Vet.Med.J.,Giza. Vol.55, No.4. (2007):1019-1033.

A COMPARATIVE STUDY ON DIAGNOSIS OF MYCOPLASMAL MASTITIS AS AND INTERRELATED TO CLINICOPATHOLOGICAL CHANGES

*SARFINAS S. ABD EL- GHANY *DALIA M. MOHSEN ; and **DINA Y. EL-SHAFEY

*Buffalo diseases Research Dept., ** Dept. of Mycoplasma
Animal Health Research Institute, Center of Agriculture Researches, Ministry of Agriculture, Egypt.

Received: 26. 8. 2007
Accepted: 5. 9. 2007

SUMMARY

Out of 100 milk samples collected from 45 buffaloes suffering from clinical mastitis and 55 milk samples collected from 19 apparent healthy buffaloes, showed that 7 samples were positive for Mycoplasma from mastitic cases meanwhile no Mycoplasma was obtained culturally from apparent healthy cases. PCR and indirect enzyme linked immunosorbent assay (ELISA) were used here in this study as a modern technique for diagnosis and results obtained showed that all isolates with the goover samples proved by conventional methods to be Mycoplasma bovis were also found to be positive by the two methods.

Clinicopathological picture was described by haematological and biochemical examination of blood and serum samples of apparent healthy and mastitic buffaloes.

INTRODUCTION

Mastitis is an economically important disease in buffaloes. Mastitis among dairy cows and buffaloes still considered as one of the major diseaseproblems in dairy farms and dairy industry (Ghadersohi et al., 2004).It reduces milk yield and consequently causes significant reduction of income to dairy cattle and buffaloes breeders. A lot of microorganisms are included as causes of bovine mastitis. Among these *Mycoplasma* organisms and several other bacteria characterized by rapid spread within the herd causing sever drop in milk production , prolonged disease course poor response to therapy and high culling rates leading to heavy economic losses in infected dairies (Gonzalez and Wilson, 2003).

In Egypt, *Mycoplasma* species were isolated

from mastitic milk of cows and buffaloes by several researchers (Bayoumi, 2003; Mohsen, 2004; El Shafey, 2005; Abd El Rahman, 2006 and Filiossis et al, 2007) *M.bovis* and *M.bovigenitalium* were the most commonly identified pathogens, mostly from pneumonic calves,but occasionally from cattle with mastitis and arthritis (Ayling et al., 2004). Delay in diagnosis of mycoplasmal infections in dairy herds can result in substantial financial loss and the establishment of chronic carriers (Wilson et al., 2007). So detection of the organism by PCR and ELISA are found to be the methods of choice when sensitivity and rapidity are needed (Svenstrup et al., 2006).

The biochemical and haematological effect of *Mycoplasma* on adult buffaloes were studied by Dacie and Lewis (1991) who studied the biochemical finding of the serum of affiected animals revealed significant changes mainly in blood glucose, cortisol, serum transamination and protein profiles. Marked decrease was noticed in total red blood count and haemoglobin percent in mastitic buffaloes compared with normal cultural (Ulvund, 1990).

The present study was aimed mainly to focus on the presence of Mycoplasma as a real cause of mastitis, In addition to apply a comparative study between traditional method of isolation and identification and that of using moderately recent tools of diagnosis including ELISA and PCR. Secondly a trial to discuss the effect of Mycoplasma on different haematological parameters using haemogram profile.

MATERIAL AND METHODS

Samples: For conducting this work 100 milk samples and 40 blood samples were collected from 40 buffaloes with clinical signs of mastitis. In addition, 55 milk samples and 19 blood samples were collected from 19 apparently healthy buffaloes.

A) Preparation of milk whey for serological testing.

Separation of milk whey was done according to Fey et al. (1976), in which milk was defatted 1:4 with sterile distilled water and the casein then precipitated by adjusting the pH 4.6 with diluted HCL (0.5 N) and removed by centrifugation. The supernatant whey was then immediately brought to pH 6.6 with NaOH (0.5 N) lastly it was kept at - 20° C till use.

B) Preparation of whole blood samples:

Five ml were allowed to flaw freely and gently into clean, dry and acid washed bottles with a tuber stoppers. The bottles contained (EDTA) as anticoagulant in concentration of 1 mg/ml. The sample was then gently and thoroughly mixed. This samples was used for evaluation of some hematological studies including total RBCs, Hb %, PCV, McH, McHC and total and differential leucocytic count.

