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SHORT COMMUNICATION

Surveillance of Johne's Diseases Bacterium, *Mycobacterium avium* Subspecies *Paratuberculosis* in Dairy Cattle in Egypt using Three Laboratory Diagnostic Techniques

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ABSTRACT

Johne's disease is a chronic infectious disease of all ruminants caused by the acid-fast bacilli, *Mycobacterium avium* subspecies *paratuberculosis*. Various laboratory diagnostic techniques were developed for the diagnosis of JD including cultural and immunological approaches or molecular biological tools. In the current investigation, 198 fecal samples of dairy cattle from four farms in different governorates in Egypt were subjected to direct Ziehl-Neelsen stain, culture in Herrold's Egg Yolk Agar media and polymerase chain reaction by amplification of IS900 gene. Results recommended that the application of ZN stain on fecal samples from clinically suspected cases should be occurred, and then PCR targeting IS900 gene should be applied on those which contain acid-fast bacilli to ensure rapid and accurate laboratory diagnosis of *Mycobacterium avium* subspecies *paratuberculosis*.

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INTRODUCTION

Paratuberculosis, also called Johne's disease, is one of the economically significant chronic devastating disease affecting ruminant animals including cattle, sheep, goats and some wild ruminants. The disease is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), which is among the slowest growing mycobacteria. It requires from 8-16 weeks for producing visible colonies on the conventional agar media. MAP is a small gram positive, non-motile, acid-fast bacillus. The bacilli occur in clumps entangled with one another by a network of intercellular filaments. The main distinguishing feature of this species is its dependency on exogenous mycobactin for *in vitro* growth in its specific media, Herrold Egg Yolk media (HEY) (Merkal *et al.*, 1987).

The lack of sensitive, specific and rapid test for detection and rapid removal of infected animals from the herd hamper the control of this disease. The early detection of infected animal is important in minimizing the economic losses caused by Johne's disease. An array of diagnostic tests have been tried and evaluated for diagnosis of the disease. Isolation of MAP from feces remains the most accurate and reliable diagnostic method (Chiodini *et al.*, 1984, Kim *et al.*, 1989). The long incubation time required for isolation of this pathogen (8-16 weeks) and the necessity of presence of more than 100 bacilli/gram of feces for successful isolation detract significantly from the diagnostic value of this approach. However, the fecal culture test is still recognized as the gold standard for diagnosis (Whitlock *et al.*, 2000).

PCR methods have the potential to be rapid and as much specific as fecal cultures. The major limitation of PCR is that it generally lacks the desired sensitivity when applied to fecal samples (Sockett *et al.*, 1992). On the other hand, former studies have developed several polymerase chain reaction (PCR) based tests to detect MAP DNA but it has been difficult to achieve a sensitivity equal to culture for detecting MAP directly from bovine feces by PCR (Vary *et al.*, 1990, van der Giessen *et al.*, 1992, Collins *et al.*, 1993, Dell'Isola *et al.*, 1994, Bauerfeind *et al.*, 1996, Secott *et al.*, 1999).

In contrast, PCR tests for detection of MAP in fecal samples have vastly improved recently, leading to an increased sensitivity of detection of low shedders, including detection level of one colony forming unit (cfu)/g feces (Nielsen *et al.*, 2001). The majority of PCR assays have been based upon the amplification of the MAP IS900 element due to its sensitive detection rather than other genes (Christopher-Hennings 2003, Green *et al.*, 1989). Therefore, the aim of the current study is to present the findings of a head to head comparison between culture and PCR tests trying to evaluate each tool used for the diagnosis of such disease divulging their advantages and disadvantages.

MATERIALS AND METHODS

Fecal samples

198 fecal samples have been collected from suspected dairy cattle showing clinical signs of Johne's disease including wasting, chronic intermittent diarrhea, emaciation, decrease in net body weight and emaciation. Samples were collected from different farms covering Alexandria, Gharbyia, Ismailia, Kafr El-Sheikh and Giza Governorates. Specimens were divided into three parts, one for direct examination by Ziehl-Neelsen's (ZN) stain, the second is for culture, and the last is for PCR.

Ziehl-Neelsen (ZN) stain

Direct smears were prepared from stool specimens and ZN was applied according to Manning and Collins protocol (Manning and Collins 2001)

Fecal culture of MAP

Fecal culture was done according to Whipple *et al.,* 1991. Briefly, one gram of feces were added to 20 ml of sterile distilled water the tubes were shaken and then allowed to stand undisturbed for 30 min. Five ml of the supernatant were mixed with 0.75% Hexadecyl pyridinium chloride (HPC) for decontamination (Sigma, USA). Herrold Egg Yolk Media (HEYM) slopes were inoculated with 0.1 ml of the undisturbed sediment. Each sample were cultured in duplicates on HEYM slopes and incubated at 37°C. Tubes were observed in a 2 weeks interval for 16 weeks for colony morphology, and ZN stain.

