

Evaluation of ELISA and The Conventional Methods Used in JOHNE' S Disease Diagnosis in Cows

Riad, E.M., Amany, N. dapgh ., and Dalia M. Mohsen

Animal Health Rsearch Institute - Dokki, Giza.

Abstract

Paratuberculosis in cattle is an infectious disease caused by *Mycobacterium avium supspecies* paratuberculosis (MAP) .Paratuberculosis or John's disease(JD) is a Chronic debilitating disease that affects a wide range of animal hosts. So, this study was designed to focus on diagnosis of paratuberculosis by traditional culture method and ELISA .

A total of 165 Egyptian cattle (more than 5 years old) were collected from some private farms in different districts at El Sharkia governorate,. All diseased (155) cattle were in poor condition with marked reduction in milk production, chronic or intermittent diarrhea and showed no fever. From which, fecal , milk and serum samples were collected as well as from other 10 contact apparently normal animals. The Fecal and milk samples were decontaminated and were inoculated onto Herrold's egg yolk medium (HEYM) slants supplemented with mycobactin j., and the slants were incubated at 37°c and observed every 2 weeks for 16 weeks.

The suspected growth represented by grayish white rough colonies were smeared and stained by Z.N stain then microscopy examined. The indirect ELISA was carried out on the serum and milk samples using commercial ELISA kit as well as the use of modified ELISA.

The obtained results revealed that out of 165 collected samples from. 13 fecal samples were positive for detection of MAP.by culture method from (155) diarrheic cows and two samples from 10 apparently healthy contact cows with a percentage of 7.9 % and 20 % respectively . The results of indirect ELISA by the using the commercial kit revealed that, 18 serum samples out of 165 (10.9%) tested cow samples were positive and were harboring specific antibodies against MAP (16 cases out of 155 diseased animals and two out of 10 apparently healthy contact cows).

Using ELISA with W. antigen detected 19 positive cases out of 165 examined caws and ELISA with S. antigen detected of (21) positive cases . The diagnostic sensitivity and specificity for JD, by the S.ELISA where greater and showed highly detection rate of paratuberculosis infection.

Key words: *Mycobacterium avium supspecies* paratuberculosis, Paratuberculosis, Johns disease , ELISA . cattle.

Introduction

Paratuberculosis in cattle is an infectious disease caused by *Mycobacterium avium subspecies paratuberculosis*. Paratuberculosis or Johne's disease is a chronic debilitating disease that affects a wide range of animal hosts, (Lombard *et al.*, (2011). and causes great economic losses in young cattle (Ott *et al.*, (1999) . It is characterized by long "subclinical" stage of infection. Most cattle shed detectable numbers of the *M. paratuberculosis* bacteria in feces up to two years of age.

Although some possible gut damage has occurred, infected cows could recover after supportive therapy and clinical signs may disappear after weeks without treatment but stress factors including pregnancy, lactation and worms infection could trigger the recurrence of signs (Bannantine *et al.*, (2002) and Allaker and Kapas,(2003).

Diagnosis of MAP is very difficult and complicated as the interference of other mycobacterial infections in the diagnostic tests which has been suggested . Also the intermittent shedding of MAP in feces affects the detection of infected animals especially the subclinical cases . (Paolietchi *et al.*, (2003).

Serum antibodies are detectable later than DTH. They may also be present in carriers that have recovered from infection. Serum antibodies are present more constantly and are of higher titer as lesions become more extensive, reflecting the amount of antigen present.(Kalis *et al.*, (1999). Other mycobacterial diseases including mammalian and avian tuberculosis can cause Delayed -type hypersensitivity DTH and the presence of serum antibodies. So, these diseases need to be differentiated from paratuberculosis by the use of specific diagnostic tests. Exposure to environmental saprophytic mycobacteria may also sensitize livestock, resulting in non specific DTH reactions. (OIE ,(2014) The diagnosis based on clinical ground confirmed by the demonstration of MAP in feces by microscopy , culture or by the PCR. The direct diagnosis of paratuberculosis includes clinical signs and bacteriological examination for isolation of MAP from feces .

The fecal culture is widely considered to be the gold standard for the diagnosis in live animals sample proce, there are several culture methods which vary with respect to media and Samples processing protocol and the cultivation of MAP is performed using special media supplemented with mycobactin j.(Nielsen ,*et al* (2004). While the detection of subclinical infection depends on the detection of specific antibodies by serology .

