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LCD array and IS900 efficiency in relation to traditional diagnostic techniques for diagnosis of *Mycobacterium avium* subspecies *paratuberculosis* in cattle in Egypt

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ABSTRACT

This study aimed to compare traditional tests (Johnin test, fecal staining and fecal culture) with advanced laboratory tests (ELISA, LCD array and IS900 PCR) for detection of Johne's disease. A total of 365 Holstein–Friesian dairy cattle (40 express profuse diarrhea unresponsive to treatment and 325 contacting them) tested with Johnin test, blood collected for ELISA and fecal samples for fecal staining as well as fecal culture, application of LCD array and PCR using IS900 on DNA extracted from *Mycobacterium paratuberculosis* bacilli (from feces and culture). Johnin test was 40/40 (100%) and 25/325 (7.69%), fecal staining was 13 (37.1%) and 2 (50%), ELISA was 35/40 (87.5%) and 4/25 (16%) for clinical cattle and apparently healthy contacting them respectively. Isolation was 12/13 (92.3%) of the (Johnin test +ve, ELISA +ve and Acid Fast Bacilli +ve) from the clinically positive cattle and 1/2 (50%) of the (Johnin test +ve, ELISA +ve and Acid Fast Bacilli +ve) from apparently healthy contacting them while LCD array and IS900 gave 100% confirming the isolation results. In conclusion, LCD array depending on 16S RNA and DNA hybridization with specific probes for detection of *M. paratuberculosis* are fast, sensitive and labor-saving when combined with IS900.

Introduction

Mycobacterium avium subsp. *Paratuberculosis* (MAP) is the causative agent of Johne's disease (JD) in cattle, an inflammatory bowel disease leading to profuse diarrhea, reduced milk yields and loss of livestock [1]. In addition, dairy producers suffer losses from repeated testing, culling false-positive animals and lost cow longevity. The annual economic impact to the dairy industry is estimated at $276 per cow [2]. Paratuberculosis is recognized as a major problem in livestock production systems–zoos, those in captivity and free-ranging wildlife. It can be found worldwide [3], and furthermore, it is highly likely to be the potential etiological agent of Crohn's disease [4].

In Egypt, JD is on the rise in domestic livestock. There were no reports on the subject until a study was done covering five regions and infection was present in 51% of the clinically sick and 11% of the apparently healthy cattle based on Ziehl–Neelsen staining of fecal samples, culture and PCR [5].

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JD may be classified into four stages. Stage I: silent infection (no evidence of disease, test positive or shedding). Stage II: subclinical disease (no evidence, shedding intermittent, and test positive or negative). Stage III: clinical disease (early signs of the disease, test positive, shedding). Stage IV: advanced clinical disease. Hence, the disease still presents a diagnostic challenge until the animal has progressed to stage III or IV of the disease [6].

The clinical presumptive diagnosis of paratuberculosis may be confirmed by the evaluation of cell-mediated immune response by a single intradermal Johnin test [7], the detection of interferon gamma test IFN-γ [8], the detection of MAP in fecal samples and the detection of antibodies against MAP [9]. The transition from a predominately cell-mediated immune to humoral response against MAP occurs at the end of stage II, and precedes the onset of clinical signs [10]. There are many humoral response-based tests such as Complement Fixation (CF) and agar gel immunodiffusion (AGID), but not as sensitive as ELISA [11].

MAP isolation begins with decontamination of fecal samples with Hexadecyl Pyridinium Chloride (HPC) 0.75% for 16–24 h and cultivation on Herrold's egg yolk medium (HEYM) that is widely used, but it requires incubation for 2–4 months [12]. Mycobactin dependency is one of the most discerning properties of MAP [13]. Traditional diagnostic approaches relied upon staining Acid-Fast Bacilli, isolation and identification using biochemical analysis; this process is considered time-consuming, labor intensive and sometimes lacks sensitivity and specificity.