C) Serum samples: Blood samples were taken in dry clean centrifuge tubes. The collected blood samples were centrifuged at 3000 r.p.m for 20

mint. Clear nonhaemolysed sera were separated by vials for ELISA test and for measuring serum ALT, AST, Alkaline phosphatase (AP), Lactate dehydrogenase, Total protein, Albumin, Globulin, Glucose, Cholesterol and serum cortisol.

Isolation of *Mycoplasma* species:

The isolation of *Mycoplasma* was carried out according to Jasper et al. (1966); 0.2 ml of milk samples was inoculated in to tubes containing PPLO broth with horse serum, DNA, yeast extract, Thallium acetate and penicillin G sodium. Tubes were incubated at 37°C for 3 days after which one loopfull was cultured at 37°C in moisture box and 10 % CO_2 for anther 3 days. Plates were then examined directly under 25 X and 100 X magnifications for typical *Mycoplasma* colonies. The negative plates condition and examined daily for the growth of *Mycoplasma*.

Digitonin Sensitivity Test:-

The test was done to differentiate between *Mycoplasma* and *Acholeplasma* which are capable to grow on the media and cannot be differentiated morphologically (Thurmond et al., 1989). The zone of inhibition of *Mycoplasma* strains ranged from 16 - 38 mm and in *Acholeplasma* from 8 - 14 mm.

Serological Identification of Mycoplasma by ELISA (Ashwani Kumar and Garg, 1996):-

The sonicated whole cell antigen was diluted to 1 in 25 using carbonate - bicarbonate buffer (pH 9.2), centrifuged at 10,000 G for 30 min and protein of supernatant was so adjusted that each well of the plate received 1.75µg /well and 3.31 µg/ well protein of *M.bovis* and *M.bovigenitalium*, respectively. Enzyme anti-globulin conjugate used was horseradishproxides conjugated anti-bovine 1Gg (KPL co.).

The substrate used was 0.2mm solution (w/v) of 40 mm 2,2 -azino-di (3 ethylbenzthiazoline- sulfonate) (ABTS), which was prepared fresh in hydrogen peroxide solution with 0.5 M citrate buffer. Disposable micro titer plates were used as test vehicle for soluble *Mycoplasma* antigen. The optimum dilutions of antigen and anti-bovine gamma globulin- horseradish peroxides conjugate were determined (Boothby et al., 1981). The optimum dilution of antigen was 1:100 for M. bovis (3.5 mg/ml) and *M.bovigenitalium* (6.6 mg/ml) while the optimum dilution of anti-bovine gamma globulin conjugate was 1: 400.

Serum samples were analyzed for *M.bovis* and *M. bovigenitalium* specific antibodies as provided earlier ,Boothby et al., (1981).

Results were expressed as provided earlier. Results were expressed as absorbance at 450 nm. With each test batch, known negative and Mycoplasmal hyper immune sears from cow and calves were included for comparison. The range of maximum absorbance of reference negative serum varied from 0.043 to 0.195 for *M. bovis* antigen

and 0.043 to 0.143 for M. bovigenitalium antigen on the basis of about 65 readings of the same negative serum in our study and taken as cut off absorbance value for calculating ELISA end fiter.

Polymerase chain reaction for diagnosis of Mycoplasma

1.Strains or isolates in 4 eppendorf tube (1 ml each).

2.Centrifuge the tubes in a micro-centrifuge at 12,000 rpm for 5 minutes

3.Wash the cell pellet twice in 100ul of PBS.

4.DNA extraction and purification:

A. DNA extraction by rapid method (Fan et al., 1995)

1.Distribute 4 ml of culture from each Mycoplasma

2.Suspended the washed pellet in 50ul PBS and heat directly at 100°C for 10 minutes in a heat block.

3.Cool on ice or refrigerator for other 10 minutes.

4. Finally, the cell suspension was centrifuged for 3 minutes at 12,000 rpm and the supernatant containing chromosomal DNA was collected and stored at 4°C.

Primer selection:

Two oligonucleotide primers were selected as one right and one left as described by Yleana et al., (1995), were prepared by Sigma (Germany).