Control of paratuberculosis is very difficult as it has long incubation period and difficult diagnosis. However fecal culture is not only complicated, time consuming, expensive, liable for contamination but also of little practical use. The indirect diagnosis of MAP depends on immunological techniques as skin allergic test (Johnin), interferon gamma assay and ELISA .

Despite of low sensitivity and specificity, ELISA is considered as a method of choice for diagnosis of Johne's disease in positive herds. (**Hendrich, *et al* (2005) and Speer ,*et al* (2006)**).

So, this study was designed to focus on diagnosis of paratuberculosis in some dairy farms in Egypt and using ELISA in comparison with traditional culture technique.

Material and Methods

A total of 165 Egyptian cattle more than 5 years old, were collected from different farms in different districts at El Sharkia governorate, A total of (155) diseased cattle were in poor condition with marked reduction in milk production, chronic or intermittent diarrhea and showed no fever. From which, fecal , milk and serum samples were collected .Also samples were collected from 10 contact apparently healthy animals.

Fecal samples were decontaminated with 0.9 HPC (Hexadecylpyridinium chloride) according to **Whipple *et al.*, (1991)**. 300 µl of each decontaminated fecal sediment were inoculated onto Herrold's egg yolk medium (HEYM) slants supplemented with mycobactin j. the slants were incubated at 37°C in horizontal position for one week with the caps loosened to allow absorption and evaporation of residual moisture. Then caps were tightened and tubes were returned to vertical position and observed every 2 weeks interval for 16 weeks. The suspected colonies were smeared and stained by Z.N stain then microscopically examined for acid fast bacilli..

Indirect ELISA test was carried out on the collected serum samples for detection of specific antibodies against (MAP) using commercial ELISA kit I.D. SCREEN Paratuberculosis indirect screening test as well as the use of modified ELISA .

1- I.D. .screening test :

Serum (milk) samples (10 microliter) as well as control positive and control negative samples (included in the kit) were prepared and diluted in diluent buffer 1/12 and incubated 45 min at 21°C

The diluted samples were added (100 microliter /well) to all coated plates and incubated for 45 min.at 21°C then wash.

The conjugate was diluted 10x to 1/10 and added as(100 microliter/ well) then incubated for 30 min.at 21°C.then wash.

Enzyme substrate was added as 100 microliter .and incubated for 15 min.at 21°C in dark.

The reaction is stopped and the color development is measured at 450 nm.

INTER PRETATION OF THE RESULTS: ELISA kit I.D. SCREEN
 Paratuberculosis indirect screening test

$$S/P \% = \frac{O.D.SAMPLE - O.D.N.C}{O.D.P.C - O.D.N.C} \times 100$$

RESULTS	STATUS
S/P % less than 60 %	NEGATIVE
S/P % 60 % - 70 %	DOUBTFUL
S/P % more than 70 %	POSITIVE

2- Modified ELISA

According to **Speer et al.,(2006)** who developed (ELISA) for the diagnosis of Johne's disease using whole bacilli treated with formaldehyde (**W. ELISA**) and surface antigens obtained by treatment of *M. avium* subsp. paratuberculosis bacilli with formaldehyde and then brief sonication (**S.ELISA**).

To optimize the S. ELISA test, various concentration (3.7 to 37%) of formaldehyde and intervals of sonication (2 to 300 s) were tested. With an increase in formaldehyde concentration and a decrease interval of sonication , there was a concomitant decrease in the non specific binding by the S. ELISA.

So, the S. ELISA was prepared by treating *M.avium* subsp. paratuberculosis with 37% formaldehyde and then a 2 seconds of sonication .

The prepared antigens (W. antigen and S. antigen) were used as coated antigens in ELISA in concentration of (5 mg/1 ml) and the next steps were completed as follow :

- Serum samples (milk samples)were added (100 microliter / well) in concentration of 1/20 and the coated plates were incubated for 1 hr. then wash
- anti- bovine IgG conjugate was used (100 microliter / well) in concentration of 1:1000 and incubated for 1 hr. then wash.
- ABTS substrate was added (100 microliter / well) and all plates were incubated for 15 minutes.
- Measure the results using spectrophotometer at wave length 405 nm.

Results and Discussion

Paratuberculosis (Johne's disease) is one of the most economically important diseases of dairy cattle and caused by *Mycobacterium avium* subsp. paratuberculosis (MAP) which cause severe economic losses in dairy production farms and believed to be a potential public health hazard. Infected cows usually suffer from weight loss, diarrhea, decreased milk production and even death.

