



ISOLATION OF JOHN'S BACILLI FROM THE MANURE OF IRAQI CATTLE

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Abstract

Paratuberculosis or Johne's disease is a chronic debilitating, enteropathy mainly of ruminants that is caused by bacterium *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The present research conducted on the basis of using (ZN) staining and (HEYA) technique for 53 fecal samples collected from cattle's with suspected clinical signs and subclinical of Johne's disease (JD) from different south/mid provinces and cities of Iraq. Staining of fecal smears showed 24 positive cases, 16 with advanced clinical disease signs, and 9 from cows subclinical /apparently healthy out of 13 that gave positive with HEYA results, 2 positive out of 24 negative with subclinical and 11 positive out of 29 suspected. The data recovered from HEYA and ZN-stain confirmed a significant role in the diagnosis of clinical and subclinical cases that play the main role of the spread of John's bacilli to the farm and other healthy animals.

Key words: John's bacilli, manure, Iraqi cattle

Introduction

Paratuberculosis (PTB), is an enteric granulomatous disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) that mainly affects ruminants worldwide (Cunha *et al.*, 2020; De Grossi *et al.*, 2020 ;Gopi *et al.*, 2020). In Iraq, during 2003 para. TB bacilli was first isolated on Egg Dorsets medium supplemented with extracted mycobactin from tuberculosis as well as sodium pyruvate and the aliment serologically used ELISA (Maytham, 2003), along with molecular and partial sequencing of Johne's bacilli in 2016 (Abdulrasool & Mahdi, 2016).

The ailment also had been detected in the milke of Iraq cattle (Abdulrasool, Hussain, & Hayawi, 2020). Para-TB bacterium is a facultative intracellular, Grams-positive, acid-fast small ($0.5 \times 1.5 \mu\text{m}$) small rod-shaped bacterium. The cell wall is thick and waxy (Rathnaiah *et al.*, 2017). Johne's bacillus shows the most oversensitive growth of all mycobacteria; incubation lasts 8 -12 weeks, with a reliance on exogenous mycobactin spatially type J (Lilenbaum, Marassi, & Oelemann 2007; Molmeret *et*

al., 2005). The disease results in reduced milk production, progressive weight loss, lower slaughter value, and premature culling, with possible impacts on fertility and udder health. Eventually, infection can lead to the clinical form that manifests as debilitation, chronic diarrhea, emaciation, and eventual death (Tiwari *et al.*, 2006). According to the OIE Worldwide Animal Health Information Database (WAHID interface), 58 out of 241 countries reported MAP infection during 2017. While not all countries report infection (Whittington *et al.*, 2019; Cunha *et al.*, 2020). Affecting the distal part of intestine as well as regional lymph nodes (Kyle, 2013). Cell mediated immune response (CMI) is higher, during the early stages of the disease with fecal shedding of the organism and serum antibody response is absent or diminished (Maroudam *et al.*, 2015). The subclinical stage is immunologically characterized by a protective Th1 immune response, and an elevated level of IFN- γ . The development of infection and appearance of clinical disease is associated with the shift from a Th1 to a non-protective Th2-mediated humoral response in the late subclinical phase (Roussey, Steibel & Coussens, 2014; Amato, 2018). Humoral response predominates with

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progressively increased shedding of the organism (Maroudam *et al.*, 2015). The Infection with PTB divided into 4 stages depending on the severity of clinical signs, potential for shedding organisms into the environment. Include silent infection, subclinical infection, clinical infection and advanced clinical infection (Fernández-Silva, Correa-Valencia & Ramírez, 2014). Diagnostic testing for para-TB remains one of the linchpins for management and successful control of this infectious disease (Aly *et al.*, 2010). The diagnosis of JD with add of the acid fast staining and culture of clinical specimen which were reliable several decade ago. Sometime ZN-staining cannot definitely differentiate para-TB organism among other mycobacteria. Bacterial culture in solid medium is still the reference diagnostic method (Dugassa & Demisie, 2014). Culture on Herrold's Egg Yolk Agar (HEYA) is more specific and had been considered the gold standard test for MAP (Collins *et al.*, 2006).

