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Bacteriological and Molecular Studies on Johne's Disease in Cattle

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ABSTRACT

Paratuberculosis or Johne's Disease (JD) is a chronic and incurable granulomatous enteric disease affecting cattle, buffaloes, sheep, goats and the other ruminants caused by *M. avium* subspecies paratuberculosis (MAP). In this study, A total of 300 cows (240 clinically diseased animals and 60 apparently healthy animals) were examined for the presence of clinical signs of johne's disease including incurable chronic diarrhoea, interment firstly then intense and continuous which is not responding to treatment, emaciation and progressive weakness. Fecal and serum samples (each of 300) were collected from the examined cows housed in 3 Egyptian Governorates (Sharkia, Kalyoubia and Giza). Fecal samples were collected then examined according to the pooling procedure and decontaminated by Hexa decylpyridinium chloride solution (HPC 0.9% prior to culturing on Herrold's Egg Yolk Medium (HEYM). MAP was isolated from 34 of the 60 pooled fecal samples tested (57%). Fecal smears were examined using Ziehl- Neelsen stain (ZN) for the presence of acid fast bacilli revealing 29 fecal smears (48%) of 60 fecal smears were positive. ELISA was conducted on serum samples to detect antibodies against MAP, 212 (71%) of serum samples were positive for antibodies against MAP. Molecular confirmation by PCR IS900 assay was carried out using specific primers directly on fecal sample, Out of the 60 pooled fecal samples, 45 pools (75%) were positive.

A phylogenetic analysis for determining the genetic difference between current infection and other infected strains in other localities was conducted.

INTRODUCTION

Johne's disease is a chronic infectious enteric disease of ruminant where *M.avium* subspecies

Paratuberculosis is the causative agent of the disease. It is a slow growing, Gram-positive and acid-fast bacterium that requires several months of incubation **(Norton et al. 2010)**. The infection is typically characterized by long incubation period followed by chronic progressive diarrhoea, decrease in milk yield, submandibular edema, anemia, loss of weight leading to cachexia and finally to death **(Cocito et al. 1994 Harris and Barletta 2001).** Animals are infected by ingestion of food and water contaminated by feces of infected animals **(Ahmed et al. 2019).** The intrauterine transmission of MAP may occur in cattle and could

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represent an important source of infection **(Belo-Reis et al. 2016).** The incidence of subclinical cases shedding organisms intermittently may be as high as 15%.Calves are susceptible but don't show signs until adulthood. JD is ranked as a notifiable disease in many countries **(Kennedy 2001).** The prolonged incubation period of the disease (2-10 years) and the act that many animals may harbor the organism without ever showing clinical symptoms have made the diagnosis of latent cases difficult. Meanwhile, the lack of any specific chemotherapeutic agent, together with the vaccination drawbacks has made the disease particularly difficult to control. **(Mckenna et al. 2006 and Barry et al. 2011)**. Studies are carried out mostly on cattle, so little is knownabout paratuberculosis in buffalo, Hence a more comprehensive studies of MAP in buffaloes is of a great value to facilitate the design of prevention and control programs. Although the diagnosis of JD remains difficult; there is a wide range of tests for diagnosis of johne's disease. Bacteriologic fecal culture remains the most definitive test for identification of both subclinically and clinically infected animals. This fecal culture is time consuming , requiring a long incubation period of 8 to more than 16 weeks for bacterial recovery. This causes problems for producers who need to make quick-decisions for the purchase or replacement animals or to cull the infected animals from their herds (**Abd-E l Malek S. et al. 2015**) The sensitivity of the Ziehl Neelsen test has been estimated at 49% (**Zimmer et al. 1999)** in clinically affected animals. The specificity of the ZN -test was estimated at 83% **(Ris et al. 1988)** in fecal culture -negative animals from paratuberculosis free herds. Concerning control programs, ELISA is widely used screening assay for the detection of anti-bodies against MAP in blood or milk because of their advantages like easy performance, high capacity and low costs. PCR technique is a more rapid test which could be an alternative to the time consuming traditional cultural method **(Douarre et al. 2010).** PCR test has led to increase specificity and sensitivity of detection of low shedders. **(Collins M.T. and Sockett D.C. 1993 Whittington 2001 Mason et al. 2001 and Behr and Collins 2010)**. Mycobacterium avium sub species paratuberculosis may have public health importance as clinical similarities have been observed between Johne's disease in animals and Crohn's disease (CD) in humans and both viable MAP and MAP genetic material have been found in some patients diagnosed with Crohn's disease despite almost 100 years of investigation, conflicting data still occur and the responsibility of MAP as the etiological agent of CD was proven in some reports and denied by others. M.avium subspecies paratuberculosis has been isolated from Crohn's patient tissue world-wide. Inspite of that , many laboratories are still reporting their inability to culture it at all , as some human isolates may take up to 6 years to grow even under standard culture and de-contamination conditions **(Collins et al. 2000 and Parrish et al. 2008).**

