# BACTERIOLOGICAL, MOLECULAR AND BIOCHEMICAL STUDIES ON CATTLE INFECTED WITH PARATUBERCULOSIS

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***Abstract***

This study was carried out on two dairy farms ,one on Alexandria desert road(N=268) and the other (N=163) on Sharkya Governorate with main objective of studying paratuberculosis by serological, bacteriological and molecular investigations in addition of clarifying humoral pattern and serum biochemical changes in affected animals. Serolgical investigations for detection of anti Mycobacterium paratuberculosis (M.paratuberculosis ) antibodies in bovine serum by Enzyme linked immunosorbant assay (ELISA) test revealed an incidence of 9.3% and 11.04% in Alex. desert road and Sharkya farm respectively .The levels of antibodies showed fluctuation up and down in repitetive samples with 2or 3 days interval up to one month for the same case. Fecal samples (N=25) of seropositive animals were cultured on Herrold's egg yolk medium with mycobactin, the results revealed isolation of mycobacterium avium subspecies paratuberculosis from 17 out of 25 samples . Moreover,polymerase chain reaction(real time- PCR) specific of *M. paratuberculosis* (IS900) has been done on fecal samples (n=25) and 17 bacterial isolates . The results indicated the positivity of all tested samples. Biochemical analysis on serum samples of some infected animals revealed hypoproteinaemia and hypoalbuminaemia, in addition of decreased serum levels of copper, iorn and zinc. The results were discussed with recommendations.

## INTRODUCTION

Paratuberculosis or Johne's disease is a chronic progressive infectious disease caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) , it affects all categories of domestic and wild ruminants including cattle, goats, camels, buffaloes (Oie 2008). The disease occurs throughout the world and is responsible for considerable economic losses. There is no therapy and it invariably leads to the death of the affected animal (Joseph et al., 2001 )

Animals are most likely to be infected through the fecal-oral route due to contamination of water and food supplies by infected feces.The disease manifests in adult cows and results in economic losses caused by premature culling, reduced milk production, and loss of body weight in cattle sold for slaughter. Detection and control of MAP is complicated due to its slow division time and its ability to persist in the environment (Raizman et al., 2004). Enzyme –linked immunosorbant assay,

bacteriological cultivation of fecal samples,and PCR are tests widely used for the antimortum diagnosis of paratuberculosis in cattle herds (Clark, et al .,2008, Nielsen,2008 and Stevenson , 2010) . Enzyme-Linked Immunosorbent Assay , is a diagnostic test based on detection of Humeral immune response to MAP. The ELISA is considered to be the most suitable serologic test for use as a screening test in subclinically infected animals. The ELISA detects anti-MAP antibodies, specifically IgG isotypes in serum ( Colgrove et al., 1989 and Collins and Sockett 1993).

Due to the limitations associated with the culture method, a number of other tests have been developed to aid and confirm the diagnosis of paratuberculosis. The technique right now that is the most focus of attention is the polymerase chain reaction (PCR). The potential value of the PCR in diagnosing *Mycobacterium avium subsp. paratuberculosis* infections has been applied in a variety of clinical samples (Joseph et al., 2001 and Remya et al., 2011 ).

Herd prevalence of bovine paratuberculosis in Europe ranges from 7% to 55%. In the United States of America, herd prevalence is strongly associated with herd size, 40% of herds of more than 300 head were found to be infected. In Australia, reported dairy herd infection rates range between 9% and 22% ( Manning,and Collins, 2001). While in Egypt, dairy herd infection rates range between 0.61% to 13.77% Abdel Moghney (2008).

Trace elements are required in small concentrations as essential components of biological enzyme systems or of structural portions of biologicall active constituents (Arinola et al., 2008). Immune cells, like all other types of cells, require an adequate supply of trace elements (Fe, Cu and Zn) to express and preserve the structure and function of key metalloproteins that participate in house keeping processes such as energy production and to protect the cell against highly toxic reactive oxygen species. Also adequate levels of Fe and Zn are required for continous generation of immune cells in bone marrow and the clonal expansion of lymphocytes in response to antigenic stimulation (Chandra, 1990).Considerable research information has been reported on the effects of iron deficiency and its relationship to microbial growth and infections in animals (Kadis et al. 1984, Dallman 1987,Dhur et al. 1989). Many species of bacteria multiply more rapidly if the serum is saturated with sufficient parenteral iron and the animal is challenge-dosed with certain bacterial infections (Klasing et al. 1980, Knight et al. 1983, Desousa 1989).

