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SCREENING OF IRAQI COWS WITH MILK ELISA FOR PARA-TUBERCULOSIS (JOHNE'S DISEASE)

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Abstract

Para-tuberculosis or the disease of Johne's referred to chronic devastating, bowl inflammation of domestic as well as wild livestock that is caused by short bacilli bacterium *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The recent research relied on using of ELISA kit for screening of 186 milk samples collected from cows with suspected clinical signs of para-tuberculosis from various cities of Baghdad & southern region. The test has positively appointed specific antibodies against Johne's bacilli in 5 milk samples, 178 negative and 3 were suspected according to O.D. values. Acid fast staining of milk smears from the above assigned cows showed 3 positive cases out of 5 reacted positive with ELISA run results, 2 positive out of 178 negative with ELISA and 1 positive out of 3 suspected. The data of ELISA kit and acid fast-stain supported the sophisticated nature of the immunity to para-tuberculosis and no precise tool for diagnosis of clinical and subclinical carrier cases which were participated in the spreading of Johne's bacilli to the farm as well as other sound animals.

Introduction

Para-tuberculosis or the disease of Johne's referred to chronic devastating, bowl inflammation of domestic as well as wild livestock that is caused by bacterium named *Mycobacterium avium* subsp. *paratuberculosis* (MAP). (Manning and Colins, 2010; Shroff *et al*., 2013; Shoor *et al*., 2014; Bates *et al*., 2019). Johne's bacilli was first successfully cultivated on cultural media in Iraq and identified serologically by ELISA (Maytham, 2003), as well as to molecular determination along with IS900 sequencing of Johne's bacilli (Maytham, 2016). Johne's bacilli are small and short bacilli, Gram-positive, obligate intracellular slowly grow acid-fast bacteria that is sub categorized under the mycobacteria species of *M. avium* (Thorel *et al*., 1990; Behr, 2008). Johne's bacilli can be discriminated from *M. avium* sub sp. *avium* and *silvaticum* by its sole dependence on mycobactin for primary isolation, protein to chelate iron for *in vivo* cultivation (Thorel *et al*., 1990) along with the presence of multiple short copies of a reoccurred DNA sequence, IS*900* (Collins and Delisle, 1986). The scenario of the infection is long term (chronic) and the late stage is denoted by clinical signs such as pipe stream like long term diarrhea, emaciation and economic losses resulted from reduced milk yield, remarkable weight loss ended with premature culling, reduced slaughter value and finally death (Ott *et al*., 1999; Fectau and Whitlock, 2009). The infected animal disseminate bacteria to the udder and muscles (Koenig, *et al*., 1993; Alonso-Hearn *et al*., 2009). Johne's bacilli infection in cows involves many stages (Cousens *et al*., 2004). In the first stage of infection, the adopted host response is a cellular immune response (CMI) and bacteria may shed in the feces at minor levels, often below the detection limit, and possibly also in milk (Stabel, 2009; Sweeney *et al*., 2012). A humoral immune (HI) response triggered with circulation of IgG1 in the blood stream, and these ailed livestock are more likely to be detected by a diagnostic aid such as ELISA that has sensitivity rate relatively about Eighty eight & three percentage in the clinically infected and about Forty eight & eight percentage in the subclinical ones and may reacts positively sometimes before the cultivation of Johne's bacilli in the manure (Balzer *et al.*, 1998; Ridge *et al*., 2002; Nielsen and Toft, 2008).

Materials and Methods

Milk samples collection

(a) Milk Samples for ELISA test

One Hundred Eighty Six (186) milk samples were obtained from cows with suspected clinically contracting para-tuberculosis from different cities of Baghdad & Wasit (Swera) southern to Baghdad. Most of target cows showed clinical ailment of chronic shooting diarrhea, diarrhea for a short period, normal appetite and some of them with emaciation, others were cachexic, lethargic as well as dirtiness of cow back side.