This study aimed to through light on paratuberculosis in Egyptian buffaloes as there is lack of data about this disease in Egypt and it was achieved through isolation of MAP on HEYM's medium from pooled fecal samples, identification of the MAP organism by Ziehl Neelsen stain of fecal smears, detection of antibodies to MAP in serum samples by indirect ELISA and IS900 PCR technique. as well as to construct a phylogenetic analysis for determining the genetic difference between current infection and other infected strains in other localities.

2. MATERIALS and METHODS:

2.1. Animals

Animals were suffering from incurable chronic diarrhoea, interment firstly then intense and continuous which is not responding to treatment, emaciation and progressive weakness and apparently healthy animals went through general clinical examination parameters including rectal temperature, heart rate, respiratory rate and body condition score.

2.2. Samples:

A total of 600 random samples (300 fecal samples and 300 serum samples) were collected from Three Governorate (Sharkia, Kalyoubia and Giza). Fecal samples were collected and either directly proceesed or kept frozen in -20◦C till processing.

Blood samples were drawn in serum vacutainer without anticoagulant and kept in icebox

waiting to be sent to the laboratory. Separated serum was kept frozen in (-20 ◦C) till examination.

2.3. Pooling, Decontamination and cultivation procedures:

Fecal samples were examined on the basis of a strategic pooling procedure **(Kalis et al. 2000).**

The pooled fecal sample was decontaminated according to **(Fernàndez-Silva et al. 2011).** Then inoculated onto Herrold's egg yolk medium (HEYM) slants supplemented with mycobactin J (Oxoid) Slants were incubated at 37◦C in horizontal position for 1 week with the caps loosened to allow ab-sorption and evaporation of residual moisture on the surface of the medium. Then, caps were tightened and the tubes were returned to vertical position and incubated for 8-16 weeks and checked at 1-2 weeks intervals. The tubes were being checked for growth of gray white rough colonies. Smears were taken from the suspected samples for microscopical examination

2.4. Identification of MAP by Ziehl - Neelsen staining of direct fecal smear Fecal

It was conducted according to **(Australian and New Zealand standard diagnostic procedures 2002).**

2.5. Indirect ELISA for detection of MAP antibodies in serum samples:

The presence of MAP antibodies was detected in serum samples using the PARACHEK2 (Mycobacterium paratuberculosis test kit), (Prionics AG Wagistrasse 27a-8952 Schlieren-Zurich, Switzerland) .

2.6. Molecular MAP confirmation by using PCR IS900:

DNA extraction was carried out using QIAamp DNA stool Mini Kit **(Qiagen, Germany).** Nucleotide sequences of PCR primers for detection of MAP-specific IS900 were defined in table (1).

Table (1) Nucleotide sequences of PCR primers for detection of MAP-specific IS900

Target gene	Oligonucleotide sequence (5'-3')	Expected amplicon size (bp)
IS900	F:CCG CTA ATT GAG AGA TGC GAT TGG R:AAT CAA CTC CAG CAG CGC GGC CTC	229 (Vary et al.,1990)

Amplification of IS900 MAP was performed on Bio-Rad T100 Thermal cycler (Hercules, CA, USA) with a 60 ng of DNA template, 12.5 µl of Emerald master mix **(Takara, Japan)** and 2 µl of forward and reverse primer for MAP IS900. PCR was started with an initial denaturation step at 94ºc for 10 minutes, then followed by 35 cycles of denaturation for 60 seconds at 94 ºc, annealing for 90 seconds at 61 ºC and extension for 60 seconds at 72 ºC. PCR products were then run on gel electrophoresis (2%) for visualization of the amplified PCR product. The required product size was optimized using a 100bp DNA ladder (Jena bioscience, Germany).