Copper-deficient animals exhibit several symptoms of immune system dysfunctions. These include an absolute decrease in the number of T cells ,especially T-helper cells (Lukasewycz et al. 1985), and a marked decrease in the T and B-cell mitogens on splenic lymphocytes (Lukasewycz et al., 1985, Flynn 1984). Copper-

deficient animals show a decrease in antibody cell response with increased susceptibility to infection (Suttle and Jones 1989).

There is a strong relationship between zinc and the immune function (Fraker et al. ,1986). A zinc deficiency results in atrophy of the thymus (Fraker et al. 1977) and an increase in leukocyte count (evidence of infectious disease) with a reduced number of lymphocytes. Immature (band) neutrophils are also elevated in zinc-deficient animals.

The objective of this work was studying paratuberculosis among some dairy herds by serological, bacteriological and molecular investigations in addition of clarifying humoral pattern and serum biochemical changes in affected animals.

## MATERIALS AND METHODS

The current study was carried out during 2009 - 2010, on two dairy farms (Fresien Holestein N=431) suffering from incurable intermittent and persistence diarrhea in some animals with typical symptoms like Johne's disease (paratuberculosis). One farm allocated on Alex., desert road (N=268) and the other one on Sharkya governorate (N=163) .The animals in both farms were fed a total mixed ration (TMR) with a quantity according to their milk production.

**I-Samples:**

**1-Blood samples:**

A total number of 431 blood samples without anticoagulant for separation of serum were collected from 2 private dairy farms, for detection of anti Mycobacterium paratuberculosis antibodies in bovine serum by ELISA test ( 268 samples from Alex.desert road farm and 163 samples from a farm on Sharkya governorate).

**Enzyme linked immunosorbant assay (ELISA) test:**

A- Massive ELISA test was performed for serum harvested from adult cows of both two farms. A commercial ELISA kit "institute POURQUIER" was used which approved by OIE (200٤) to analyze serum samples. All experimental samples were analyzed and measured spectrophotometrically in duplicate wells following the manufacturer's instructions. The results were then converted to S/P ratios using the formula provided by the manufacturer.

B- Repetitive ELISA testing was performed for serum samples of 8 positive cows (Alex.desert road farm) with 2 or3days interval for one month to clarify pattern of humoral immunity profile of each positive case.

C-Biochemical analysis : From Alex.desert road Farm, 18 serum samples (10 seropositive animals clinically& subclinically and 8 from seronegative animals) were

analysed for total protein, albumin and , globulin according to the method of Friedman and Young (1997). Copper, iron and zinc. were analyzed according to tietz(1990).

**2-Fecal samples**: Facal samples were collected from seropositive animals of Alex.farm for bacteriological culture and real time polymerase chain reaction , 1-2gm of fecal matter used for bacteriological culture and the remaining sample kept at -20 °C till the PCR investigation.

II-Identification of mycobacterium paratuberculosis :

**a) Isolation and identification of the organism** by using culture onto specific media and staining was performed according to OIE terrestrial manual 2008.

**b) Ziehl-Neelsen stain for isolated bacteria:**

Suspected colonies were stained by Ziehl-Neelsen stain for demonstration of acid fast bacilli.

**c) Real time-polymerase chain reaction,** according to (Collins et al., 1993):

Extraction of DNA were performed according to instructions of QIAamp DNA Mini Kit. Target DNA was amplified with ADIAVET® PARATB REALTIME PCR Kit which uses primers and a TaqMan probe labelled with FAM, specific of *M. paratuberculosis* (IS900). Amplification of this IS*900*-based PCR was conducted in 25 µL and under the following conditions: 1 initial cycle of denaturation and activation at 50 °C for 2 minutes., another cycle of denaturation at 95 °C for 10 minutes and 45 cycles of denaturation at 95°C for 30 sec., annealing, extension and quantification at 60°C for 1 minute on thermocycler apparatus "Stratagene".