Primer (1): 5 CCT TTT AGA TTG GGA TAG

Primer (2): 5 CCG TCA AGG TAG CAT CAT TTC CTA T3

The total reaction volumes were 25 µl containing 50ng of template DNA, 2U of DNA Taq polymerase (Phaemacia), 0.5µl of 10mM DNTP and 200 ng of the *M.bovis* primer (1µl). PCR was performed on a Progene Thermal Controller (U.K.). The reaction conditions were as follow 3 cycles of 15 seconds at 94°C (denaturation), 2 minutes at 28°C (annealing), 3 minutes at 74°C (extension) and then for 35 cycles of 15 seconds at 94°C, 2 minutes at 45°C, 3 minutes at 74°C. Ten micro liter aliquots of amplified DNAs were electrophoresed in 2% agarose dels in TBE containing 0.5% ethidium bromide at 100V. Five micrograms of 100bp DNA DNA ladder (Pharmacia) were also rum in each gel as a standard for size determination of DNA fragments the DNA was visualized under ultraviolet illuminator and photographed. Visualization was done in a transiluminator (Spectroline, Model 312 A, 312 nm Ultraviolet, USA) and photographs were taken by UV camera (Polaroid DS 34 direct screen instant camera, England).

Blood hematology:-

The hemoglobin content was estimated by Drabkins methods which described by Benjamin (1970). The packed cell volume was carried out using microhaematocrit tube after Coles (1986). Total Red Blood Cell Count (RBCs) and total leucocytic count carried out using haemocytometer after Coles (1986).

Biochemical analysis:-

1) Determination of serum aspartate amino transferrase (Ast) and Alanine amino transferase (ALT) were described by Reitman and Frankle (1957).
2) Determination of total protein and albumin were described by Sonnen wirth and Jarett (1980) and Drupt (1974) respectively.
3) Determination of blood glucose using test kits Sonnen wirth and Jarett, (1980)
4) Determination of blood serum cholesterol according to Richmound (1973).
5) Determination of serum cortizole using radioimmunoassay kits according to Calstonr et al., (1988).
6) Determination of serum lactate dehydrogenase (LDH) according to Cabaud and Worblewski (1958).

RESULTS

Out of 100 milk samples collected from 40 buffaloes suffering from clinical mastitis and 19 apparently healthy buffaloes, results obtained reeled that (7) samples were positive from mastitic buffaloes where no Mycoplasma isolates found from the apparently healthy buffaloes.

Table(1):Prevalence of mycoplasmal mastitis from apparently healthy and mastitic buffaloes.

Animal	No. of animals	No. of samples		Mycoplasma species	
		Milk	serum	*M.bovis*	*M.bovigenitalium*
Apparently healthy buffaloes	19	55	19	0	0
Mastitic buffaloes	40	100	40	5	2

Serological identification of mycoplasma isolates revealed 5 *M. bovis* strains and 2 *M. bovigenitalium* by agar gel precipitation test.