2.7 Sequencing and analysis :

Purification of DNA fragment was carried out with Gene JET Gel extraction kit (Thermo Scientific, Lithuania). The purified DNA products were then sent for sequencing in 3130 X DNA Sequencer (Genetic Analyzer, Applied biosystem, Japan) with both forward and reverse primer used in PCR.

The obtained nucleotide sequence was aligned, trimmed with the aid of Clustal W that was integrated with MEGA 6 softwar. The trimmed DNA samples were checked on Genbank for determining similarity index between other species of MAP on Genbank.

3. RESULTS

3.1. Results of Mycobacterium avium sub-species paratuberculosis Isolation:

MAP was isolated from 34 of the 60 pooled samples tested (57%). After 16 weeks of incubation, Typical colonies of MAP were observed; very small, convex (hemispherical), soft, non-mucoid and initially colorless and translucent.

Table (2) Results of isolation of fecal culture of MAP on HEYM

Governorate	No. of pooled fecal samples		Total No. of pooled fecal samples	No. and percentage (*) of Positive culture		Total No. and percentage of positive culture
	Diseased animals	Apparently healthy		Diseased	Apparently healthy	
Sharkia	16	4	20	12 (60%)	1(5%)	13(65%)
Kalyoubia	16	4	20	9 (45%)	1 (5%)	10 (50%)
Giza	16	4	20	11 (55%)	0 (0%)	11(55%)
Total	48	12	60	32	2	34 (57%)

(*) percentage was calculated according to the total number of the pooled fecal samples.

3.2. Results of Microscopic examination of Ziehl-Neelsen stained fecal smears:

MAP showed red acid alcohol-fast bacilli. Stained direct fecal smears revealed 29 (48%) of 60 pooled samples were positive as shown in table (3) .

Table (3) Results of ZN fecal smear staining :

Governorate	No. of pooled samples		No. and Percentage (*) of positive ZN stained fecal smear		Total No. of Positive ZN stained fecal smear and Percentage (%)
	Diseased animals	Apparently healthy animals	Diseased animals	Apparently healthy animals	
Sharkia	16	4	9 (45%)	1 (5%)	10 (50%)
Kalyoubia	16	4	8 (40%)	0 (0%)	8 (40%)
Giza	16	4	10 (50%)	1(5%)	11 (55%)
Total	48	12	29		29(48%)

(*) percentage was calculated according to the total number of the pooled fecal samples.

Table (4) Results of indirect ELISA test:

Governorate	No. of tested serum samples		Total No. of serum samples	No. and percentage (*) of Positive ELISA		Total No. and percentage of Positive ELISA
	Diseased	Apparently healthy		Diseased animals	Apparently healthy	
Sharkia	80	20	100	70 (70%)	0 (0%)	70 (70%)
Kalyoubia	80	20	100	60 (60%)	1 (1%)	61 (61%)
Giza	80	20	100	80 (80%)	1 (0%)	81(81%)
Total	240	60	300	210	2	212 (71%)

(*) percentage was calculated according to the total number of the pooled fecal samples.

3.4. Results of IS900 PCR:

Out of 60 pooled fecal samples, 45 pools (75%) as shown in table (4). Representative positive samples are shown in figure (1) were L2 and L3 (from Sharkia), L4 and L6 (from Kalyoubia) , L7 and L8 (from Giza).