Milk samples were collected in a 100 ml sterile containers and then stored in aliquots into micro tubes and at -20ºC until used for ELISA test and for Ziehl-Neelsen (ZN) staining protocol.

(b) Milk Samples for ELISA test

One Hundred Eighty Six (186) milk samples were stained directly with ZN staining technique.

Determination of antibodies against Johne's bacilli in milk samples

The protocol of PARACHEK® 2 (Prionics Ag, Switzerland) described as *in vitro* Enzyme Linked Immuno Sorbent Assay was followed literally according to the manufacturer instruction for detection of antibodies against Johne's bacilli in milk of cattle.

Observation of Johne's bacilli in milk samples with ZN stain

The staining set of acid fast bacteria (Institute of Serums and Vaccines/MOH/IRAQ) was been applied for all of the collected 186 milk samples according to the manufacturer protocol.

Results

Determination of antibodies against Johne's bacilli in milk samples

One hundred and Eighty Six milk samples collected from cows with suspected clinical symptoms of JD have been positively reacted against Johne's bacilli in 5 milk samples, 178 appeared negative and 3 suspected according to O.D. reads recovered from ELISA spectrophotometer as detailed (Table 1).

The number 0.15 was dependent as the cut-off value recovered from the run of the kit. All samples showed optical density (O.D.) ≥ 0.15, 0.140-0.149 and < 0.15 considered positive, suspected and negative respectively. The validity of that assay verified was adopted on the average of the O.D. data reads got from positive and negative controls of the test that were 1.191 as well as 0.090 respectively.

Observation of Johne's bacilli in milk samples with ZN stain

Direct acid fast staining technique has been performed for 186 milk samples; appearing 3 positive, 2 of them with apparent clinical signs out of 5 that revealed positive ELISA run, 2 positive among 178 negative in ELISA and 1 appeared positive within 3 suspected in ELISA run (Table 2).

Clumps of bacteria were observed in the milk slides in the suspected ailed ones with characteristic red, stumpy thick cocobacilli arraying in nests, as well as the background looks faint blue including fat droplets Fig. 1.

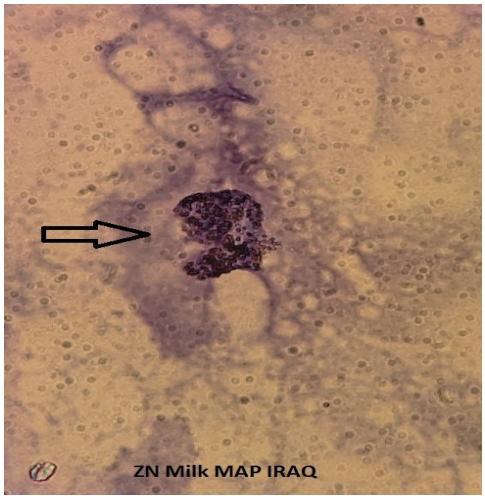

Fig. 1 : Milk sample revealed arrangement Johne's bacilli clump of in acid fast slide of advanced clinical ailed cow (Magf. X1000).

The microscopic existence of ideal Johne's bacilli clumps varies on the level of quantity within fields vary from scanty to vast in other samples of milk.

Discussion

The divulging of data about JD in IRAQ are not published until 2003 when first diagnosed and verified with MAP isolation & sero-positivity of infected animals with ELISA kit in 2003 in cattle (Maytham, 2003) as well as to MAP partial genome sequencing (Maytham, 2016).