Diagnosis of MAP usually based on detection of MAP itself or the host's immune response against it. (Timms *et al* ,(2011). The control of paratuberculosis disease is very difficult due to long incubation period and difficult diagnosis. Bacteriological culture of MAP is still considered the golden standard for diagnosis of the disease(Debroy *et al.*, (2012).

The isolation of *M. paratuberculosis* from an animal provides the definitive diagnosis of infection with the organism. Although culture is technically difficult and time-consuming to carry out, it is the only test that does not produce false-positive results (100% specificity).

The fecal culture is the best test available for the diagnosis of paratuberculosis in live animals. It is believed that the fecal culture method involving the double incubation method for decontamination of samples and cultivation on solid media detects about 30–40% of infected cattle . The fecal culture is able to detect most animals in advanced stages of the disease but identifies only a few animals in early stages of infection. It will detect infected animals 6 months or more before they develop clinical signs and during the clinical stage its sensitivity approaches 100%.

Bacteriological examination was carried out on the collected samples from (165) diseased and apparently normal cattle for detection of MAP. The obtained results of culture method showed that 13 fecal samples were positive by culture method from(155)diarrheic cows and (2) samples from apparently healthy contact cows were positive with a percentage of isolation reached 8.4 % and 20 % respectively (Table 1) .

The morphological examination of suspected growing colonies revealed a typical grayish white rough colonies of MAP after 12 week of culture on (HEYM) slants and The microscopic examination of positive culture stained by Z.N stain was appeared as red acid fast bacilli. in clumps (.photo 1 and 2) .

The results of bacteriological examination of the serum samples by indirect ELISA by the using the commercial kit revealed that, 18 serum samples out of 165 (10.9%) tested cow samples were positive and were harboring specific antibodies against MAP (16 cases out of 155 diseased animals and 2 from 10 apparently healthy contact cows were hurbering the specific antibodies against MAP (Table 2),

On the other hand , the use of ELISA with the prepared W. antigen (WHOLE CELL CULTURE ANTIGEN) can detect 19 positive cases out of 165 examined cow samples with the mean optical density reached 1.4. While ELISA with the prepared S. antigen (SURFACE ANTIGEN) showed (21) positive cases with the mean optical density reached 1.4. (.Table 2).

In this study 13 examined fecal samples were positive for MAP including 155

diarrheic cows and 2 out of 10 apparently healthy cows, this may explain the long incubation period of the disease and shedding of organism without showing clinical signs of the disease. As shown in **Table (1)**.

The obtained results are different from the result obtained by **Salem *et al.*, (2005)**, who found that, the highest prevalence rate of the disease among the examined farms. The differences in result within the examined farms may be attributed to size of sample collected and method of diagnosis. Also **Asmaa, (2014)** reported that the result of culture was 34 of 50 pooled samples tested (68%) and explained the relatively high incidence of the disease due to the pooling strategic used in the study. The culture method has many obstacles for diagnosis of MAP as it is time consuming, laborious and expensive so, immune-based diagnostic tests are alternative to fecal culture. (**Merkal and McCullough (1987) and Mikkelsen *et al.*, (2011)**).

The ELISA is at present, the most sensitive and specific test for serum antibodies to MAP in cattle (**Milner *et al* (1988), Cox *et al* (1991) and OIE,(2014)**). Despite of low sensitivity and specificity, ELISA is considered a method of choice for diagnosis of Johne's disease positive herds. This due to the ease of sample collection, rapid procedure, low cost and possibility of testing a large number of samples in a short time. (**Collin's *et al* ,(2005)** as well as the elimination of non specific reactions. (**Hope *et al* (2000), Hendrich *et al*, (2005) and Salgado *et al* ,(2005)**).

Concerning the sero-diagnosis, 16 serum samples and milk samples were positive for MAP using indirect ELISA by using the commercial kit. from diarrheic cows and 2 from apparently healthy cows. (**Table, 2**). The obtained results are slightly higher than the result obtained by (**Sergeant *et al* ,(2003), Mohan *et al* ,(2009) and Wadhwa *et al* ,(2012)** who studied the seroprevalence of paratuberculosis in serum and milk samples of cattle as they examined 235 cattle collected from 5 districts using the indirect ELISA and the results revealed that 16 (6.8%) of the 235 cattle samples were positive. The difference may be attributed to the sample size and method of diagnosis.

The higher prevalence rate was recorded by **Asmaa (2014)** who examined 340 serum samples by indirect ELISA and the results proved that 63 (18.5%) tested samples were positive.

So, for improving the indirect ELISA, the S. ELISA antigens was prepared by treating *M. avium* subspp. paratuberculosis with 37% formaldehyde and then a 2 seconds of sonication.

