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Microscopical and Serological diagnosis of Bovine Paratuberculosis (Johne's Disease) in Iraqi Cattle

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Abstract

Paratuberculosis or Johne's disease is a chronic debilitating , enteropathy of cattle and ruminants that is caused by bacterium *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The current research conducted on the basis of using PARACHEK® 2 Enzyme Linked Immuno Sorbent Assay (ELISA) kit for screening of 33 serum samples collected from cows with suspected clinical signs of paratuberculosis from different south/mid provinces and cities of Baghdad. The kit has positively detected specific antibodies against MAP in18 serum samples, 11 negative and 4 were suspected according to O.D. values. Ziehl-Neelsen staining of fecal smears showed 12 positive cases ,8 with advanced clinical disease signs out of 18 that gave positive ELISA results, 1 positive out of 11 negative with ELISA and 3 positive out of 4 suspected with ELISA kit. The data recovered from ELISA kit and ZN-stain confirmed the sophisticated nature of the immune response to Paratuberculosis and there is no precise parameter for diagnosis of clinical and subclinical carrier cases which play the major role of Johne's bacilli spreading to the farm and other healthy animals.

Keywords: Paratuberculosis, MAP, Ziehl-Neelsen staining, ELISA.

Introduction

Paratuberculosis or Johne's disease (JD) is a chronic, enteric infection of cattle and ruminants that is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Manning and Collins, 2010 ; Shroff *et al*., 2013; Shoor *et al*., 2014).

Mycobacterium avium subsp. *paratuberculosis* was first detected serologically by ELISA and isolated on cultural media in Iraq in 2003 (Maytham, 2003). MAP are small, Gram-positive, an obligated intracellular slow growing acid-fast bacteria that belong to the mycobacterial species of *Mycobacterium avium* (Thorel *et al*., 1990; Behr, 2008). MAP can be differentiated from *Mycobacterium avium* subsp. *avium* and *M. avium* subsp. *silvaticum* by its dependence of mycobactin, an iron-chelating agent for *in vivo* growth (Thorel *et al*., 1990) and by the presence of multiple copies of a repetitive DNA sequence, IS*900* (Collins and Delisle, 1986).The infection is chronic and the late stage of infection is characterized by clinical signs such as diarrhea and economic losses because of reduced milk yield, chronic weight loss lead to premature culling and reduced slaughter value (Ott *et al*.,1999; Fecteau and Whitlock, 2009).The infected animal may have disseminated infection including spread to udder and muscle tissues (Koenig, *et al*.,1993, Alonso-Hearn *et al*., 2009).MAP infection in cows follows different infection stages (Coussens *et al*., 2004).In the early stage of infection, the host response is predominantly a cell-mediated immune response (CMI) and the cow may shed MAP in feces at low levels, often below the detection limit, and possibly also in milk if disseminated infection has occurred (Stabel, 2009 ; Sweeney *et al*., 2012).

During the progression of infection a humoral immune (HI) response raised and occurrence of IgG1, and these cows are more likely to be detected by a diagnostic test such as ELISA with sensitivity rate of about 88.3% in the clinically infected animals and about 48.8% in the subclinical animals and give a positive results sometimes before the isolation of MAP in fecal specimens (Hietala,1992; Balzer *et al*,1998; Ridge *et al*.,2002; Nielsen and Toft, 2008).

Materials and Methods

1.Samples Collection

a. Blood Samples

Thirty three blood Samples were collected from group of cows collected from cows with suspected clinical signs of paratuberculosis from different southern, middle provinces and cities of Baghdad. Most cows showed clinical manifestations of chronic shooting diarrhea and emaciation with normal appetite or with diarrhea for a short period, others showed advanced stage of infection with cachexia , malaise and dirtiness of cow back side and un-responsiveness as illustrated in Fig.1.

Blood specimens were collected by using a 10 ml sterile disposable syringes with 18-gauge needle, allowed to clot at room temperature, then centrifuged at 3000 - 5000 rpm for 10 minutes at 18 C°. Serum samples were carefully transferred to micro tubes and stored in aliquots at -20ºC until used for ELISA test.

