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Phylogenetic Analysis of *Mycobacterium avium subsp. paratuberculosis* of Some Egyptian Isolates Isolated from Clinically Infected Dairy Cattle

Shaymaa Abd El-Malekand and Kh. F. Mohamed

Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt

Abstract: Three Egyptian *M. avium subsp. paratuberculosis* isolates were isolated from feces of clinically affected dairy cattle and had been subjected to phylogenetic analysis using IS*900* gene sequencing in comparison to a standard Japanese strain. The three isolates were cultivated on a modified HEYM supplemented with mycobactin J for 16 weeks, stained with Kinyoun stain and then confirmed using IS*900* PCR. A new Egyptian sequevar of *M. avium subsp. paratuberculosis* (SEQ1) was detected for the first time in Egypt as demonstrated in the phylogenetic tree. The new Egyptian sequevar showed homology of 98.2% in comparison to the standard Japanese strain (AB052552) and 98.7% in comparison to the Netherlandish strain (AF416985). The other two Egyptian sequevars (SEQ2 and SEQ3) were similar to the standard Japanese strain with homology of 100%.

Key word: Cattle · *M. avium* · *Paratuberculosis* · Phylogenetic tree

INTRODUCTION

Paratuberculosis, also called Johne's disease, is a chronic devastating disease affecting ruminants including cattle, sheep, goats and some wild ruminants [1].

The disease is caused by *Mycobacterium avium subspecies paratuberculosis (Map)*, which is among the slowest growing mycobacteria. It requires from 8 to 16 weeks for producing visible colonies on the conventional agar media [1]. The organism is closely related to *Mycobacterium avium intracellulare (MAI) complex* which belongs to the Runyon group III [2, 3].

The clinical symptoms of the disease appear months or years after the beginning of the fecal shedding of the bacilli [4]. These clinical symptoms include chronic or intermittent non-responsive diarrhea, weight loss and emaciation and may terminate with death [5].

The mycobacterial species *M. avium* is currently subdivided into three subspecies: *M. avium subsp. avium (M. avium), M. avium subsp. paratuberculosis* and *M. avium subsp. silvaticum (M. silvaticum)*. The subspecies designation of *M. paratuberculosis* is based on DNA-DNA hybridization studies and numerical taxonomy analysis. Although commonly grouped with *M. avium* in the *Mycobacterium avium intracellulare complex*, *M. intracellulare* is a genetically distinct species. At the subspecies level, *M. paratuberculosis* can be differentiated phenotypically from *M. avium* and *M. silvaticum* by its dependence on mycobactin and genotypically by the presence of multiple copies of an insertion element; IS*900* [6].

In the present work, investigations were carried out on phylogenetic analysis of *Mycobacterium avium subsp. paratuberculosis* of some Egyptian isolates obtained from clinically infected dairy cattle.

MATERIALS AND METHODS

Samples: *Only 3* Egyptian *M. avium subsp. paratuberculosis* isolates had been isolated from feces of clinically affected dairy cattle showing clinical signs of Johne's disease including wasting, chronic or intermittent diarrhea, decrease in body's net weight and emaciation with a bottle jaw.

Mycobacterium avium subsp. paratuberculosis standard Japanese strain (AB052552) was kindly supplied by Dr. Bou (Professor of Microbiology, Harbin Veterinary Research Institute (HVRI) - Beijing - China).

Cultivation of *M. avium subsp. paratuberculosis* from Bovine Fecal Specimens[7]

Samples Preparation: Fecal specimens and suspensions of organisms have been manipulated. Feces were collected rectally from each animal using a clean and dry

Corresponding Author: Kh. F. Mohamed, Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt, E-mail: kelamry@daralfarouk.com.eg.

examination glove. About 10 g of feces were placed into a clean, sealable sample container that had been labeled to permit unequivocal identification of the animal from which the sample was collected. Samples have been packed in an insulated container with refrigerated (not frozen) cool packs for protection from temperature extremes during transport, then sent to the laboratory by overnight delivery. Samples have been processed within 96 hrs of collection. Frozen samples have been rapidly thawed by placing in a 35°C water bath or incubator. Phenolic-based compounds, such as 2% Amphyl, were used as effective disinfectants.

Cultivation on a Modified HEYM: Only 1 g of feces was placed in a 50 ml centrifuge tube with 35-40 ml sterile, deionized or distilled water. Samples were shaken and vortexed to make a uniform suspension. Samples may have been shaken for 30 minutes on a horizontal shaker. The suspension had been set upright and allowed to settle for 30 minutes. Only 5 ml were transferred from the upper half of the supernatant to another 50ml centrifuge tube containing 25 ml of 0.9% HPC (final concentration was 0.75% HPC). Tubes were inverted to mix the sample suspension with decontaminant, then tubes were set upright at room temperature. After overnight (16-24 hours) decontamination at 37°C, centrifugation was used at 2500 xg for 15 minutes. Tubes of HEYM were inoculated with 0.1 ml of the sediment that was formed at the bottom of the sample tube. Inoculated tubes were placed in a horizontal position with loosened caps for at least 1 week at 37°C. When no visible moisture remained on the surface of the medium, caps were tightened and incubation was continued in a vertical position.