## RESULTS AND DISCUSSION

Mycobacterium avium subspecies paratuberculosis is the etiologic agent of Johne's disease in cattle. The disease causes diarrhea , reduced milk production, poor reproductivity, emaciation, and eventually death. In this study , serum ELISA is used to screen a 2 private dairy herds for Johne's disease , but positive tests of one farm confirmed culturally and by real time PCR . In Alex. farm the incidence of paratuberculosis was 9.3% while in Sharkya farm it was 11.04% (table .1) . The higher value of S/P in Sharkya farm was 310 versus 146.24 in Alex. farm denotes that farm may be infected since long time or the infected cows are high shedders for the causative microorganism. Pathogen antigens and activated helper T cells would stimulate B cells to differentiate and begin production of IgM and IgG1 antibodies against M.avium subsp. paratuberculosis antigens. The precise timing of antibody

production likely depends upon the route of entry and dose with higher initial doses of M.avium subsp.paratuberculosis leading to more rapid production of antibody (Waters et al., 2003)

Eight ELISA positive cases were selected ,5 of them were non pregnant and the other 3 were pregnant, taking into account that each group nearly equal in ELISA (S/P) reading, to indicate the extent of change that may be happen during the experiment time (one month). Results (table 2&3) and figure (1) showed that fluctuation in levels of antibodies up and down to the same case in addition of some cases shifted to seronegative then turned again to positive which illustrate the immune privacy of infected cases with paratuberculosis. Variation in seropositivity within repeated samples per cow was noted on several occasions, consistent with findings of Hirst et al., (2002) and Abdel Moghney, 2008). Also, Barrington et al., (2003) reported daily variation in serum ELISA results, which, suggests that the measurable humoral immune response to *Map* can vary widely from day to day. The mechanism causing this daily variation may be the infrequent shedding of MAP stimulates a low-level humoral response, with circulating antigen-specific immunoglobulins including IgA, IgM and IgG (Coussens, 2001).

Culture methods are considered the definitive test for the diagnosis of Johne's disease (Gold standard method) Collins, (1996). In this study, 25 fecal samples from 25 seropositive animals were cultured on Herrold's egg yolk media with mycobactin (HEYM).The cultured media were observed weekly up to 4 months , 17 out of 25 samples (table, 4) revealed typical colonies and when stained with Ziehl_Neelsen denoted acid fast bacilli. By using culture and staining method, we can confirm the obtained results, as organisms were identified as M.paratuberculosis comprise a homogenous group of organisms that cannot be differentiated by biochemical or serological techniques. Isolates were identified as M.paratuberculosis if they were acid fast and mycobactin dependent and required 8 to 16 weeks for primary isolation (Chiodini et al ., 1984). Moreover, the remained 8 samples were negative for colony growth, the explanation of that may because most cattle with Johne's disease typically shed the microorganism intermittently in their feces and in low numbers, particularly in the early stages of disease, (Sockett et al., 1992).

In the past few years molecular approaches to diagnosis have been transforming the investigation of infectious disease. The introduction of real time PCR has greatly reduced identification time and improved the level of detection in clinical specimens.

Real time polymerase chain reaction was done on 25 fecal samples collected from 25 seropositive animals and 17 grown colonies resembles 17 seropositive animals The results of the real time PCR as shown in the normalized melt curves confirming that

fecal extracted DNAs (were M. avium subspp. Paratuberculosis (Fig, 5) as the detection in PCR is based on enzymatic gene ampilification and primers and a Taqman probe labeled with FAM, specific of mycobacterium paratuberculosis (IS900). The results clarified that each resemble sample of the animal (fecal-colony) was differ in its Cycle threshold ( CT) . The colony's CT was earlier than fecal e.g sample no. 5536 fecal CT was( 32) while colony's CT was ( 17.72 ) that is due to DNA concentration in colony sample higher than fecal sample.

In this study within 25 fecal samples one case was purchased from Germany, the farm examined all the purchased animals after their arrival from the quarantine. The surprising result was one case (cow no.1042) revealed positive by ELISA test (S/P= 72 ) , moreover its fecal and isolated colony real time PCR CT was 39.64 and 38.76 respectively (Fig. 5 ). The obtained results considered a strong alarm for the competent authority commitment toward our animal health as testing imported animals should be obligatory for paratuberculosis. Since quantification of MAP DNA in feces by quantitative PCR may provide immediate information to estimate the stage of infection as well as the risk of transmission from infected animals (Kawaji et al 2011).