Figure 1. Cow with advanced clinical Paratuberculosis infection revealing cachexia, malaise and back part dirtiness.

b. Fecal Samples

Fecal specimens were collected from the same 33 cows that showed suspected Paratuberculosis clinical signs of emaciation , either with chronic shooting or intermittent diarrhea and from cows living with the same population. The specimens were stained directly with ZN staining technique.

Finger scraping specimen collected from corrugated rectal mucosa of one of the strongly infected cow. Table 1 below is showing the numbers of fecal and blood samples that have been collected from the target animals.

Table 1. The numbers of fecal and blood samples that have been collected from the target animals:

Samples of clinically suspected cows	Total Samples
Blood	33
Feces	33

2. Detection of specific antibodies against *Mycobacterium avium* subsp. *paratuberculosis* in Serum samples

PARACHEK® 2 is an *in vitro* Enzyme Linked Immuno Sorbent Assay kit (Prionics Ag, Switzerland) for detection of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* in serum of cattle, has been used to screen the cows suspected of being infected with Johne's disease.

PARACHEK® 2 KIT CONTENT 480		
Microtiter Plates coated with MAP Antigen	5	Plates
Positive Control	1	Vial
Negative Control	1	Vial
Green Diluent (Sample Diluent Buffer)	1	Vial
Wash Buffer – 20X Concentrate	1	Vial
Conjugate – 100X Concentrate (Horseradish Peroxidase labeled anti-bovine IgG)	1	Vial
Blue Diluent (Conjugate Diluent Buffer)	1	Vial
Enzyme Substrate	1	Vial
Enzyme Stopping Solution	1	Vial

* Assay Procedure

All frozen serum samples that were collected from the defined animals thawed and prepared to be run by PARACHEK®2 ELISA kit according to the kit instruction as follows:

1- All kit reagents were equilibrated to room temperature except the conjugate 100X concentrate kept cooled.
2- Serum samples were diluted 1:20 in the *Mycobacterium phlei* green diluent at room temperature in dummy plate with thoroughly pipetting up and down several times. The reaction time extended up to 24 hours.
3- The 100 µl of test samples and controls transferred to the corresponding wells of the kit plate. The positive and negative controls were done in duplicate.
4- The plate covered with its lid and incubated at room temperature for 30 minutes.
5- The plate is washed manually with 3X300 µl of the prediluted 1:20 washing buffer in pure distilled water .Non specific unbound antibodies washed off .At the end of washing step, the plate tapped face down several times on absorbent filter paper pad to remove as much as can the remaining washing buffer.
6- The 100 µl of freshly prepared conjugate solution were added to each well.
7- The plate was covered and incubated at room temperature for 30 minutes.
8- The plate was rinsed as in step 5.
9- The 100 µl of ready to use enzyme substrate was pipette into each wells;then the plate was shake gently , covered and incubated for 20 minutes at room temperature.
10- The 50 µl of enzyme stopping solution was pipette to each well with gentle agitation of the plate.
11- The optical density (O.D.) was read at absorbance of 450 nm by ELISA reader within 30 minutes.

3. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in fecal samples

The ZN staining kit (Institute of Serums and Vaccines/MOH/IRAQ) has been performed to all of the collected fecal specimens and according to manufacturer protocol as below:

1- Fecal smear made from each fecal specimen on a clean slide.
2- The smear heat fixed by passing each slide 3 times over Bunsen burner flame.
3- The concentrated carbol fuchsine was poured over fecal smear with heating at intervals up to 7 minutes with avoiding of boiling.
4- The slide cooled down and washed with tap water.
5- The smear was decolorized via 20% Sulphuric acid for 15-30 second and then washed with tap water to stop de-colorization.
6- Methylene blue was 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 beyond adding few drop of oil-immersion.

Results

1. Detection of specific antibodies against *Mycobacterium avium* subsp. *paratuberculosis* in serum samples

PARACHEK® 2 ELISA kit is used in this research for screening of 33 serum samples collected from cows with suspected clinical signs of Paratuberculosis from different southern provinces and cities of Baghdad.

The kit has positively detected specific antibodies against MAP in 18 serum samples, 11 negative and 4 were suspected according to O.D. values obtained by ELISA reader as described in details (Table 2).