The milk samples were collected according to the observations as well as to the history of the case recorded from the owners and clinical findings from some of the PTB suspected infected cows which showed the typical clinical manifestations of the disease which included chronic, from time to time pipe stream shooting un-painful pea-like soap diarrhea (Radostitis *et al*., 2010). Four adults ailed cows their age lies between 7-9 years old appeared the advanced clinical signs of para-tuberculosis with edema of the submandibular region, lethargy sunken eyes and cachectic with reduction in milk production except one cow with no depletion in milk yield as this may be due to individual variation along with breed resistance to MAP infection (Botaro *et al*., 2017). One of these cows suffered from mastitis & this may give an indication of the stage of disease ongoing in the same dam, hence JD may influence the occurrence of mastitis (Garcia and Shalloo, 2015). A heifer with 3 yrs old borne from the same dam with mastitis which its milk reacted positively with ELISA run indicated this heifer contract the disease via intrauterine route (Whittington and Windsor, 2009; Whittington *et al*., 2019).

The assay of adopted, on the Indirect solid phase with prior serum samples absorption collected from clinically, sub-clinically as well as overtly healthy animals which amplify the assay sensitivity up to Eighty percentage and specificity up to Ninety Two percentage (Crabb *et al*., 1999). The assay sensitivity amplified thru the run to detect the specific immunoglobulins especially IgG1 to Johne's bacilli in the milk with pre absorption of the non specific sera due to common shared antigens (Wayn and Kubica, 1994; Ridge *et al*., 2002; Gardner *et al*., 2011) and recorded of vale as diagnostic aid in defining of bovine Para-tuberculosis in the herds involved a previous background of illness with JD and affordable to screen as well as to eliminate any positive threats.

The sensitivity of protocol of ELISA test run for sub-clinically ailed animals within the first phases of JD, is also triggered by the dynamic of immunoglobulins secretion and hence some animals gave positive results with milk ELISA (Gardner *et al*., 2011; Bates *et al*., 2019).

Despite the immune reaction to is still paradoxical and did not fully outlined till now and demands the next steps for investigation (Begg *et al*., 2011; Munir *et al*., 2014; Luttikholt *et al*., 2019) but the first response of immunity in JD ailed ruminants has been so far cellular (CMI) especially within the first phase of the illness with a shift to humoral later while the disease is ongoing. In late-stage of the infection, beyond the cytotoxic regulatory cells of suppression, the major observed response of defence to Johne's bacilli would be production of IgG1 which elucidate T helper 2 humoral induction response (Coussen, 2004; Wu *et al*., 2007; Wadhwa *et al*., 2013). In similar, we found the latently infected dams positively reacted and others negatively to ELISA have a decline in milk production only observed with high-shedding of MAP in their milk (McAloon *et al*., 2015). Only animals with advanced stage of the disease and have elevated levels of anti MAP antibodies may increase the sensitivity of ELISA data recovery (Rathnaiah *et al*., 2017).

For the above reason certain suspected cows lower to the years Four of age that appeared low or below detectable immunoglobulins circulating within the blood whereas cellular compartments should be observed mainly Interferon-γ; and energy phenomenon may explain un detectable antibodies in one of the adults over 7 years.

Suspected ELISA records along with positive ZN stain as well as the presence of variable numbers of Johne's bacilli aggregations per field of their milk; this accorded definitely with what recorded (Radostitis *et al*., 2006). However, the microscopical sensitivity as well as specificity of the examination of Johne's bacilli in milk have been of doubt. The smears should be examined from time to time to ensure a positive data; in special when the suspected ailed ones tentatively manifested clinically and healthy animals existed together with ailed ones; along with professionally handling on examination of morphological Johne's bacilli characteristics could be relied on for detection of the disease.

The availability of Johne's bacilli clumps with more than three organisms of small, powerful acid-fast short bacilli in stained smears of any biological samples, is a presumptive diagnosis (O.I.E., 2014).

It is extremely crucial to eliminate positive ZN -positive cases especially strong shedders out of the herd showing or not showing the clinical symptoms (Hüseyin *et al.*, 2012). The phase of the ailment within the studied animals could influence the degree of Johne's bacilli shedding into milk which is not at all the time concur the ELISA readings (Gardner *et al*., 2011). The elimination of the ailed away from any herd will cut the way of MAP transmission to their offspring via colostrum or milk.