Sometime, PCR technique on fecal DNA showed lake of sensitivity due to the extreme difficulty of removing PCR inhibitors when preparing DNA from fecal extracts, but may also be due to the difficulty of lysing these organism.

Several methods for the detection of *M. paratuberculosis* by PCR are commercially available. One of these tests was Adiavet® Paratub, Adiagene, Saint Brieuc, France) which recommends use of the QIAamp DNA Mini Kit (QIAGEN) for extraction of DNA from fecal samples. The QIAamp DNA extraction kit was able to remove PCR inhibitors and increase the sensitivity of the test (Chevallier et al., 2002).

Moreover, The kits detection is based on primers and a TaqMan probe labelled with FAM, specific of *M. paratuberculosis* (IS900). The insertion sequence IS*900* is a repetitive DNA insertion element of 1,5 kb and is considered to be unique to *Mycobacterium avium* subsp. *Paratuberculosis*. Also, IS*900* is present in multiple copies (15 – 20) within the *M. avium* subsp. *paratuberculosis* genome. Therefore it is an ideal target for identification.

Real time PCR has several advantages : increases the specificity by including an internal hybridization probe, reduces cross contamination by including UNG (dUTP N-glycosylase) and eliminating post-PCR processing, determines the starting concentration of target sequence in the sample, and is less sensitive to PCR inhibitors. Other advantages of real time PCR was cost analysis, where cost analysis including material and labor, indicated an approximately 50% higher cost per test for bacteriological culture than for the conventional and real-time PCR tests. Furthermore,

the real-time PCR method is relatively simple and robust, and results can be achieved within 24 h. (Sangeeta,et al.,2004). In the current test of real time PCR (Figure 5) , it takes maximum 6 hours, this advantage will help in national control plan of the disease within short time and eliminating risk of shedding MAP pathogens within infected farm and inter between farms.

Real-time PCR is a sensitive method where Schlederer and Raberger (2005) mentioned that one MAP bacteria contains 5 fg DNA and 17 repetitive IS*900* elements. Real time PCR was able to detect a single copy of the IS*900* element.

In late stage of paratuberculosis the ileum becomes thickened by granulomatous inflammation, the animal will develop a protein-losing enteropathy. It will have diarrhoea, impaired absorption of protein, and depletion of the body protein. In particular, there may be marked hypoalbuminaemia, which, in turn, impairs the ability of the animal to retain fluids within the vasculature leading to oedema, which may be most noticeable in the submandibular region as "bottle jaw" (Collins,2003). The clinical cases (table,5) showed marked reduced value in both total protein and albumin, these cases had chronic diarrhea and emaciation with submandibular oedema (figure ,3&4) due to a destructive granulomatous inflammatory response that eventually led to intestinal malabsorption and protein lose enteropathy (Sweeney,2011).

Although the cows were fed balanced ration with feed additives of microelements as recommended, the results in clinical paratuberclosis cases showed marked decrease in both copper and iron concentrations in serum (table,5) that may be due to mycobacterium avium subsp.paratuberculosis metabolism which may lead to serum deficiency or due to malabsorption of such elements in advanced clinical cases which suffer from chronic profuse diarrhea, but it needs more investigations to clarify. The absorption of iron is inhibited by profuse diarrhoea, malabsorption syndrome, achlorohydria, dissertion of small intestine and partial or total gastrectomy (Malhotra,1998). While Engle et al., (2001) studies verified the thesis that no significant correlation exists between copper intake and copper concentration in serum when the cows were fed as recommended.

The studies of Markus et al.,( 2010 ) revealed that trace element (copper, cobalt, iron, Manganese) analysis on liver biopsy of confirmed clinical paratuberculosis cases showed a significantly ($p<0.05$) lowered level in contrast to non-infected healthy cattle, which was in complete agreement of our results (table,5).

Trace elements are thought to play a key role in enzymes that control a series of events required to initiate the immune response. Deficiencies in trace elements such as Copper can decrease an animal's immune response, thereby increasing

susceptibility to disease (Suttle and Jones, 1986). One of the earliest functional defects in cattle undergoing copper depletion is failure of microbiocidal defense mechanisms. Circulating neutrophils retain their capacity for phagocytosis of foreign organisms , but microbiocidal activity declines steadily (Boyne and Arther,1981).