The cut-off value obtained from the run of the PARACHEK® 2 kit was 0.15. All samples gave optical density (O.D.) ≥ 0.15 considered positive (according to test protocol) , any samples O.D. lied within 0.140-0.149 range considered suspected and the rest were < 0.15 below the suspected range considered negative. Assay validity checked according to the mean O.D. data reading obtained for positive control and negative control of the kit which gave 1.195 and 0.101 respectively; these results confirmed that the kit is valid for run as advised by kit protocol.

Table 2. PARACHEK® 2 ELISA kit results of the serum samples of cows clinically suspected of being infected with Johne's Disease.

Province/ City	ELISA TEST	Clinically Suspected Cows		
	PARACHEK® 2 ELISA kit/Serum samples tested	Positive O.D. ≥ 0.15	Negative O.D. < 0.15	Suspected Range 0.140-0.149
Baghdad	2	1	0	1
Wasit	3	2	0	1
Swera	7	5	1	1
Nomania	2	1	1	0
Zubaidia	2	1	1	0
Basra	2	1	1	0
Nasiria	2	0	2	0
Dewania	2	1	1	0
Babil	2	1	1	0
Mahmodia	2	0	2	0
Tagi	4	3	0	1
Abu-Ghraib	3	2	1	0
Total	33	18	11	4
Percentage %		54.54 %	33.33 %	12.12 %

2. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in fecal samples

In the present research, direct ZN staining technique applied for sum of the 33 fecal samples collected from the same 33 cows from different south and mid provinces/cities of Iraq; that revealed 12 positive cases , 8 with advanced clinical disease signs out of 18 that gave positive ELISA results, 1 positive out of 11 negative with ELISA and 3 positive out of 4 suspected with ELISA kit. The positive results for 8 fecal samples obtained from dams cows with an age average of (7-11) years old showed clinical Paratuberculosis symptoms of normal appetite , un-feverish ,chronic shooting diarrhea and 3 daughters of the above dams with intermittent shooting diarrhea with an average age of 2-4 years (Table 3).

Typical clumps of *Mycobacterium avium* subsp. *paratuberculosis* were detected in the fecal smears examined from the suspected cows with characteristic red to pink short , thick cocobacilli 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. 2.

Table 3. Direct Ziehl Neelsen (ZN) staining of fecal samples smears results of all of cows Suspected of being infected clinically with Paratuberculosis.

Province/City	Direct Smear Technique	Clinically Suspected Cows	
	ZN-Stain	Positive	Negative
Baghdad	2	1	1
Wasit	3	1	2
Swera	7	4	3
Nomania	2	1	1
Zubaidia	2	1	1
Basra	2	0	2
Nasiria	2	1	1
Dewania	2	1	1
Babil	2	0	2
Mahmodia	2	0	2
Tagi	4	1	3
Abu-Ghraib	3	1	2
Total	33	12	21
Percentage %		36.36 %	63.63 %

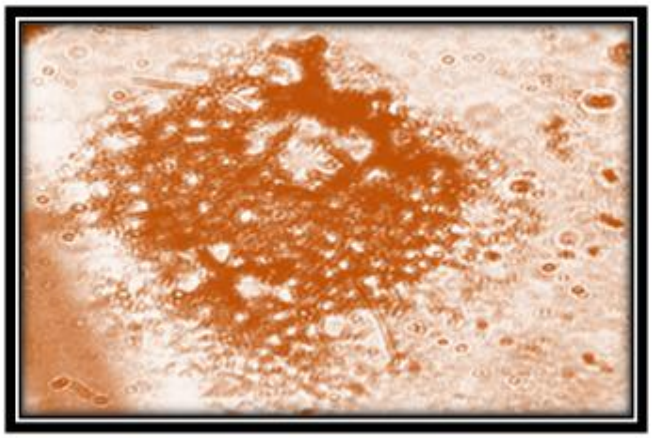

Figure 2. Large typical clump arrangement of *Mycobacterium avium* subsp. *paratuberculosis* from ZN-positive fecal sample of cow with advanced clinical Paratubeculosis (Magf. X1000).

The ZN-positive cases showed microscopically varies existence on the level of quantity of *Mycobacterium avium* subsp. *paratuberculosis* typical clumps in each field from scanty to vast in other fecal specimens.