This tested antigen produced the greatest value over that obtained by commercial kit or W. ELISA between *M. avium* subspp. paratuberculosis negative and positive serum samples. (**Hope *et al* ,(2000) and Jubb *et al* ,(2004)** as well as in

milk samples .

Salgado et al, (2005) and **Speer et al ,(2006)** proved that , the diagnostic sensitivity and specificity for JD. by the S.ELISA where greater than 95% and the S. ELISA showed specific detection of M. avium subspecies paratuberculosis infection in calf experimentally inoculated with M.avium subspecies paratuberculosis or other mycobacterium based on diagnostic sensitivity and specificity .

Now, there is no doubtful that ,ELISA is considered a method of choice for diagnosis of Johne's disease positive herds. This due to the ease of sample collection, rapid procedure, low cost and possibility of testing a large number of samples in a short time.

Conclusion

Paratuberculosis represent a great issue in animal farms nowadays. The use of new antigens like WHOLE CELL ANTIGENS or SURFACE ANTIGENS yield a clear significant difference and detectable value in diagnosis of paratuberculosis infection .

Based on diagnostic sensitivity and specificity , the use of S. ELISA appear superior to the commercial ELISA as routinely used for diagnosis of Johns disease and more convenient than culture method as well as for rapid detection of subclinical infected animals .

Control of JD could be achieved by good herd management practices, and diagnosis.

Table (1) the results of bacteriological examination of collected samples from diseased and apparently normal cattle by cultural method

Type of samples	Number of tested samples	Cultural method			Total	
		Diseased animals		Apparently healthy animals	no	%
		No.	%			
Feces samples	165	13/155	8.4%	2 /10	15/165	9%

(Table 2) Results of ELISA on milk and serum samples collected from diseased and apparently normal cattle.

Type of samples	Number of tested samples	ELISA					
		Commercial. Kit		Prep. Antigen.			
		Positive	Mean O.D	W. ELISA			S. ELISA
				Positive	Mean O.D	positive	Mean O.D.
Milk samples	165	18	0.82	19	1.3	21	1.3
Serum samples	165	18	0.84	19	1.4	21	1.4

Table (3) comparison between the bacteriological findings of MAP infection by cultural method and ELISA. (165 -animals)

Cultural method			ELISA					
			W. EIISA			S . EIISA		
Diarrheic	Contact animals	Total	Diarrheic	Contact animals	Total	Diarrheic	Contact animals	Total
13/155	2 /10	15 /165	17 /155	2/10	19/165	18 /155	3/10	21/165

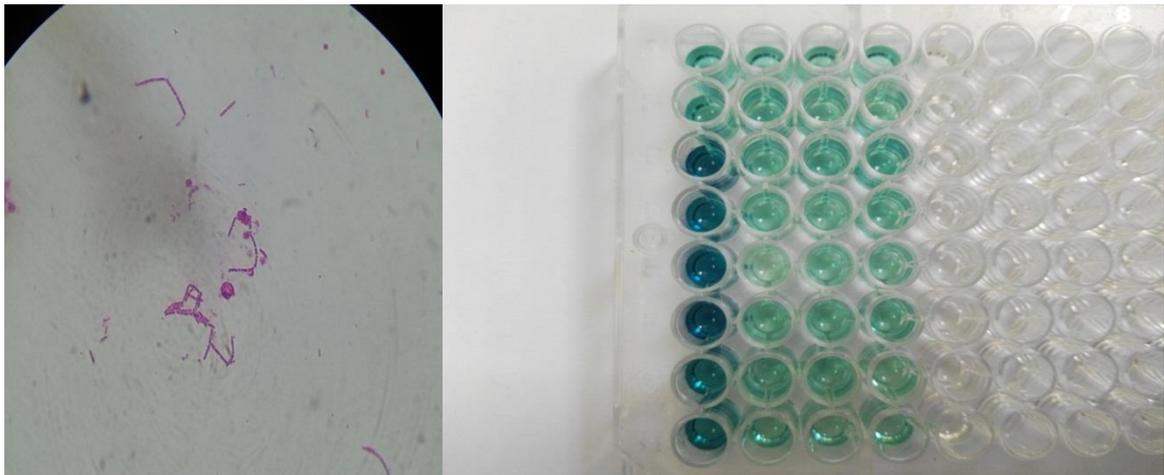


Photo 1 : Acid fast bacilli of isolated strain stained with Z.N.stain

Photo 2 : The results of ELISA of positive and negative serum samples

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