Zinc is needed for tissue repair and wound healing, plays a vital role in protein synthesis and digestion, It is an important constituent of plasma (Malhotra, 1998, Murray et al., 2000). The lowered zinc values in both clinical and subclinical cases (table,5) may be correlated with the stage of disease (clinical with profuse diarrhea or intermittent) also may be correlated with protein and albumin concentrations, where a large percentage, about 90%, of the total plasma zinc concentration is associated with albumin, <10% with alpha-2 macroglobulin (Gordon, 1977), that may explain the lower zinc values in clinical cases with hypoalbuminea due to this closed correlation.
In conclusions Immunologically-based tests for Johne's disease are rapid but lack of specificity and sensitivity. In this context, real time PCR showed a useful role to play in combination with ELISA as diagnostic tools for the control of Johne's disease. The establishment of that diagnostic tool in controlling paratuberculosis among infected dairy herds and protection of new entrance of infected agents within imported animals must be encourage.

Table 1. Results of anti mycobacterium paratuberculosis antibodies in bovine serum by ELISA test .

| Farm | No. of tested animals (431) | Negative | | Suspect% | | Positive% | | S/P | |
|---|---|---|---|---|---|---|---|---|---|
| | | N0. | % | N0. | % | No. | % | Minimum | maximum |
| Alex.farm | 268 | 241 | 89.99 | 2 | 0.7 | 25 | 9.3 | 115 | 146.24 |
| Sharkya farm | 163 | 143 | 87.73 | 2 | 1.22 | 18 | 11.4 | 85.5 | **310** |

1. S/P=Sample positive ratio
2. Positive value (S/P ≥70)
3. Suspected value (S/P<70-60)
4. Negative value(S/P below 60)

Tabl e 2. Pattern of humoral immunity (S/P) of 8 positive cows during one month with 2 days interval testing

| cow no | 29th oct | 1st nov | 4th nov | 6th nov | 8th nov | 11th nov | 13th nov | 15th nov | 18th nov | 20th nov | 22th nov | 25th nov | 27th nov |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 4084 | 145.99 | 189.45 | 193.39 | 181.09 | 209.83 | 171.18 | 168.86 | 208.18 | 207.16 | 207.16 | 201.4 | 182.92 | 183.48 |
| 4835 | 145.2 | 204.7 | 206 | 204.4 | 187.7 | 201.2 | 181.5 | 205.4 | 203.7 | 203.5 | 204.5 | 206 | 202.2 |
| 5159 | 146 | 203.8 | 187.2 | 190.4 | 204.4 | 161.8 | 166.2 | 203.3 | 194.7 | 204.7 | 204 | 183 | 3 |
| 5079 | 145.9 | 203.5 | 204.3 | 206.3 | 206.8 | 200.7 | 203.2 | 203.9 | 203.8 | 205.9 | 204.4 | 203.8 | 203.5 |
| 5424 | 146.2 | 3.3 | 2.3 | 5.6 | 99.7 | 2.9 | 6.6 | 6.1 | 4.1 | 7.6 | 11.5 | 7.8 | 206 |
| 5536 preg | 295.9 | 199.1 | 186.9 | 173.9 | 173.9 | 169.5 | 177.7 | 198.2 | 202.7 | 202.3 | 204 | 200.9 | 203.7 |
| 5720 preg | 295.5 | 205.2 | 201.6 | 208.1 | 180.8 | 172.3 | 165.4 | 194.7 | 204.6 | 3.8 | 199.6 | 186.4 | 194 |
| 5724 preg | 293.2 | 197 | 199 | 183 | 189.1 | 188.3 | 172.5 | 204.4 | 204.4 | 204.4 | 204.6 | 207.6 | 160.9 |