Finger scraping specimen collected from corrugated rectal mucosa of one of the strongly infected cow that revealed strong ZN-positive fecal samples and ELISA kit reading.

Discussion

Bovine Partuberculosis (PTB) orJohne's disease is one of the most hidden diseases or also called the ghost disease which affected the livestock industry with massive economic losses and although Bovine PTB found in many countries worldwide; It is worth mentioning that, the available data on the incidence and prevalence of the diseases in IRAQ are limited and very not published until 2003 when first diagnosed and confirmed in 2003 in cattle (Maytham, 2003). Both Johne's Disase (JD) and Crohn's Disease (CD) are chronic devastating granulomatous intestinal syndromes. The JD causes enormous economic losses for the dairy industry. The office International des Epizooties (OIE) considers this disease of major global importance and categorizes the disease as list B transmissible disease. OIE also considered it of socioeconomic and public health importance within countries and is significant for the trade of animals and their products (OIE manual 2008).

The first step in the schedule of the present study, was screening of the cows suspected of having Paratubeculosis depending on the case history obtained from farmers and clinical findings. On the above mentioned basis serum samples collected from cows showed the suspected clinical manifestation of Johne's disease that recorded on 33 of the affected cows ,most cows with chronic and intermittent pipe stream shooting un-painful diarrhea with pea-like soap appearance (Aiello and Mays,1998 ; Radostitis *et al*., 2006). Three cows with an average of 9-11 years old showed the advanced clinical signs of Paratuberculosis with submandibular odema , sunken eyes, lethargy and cachectic. Feces was homogenous water sometimes slightly thick, with no foul odor or mucous or blood and this agreed with what observed by (Gayand Sherman, 1992).

PARACHEK® 2 Enzyme Linked Immuno Sorbent Assay (ELISA) kit used in this study that based on the Indirect solid phase of Johne's absorbed ELISA with pre absorption of serum samples collected from clinically,subclinically and overtly healthy cows which increased the sensitivity of the assay up to 80% and specificity up to 92% (Crabb *et al*.,1999).The kit run detected specific antibodies against MAP in 18 with 54.54% of the tested serum samples out of 33. Eight out of 18 positive serum specimens showed clinical disease signs revealed positive ZN-stained fecal smear, out of 11 negative 1 revealed positive ZN-stained fecal smear and out of 4 suspected 3 of them revealed positive ZN-stained fecal samples. One of the dams gave negative reaction to ELISA test despite of the positive ZN staining with the presence of large numbers of MAP bacilli nests in their fecal smear .The suspected cows with an age averaged 2-5 years were borne from infected damsdams revealed suspected results and this observation indicative of intrauterine infection (Merkal *et al*.,1982,Sockett*et al*.,1992;Sweeny *et al*.,1992).Assay validity checked according to the O.D. data reading obtained for positive control and negative control of the kit which gave 1.195 and 0.101 respectively; these results confirmed that the kit is valid for run as advised by kit protocol.PARACHEK®2 as ELISA kit considered highly specific and sensitive and the positivity value proportionally related to the O.D. reading of each positive samples data recovery with high qualitative value of the kit. Some serum samples showed elevated positive value along with advanced clinical signs of Johne's disease and this gave an indication of strong positive case as confirmed by Collins (2002a) who denoted that any cows serum samples showed O.D. value over the cut-off value of ELISA kit gave an indication of strong positive case of bovine Paratuberculosis and any case low below the cut-off value with little bit considered suspected. Crabb *et al*., (1999) referred that the test should be repeated each 3 months to confirm the result of first run of the conventional ELISA kit for suspected results. Collins *et al*.,(1999) study conducted emphasized that it is possible to rely on the result of absorbed ELISA kit when the real percentage of morbidity is over 3%.Collins (2002b) also confirmed that absorbed ELISA test could be used as valuable diagnostic tool in detection of bovine Paratuberculosis in cattle herds with a previous history of infection with Johne's Disease and considered as economic test to screen the herds and to eliminate any positive case.

Absorbed ELISA kit is highly specific due to incubation of serum specimens first with *Mycobacterium phlei* antigen buffer to exclude all non-specific cross-reactant antibodies of MAP with *Mycobacterium avium*, *Mycobacterium tuberculosis* (Wayne and Kubica,1994; Ridge *et al*., 2002).