The risk of transmission of MAP thru consuming milk products from JD dairy cattle to human and contracting the disease of Crohn's (CD) that is a devastating chronic granuloma syndromes of the bowl. Para-tuberculosis causes devastating financial losses for the dairy production and enrolled as grade B within the list of OIE transmissible diseases because it is considered one of the top priority on public health inside countries as well as to its influence on the trade of animals and their stuffs (O.I.E. manual 2008).

Table 1 : ELISA kit data recovered results of the milk of JD suspected cows.

Governorate/ City	ELISA TEST	Clinically Suspected Cows		
	ELISA/milk	+	-	±
Baghdad	165	2	162	1
Wasit/Swera	21	3	16	2
Total	186	5	178	3
Percentage %		2.68	95.69	1.61

+ = positive, **-** = negative, **±** = suspected

Table 2 : Direct acid fast bacilli staining of milk smears results of all of JD suspected cows.

Governorate	Direct milk smear	Clinically Suspected	
	Acid-fast Stain	+ve	-ve
Baghdad	165	2	163
Kut (Swera)	21	3	18
Total	186	5	181
Percentage %		2.68	97.31

+ve = positive, -ve = negative

References

Alonso-Hearn, M.; Molina, E.; Geijo, M.; Vazquez, P.; Sevilla, I.; Garrido, J.M. and Juste, R.A. (2009). Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from muscle tissue of naturally infected cattle. Foodborne Pathog. Dis., 6: 513-518.

Balzer, S.E.; Teubert, D.G. and Collins, M.T. (1998). Temporal study to evaluate serum antibody by ELISA, gamma interferon test kit and radiometric fecal culture for diagnosis of paratuberculosis in naturally infected adult dairy cattle Proc.4th. Intl.Coll.paratuberculosis. PP:54

Bates, A.; O'Brien, R.; Liggett, S. and Griffin, F. (2019). Control of *Mycobacterium avium* subsp. *paratuberculosis* infection on n New Zealand Pastoral dairy farm. BMC Veterinary Research, 15: 266.

Behr, M.A. (2008). *Mycobacterium* du jour: what's on tomorrow's menu? Microb. Infet., 10: 968-972.

Botaro, B.G.; Ruelle, E.; More, S.J.; Strain, S.; Graham, D.A.; O'Flaherty J. and Shalloo. L. (2017). Associations between paratuberculosis ELISA results and test-day records of cows enrolled in the Irish Johne's Disease Control Program. J. Dairy Sci., 100: 7468–7477.

Christie, G.J. (1950). A further note on the examination of fecal samples. The Vet. Rec., 62(30): 438-440.

Collins, D.M. and Delisle, G.W. (1986). Restriction endonuclease analysis of various strains of *Mycobacterium paratuberculosis* isolated from cattle. Am. J. Vet. Res., (47): 2226-2229.

Coussens, P.M. (2004). Model for Immune Responses to *Mycobacterium avium* Subspecies paratuberculosis in Cattle. Infection and Immunity, 72(6): 3089–3096.

Crabb, J.H.; Sweeny, R.W.; Cressman, H.; McAdams, S. and Whitlock, R.H. (1999). New rapid diagnostic test for Johne's disease in cattle. The Bovine Proceeding, September, No.32: 247-249.

Fecteau, M.E. and Whitlock, R.H. (2009). Paratuberculosis in cattle. Pages 144 in Paratuberculosis: Organism, Disease, Control. M.A. Behr and D.M. Collins eds. CAB International, UK.

Garcia, A.B. and Shalloo, L. (2015). Invited review: the economic impact and control of paratuberculosis in cattle. J Dairy Sci., 98(8): 5019–5039.

