease-infected cattle.

Source of specimens	No. of examined samples	Culture		Polymerase chain reaction	
		Positive samples	Prevalence rate	Positive samples	Prevalence rate
University herd	35	1	2.9%	2	5.7%
Private herd	37	4	10.8%	7	18.9%
Total	72	5	6.9%	9	12.5%

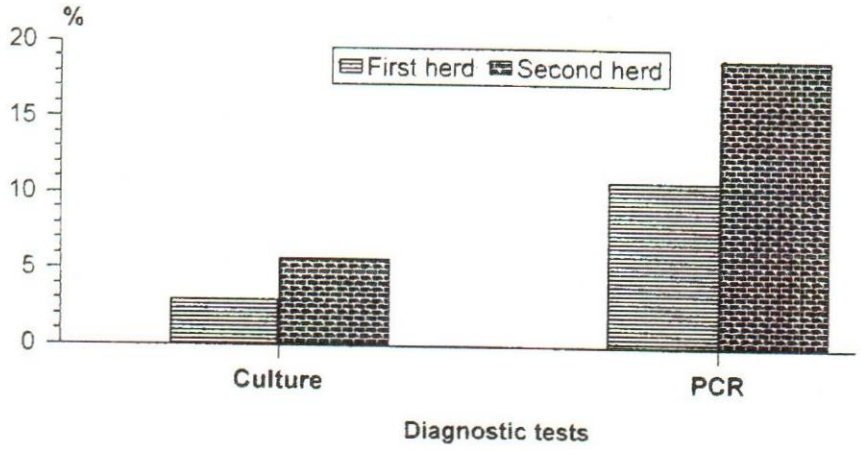

Fig. 1: Prevalence rates of paratuberculosis as indicated by faecal culture and PCR.

Table 2: Sensitivity of the PCR and culture technique in detection of the causative agent from bovine faeces.

Test result						
Effect of testing					Sensitivity percentages*	
In series				In parallel	Culture	PCR
Agreement		Disagreement				
C+/P+	C-/P-	C-/P+	C+/P-	C+ and/or P+		
5/9	63/ 72	4/ 9	0/ 9	9/ 72	55.6%	100%

C, culture; P, PCR; * No. of positive specimens by a test/ total positive specimens to either test.

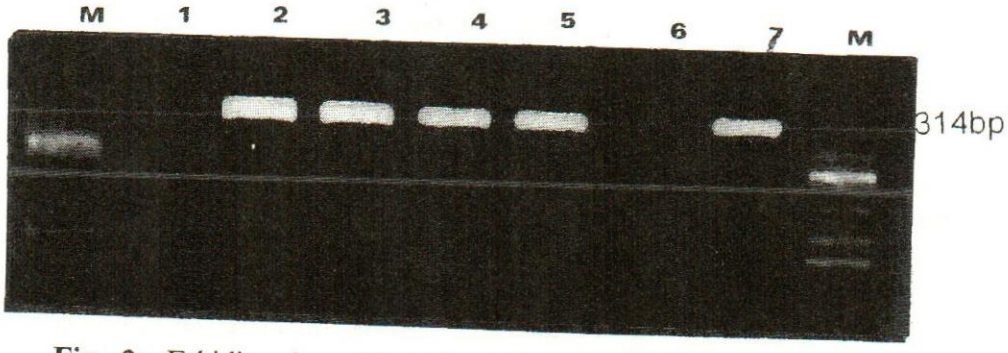

Fig. 2: Ethidium bromide stained 2% gel of PCR amplified products, M. Standard marker, lane 1, PCR-negative; lanes 2,3,4 and 5 are PCR-positive; lane 6, negative control; lane 7, positive control.

DISCUSSION

Since the primers used are complementery for the *IS*900, the results appeared more reliable because this insertion sequences are stable, highly specific and present in multiple copies (15-20) per *M. paratuberculosis* chromosome (McFadden et al., 1987; Green et al., 1989; Vary et al., 1990, and Collins et al., 1995). By using such primers, few organisms could be detected (Giessen et al., 1992).

The results recorded in Table (1) revealed that, 5.7% (2/35) and 18.9% (7/37) were positive by PCR for the University and private herd, respectively. On the other hand, the prevalence rates as recorded by culture technique were 2.9% (1/35) and 10.8% (4/37) for the University and private herds, respectively (Fig.1). The overall prevalence rate was 6.9% and 12.5% by the culture and PCR, respectively.

The results illustrated that, PCR gave more or less double prevalence rate as that recorded by the culture method. This could be attributed to the high sensitivity of PCR to amplify a few femtogram quantities of DNA of the organism under amplification. It was recorded that as low numbers of *M. paratuberculosis* as 50 organisms/g of faecal matter could be detected by PCR technique (Collins et al., 1995). Lower prevalence rates by the culture technique may be attributed to the use HPC as a decontaminant agent. It was stated that 100-fold loss of the organisms by such treatment was recorded before (Lambrecht et al.,

1988, and Mokresh et al., 1989). This reduced the culture sensitivity specially for specimens obtained from animals normally shedding low numbers of Mycobacteria. However, there are two factors adversly affecting *M. paratuberculosis* detection in the faecal specimens: organism numbers and the commercial antibiotic coktail used to control non-mycobacterial microflora (Collins et al., 1995).

The obtained results may not give the real prevalence rate of the infected animals by either tests. This could be attributed to the intermittent or sporadic shedding pattern of the organism from the infected animal. In this respect, it was reported that only 29.0% of paratuberculosis-infected ruminants were shedding the organism (Buergelt et al., 1977). The previous study on the shedding pattern of *M. paratuberculosis* revealed that animals with few colony counts were not detected by the culture technique over two years while others had a more consistent increase in their colony count over time (Whitlock et al., 1994). The same study showed that less than 40% of the faecal culture-positive animals were detected on the first trial culture and the remainder were detected on subsequent herd culture over four years period. The sensitivity of the faecal culture technique was an identification of approximately one-third of the infected animals in the inital herd test.

Although the PCR is sensitive technique, but false-negative results will be suspected. This could be attributed to the PCR-inhibitors co-purifying with DNA extracts which were not removed by the current extraction procedures (Vary et al., 1990; Brisson et al., 1991; Giessen et al., 1992, and Guillon et al., 1994). Moreover, inefficient extraction of mycobacteria from the specimens, particularly when few organisms are involved may be the other cause.

Since diagnostic accuracy has two components, sensitivity and specificity, precise determination of these values are essential for the interpretation of the test results.

Test sensitivity: means the percentage of infected cattle that have positive test result. By considering the positive result to either test as a positive one, the culture sensitivity was 55,6% while it was 100% for PCR as all culture-positive samples were also positive by PCR technique (Table 2). The higher sensitivity of PCR over culture in our result may be attributed to using primers complementary to *IS*900 which is highly specific for *M. paratuberculosis*. Moreover, efficient amplification procedures enormously increase DNA and inturn increase the technique sensitivity.

Test specificity: It means the percentage of non-infected animals that have negative test results for the disease. However, no positive results were recorded either by the culture or PCR out of 33 samples obtained from certified paratuberculosis-free animals. Moreover, out of the 72 examined samples, no culture growth was shown in all PCR-negative samples (table 2), indicating that PCR (using MP3 and MP4 primers) as well as the culture methods are 100% specific.

It could be concluded that PCR is a time-safer, specific and highly sensitive than the conventional culture method. PCR is considered the method of choice for diagnosis of Johne's disease and a useful tool for culling programs. In order to overcome the false-negative results due to the intermittent shedding pattern of the causative agent, the test must be repeated at time intervals.

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