MAP DNA extraction from fecal samples (Christopher-Hennings *et al.*, 2003)

HerdChekt® *Mycobacterium paratuberculosis* DNA Test Kit (IDEXX Laboratories, Westbrook, ME, USA) was used according to insert instructions. Only one gram fecal sample was suspended in 25 ml of sterile distilled water mixed with 10 N NaOH and an extraction detergent and vortexed with polycarbonate beads for 2 min. The suspension was allowed to settle for 30 min, and the supernatant was then transferred to a clean tube and centrifuged at 1 750 X *g* for 30 min. The pellet was washed, vortexed, and centrifuged again at 1 750 X *g* for 30 min. After then, the supernatant was removed and 3 lysing reagents were added to the pellet, and the bacterial lysate was placed in a lysing matrix tube. Mechanical interference of the sample was done for 30 sec at 5.5 m/sec in a homogenizer to release of intact DNA. After homogenization, the tube was centrifuged at 8 000 X *g* for 5 min in cooling centrifuge and the supernatant was transferred to another clean tube and centrifuged again at 16 000 X *g* for 10 min. To purify DNA, a matrix-binding solution was added to 700 µl of supernatant and allowed to incubate at room temperature. After centrifugation, the matrix was washed, a matrix elution buffer was added, and the solution was incubated at 37 C for 10 min. The supernatant obtained after centrifugation was added successively to each of 3 DNA column purification buffers. The final DNA product was eluted from the column, precipitated with ethanol, washed, re-suspended in a suspension solution, and incubated at 37 C for 30 min. The product after then was preserved at -80°C until use.

Polymerase Chain Reaction (Christopher-Hennings *et al.,* 2003, Stabel *et al.*, 2004)

Reaction tubes contained a total volume of 50 µl consisting of Ultrapure distilled water (DNAase, RNAase-free), GeneAmp 10X PCR buffer II, 2.5 mM $MgCl_2$, 0.25 mM deoxyribonucleoside triphosphates, 0.25 mM primers, and 2 U of AmpliTaq gold. Primer sequences for the *M. paratuberculosis*–specific genetic element, IS*900*, were used in the reaction mixture as follows: 5`-CCGCTA ATTGAGAGATGCGATTGG-3`, forward primer, and 5`-AATCAACTCCAGCAGCGCGGCCTCG- 3`, backward primer, to amplify a 229 bp gene sequence. Controls consisted of reaction mixture alone and positive control contained 1 µl of genomic DNA from *M. paratuberculosis* (strain 19698). A PCR analysis on DNA was run from undiluted fecal supernatants. Samples were run according to this protocol: 1 cycle at 94 C, 10 min; 50 cycles at 94 C, 59 sec, 60 C, 30 sec, and 72 C, 59 sec; followed by a final extension cycle at 72 C, 10 min. Polymerase chain reaction amplificates and a 100 bp gene ruler marker were then electrophoresed in agarose gel. Gels were then observed on a UV transilluminator for bands that appeared at 229 bp.

RESULTS

Table 1 shows the result of positive cases using different diagnostic methods. Ziehl-Neelsen stain was applied directly on the fecal samples and showed 90 positive cases. The stain was also applied on the colonies of the 16 positive cultures (Fig. 1). PCR (Fig. 2) was applied directly on the fecal samples and showed 35 positive cases and was also applied on the 16 positive cultures for confirmation.

DISCUSSION

Mycobacterium avium subspecies *paratuberculosis* (MAP), an extremely slow growing mycobactin-dependent bacterium, is the etiologic agent of Johne's disease (JD) in cattle. The organism has been also isolated from sub-human primates suffering from chronic ileitis and from patients with Crohn's disease (CD) in humans. Both JD and CD are chronic devastating granulomatous intestinal syndromes. The JD causes enormous economic losses for the dairy industry. The office International des Epizooties (OIE) considers this disease of major global importance and categorizes the disease as list B transmissible disease. The OIE considered it of socioeconomic and public health importance within countries and is significant for the trade of animals and animal products (OIE manual 2008). Various laboratory diagnostic techniques were developed to be used for the diagnosis of JD including immunological approaches (Manning *et al.*, 2003). In the present investigation, Ziehl-Neelsen stained fecal smears were examined micro-scopically and revealed that 90/198 (45.5%) were positive for acid fast bacilli. Nevertheless, only 16/198 (8%) were positive when cultured in HEYA media and then colonies were confirmed by PCR. Those 16 samples showed positive acid-fast bacilli when stained with ZN in the previous step. The disadvantage of applying ZN directly on fecal samples is that it does not differentiate among