Material and Method

Isolation of MAP from fecal samples

1. Fecal Samples collection

Fecal samples were collected from most of the cows that showed suspected JD Clinical signs of emaciation, either with chronic shooting or intermittent diarrhea same animal showed normal appetite, thirsty with sunken eye. And from subclinical cows living with the same population the samples were kept at -20 °C for further staining ZN stain and culture. Table 1.1. Below is showing the number of fecal samples have been collected from the target animals.

Table 1.1.: The number of fecal sample that have been collected from the target animals

Samples collected	Number
Clinical cows	29
subclinical cows	24
Total samples	53

2. Detection of Johne's bacilli in the fecal sample by acid fast stain

The ZN staining was performed on all of the collected fecal samples and according to manufacturer protocol (Institute of Serums and Vaccines/MOH/IRAQ) as below:

1. Fecal smear made from each fecal sample on a clean slide.
2. The smear heat fixed by passing each slide 3 times over a Bunsen burner flame.
3. Pour of concentrated carbol fuchsin over fecal smear with heating until steaming at intervals up to 7

minutes with avoiding of boiling.

4. Slide cooled down and washed with tap water.
5. The smear decolorizes with 20 % Sulphuric acid for 15-30 seconds then washed with tap water.
6. Methylene blue added as counter stain for 1 minute.
7. The slide was rinsed with tap water gently.
8. Slide was air-dried and examined under oil-immersion lens.

3. Isolation of the bacteria on Herrold's Egg Yolk Agar (HEYA) supplemented with Mycobactin J plus sodium pyruvate and on HEYA without Mycobactin J or s sodium pyruvate (Control)

Isolation the MAP on HEYA was performed on all of the collected fecal samples and according to manufacturer protocol (Remel Inc/USA) as below:

A. Decontamination

The method was conducted according to (Quinn *et al.*, 1998):

1-One gram of feces was mixed in a (40) ml of distilled water, then mixture shaken for 30 minutes and then left for one hour to precipitate.

2-An amount of (5)ml of supernatant is taken and placed in 30 ml of sterile distilled water containing a substance Benzalkonium chloride concentration (0.3%).

3-The mixture left for 36 hours at room temperature.

4-(2) ml the supernatant was eliminated of precipitate was removed and mixed well with (4) drops of amphotericin B solution at a concentration of (5mg / ml).

B. Centrifugation of the last deposit at speed of (5000-6000) r.p.m for 15 min as modified by (Whitlock *et al.*, 2000).

C. Inoculation of about 0.1 ml of centrifuged deposit onto (HEYA) plus mycobactin J plus sodium pyruvate, the media were incubated at 37°C and check weekly up to 20 weeks.

D. The same amount of centrifuged deposit was inoculated onto control media.

Result

1. Detection of Johne's bacilli in the fecal smear by acid fast stain

Direct (ZN) staining technique applied to the sum of 53 fecal samples collected from cows with suspected clinical signs of PTB and asymptomatic different south/ mid provinces and cities of Iraq. Staining of fecal smears showed 25 positive cases, 16 with advanced clinical

disease Signs and nine from cows subclinical /apparently healthy (Table 1.2.). The microscopic finding revealed the presence variable number of typical clumps of Para-TB in the fecal smears examined from the suspected cows with a characteristic red to pink short, thick coco bacilli arranged in myriads of nests, small aggregations as well as to singles scattered in the field and the background looks with faint blue discoloration as illustrated in Fig. 1.1.

2. Isolation of MAP on Herrold's Egg Yolk Agar (HEYA) with mycobactin J plus sodium pyruvate

Johne's bacilli were successfully isolated on HEYA for 13 samples out of 25 that were positive with acid fast stain (Table 1.3.).