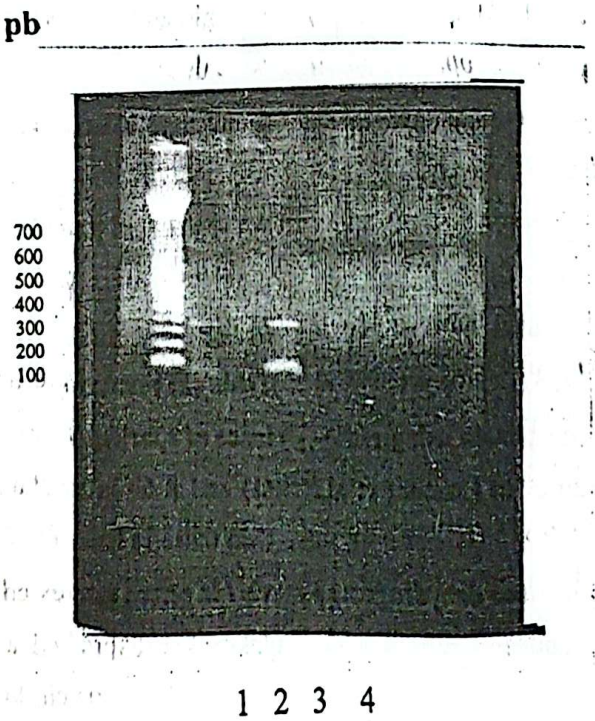
Seroprevalence by ELISA:-

Table (2) reveled a higher antibodies seroprevalence of specific M. bovis in serum samples (52.5 %) and 8 % in milk whey in mastitic buffaloes than those in healthy buffaloes (5.5 %) for M. bovis / in serum samples and 0 % in milk whey samples. Seven of 40 (17.5 %) cases of mastitic buffaloes had sero-diagnostic / suspect M. bovigentalium in serum samples and 5 of 100 (5 %) in milk whey samples Comparing by (11.7 %) of serum samples and (1.81 %) for milk whey samples in case of apparently healthy buffaloes.

Table (2): Seroprevalence of *M. bovis* and *M. bovigenitalium* antibodies in serum and milk whey collected from Egyptian Buffaloes by ELISA.

Animal		*M. bovis*			*M. bovigenitalium*		
		+ Ve	- Ve	%	+ Ve	Ve -	%
App. H	S	1	18	5.5	2	17	11.7
	M.W	0	55	0	1	54	1.81
Mastitic	S	21	19	52.5	7	33	17.5
	M.W	8	92	8	5	95	5

Results of PCR:



Lane 1: Low Molecular weight (Pharmacia)
Lane 2 : positive sample to *M.bovis*
Lane 3: Negative sample to *M.bovis*.
Lane 4: Positive sample to *M.bovis*.

Hematological profile and biochemical analysis:-

The results of hematological and biochemical examinations of apparently and mastitic buffaloes are illustrated in tables (3 -7). The results of hematology contain haemogram and leukogram.

A) Haemogram: The mean values of total red blood corpuscles (TRBcs), hemoglobin content Hb %, packed cell volume (PCV) % mean corpuscular haemoglobin MCH and Mean Corpascular hemoglobin concentration (MCHC) are presented in table (3).

Total RBCs Hb, McH and McHC showed significantly decreased in mastitic buffaloes when compared with the corresponding values of healthy one, while PCV and MCV revealed non significant variation.

B) Leukogram: The mean values of total whit blood cells and differential leucocytic count in apparently healthy and Mastitic buffaloes are presented in table (4). The analysis of data revealed no significant leucopenia, eosinopenia and significant monocytosis in mastitic buffaloes than healthy ones.

Biochemical Examination:-

A) Table (5) shows the mean values of serum enzyme activities of aspartate aminotransferase (AST), alaninaminotransferase (ALT), Alkaline phosphatase (AP) and Lactic dehydrogenase (LDH) in apparently healthy and mastitic buffaloes. There are highly significant in all parameter in the mastitic animal compared with healthy one.

B) The mean values of total serum protein albumin, globulin and A/G ratio in healthy and diseased mastitic buffaloes are tabulated in table (6). There was a significant decrease in serum protein, albumin and A/G ratio of mastitic buffaloes than healthy one, while serum globulin level showed no significant increase in the same animal than healthy buffaloes.

C) The mean values of blood serum glucose, cholesterol and cortisol activity in healthy and mastitic buffaloes are illustrated in table (7). There were significant decrease in serum glucose and significant increase in serum cortisal concentration in mastitic buffaloes when compared with healthy one.

Table (3): Haemogram profile in both apparently healthy and Mycoplasmal mastitic buffalo.

	Apparently healthy	Mastitic
Total RBcs 10^6/ cum	6 ± 1.1	5.01 ± 2.3*
Haematocrit Pcv	31.6 ± 0.72	30.17 ± 1.5
Hemoglobin g/dl	8.4 ± 0.13	7.9 ± 0.55*
MCv	52.5 ± 0.4	53.5 ± 0.9
McH	14.0 ± 0.17	11.58 ± 0.31**
McHc	26.3 ± 0. 8	22.00 ± 0.66*

Table (4): Mean values of total WBCs and differential leucocytic count in both apparently healthy and Mycoplasma mastitic buffalo.