5. S/P=Sample positive ratio
6. Positive value (S/P ≥70)
7. Suspected value (S/P<70-60)
8. Negative value(S/P below 60)

Table 3 . Mean ±SD of S/P antibody values for repeated samples of 8 positive cows during one month with 2 days interval

| Cow number | S/P (Mean ±SD) | Minimum S/P value | Maximum S/P value |
|---|---|---|---|
| 4084( nonpregnant) | 188.46±19.00423 | 145.99 | 209.83 |
| 4835(nonpregnant) | 196.62±17.17919 | 145.2 | 206 |
| 5159(nonpregnant) | 173.26 ±54.53868 | 3 | 204.7 |
| 5079(nonpregnant) | 199.69 ±16.23651 | 145.9 | 206.8 |
| 5424(nonpregnant) | 39.20 ±67.18801 | 2.3 | 206 |
| 5536(pregnant) | 199.13 ±31.87958 | 169.5 | 295.9 |
| 5720(pregnant) | 185.56 ±63.03617 | 3.8 | 295.9 |
| 5724(pregnant) | 200.64 ±31.16832 | 160.9 | 293.2 |

Table 4. Results of fecal culture and real time PCR for the seropositive samples

| No. of fecal samples tested in Alex. farm | Fecal culture | | Real time PCR | |
|---|---|---|---|---|
| | Positive | negative | Positive | negative |
| 25 | 17(68 %) | 8( 32 %) | 25(100%) | Zero |

Table 5 . Biochemical profile of total protein, albumin, globulin, copper, iorn and zinc in sera of clinical and subclinical paratuberculosis cases.

| Test / Case type | Total proteins g/dl Mean ±SD | Albumin g/dl Mean ±SD | Globulins g/dl Mean ±SD | Copper µmol/l Mean ±SD | Iorn µmol/l Mean ±SD | Zinc µmol/l Mean ±SD |
|---|---|---|---|---|---|---|
| Seropositive Clinical cases No.=5 | $^{a}$ 6.03±0.92 | $^{a}$ 2.31±0.27 | $^{a}$ 3.71±1.15 | $^{a}$ 0.716±0.08 | $^{a}$ 16.26±1.71 | $^{a}$ 4.84±0.73 |
| Seropositive Subclinical cases No.=5 | $^{b}$ 8.55±1.20 | $^{ab}$2.66±0.75 | $^{b}$ 5.88±1.23 | $^{c}$ 0.918±0.05 | $^{b}$ 22.51±5.45 | $^{ac}$5.19±1.01 |
| Control (seronegative) N0.=8 | $^{b}$ 8.89±0.38 | $^{b}$ 3.1±0.46 | $^{b}$ 5.78±0.54 | $^{b}$ 1.18±0.27 | $^{ab}$ 19.81±5.01 | $^{b}$ 6.01±0.36 |

Litters with different superscripts within columns are significantly different. (P<0.05)

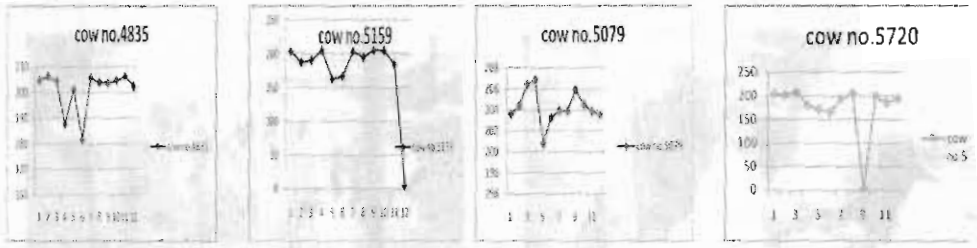


Fig. 1. Results of S/P values for repeated samples of individual cow during one month with 2 days interval

Fig. 2. Typical colonies of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) on" Herrold's egg yolk medium with mycobactin and acid fast bacilli after staining with Ziehl-Neelsen stain