The colonies were observed on this media at the week 8 from the beginning of the inoculation and were very small in size like a pinhead and colorless while the aging of the culture; the size of the colony became larger exceeds 0.5 mm at the week 13 and the color darker tinge white with gray color then at week 17 their diameter reach up to 1mm (Figs. 1.2, A and 1.2, B)

Table 1.2.: Direct Ziehl Neelsen (ZN) staining of fecal sample smears result of subclinical cows and suspected of being infected clinically Cows with paeatuberculosis

Province/City	Direct smear technique	Clinically Suspected Cows		Subclinical Cows	
		Positive	Negative	Positive	Negative
	ZN-Stain				
Diyala	8	4 (50.00%)	0 (0.00%)	1 (12.50%)	3 (37.50%)
Hilla	16	3 (18.75%)	5 (31.25%)	4 (25.00%)	4 (25.00%)
Abu Ghrab	3	1 (33.33%)	0 (0.00%)	0 (0.00%)	2 (66.67%)
Radwaniyah	5	3 (60.00%)	0 (0.00%)	0 (0.00%)	2 (40.00%)
Arab jabour	10	3 (30.00%)	4 (40.00%)	3 (30.00%)	0 (0.00%)
AL-Dora slaughter house	11	2 (18.18%)	4 (36.36%)	1 (9.09%)	4 (36.36%)
Chi-Square (χ^2) ⁽¹⁾	—	12.338 **		9.541 **	
Total	53	16	13	9	15
Percentage%		30.18%	24.52%	16.98%	28.3%
Chi-Square (χ^2) ⁽²⁾		2.073 NS		5.319 **	
Chi-Square (χ^2) ⁽³⁾		5.188 *			
* (P≤0.05), ** (P≤0.01).					

Statistical Analysis: The Statistical Analysis System- SAS (2012) program was used to detect the effect of difference factors in study parameters. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study.

Table 1.3.: Isolation of MAP on Herrold's Egg Yolk Agar (HEYA) of fecal sample smears result of subclinical cows and suspected of being infected clinically Cows with paeatuberculosis

Province/City	Number of sampls culture on	Clinically Suspected Cows		Subclinical Cows	
		Positive	Negative	Positive	Negative
	HEYA				
Diyala	8	3 (37.50%)	1 (12.50%)	0 (0.00%)	4 (50.00%)
Hilla	16	2 (12.50%)	6 (37.50%)	1 (6.25%)	7 (43.75%)
Abu Ghrab	3	1 (33.33%)	0 (0.00%)	0 (0.00%)	2 (66.67%)
Radwaniyah	5	2 (40.00%)	1 (20.00%)	0 (0.00%)	2 (40.00%)
Arab jabour	10	2 (20.00%)	5 (50.00%)	1 (10.00%)	2 (20.00%)
AL-Dora slaughter house	11	1 (9.09%)	5 (45.45%)	0 (0.00%)	5 (45.45%)
Chi-Square (χ^2) ⁽¹⁾	—	9.805 **		9.172 **	
Total	53	11	18	2	22
Percentage%		20.75%	33.96%	3.77%	41.50%
Chi-Square (χ^2) ⁽²⁾		5.276 *		10.652 **	
Chi-Square (χ^2) ⁽³⁾		6.549 **			
* (P≤0.05), ** (P?0.01)					

Statistical Analysis: The Statistical Analysis System- SAS (2012) program was used to detect the effect of difference factors in study parameters. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study.

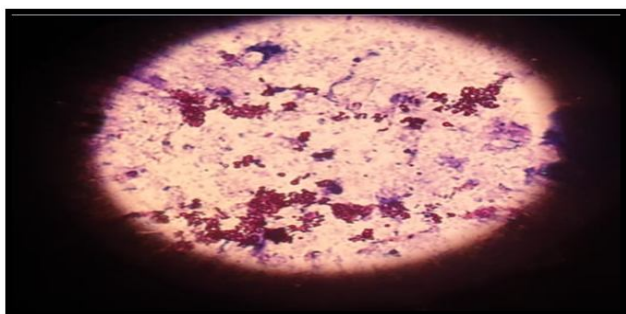


Fig. 1.1.: Large typical clump arrangement of para-TB from ZN-positive fecal sample A. cow with advance clinical PTB /B, subclinical cows ofPTB (Magf.X1000)

Comparative statistical analysis has been applied between the isolation of Johne’s bacilli on HEYA and the direct smear technique from the fecal sample that have been collected from Clinically Suspected/ Subclinical Cows (Table 1.4.)