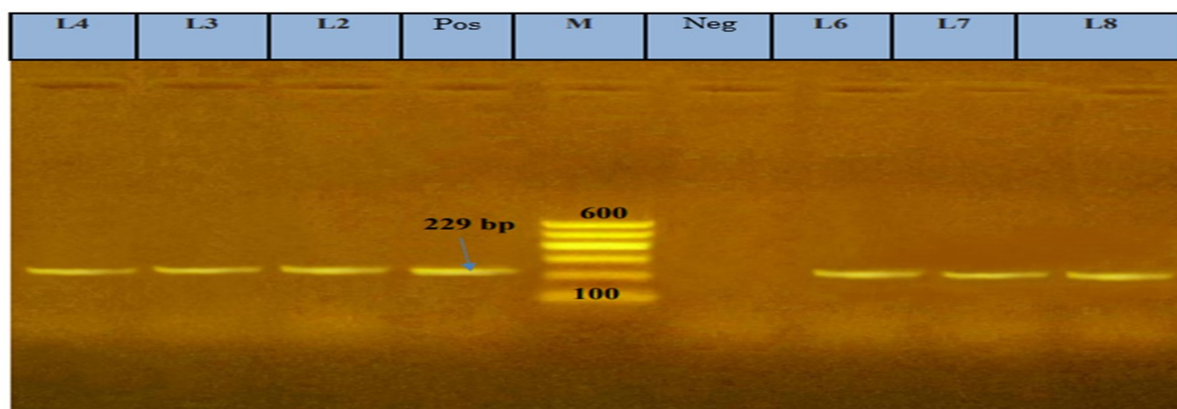

Figure (1) Showing results of IS900 PCR amplicons of fecal samples
M: Marker 100pb. Pos: Control positive . L2, L3, L4, L6, L7 and L8: Positive samples.
Neg: Negative control.

Table (5) Results of amplification of MAP IS900 gene by using PCR:

Governorate	No. of examined samples			Positive PCR (N)		
	Diseased animals	Apparently healthy animals	Total No. of Examined samples	Diseased animals	Apparent healthy animals	Total No. of positive PCR
Sharkia	16	4	20	14 (70%)	1 (5%)	15 (80%)
Kalyoubia	16	4	20	12(60%)	2 (10%)	14 (70%)
Damietta	16	4	20	15 (75%)	1 (5%)	16 (80%)
Total	48	12	60	41	4	45 (75%)

(*) percentage was calculated according to the total number of the serum samples in each governorate.

3.5. Results of IS900 sequencing:

The amplification of MAP DNA samples was sequenced, matched and deposited on gen-bank with accession number of MH663496 and named as Mycobacterium avium subsp. par tuberculosis Egy 1. The aligned MAP species are (KY587112, KT075353, KT075351, KT075350, MH663496, KJ173784, JQ837281, EU130943, EF514831, EF536056, EF536046, S74401, AF416985, AF305073, AJ250018, AJ011838, AB052552, AJ250023, AJ251434, AJ250022, X16293, EF536047, AY974347, KT275243, EF536041, FJ775182, AY974346, JN983503, EU232747, EF514825, AF455252, EF536048, EU714039, EF514828, KJ173781, EU232756,EU714041, EU232748, HQ830160, EU057153,EU057170, EU714040, EU232755, KJ882903, HM172613, GQ144322). It showed a 100% identity with MAP strain KVAFSU_S196. Phylogenetic tree (Fig. 2) was divided into two clades (clade A and B) where our isolated species of MAP was located in subclade B among other related species of *M.avium* (MAP strain KVAFSU_S196, MAP strain 25, MAP strain steroyl ACP desaturase, MAP strain B42, MAP strain 25C) that was isolated from India. Moreover, pairwise distance showed 0.0009 with our isolated strain. The Indian strain and our Egyptian strain were shown a close genetic distance with high node support. The highest pairwise difference was observed in JQ837281 (1.3 EU130943 (1.4), S74401 (1.3), AJ011838 (1.3) and KT275243 (1.4). Those species were located in different localities in Australia, India, United Kingdom and in Egypt..