Tubes were examined at least once every 4 weeks for at least 12-16 weeks and then examined for small pinpoint creamy white colonies.

The isolates were stained using Kinyoun stain according to Garcia [8] *to show the acid-fast bacilli of Map isolates surrounded with non acid-fast microorg*anisms.

DNA extraction, IS*900* PCR and Electrophoresis [9]: The colonies were harvested into 1x TE buffer. They were washed 3 times. The supernatant was removed; pellet was taken and suspended in 1x TE buffer. Heat block was used at 100°C for 10 minutes for DNA release.

Components were mixed in PCR Eppendorf:

Extracted DNA, 2 μl plus 10 μl 2X Master mix and 1 μl of each primer used to amplify 229 bp.

Deionized water up to 20 μl.

Forward: 5'-CCGCTAATTGAGAGATGCGATTGG-3'.
Reverse: 5'-AATCAACTCCAGCAGCGCGGCCTCG-3'.

The electrophoresis grade Agarose was prepared in 1x electrophoresis buffer to reach the required 1.5% concentration. The Agarose was cooked in a microwave with agitation till being clear. The Agarose was allowed to cool, then 0.5 μg/ml ethidium bromide was added. The Agarose was poured in the electrophoresis mould to make 4 mm depth. The comb was inserted and left to solidify. The comb was removed gently. The TAE buffer was poured until covering the gel. The sample was injected with loading dye and sunk in the well. The cathode and anode were matched with power supply at 100 volt. The current was stopped when the loading dye reached 2:3 to the gel. The transilluminator was used to detect the desired 229 bp band.

DNA Sequencing of Positive Local Isolates by PCR and Phylogenetic Analysis (Bioinformatics Analysis) Using Sequence Alignment (DNASTAR Megaalign Software Program by Jotun Hein Method [10]: DNA sequences were aligned via pairwise and multiple sequence alignment algorithms including Jotun Hein and Dotplot analyses. Sub-alignments were easily created by selecting ranges of longer alignments to create neat blocks of similarity or to remove the 'ragged' termini due to length variance in data. Sequences were downloaded directly from NCBI or a database using accession numbers, BLAST and text searches were made to match the highlighted segment of current sequence and to add a search result to the project.

Phylogeny: Evolutionary relationships predicted from multiple sequence alignment were viewed. The length of each pair of branches represented the distance between sequence pairs, while the units at the bottom of the tree indicated the number of substitution events. The Bootstrapping method was used to test for consistency and robustness of a tree created using a Clustal methodology. The branch patterns with higher values were statistically more likely to occur and give a measure of confidence in the optimal tree structure. Sequence similarity and distance were calculated in relation to the constructed phylogeny.

Analysis of Alignment Results: The final alignment was edited and adjusted by inserting and moving gaps. The alignment results were customized by highlighting residues that matched/mismatched the consensus, or other sequences in the project. A custom consensus was defined and the consensus strength was displayed as a

color-coded histogram. The alignments were exported into several popular formats. They were exported as a GenVision project for creation of publication-quality images.

RESULTS AND DISCUSSION

Three isolates obtained on HEYM medium after 16 weeks of incubations at 37°C(Fig. 1). The three isolates were confirmed using Kinyoun's stain showing acid-fast bacilli *of* Map isolates surrounded with non acid-fast microorganisms added *in vitro,* (Fig. 2). IS*900* PCR results confirmed the 229 bp in the three isolates of *M. avium subsp. paratuberculosis* as shown in fig. 3.

Phylogenetic analysis was applied by IS*900* gene sequencing for three isolates and a standard strain using DNASTAR Mega alignment program using J. Hein method (Hein,2001). A new Egyptian sequevar of *M. paratuberculosis* (SEQ1) was detected for the first time in Egypt - as far as it is known - as demonstrated in the phylogenetic tree which is shown in fig. 4. The new Egyptian sequevar showed homology of 98.2% in comparison to the standard Japanese strain (AB052552) as shown in fig.4 and 98.7% in comparison to Netherlandish strain (AF416985) according to the obtained data from the Gene Bank using DNASTAR MEGA 4 alignment program The other two Egyptian sequevars (SEQ2 and SEQ3) were similar to standard Japanese strain with homology of 100% as shown in Fig. 4. The sequence analysis can provide new insights for better understanding the evolutionary events of these isolates, help in improving drugs,vaccines, diagnostics tools and epidemiological tracing for controlling the Mycobacterial diseases [11-14].