Discussion

Johne’s disease primarily affects ruminants and is caused by the slow-growing para-TB bacilli.as well as found in many countries worldwide; it is called the ghost diseases. In 2003 when first diagnosed and confirmed in Iraq; the available data on the incidence and prevalence of the diseases area absent before this period (Abdurasool & Mahdi, 2016). Direct microscopic smear for 53 cattle fecal stand by ZN staining revealed the presence of typical clumps of acid-fast bacteria bacilli that also could be considered an alternative to fecal culture (Constable *et al.*, 2016). However, the sensitivity and specificity of the microscopical examination have always been in misgiving (Ris, Hamel & Ayling, 1988; Abdurasool & Mahdi, 2016). And this requires a professional veterinarian’s skill of adaptation on examination of MAP typical microscopically morphological characteristics could be considered as a diagnostic parameter for Bovine

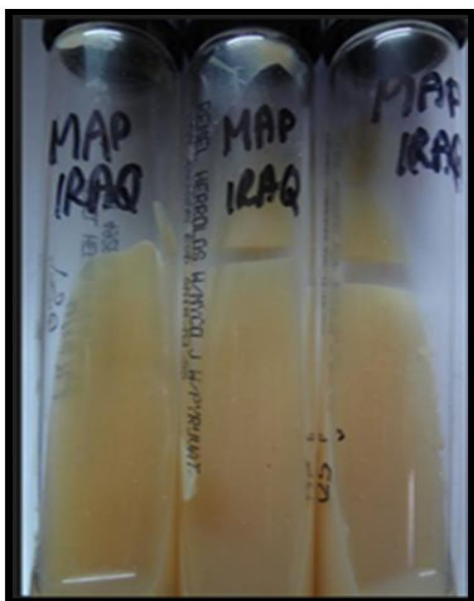


Fig. 1.2.A.: Colony on HEYA tube



Fig. 1.2.B.: Close colonies are White-gray in color

Table 1.4.: Comparison between HEYA and ZN-Stain

Method	Clinically Suspected Cows		Subclinical Cows		Chi-Square (χ ²)
	Positive No. (%)	Negative No. (%)	Positive No. (%)	Negative No. (%)	
HEYA(No. =53)	11 (20.75%)	18 (33.96%)	2 (3.77%)	22 (41.50%)	6.337 **
technique ZN-Stain (No. =53)	16 (30.18%)	13 (24.52%)	9 (16.98%)	15 (28.30%)	5.592 *
Chs-Square (χ ²)	4.672 *		5.086 *		—
* (P≤0.05), ** (P≤0.01).					

Statistical Analysis: The Statistical Analysis System- SAS (2012) program was used to detect the effect of difference factors in study parameters. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study.

PTB. Also, it may be needful to examine smears on several occasions to confirm the results. ZN staining of fecal samples showed positive results for 16/29 samples (30.18%) from cows with an age average of 3-5 years that were showed suspected clinical PTB symptoms of un-feverish, chronic shooting, normal appetite and intermittent diarrhea. 15 fecal samples were obtained from cows and one from an ox. As well as obtained positive results for 9/24 from 8 subclinical cows fecal samples and one from an ox. The fifteen daughters of the above suspected dam cows with intermittent shedding diarrhea revealed positive ZN staining while 13 samples were negative by (24.52 %). It was so clear that the number of PTB nests detected in the manure of each positive field sample vitiated between samples from scanty to vast and this could be interpreted due to the presence of different phases of the disease, so the infected animals some are non-shedders, some are light shedders (less than 100 organisms/g of feces) and some are heavy shedders (more than 1000 organisms/g of feces) and, of these, the heavy shedders may develop clinical disease (Mitchell *et al.*, 2015). In general, the microscopical examination of fecal smears for the presence of acid-fast clumps are an unreliable method of detecting PTB in bovine feces alone and should be backed up with a culture which is superior but it takes a long incubation period, it is considered golden test (Clark Jr *et al.*, 2008 ;Keller *et al.*, 2014). Therefore, in ZN test-positive cattle showing or not showing the clinical signs of the disease, it is extremely important to eliminate positive once especially shedders from the herd. The perfect handling is to repeat sample investigation per animal due to the chronic nature of the disease, intermittent shedding of the bacteria, and possible disagreement between fecal samples results and this provide superior detection ability for fecal samples. In addition, the disease stage of ailed animals within studied groups could affect the degree of bacterial shedding into fecal samples (Bates *et al.*, 2019).