Gardner, I.A.; Nielsen, S.S.; Whittington, R.J.; Collins, M.T.; Baökker, D.; Harris, B.; Sreevatsan, S.; Lombard, J.E.; Sweeney, R.; Smith, D.R.; Gavalchin, J. and Eda, S. (2011). Consensus-based reporting standards for diagnostic test accuracy studies for paratuberculosis in ruminants. Prev. Vet. Med. 101: 18-34.

Gardner, I.A.; Nielsen, S.S.; Whittington, R.J.; Collins, M.T.; Bakker, D.; Harris, B.; Sreevatsan, S.; Lombard, J.E.; Sweeney, R. and Smith, D.R. *et al.* (2011). Consensus-based reporting standards for diagnostic test accuracy studies for paratuberculosis in ruminants. Prev Vet Med., 101(1–2): 18–34.

Hüseyin, C.; Zafer, M.; Gülşah, D.; Ethem, M.T. and Sezgin, S. (2012). Evaluation of Faecal Shedding of Acid-fast *Mycobacterium avium* subsp. *paratuberculosis* (map) in both Intradermal Johnin Test- and Serologically (Elisa) Map positive Cattle. J. Biol. Environ. Sci. 6(18): 281-283.

Koenig, G.J.; Hoffsis, G.F.; Shulaw, W.P.; Bech-Nielsen, S.; Rings, D.M. and St-Jean, G. (1993). Isolation of *Mycobacterium paratuberculosis* from mononuclear cells in tissues, blood, and mammary glands of cows with advanced paratuberculosis. Am. J. Vet. Res., 54:1441-1445.

Luttikholt, S.; Lievaart-Peterson, K.; Gonggrijp, M.; Aalberts, M.; van Schaik, G. and Vellema, P. (2019).

Mycobacterium avium subsp. paratuberculosis ELISA Responses in Milk Samples from Vaccinated and Nonvaccinated Dairy Goat Herds in The Netherlands. Vet. Sci., 6: 58.

Manning, E.J.B. and Collins, M.T. (2010). History of paratuberculosis. Pages 1-9 in Paratuberculosis: Organism, Disease, Control. M.A. Behr and D.K. Collins eds. CAB International, UK.

Maytham, I.A. (2003). Diagnostic Study of Bovine Paratuberculosis (Johne's Disease) In Iraq. Master of Science Thesis. University of Baghdad, College of Veterinary Medicine.

Maytham, I.A. (2016). Detection and genotyping of *Mycobacterium avium* subsp. *paratuberculosis* strains in Iraqi cattle. PhD Dissertation. University of Baghdad, College of Veterinary Medicine.

McAloon, C.G.; Whyte, P.; More, S.J.; Green, M.J.; O'Grady, L.; Garcia, A. and Doherty, M.L. (2015). The effect of paratuberculosis on milk yield-a systematic review and meta-analysis. J. Dairy Sci., 99: 1449–1460.

Munir, M.T.; Munir, A.R.; Murtaz, H. and Abubakar, M. (2014). Epidemiology, diagnosis and control options of johne's disease in endemic situations. Res. J. Vet. Pract., 2(5): 84–90.

Nielsen, S.S. and Toft, N. (2008). Ante mortem diagnosis of paratuberculosis: a review of accuracies of ELISA, interferon–g assay and faecal culture techniques. Vet. Microbiol. 129: 217–235.

Nielsen, S.S. and Toft, N. (2008). Ante mortem diagnosis of paratuberculosis: A review of accuracies of ELISA, interferon-γ assay and faecal culture techniques. Vet. Microbiol. 129: 217–235.

Nielsen, S.S.; Krogh, M.A. and Enevoldsen, C. (2009). Time to the occurrence of a decline in milk production in cows with various paratuberculosis antibody profiles. J. Dairy Sci., 92: 149–155.

OIE Terrestrial manual, Paratuberculosis (John's Disease). (2014).Chapter 2.1. 11: 1-16.

Ott, S.L.; Wells, S.J. and Wagner, B.A. (1999). Herd-level economic losses associated with Johne's disease on U.S. dairy operation. Preventive Vet. Med., 40: 179-192.