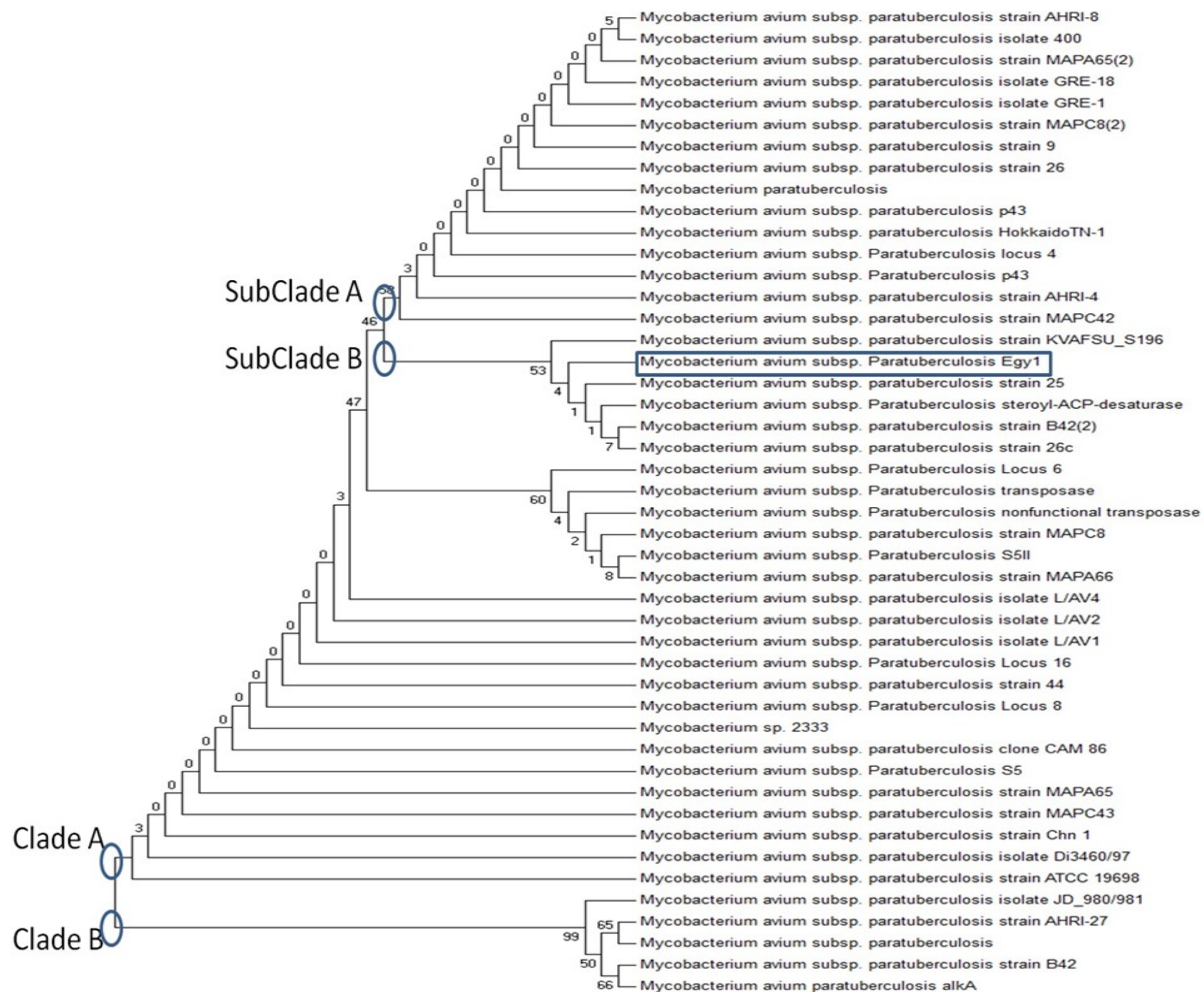

Fig 2 Phylogenetic analysis of different strains of MAP infection with our isolated strain "blue rectangle". phylogenetic tree was divided into two clades; clade A and clade B. Clade A was subdivided into two sub clades A and B where our isolated sequence (Mycobacterium avium subsp paratuberculosis EGY1) was located (sub-clade B)

DISCUSSION

Johne's disease (JD) is caused by the bacterium Mycobacterium avium subspecies paratuberculosis. Infections normally affect ruminants but have also been seen in a variety of nonruminant species, including rabbits, foxes, birds, horses, dogs, and non-human primates have been infected also. JD primarily affects the intestine of all wild and domestic ruminants including cattle, goats, sheep, camels, Ilamas, giraffes, bison, buffaloes, deer and antelopes (**Whittington et al. 2011) and (Kamel et al. 2016**). The disease has been also recorded in a variety of non-ruminant species including rabbits, foxes, birds, horses, donkeys, dogs, cats, pigs, and non-human pri-mates **(Beard et al. 2001 Glanemann et al. 2008 Stief et al. 2012 and Ahmed et al. 2019**). Clinical signs of JD including wasting, chronic intermittent diarrhoea ,emaciation, decrease in net body weight and milk yield. Animals are infected by ingestion of food and water contaminated by feces of infected individuals **(Philip Rasmussen et al. 2021).** Shedding of infectious agent usually occured from clinical cases in spite of the incidence of subclinical cases shedding organisms intermittently may be as high as 15%.Studies are carried out mostly on cattle. Understanding the dynamics of the disease is the key element of control as there's lack of practical therapeutic approaches and lack of a vaccine that prevents transmission and the complexity and difficulty of the on farm control strategies genders the prevention of infection **(Behr and Collins 2010).** More recently evidence has accumulated for an association of MAP with Crohn's dis-