The primary immune response of ruminants to Johne's disease has been long associated with a cell mediated immune (CMI) response in the early stages of infection with a switch to an antibody response later as the disease manifests (Begg *et al*., 2011).In late-stage infection, following development of the proposed suppressor and cytotoxic immune-regulatory cells, the major observed immune response to *M. avium* subsp. *paratuberculosis* would be production of IgG1 as an

indication of Th2 (humoral) response (Coussens, 2004; Wu *et al*., 2007 ; Wadhwa *et al*., 2013).But the later stage response doesn't prove sufficient to check infection while reported shedding of MAP in feces and this reflected the obscured and sophisticated immune response against MAP which did not fully understood till now and requires further investigation (Munir *et al*., 2014).For this reason some of the suspected cows were under 4 years of age which with low or undetectable antibodies circulating in their blood while cellular parameters should be available especially Interferon-Gamma ; and one of the adult over 7 years of age revealed un detectable antibodies maybe due to energy state of immunity.

Most typical signs were obvious in adult cows with an age over 3 year old, intermittent diarrhea seen on cows with an average age of 2-4 years which showed suspected ELISA result and positive ZN stain with presence of very variable numbers of MAP clumps per field of their fecal samples and this agreed completely with what denoted by (Radostitis *et al*., 2006).

Direct method used for microscopic examination of Ziehl-Neelsen (ZN) stained smears of feces revealed the presence of typical small coco-bacillary clumps of acid-fast MAP also considered an alternative to fecal culture. However, the sensitivity and specificity of the microscopical examination have been in doubt. Also it may be necessary to examine smears on several occasions to obtain a positive result. But when the suspected animals manifested with JD clinical signs and animals lived together with infected ones along with professional veterinarian skill of adaptation on examination of MAP typical microscopical morphological characteristics could be considered as diagnostic parameter for Bovine Paratuberculosis.

Ziehl-Neelsen staining of fecal samples showed that 12 fecal samples were positive out of 33 , 8 fecal specimens were obtained from dams cows with an age average of (7-11) years showed clinical paratuberculosis symptoms ,4(3 out of were daughters of the above dams with intermittent diarrhea) and 21 were negative. The presence of typical MAP clumps varied from sample to sample but most of positive ones were with heavy shedding. Clumps of acid-fast MAP bacteria are more likely to be observed during a diarrheic phase in the epithelial cells are of diagnostic value and, when epithelial cells are more likely to be shed, than in a period when feces are normal. In general, the microscopical examination of fecal smears for the presence of acid-fast clumps is an unreliable method of detecting MAP in bovine feces alone and should be backed up with culture which is superior but time consuming ,ELISA test which support the result of fecal smear or with direct fecal PCR (O.I.E.,2014).

One of the older cow which aged about 11 years showed the aggregation of MAP clumps from rectal biopsy specimen obtained during sampling with obvious thickening of rectal mucosa which indicated that the target dam is in the late stage of Paratuberculosis. This observation data confirmed when pinch biopsy collected with the fingernails, or scrapings of rectal mucosa, are of great advantage along with fecal smears, as it is only in the late clinical stages that the rectal mucosa is invaded. If rectal scrapings or rectal pinch biopsy are also used as a positive finding if clumps of acid-fast bacilli is presented in epithelial cells or macrophages (Radotitis, 2006; O.I.E., 2014).

Ziehl–Neelsen-stained smears of feces or intestinal mucosa that are examined microscopically is a presumptive diagnosis of Paratuberculosis if clumps (three or more organisms) of small, strongly acid-fast bacilli are found. (O.I.E. 2014).

ZN test-positive cattle showing or not showing the clinical signs of the disease, it is extremely important to eliminate positive and especially shedder animals from the herd (Hüseyin *et al.,* 2012). Perfect handling, is the repeated samples per cow due to the chronic nature of the disease, intermittent shedding of the bacteria, and possible disagreement between fecal specimens results and also with other diagnostic tools like ELISA test, this provide superior detection ability for fecal samples. In addition, the disease stage of cows within study groups could affect the degree of bacterial shedding into fecal samples which maybe not always compatible with ELISA values (Nielsen and Toft , 2008 and Gardner *et al*., 2011).

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