Role of *Mycobacterium avium* sub species *paratuberculosis* in persistant diarrhoea in Egyptian buffaloes

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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 buffaloes (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 buffaloes housed in 3 Egyptian Governorates (Sharkia, Kalyoubia and Damietta). 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. This study aimed to through a light on paratuberculosis in Egyptian buffaloes as there is lack of data about this disease in Egypt

1. Introduction

Paratuberculosis or johne's disease (JD) is a chronic and incurable granulomatous enteric disease affecting cattle, buffalo,sheep, goats and the other ruminants caused by *M. avium* subspecies *paratuberculosis* (MAP). 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. The intrauterine transmission of Map may occur in buffaloes and could 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, 2011**). 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 known about 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 (**Shaymaa *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 antibodies 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 *et al.*, 1993; Whittington, 2001; Mason *et al.*, 2001 and Behr and Collins, 2010**).

Mycobacterium avium sub species *paratuber-culosis* 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 worldwide. 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 decontamination 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.

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 3 Governorate (Sharkia, Kalyoubia and Damietta). Fecal samples were collected and either directly proceesed or kept frozen in - 20 C till processing

Blood samples were drawn in serum vaccutainer without anticoagulant and kept in ice

box 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 by weighing 2 g of feces from each of 5 animals into sterile mortar. A small amount of sterile water (approximately 5ml) was added to make it possible to thoroughly mix the 5 samples with a sterile pestle.

After mixing, a 3g aliquot (2 g feces and 1 ml water) was weighed out and transferred to a second sterile mortar (**Kalis *et al.,* 2000)**. The pooled fecal sample was decontaminated with 0.9% Hexadecylpyridinium Chloride solution (HPC) for 24 h. 3 g of feces were added to a 50mL sterile tube containing 30ml of a 0.9% HPC. This suspension was manually mixed by shaking and vortexing and let in vertical position for 30 min at room temperature to allow the precipitation and sedimentation of big particles. Approximately 20ml of the upper portion of the supernatant was transferred to another 50ml sterile tube. Tubes were placed in vertical position in dark place for 24 h at room temperature. Then, centrifugation of the tubes for 10 min by 3000 rpm. (**Fernàndez-Silva *et al*., 2011)**. Supernatant was discarded and Herrold's egg yolk medium (HEYM) slants which are supplemented with mycobactin J (Prepared Culture Media) were inoculated with 300 µl of the decontaminated pellet. The slants were incubated at 37 C in horizontal position for 1 week with the caps loosened to allow absorption 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 **(Fernàndez-Silva *et al*., 2011).**

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

Fecal smears were prepared and air dried for 10 minutes then heat fixed at 60-70ºc for 2hrs .Each slide was flooded with carbol fuchsin and heated gently until a small amount of steam rises .boiling was avoided. The slides were left for 5-10 minutes then rinsed with tap water. Acid alcohol was added for at least 1-2 minutes then slides were rinsed thoroughly with tap water. The slides were flooded with Methylene blue as a counterstain and were left for 1-2 minutes then rinsed with tap water and air dried. The slides were examined under oil immersion lens. (**Australian and New Zealand standard diagnostic procedures, 2010).**

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) Results were interpreted according to manufacturer's instructions.

2.6. Molecular MAP confirmation by using PCR IS900

DNA extraction was carried out   using) **QIAamp DNA stool Mini Kit (Qiagen, Germany)** according to manufacturer's instructions. The PCR was carried out using primers to detect the presence of MAP-specific IS900 DNA (Table-1).

Table (1). Nucleotide sequences of PCR primers

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 G	229 **(Vary *et al.*,1990)**