ease in humans, adding to the pressure on animal health authorities to take precautions to control paratuberculosis. JD has been reported in almost every continent. Studies from various parts of the world have shown the widespread distribution of the disease. It is mainly a problem reported in Australia, Canada, Argentina, USA, Mexico, Brazil, New Zealand, Denmark, Belgium, Norway, Switzerland, Netherlands, France, Spain, Germany, England, Scotland, Ireland, Italy, Greece, Thailand, India, Japan, Saudi Arabia, Iran, Egypt, Morocco and South Africa. However, Sweden and some states in Australia are the only regions that claimed to be free from the disease (**Bauerfeind et al. 1996 Moreira et al. 1999 Pavlik et al. 1999 Machackova et al. 2004 Salem et al. 2005 Palmer et al. 2005 and carvalho et al. 2009**). Fecal culture is the most practical mean of confirming infection in herd screening programs. Although fecal culture is technically difficult and time consuming (**Van schaik et al. 2003**). The pooling of fecal samples from more than one animal for culture is a logical way to reduce the cost of detection of MAP at herd level and this observation is in accordance with that mentioned by (**Whittington et al. 2001 Van Schaik et al. 2003 and Kalis et al. 2004).**
Current diagnostic tests consider the culture as the gold standard method (**Paolicchi et al. 2003).**

One of the goals of this study was to evaluate diagnostic methods for MAP diagnosis in cattle. The tests used to diagnose MAP were carried out in parallel to each other. Pooled fecal samples were cultured using Herrold's egg yolk medium (HEYM) supplemented with mycobactin J as the egg based media (LJM & HEYM) are the first choice for culture of clinical and environmental samples (**Moravoka et al. 2012).** The result of the culture was 34 positive slants of the 60 pooled fecal samples tested (57%) which were collected from three Egyptian governorates (Sharkia, Kalyoubia and Damietta). After 16 weeks of incubation on herrold's media containing egg yolk slope, Positive slants show typical colonies of MAP; very small, convex (hemispherical), soft, non mucoid and initially colorless & translucent. Colonies become bigger, more raised, opaque, off-white cream to buff or beige coloured as incubation continues. Negative slants should not be disposed until 10 months pass as some slants, took about 9 months to observe typical colonies. and our result was almost near the findings of **Gamaal (2014),** as she reported the result of culture was 34 of the 50 pooled samples tested (68%). The low sensitivity of fecal culture could be due to intermittent shedding of causative agent in the feces (**Stabel 1997)** or due to substantial decrease in the bacterial load during specimen decontamination protocol (**Reddacliff et al. 2003)** and as reported by **Visser (1999)** fecal culture may not necessarily give positive results in animals shedding low numbers of MAP in their feces. Stained fecal smears using Z.N stain were examined microscopically and revealed that 29 (48%) of the 60 pooled samples were positive as it showed the presence of acid fast bacilli at microscopic examination. Despite the noticeable low figures of sensitivity and specificity of ELISA (Enzyme-linked immunosorbent assay). It is considered as the method of choice for the detection of J.D. positive herds. This is due to the suitability of samples collection, rapid laboratory turnaround time. Low cost, and possibility to test a large number of samples in a short time (**Muskens et al. 2003 Böttcher and Gangl 2004 Collins 2011).** In this study, Serum samples of diseased individuals and apparently healthy animals were examined by indirect ELISA for detection of antibodies against MAP where 212 (71%) out of 300 serum samples were positive and 88 serum samples were negative at a percentage of (29%) . All individual animals which were positive ELISA for MAP were in the positive pooled fecal cultures. (**Weber et al. 2009)** reported that the result of positive ELISA. serum samples were 57%. The use of ELISA in longstanding infected herds reveals reasonable sensitivity as the antibodies become more abundant in later stages of the disease (**McKenna et al. 2006).** The specificity of the test is markedly increased by the absorption with sonicates from other mycobacteria like Mycobacterium phlei in the so-called absorbed ELISA where specificity reaches 99-100% and sensitivity reaches 53-55% (**Böttcher and Gangl 2004 and Shin et al. 2008).**