John's bacilli have been isolated on HEYA with mycobactin J plus sodium pyruvate; the colonies observed on this medium after (100) days from the beginning of the inoculation were very subtle, tiny, colorless and their size very small like a pinhead, In Contrary to what observed by Maytham (2003), the colonies appeared after (115) days on Egg Dorsets medium supplemented with mycobactin extracted from tuberculosis as well as sodium pyruvate. Hence HEYA is more specific and had been considered the perfect media to be used for cultivation of MAP until now(Collins *et al.*, 2006). While the aging of the culture; the size of the colonies became larger

exceeded 0.5 mm and at the week 13 and the color became darker tinged with white grayish discoloration, their surface looked smooth as well as shiny then at the week 17 their diameter reach up to 1mm. Whereas the colonies did not appear in the HEYA control tube this is a definitive evidence of their reliance on mycobactin type J (De Juan *et al.*, 2006). Isolation investigation of the fecal samples revealed positive 11 (20.75%) from cows' ages 4-5 years, 10 dams, one an ox; were showed the suspected clinical John's disease symptoms with chronic shooting or intermittent diarrhea same animal showed normal appetite, thirsty with sunken eye (Gay & Sherman, 1992; O.I.E., 2014). Whereas 2 samples (3.77%) were positive cows from subclinical case, 22 samples (41.50%) were negative. The best results of isolation were obtained by the centrifugation of the decontaminated fecal samples at a rate between (5000-6000) r.p.m for 15 min. depositing large numbers of John's bacilli found in the supernatant, confirms with Whitlock *et al* (2000).

A Comparison between HEYA and ZN-Stain was clear that there are significant differences ($P_d \leq 0.05$) between HEYA and ZN-stain in the positive percentage when samples are clinically suspected, as they were higher in the ZN-Stain method and lower in HEYA, as the positive percentage in the two methods reached (30.18%) and (20.75%), respectively there were also significant differences between the two methods for Subclinical samples in the same direction and the highest percentage was ZN-Stain (16.98%) and the lowest HEYA (3.77%). Associated with disruption of protective immunity during the latent stage of infection (Olsen *et al.*, 2009; Singh *et al.*, 2014) .On other hand persistent shedding was associated with development of clinical disease (Waters *et al.*, 2003; Koo *et al.*, 2004). Sometime the bacteria may shed in the feces at minor levels, often below the detection limit (Sweeney *et al.*, 2012).

As well as shows that there are highly significant differences ($P_d \leq 0.01$) between clinically suspected and subclinical positive samples for the HEYA method and the ratios were 20.75% and 3.77%, respectively. The differences were significant ($P_d \leq 0.05$) between clinically suspected and subclinical for positive samples of the ZN-Stain method, and the ratios were 30.18% and 16.98%, meaning that positive samples were higher in clinically suspected samples in both methods. It is estimated that each animal has distinct clinical symptoms, at the same time in the field, between four and eight animals have a subclinical shape and are therefore symptomatic (De Grossi *et al.*, 2020). As well as can survive freely in the

environment for up to one year, when the animals live together with infected ones, the suspected animals showed the JD clinical sign (Manning & Collins, 2001; Chacon, Bermudez & Barletta, 2004; De Grossi *et al.*, 2020). This study supported Collins' (2001) that isolating the bacterium from the feces of any animal is conclusive evidence of ailment, but the problems of using microbial isolation that it is a costly method with prolong duration of incubation, as well as about the problem of contamination of the culture (Collins & Manning, 2001; Grant *et al.*, 2001). Beside waste the time required sometime to obtain the fecal sample.

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