The amplification product was analyzed using gel electrophoresis on 1.5% agarose gel (**Sambrook *et al*, 1989**) and was subsequently visualized by UV illumination after ethidium bromide staining and was photographed by a gel documentation system.
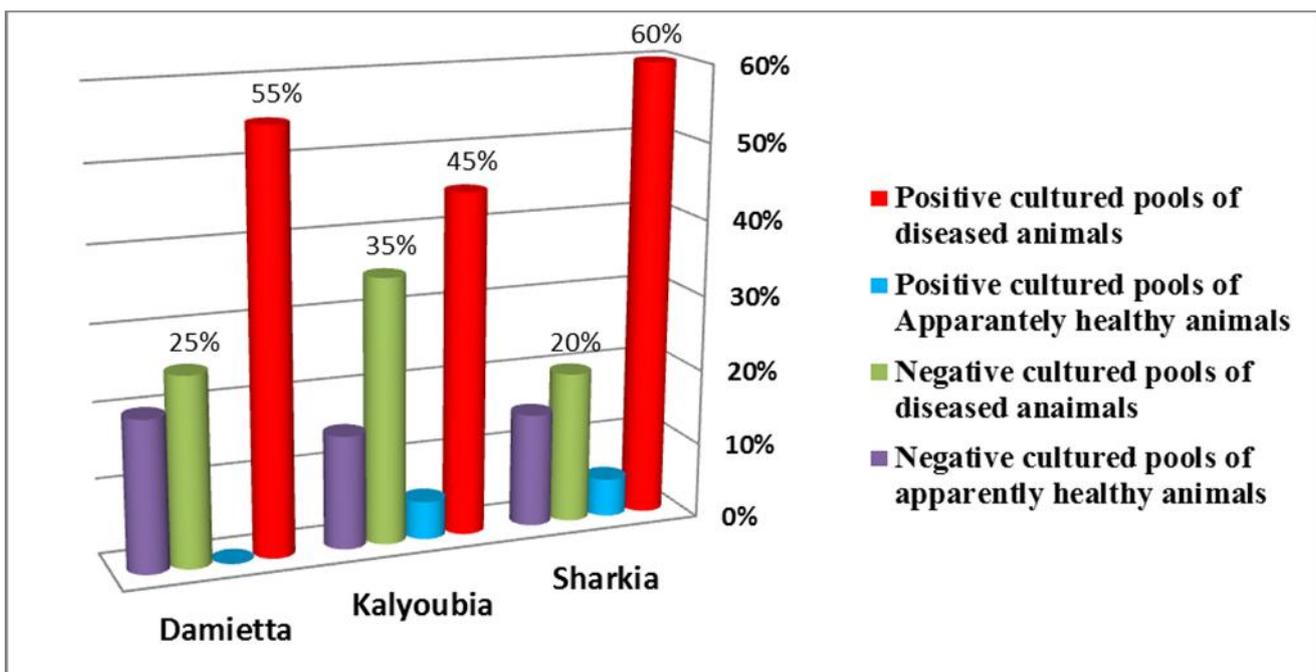
3. Results
3.1. Isolation of Mycobacterium avium subspecies *paratuberculosis*

The culture results for all pooled fecal samples tested during this study are shown in table (1). MAP was isolated from 34 of the 60 pooled samples tested (57%). After 16 weeks of incubation on Herrold's media containing egg yolk slope, Typical colonies of MAP were observed; very small, convex (hemispherical), soft, non-mucoid and initially colourless and translucent. For some slants, it took about nine months to observe typical colonies

Table (1). 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%)
Damietta	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.

Figure (1). Results of isolation of foecal culture of MAP on HEYM

3.2. Microscopic examination of Ziehl–Neelsen stained fecal smears

Microscopic appearance of MAP showed red acid alcohol-fast ZN staining bacilli. Staining of direct fecal smears revealed 29 (**48%**) of 60 pooled samples were positive as shown in table (2)

Table (2). 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%)
Damietta	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.

3.3. ELISA assay results

ELISA was carried out individually to all serum samples of culture-positive animals and culture-negative animals. The ELISA results are shown in table (3). In total, out of 300 serum samples of clinically diseased animals and apparently healthy animals tested using indirect ELISA for detection of antibodies against

Table (3). 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%)
Damietta	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. Amplification of MAP IS900 gene by using PCR

Out of 60 pooled fecal samples, 45 pools (75%) were positive.