Radostits, O.M.; Gay, C.C.; Hinchcliff, K.W. and Constable, P.D. (2006). Veterinary Medicine. 10th ed. London: Bailler Tindall. 1017-1044.

Rathnaiah, G.; Zinniel, D.K.; Bannantine J.P.; Stabel J.R.; Gröhn Y.T.; Collins, M.T. and Barletta R.G. (2017). Pathogenesis, Molecular Genetics, and Genomics of *Mycobacterium avium* subsp. *paratuberculosis*, the etiologic Agent of Johne's Disease. Front. Vet. Sci. 4: 187.

Ridge, S.E.; Morgan, I.R. and Condron, R.J. (2002). Comparison of the Johne's absorbed EIA and the complement fixation test for the diagnosis of Johne's disease in cattle. Proc. 3rd Intl. Coll. Paratuberculosis. PP: 22.

Shoor, V.S.; Saurabh, G.; Kundan, K.C.; Krishna, D.R.; Naveen, K.; Jagdip, S.; Sohal, S.S.; Ruchi, T.; Sandip, C. and Kuldeep, D. (2014). Johne's Disease (JD) in a High Yielding Holstein Friesian Cattle Dairy Farm in India. Journal of Biological Sciences, 14: 195-203.

Shroff, S.; Chandel, B.S.; Dadawala, A.I.; Singh, S.V. and Bhagat, A.G. (2013). Evaluation of Indigenous vaccine in Patanwadi sheep naturally infected with clinical Johne's disease. Res. Opin. Anim. Vet. Sci., 3: 322-329.

Stabel, J.R. (2009). Immunology of paratuberculosis infection and disease. Pages 230 in Paratuberculosis: Organism, Disease Control. M. A. Behr and D. M. Collins eds. CAB International, UK.

Sweeney, R.W.; Collins, M.T.; Koets, A.P.; McGuirk, S.M. and Roussel, A.J. (2012). Paratuberculosis (Johne's Disease) in cattle and other susceptible species. J. Vet. Internal Med., 26: 1239-1250.

Sweeney, R.W.; Uzonna, J.; Whitlock, R.H.; Habecker, P.L.; Chilton, P. and Scott, P. (2006). Tissue predilection sites and effect of dose on *Mycobacterium avium* subs. *paratuberculosis* organism recovery in a short-term bovine experimental oral infection model. Res. Vet. Sci., 80: 253-259.

Thorel, M.F.; Krichevsky, M. and Vincent Levy-Frebault, V. (1990). Numerical taxonomy of mycobactin-dependent *Mycobacteria*, emended description of *Mycobacterium avium* and description of *Mycobacterium avium* subsp. *avium* subsp. nov., *Mycobacterium avium* subsp. *paratuberculosis* subsp. *nov.* and *Mycobacterium avium* subsp. *silvaticum* subsp. *nov.* Int. J. Syst. Bacteriol. 40: 254-260.

Wadhwa, A.; Kumar, N.; Velasco–Villa, A. and Eda, S. (2013). Overview of Johne's disease immunology. Vet World. 6(11): 901–904.

Wayne, L.G. and Kubica, G.P. (1994). Family: Mycobacteriaceae. In: Bergeys Manual Of Systematic Bacteriology. Sneath, P.H.; Mair, N.S.; Sharpe, M.E.andHolt, J.G. Eds. Vol. II. Williams and Wilkins, London. PP: 1436-1457.

Whittington, R. and Windsor, P. (2009). In utero infection of cattle with *Mycobacterium avium* subsp. *paratuberculosis*: a critical review and meta-analysis. Vet J., 179(1): 60–69.

Whittington, R.; Donat, K. and deWaard, J. (2019). Control of paratuberculosis: who, why and how: A review of 48 countries. BMC Veterinary Research, 15: 198.