Fig. 3. Submaxillary oedima in paratuberculosis clinical case

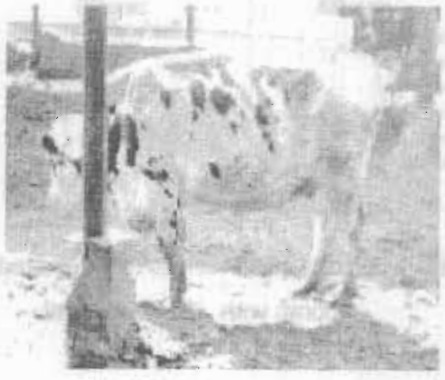

Fig. 4. advanced clinical case

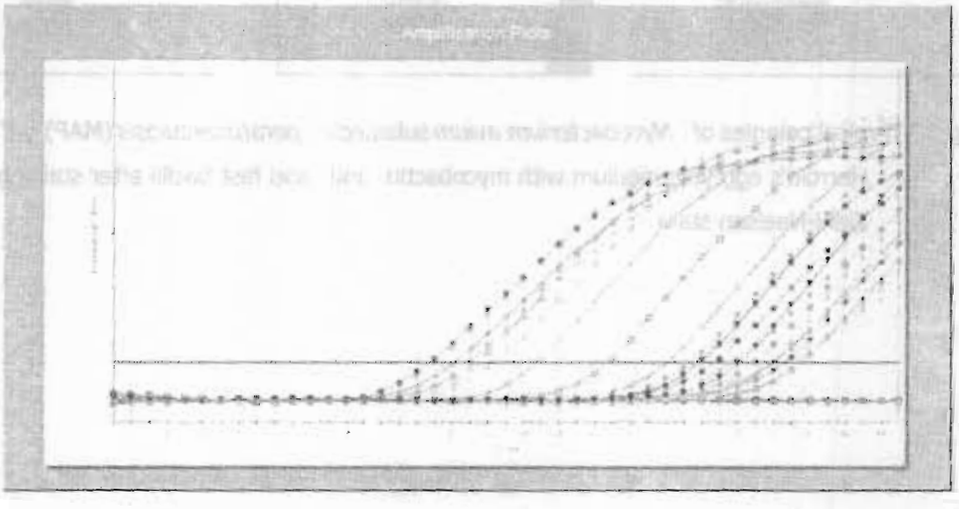

Fig. 5. Amplification plot of fecal samples and mycobacterium paratuberculosis isolates by using ADIAVET® PARATB REALTIME PCR Kit .

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# دراسات بكتريولوجيه و جزيئية وكيميائية حيوية على الأبقار المصابة بالبارا تيوبيركيلوزيز

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أجريت تلك الدراسة على مزرعتين للألبان احداهما بطريق الإسكندرية الصحراوى وعددها ٢٦٨ راس حلاب والأخرى بمحافظة الشرقية وعددها ١٦٣ راس حلاب وذلك لإستبيان إصابتهما بمرض البارا تيوبيركيلوزيز من خلال بعض الإختبارات السيرولوجية إضافة الى محاولات العزل البكتيرى من براز بعض الحيوانات وتطبيق تقنيات البيولوجيا الجزيئية لتحديد الميكروب المسبب وعمل بعض الفحوصات الكيميائية الحيوية على مصل بعض الحيوانات .

ودلت نتائج الفحوص السيرولوجية للكشف عن الأجسام المضادة للميكروب المسبب وذلك بإختبار الإليزا على إصابة ٩.٣% و ١١.٠٤% بمزرعة الاسكندرية الصحراوى والشرقية على التوالى ودلت الاختبارات السيرولوجية أيضا على تذبذب مستويات الأجسام المضادة صعوداً وهبوطاً لنفس الحيوان لعينات تم سحبها بفترة بينية مدتها يومان ولمدة شهر . هذا وقد تم محاولة العزل البكتيرى وذلك بزرع عينات براز (عددها ٢٥) من حيوانات ايجابية للفحص السيرولوجى على المستنبتات البكتيرية الخاصة ودلت النتائج على عزل الميكروب المسبب من عدد ١٧ عينه

تم اجراء اختبار تفاعل انزيم البلمرة المتسلسل ذو الوقت الحقيقى على كل من عينات البراز السابقة (عدد٢٥) وكذلك على الميكروب المعزول منها (عدد ١٧) وذلك للكشف عن جين IS900 الدال على ميكروب mycobacterium aviam subspecies paratuberculosi وأظهرت النتائج إيجابية جميع العينات.

أما الإختبارات الكيميائية الحيوية على مصل بعض الحيوانات فأظهرت نقصاً فى مستويات البروتين و الألبيومين وعناصر النحاس والحديد والزنك فى مصل الحيوانات فى المراحل السريرية.وقد تم مناقشة النتائج ووضع التوصيات اللازمة.