All positive culture fecal pools were PCR positive, although not every PCR positive sam-

ple was positive culture pool. Results of PCR is summarized in table (4). Representative positive samples are shown in photo (1)
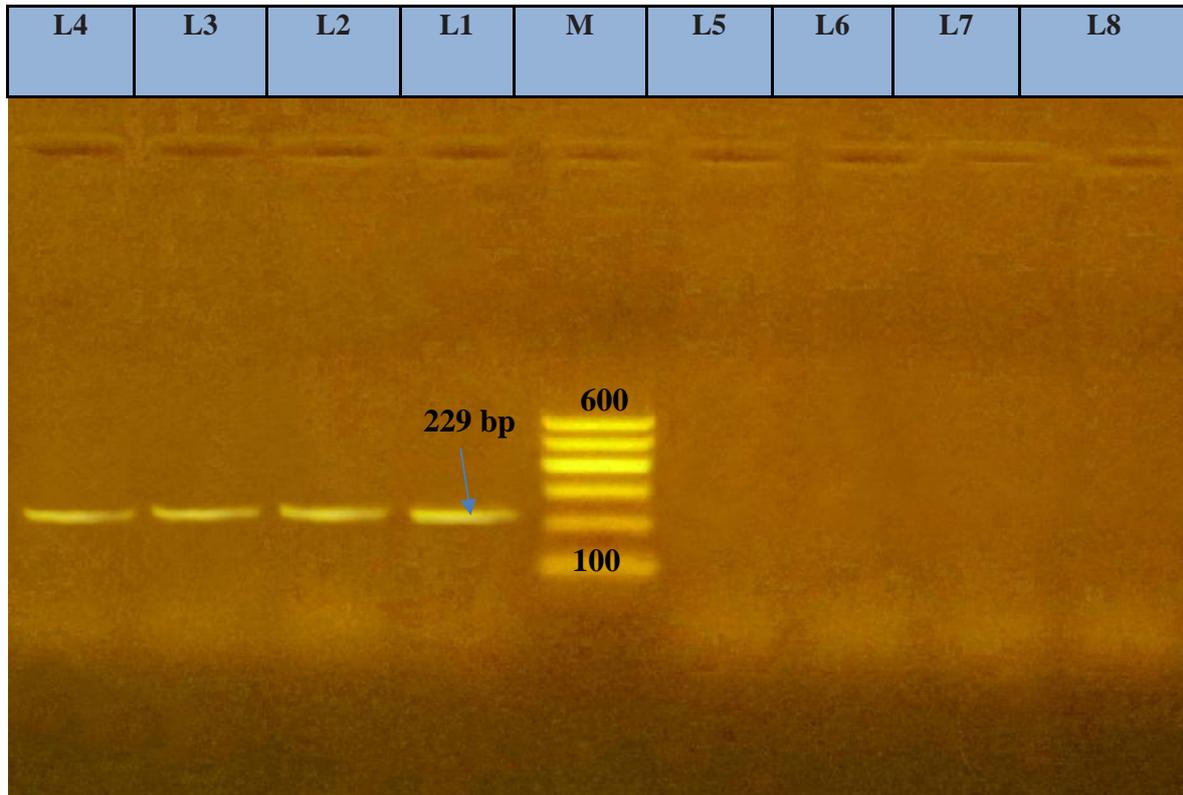
Photo (1): Showing results of IS900 PCR amplicons of some fecal samples
M: Marker 100pb
L1: Control positive
L2, L3 and L4: Positive samples
L5: Control Negative
L6, L7 and L8: Negative samples

Table (4). Results of IS900 PCR

Governorate	No. of examined samples		Total No. of Examined samples	Positive PCR (N)		Total No. of positive PCR
	Diseased animals	Apparently healthy animals		Diseased animals	Apparently healthy animals	
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.

4. 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 non-ruminant 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**).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 primates (**Beard *et al*. 2001; Glanemann *et al*. 2008 and Stief *et al*. 2012**).

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. 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, So little is known about Paratuberculosis in buffaloes, Hence, a more comprehensive knowledge of *Mycobacterium avium* subsp. *paratuberculosis* in buffaloes is of great value to facilitate the design of prevention and control programs. 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 disease 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 Egyptian buffaloes. The tests used to diagnose MAP in buffaloes 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 & 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 **Asmaa (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 *et al*. 2006).** Many publications 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.

It can be concluded that
•Some points should be considered as the intermittent shedding of the microorganism and sometimes, the low numbers of MAP in feces

and the loss of the microorganism during the exhausting decontamination protocol .

•Identification through ZN staining for fecal smears revealed that 29(48%) out of the 60 pooled samples were positive.

•By ELISA, out of the 300 serum samples, 212 (71%) of tested serum samples were positive. It's unfair to say that it is a true sensitivity; As ELISA in this study was performed on all individual animals serum samples unlike fecal culture was performed according to pooling procedure and the only way to determine that the animal is infected with MAP or not is by reculturing it individually.

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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