Leucogram		App. healthy	Mastitic
WBcs total (10^3 µl)		11.45 ± 0.02	10.8 ± 0.10
Differential	Neutrophiles %	37.46 ± 1	35.18 ± 0.11
	Eosinophils %	6.91 ± 0.32	4.23 ± 0.20
	Basophiles %	0.44 ± 0.18	0.08 ± 0.10
	Lymphocytes %	60.00 ± 2.0	60.01 ± 3.0
	Monocytes %	4.88 ± 0.46	6.50 ± 0.51*

Table (5): Serum activity of AST, ALT, AP and LDH in both apparently healthy and Mycoplasma mastitic buffalo.

parameter	App. healthy	Mastitic
AST IU/L	32.19 ± 0.91	75.01 ± 4.16**
ALT IU/L	13.52 ± 1.17	17.42 ± 5.01*
AP IU/L	87.0 ± 0.50	112.1 ± 0.31**
LDH IU/L	417.01 ± 9.2	690 ± 17.1**

Table (6): Mean values ± sE of serum T.P. a/b. glob and A/G ratio in both apparent healthy and Mycoplasma mastitic buffalo.

parameter	App. Healthy	Mastitic
Total protein g/dl	6.62 + 0.63	5.81 + 1.9*
Albumin g/dl	3.53 + 0.91	2.29 + 0.31*
Globulin g/dl	3.09 + 0.1	3.52 + 0.34
A/G ratio %	1.14 + 0.43	0.66 + 0.10*

Table (7): Mean values (± st) serum glucose, cholesterol and cortisul.

parameter	App. Healthy	Mastitic
Glucose mg/100nl	60.61 ± 1.05	41.76 ± 3.6**
Cholesterol mg/100nl	141.76 ± 3.6	138.6 ± 4.6
Cortisul mg/100nl	5.51 ± 0.13	8.01 ± 3.06**

* Significant $p < 0.05$

** Highly significant $p < 0.01$

Ast : Aspartate amino transaminase.
ALT: Alanine amino transaminase.
AP: Alkaine phosphatase.
LDH: Lactate dehydrogenase.

DISCUSSION

Mycoplasmal mastitis is a potential highly contagious disease that can cause sever economic losses in affected herds. Moreover, control of mycoplasmal mastitis requires testing and culling of mycoplasmal positive animals as soon as possible Gonzalez and Wilson, (2003).

The present study was designated for screening the prevalence of mycoplasal mastitis infection and evaluation of the current and recent diagnostic techniques.

Fristly using of conventional method revealed that 7out of 100 mastitic milk samples were bacteriologically positive.

Table(1) reveled that the isolation trials of Mycoplasma species from clinically mastitic buffaloes including *M.bovis* 5(5%) and *M.bovigenitalium* 2(2%) and this incidence of isblution was found to be ultimately near to that obtained by Filloussis et al.(2007) who detected Mycoplasma in mastitic cases with percentage of 8.2%. On the other hand Pal et al ., (1984) and El Shinawy et al., (1993) recorded higher percentage of *Mycoplasma species* from clinical mastitis . These variation in the percentage of positive cases may be attributed to breeding system , hygienic measures, immunological states and rate of exposure of such animals to pathogenic organisms as well as other factors. Zaitoun et al., (1991). Current method used to diagnose *Mycoplasma species* infections are inadequate because they lack speed, sensitivity and specificity Simecha et al., (1992), Mohsen (2004), Abd el Rahman (2006) and delay in diagnosis of mycoplasma infections in dairy herds can result in substantial financial loss and the establishment of chronic subclinical carriers Wilson et al., (2007).

The commonly used serological assays for Mycoplasma are complement fixation, indirect haemagglutination, haemagglutination, each is relatively insensitive for detection of the primery immune response, Regalla (1995). So the demand of moderate recent technique for diagnosis become urgent.

The Indirect ELISA for detection of Ab to mycoplasma infection is suggested to be an inexpensive and sensitive method for rapid screening large number of animals Davidson et al., (1981) and Busolo et al., (1983). We report here the production of Indirect ELISA to the serological diagnosis of *M. bovis* and *M. bovigenitalium* infection in Egyptian buffaloes.

The seroprevalence of *M. bovis* specific ELISA antibodies was higher in serum and milk whey samples of mastitic buffaloes (52.5 % and 8%) than apparently healthy buffaloes (5.1% and 0 %). Similarly, *M. bovigenitalium* seroprevalence in mastitic buffaloes (17.5 % and 5%) respectively were higher than those of apparently healthy buffaloes (11.7 % and 1.81 %). It was obvious that the serum antibodies were more clearly presented than in milk whey samples (Table 2).