Detection of mycobacteria in clinical specimens by PCR amplification of 16S rRNA and then screening was done with genus highly discriminating probes or nucleic acid sequencing considered an efficient strategy to detect and identify multiple mycobacterial species [14]. IS900 is a unique MAP gene [15]. This insertion sequence is a 1451 bp repeated 15–20 times in the MAP genome [16]; 97% of DNA homology was recorded amongst most isolates of MAP, and *M. avium* subspecies *avium* has been reported [17]. Sequences related to IS900 were found in Wood Pigeon mycobacteria (IS902) and *M. avium* subspecies *avium* (IS901). IS1626 (found in *M. avium* subspecies *avium* and *Mycobacterium intracelluare*) is another closely related insertion sequence of IS900 [18]. The f57 sequence is specific for the JD agent and is not found in any other mycobacterial species. It is a 620 bp [19]. In this study, detection of *Mycobacterium paratuberculosis* from infected cattle and a comparison among traditional diagnostic techniques like Johnin test, fecal smears and isolation, as well as advanced diagnostics such as ELISA, DNA hybridization and PCR using IS900, was undertaken.

Material and methods

Johnin and ELISA testing of animals

365 Holstein–Friesian dairy farm cattle presenting with profuse diarrhea unresponsive to treatment were subjected to a single intradermal Johnin test [7] by injecting 0.1 ml of Johnin (ID-Lelystad, the Netherlands) on the mid-neck area; skin thickness was measured immediately before and 72 h after injection after approval from farm owners (testing animals in compliance with the ethics of the International Guiding Principles for Biomedical Research Involving Animals [1985]). An increase in skin thickness of $\geqslant$4 mm and appearance of edema and pain on palpation of the site of injection were considered as a positive reaction. ELISA test using 4 plates ready to use (Prionic USA ELISA kits) was applied (upon serum samples collected from all cattle before Johnin injection) according to the manufacturer; the results were measured using a Biotek ELx800 reader at 450 nm, and cases considered suspected positive when being Johnin and ELISA positive.

Fecal smears and fecal culturing

Three grams of fecal samples collected from Johnin and ELISA positive cases were mixed with 30 ml of 2% HPC solution homogenized and shaken for 30 s and then incubated overnight at room temperature. The supernatant was slowly discarded and 200 μl from the sediment using a sterile platinum loop was used to inoculate two tubes of Herrold's egg yolk medium (HEYM) media; one tube contained mycobactin J (1 mg/l) and the other tube was without mycobactin J. There were then incubated at 37 °C [12]. Smears were also made from the decontaminated fecal samples and examined according to [20] Acid Fast Bacilli (AFB) scoring criteria: no AFB in at least 100 microscopic fields was scored as negative (−); 1–9 AFB in 100 microscopic fields was scored as doubtful (?); and 10–99 AFB in 100 microscopic fields was scored as positive (+).

DNA extraction and molecular examination

DNA extraction was obtained from decontaminated fecal samples and from positive cultures (the extraction was mainly performed by QIAamp DNA extraction Mini prep Kit for extraction of DNA, especially from Gram-positive bacteria). The decontaminated fecal samples were transferred to a screw-capped centrifuge tube and then centrifugation was commenced for 10 min at 5000*g* (7500 rpm) until the bacteria formed a whitish thin layer upon the concentrated fecal sample. Then the bacterial interface was pipetted from this formed layer and washed three times with PBS with alternative centrifugation, and then it was pelleted by centrifugation for 10 min at 5000*g* (7500 rpm); the bacterial pellet was then re-suspended in 180 μl Buffer ATL (supplied in the QIAamp DNA Mini Kit). Also from the obtained cultures, three colonies were collected and re-suspended in 180 μl Buffer ATL and the manufacturer's protocol for extraction was followed. DNA hybridization is mainly utilized for the preliminary detection of the presence of *M. avium*; this processed is performed by using MYCO Direct1.7 LCD-Array Kit DNA based identification of *Mycobacterium tuberculosis complex* (MTUB) and other Mycobacteria (MOT); these kits are mainly dependent upon the utilization of two primer sets. Primer Mix A: Genus Amplification of rRNA gene region (ITS) from most members of the genus Mycobacteria Fragment size: 265–225 bp dependent on the species. Primer Mix B: TUB Amplification of a fragment from the repetitive element IS6110 from members of the