Table 1: Positive MAP cases using different techniques. Samples were collected from five regions in Egypt

Governorate	Alexandria	Ismailia	Kafr El-Sheikh	Gharbia	Giza	Total	%
Total No. of collected specimens	100	18	25	28	27	198	100
No. of cases positive by ZN	50	4	11	13	12	90	45.4
No. of cases positive by culture in HEYA	7	1	2	2	4	16	8
No. of cases positive by PCR using IS900	20	1	5	4	5	35	17.67

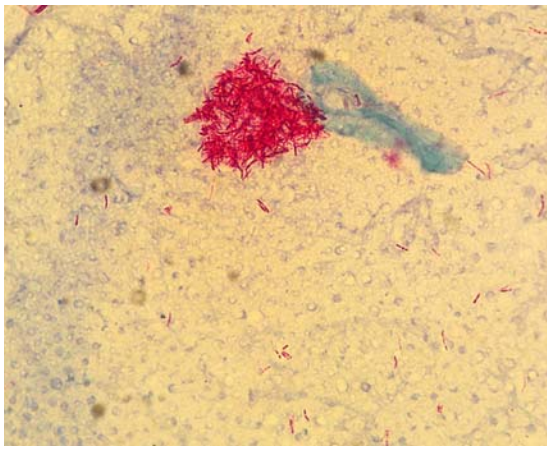

Fig. 1: ZN stain showing red acid fast bacilli

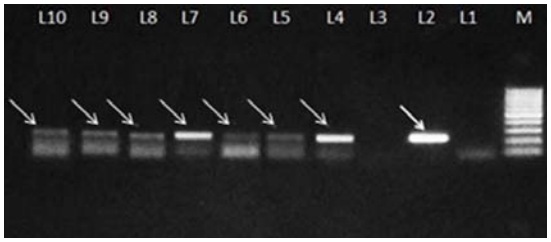

Fig. 2: M: Marker (1000 bp); L1: Negative control; L2: Positive control (DNA); L3: Negative sample; L4 to L10: Positive samples; **Notes:** 1. Arrows point out the bands of PCR products; 2. The bands differ in intensity due to the differences between strains

other atypical rapid growing mycobacterial species and this may explain also why cases contained acid-fast bacilli were culture negative on HEYA media (OIE manual 2008).

On the other hand, although fecal culture is actually considered the "gold standard" for the diagnosis of bovine paratuberculosis for its good specificity, its extensive use in laboratory diagnosis of the diseases has been hampered due to the long incubation period (Paolicchi 2003, 32). The long generation time of *M. paratuberculosis* requires up to 20 weeks to produce visible colonies on solid medium composed mostly of Herrold egg yolk (HEY) agar with mycobactin (Kim *et al.,* 2004). This creates management problems for producers who need to make quick decisions for the purchase of replacement animals or to cull infected animals from their herd. However, the culturing method remains the gold standard despite its logistical and practical limitations (Douarre *et al.,* 2010). In recent years, advances have been made in the improvement of methods for the detection of *M. avium* subsp. *paratuberculosis* DNA by PCR (O'Mahony and Hill 2002, Kim *et al.,* 2002, Fang *et al.,* 2002, Christopher-Hennings *et al.,* 2003, Khare *et al.,* 2004, Taddei *et al.,* 2004). The main advantage of such technique is the rapid detection and confirmation of MAP (2-3 days). Therefore, when PCR was applied on the fecal specimens under investigation, to detect IS900 gene, the positive cases were 35/198 (17.67%). IS*900* primer was used in the reaction because the IS*900* element is present at approximately 17 copies in the genome (Christopher-Hennings *et al.,* 2003), and PCR that detects this element thereby has a higher level of sensitivity of detection than tests that detect other genes. The 35 PCR positive samples were also positive when stained with ZN. Therefore, from the obtained results, application of ZN stain on fecal samples from clinically suspected cases should be occurred, and then PCR targeting IS900 gene should be applied on those which contain acid-fast bacilli to ensure rapid and accurate laboratory diagnosis of MAP. The use of serological methods was eluded in the present study because of the doubtful and the lack of reliance and accuracy in their results and their inability to identify subclinical cases (Harris and Barletta 2001, Stabel *et al.,* 2002, Huda and Jensen 2003, Muskens *et al.,* 2003).

It is worth mentioning that although JD could be found in many countries worldwide, the available data on the incidence and prevalence of the diseases in Egypt are limited and very few reports regarding JD had been published until now (Salem *et al.,* 2005). Thus, the present article can add to our knowledge the information on the presence of JD in Egyptian dairy cattle.

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