Fig. 1: The typical colonial morphology of *M. avium* subsp *paratuberculosis* on HEYM medium supplemented with mycobactin Jshowing small, pinpointed, white, dull and rough colonies

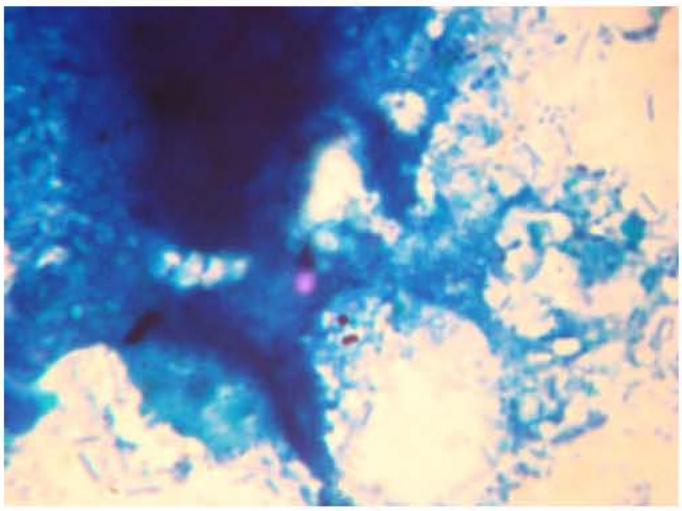

Fig. 2: Acid-fast bacilli showing *Map* isolates surrounded with non acid-fast microorganisms .

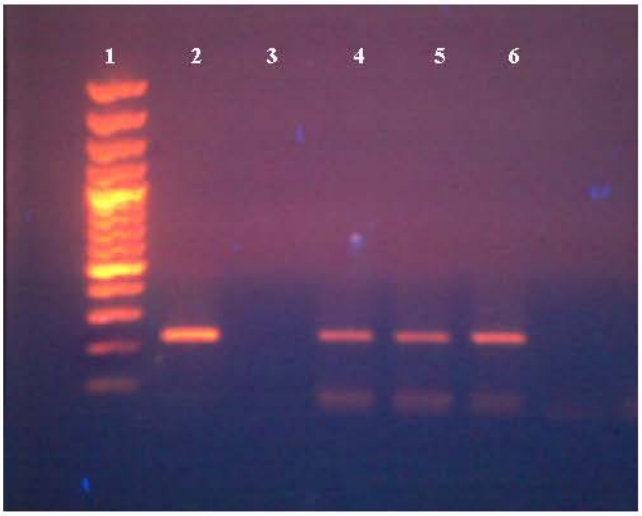

Fig. 3: Gel electrophoresis confirming 3 positive Map using IS*900* PCR and revealing a band of 229 bp.

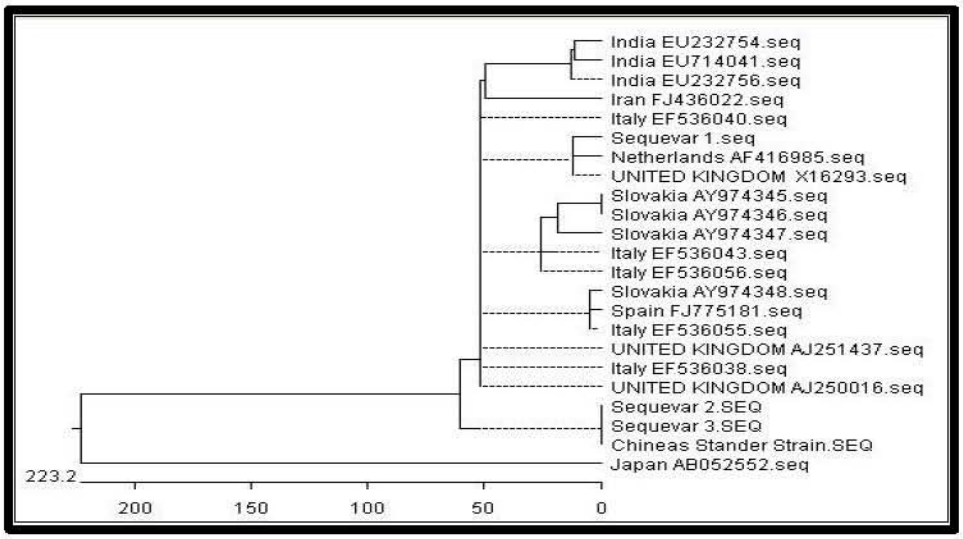

Fig. 4: Phylogenetic tree of *M. avium subsp. paratuberculosis* strains of IS*900* gene sequencing, including relations between the Egyptian isolates and the standard Japanese strain and with correlation with the recorded strains in Gene Bank.

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