El Gedawy et al. (2015) recommended the

use of both fecal culture method and indirect ELISA as laboratory methods in diagnosis and control programmes of paratuberculosis. In this study, Molecular MAP confirmation was done by Conventional PCR technique using specific IS900 primers. Recently, the PCR technique as a powerful tool in the microbiological diagnosis of different pathogens led to its extensive application in the diagnosis of JD .The IS900 is commonly used as an abundant reference marker for the molecular detection of M. avium subspecies paratuberculosis (**Herthnek and G. Boelske 2006).** Many publication considered the PCR assay as rapid and powerful tool to amplify DNA of MAP and as a confirmatory test (**Whittington, 2001 and Mason et al. 2001).** In this study, From Each governorate, 2o pooled fecal samples representing apparently healthy cases and pooled fecal samples representing the diseased cases were examined. Out of the 60 pooled fecal samples,45 pools (75%) were positive; as the DNA amplification gave a fragment size of 229 on 1.5% agarose electrophoresis) (photo (1). All positive cultured fecal pools were PCR positive although not all PCR positive samples were culture positive. Reduced sensitivity of PCR results can be due to inefficient extraction of mycobacterial DNA from fecal samples, particularly in low count microbial loaded samples and /or as a consequence of the presence of PCR inhibitors. **(Thornton and Passen 2004).** Also, it is known that fecal samples are very difficult to process and IS900 sequence was detected also in mycobacterial strains other than M. avium subspecies paratuberculosis. **(Englund et al. 2002).** So the specificity of IS900 diagnostic PCR has been questioned since some reports indicated the presence of IS900-like sequences in mycobacterial species other than MAP (**Satoko Kawaji et al. 2020**).

The amplified MAP DNA sequence showed a 100% identity with MAP strain KVAFSU_S196. Phylogenetic tree (Fig. 2) was divided into two clades (clade A and B) where our isolated species of MAP was located in subclade B among other related species of M.avium (MAP strain KVAFSU_S196, MAP strain 25, MAP strain steroyl ACP desaturase, MAP strain B42, MAP strain 25C) that was isolated from India. Moreover, pairwise distance showed 0.0009 with our isolated strain. The Indian strain and our Egyptian strain were shown a close genetic distance with high node support. The highest pairwise difference was observed in JQ837281 (1.3) EU130943 (1.4), S74401 (1.3), AJ011838 (1.3) and KT275243 (1.4). Those species were located in different localities in Australia, India, United Kingdom and in Egypt.

The obtained sequence of IS900 was identical to a sequence that was isolated from India (KY587112), while it was showing a higher difference when compared with other strains from Egypt (KJ173784 and KJ173784), although they were isolated from the fecal matter of cattle. In the two strains from Egypt, our isolated strain of MAP showed 98% similarity index, but was named as transposase sequence where it is responsible for transposition of gene in prokaryotes. Insertion sequence regulates gene transposition . On the contrary, another isolated sample from Egypt (JN983503) was isolated from fecal matter of bovine species would find to share higher than 99% similarity index with our obtained sequence. **Ahmed et al. 2019** concluded that the use of IS900 had been used extensively for studying MAP in domestic animals . PCR positive result of MAP for IS900 can be used correctly in the identification of the causative agent for Johne's disease . Different sequences for IS900 were deposited in Genbank, besides the original sequence isolated from United Kingdom (X16293) with a product size of around 1500 bp which showed a change in a nucleotide sequence of our obtained sequence with a pair wise genetic difference of 0.009, **Philip A Robinson (2020) .**

5.CONCLUSSION:

It could be concluded that the infection with the clinical form has a serious significant impact on the health of cows and the production of Egyptian dairy farms. Studying the disease economic impact might provide a greater impact with an insight into the cost efficiency of detection and screening for subclinical infections, which could help in prevention the new infections and also improve the health and productivity of the herd. The use of both fecal culture method and indirect ELISA as

laboratory methods in diagnosis and control programmes of paratuberculosis is recommended. Molecular MAP studies using specific IS900 primers are commonly used as an abundant reference marker for the molecular detection of M. avium subspecies paratuberculosis.

Further investigations and more studies are needed to understand the dynamics and the epidemiology of the disease, helping in the development of the on farm control strategies to prevent the infection and limit this hidden threat as it cause important economic losses in ruminants losses due to examination, treatment costs, loss of milk production and animals culling. Veterinary authorities should work on offering data about the prevalence of Paratuberculosis on the national scale for the different animal species.

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