One of the goal of this study is to make a comparative study as mentioned before and it is worthy to mention that PCR was found to be carried fast and accurate tool for diagnosis and this observation was in accordance to that mentioned by DNA- based techniques, especially PCR can yield rapid and specific diagnosis of infections caused by *M.bovis* Hirose et al ., (2001).

PCR result had confirmed that the isolates were *M.bovis* by the presence of the specific band at 360 bp. Similar results were obtained by Yleana et al.,(1995) who used the M.bovis PCR system to

detect the microbe in nasal samples of calves from a herd suffered from an outbreak of pneumonia as well as Eissa et al. (2007). Also Ghaderoshi etal.(1997) concluded that the PCR assay was 10 times more sensitive than dot blot hybridization (Fig 1).

In comparison to culture, the sensitivity and specificity of the PCR method were 96.2% and 99.1% for individual cow milk .However, in discrepant cases where PCR was positive and culture was negative, the PCR test was correct, sub sequent PCR tests and culturing of the individual cow's milk yielded positive results these results recorded by Cai (2005).

Concerning laboratory studies on hematological profile including haemogram and leucogram in clinically healthy and mastitic buffalo suffering from clinical signs of *Mycoplasma* infection table (3) revealed the mean values of the selected haemogranic parameters. The results presented in table (3) indicated that the haematological profile of mastitic buffaloes showed significant decrease in total RBC s count and hemoglobin value. It is obvious from these results the mastitic animal were suffering from variable degree of anemia as reported by Dacie and Lewis (1991). Also results in table (6) showed that there was significant decrease in total protein among mastitic buffaloes which may be recorded as a matter of dietary disorder where plasma protein are sensitive to nutritional influence as recorded by Kaneko et al. (1997). Also UIvund (1990) observed hypoalbuminaemia could by attributed in adequate protein synthesis as a result of mastitis which badly affected the hepatic parenchyma leading to the failure of protein synthesis as recoded by Coles (1986). On the other hand, the estimation activities of ALT, AST, LDH and Alk.Ph. Enzymes (Table 5), showed high significant elevation which may be due to alternation in the metabolic rate resulted from nutritional deficiency in mastitic buffaloes UIvund (1990). It was clear from the obtained data shown in table (7) that serum glucose level was significantly decreased. The lowered serum glucose level may be attributed to anorexia of mastitic buffaloes and depraved metabolic processes which consequently reflect up on glucose metabolism and consequently on its level as mentioned by Coles (1986) and El-Sangary (1999).

From the obtained data shown in table (7) the serum cortisol level was significantly increased. The high serum cortisol in mastitic buffaloes may be attributed in malnourished animals to reduce clearance rates or to increase secretion rates of the hormone due to the decrease in insulin and T3 level resulting from fatting and malnutrition (Henley and Judith, 1985).

In conclusion, results obtained indicate that with no doubt that using of recent techniques as a tool

for diagnosis shouldn't obliterate the use of conventional culture method and this observation was confirmed by the results obtained which indicated that although there is variation in incidence of isolation with the use of but it is not go to the extent that obligate to use a method at the expense of other methods.

REFERENCES

Abd-El Rahman, M. Aliaa,.(2006):Application of some biotechnological methods for the diagnosis of Mycoplasmal and bacterial mastitis in cows and buffaloes. Ph.D. Vet. Thesis (Bacteriology) Zagazig Uni.

Ashwani, Kumar and Grage, D.N. (1996): Detection of serum antibodies to M. bovis andM. bovigentalium in mastitic cows and buffaloes by ELISA, IHA and IGIPT. Indian Vet. J. 73 (6), 603-606.

Ayling R D , Bashirudddin S.E, Nicholas R A (2004): Mycoplasma species and related organisms isolated from ruminants in Britain between 1990and 2000. Vet. Rec.2004 Oct. 2: 155(14) 413-6.

Bayoumi, F.A.(2003):Clinical immunological aspects of Mycoplasmal mastitis Ph.D. Vet. Thesis (Infectious diseases),Benisuef Cairo Uni.

Benjamine, M. (1970): Veterinary Clinical Pathology. 2nd Ed. USA

Baird SC, Carman J, Dinsmore RP, Walker R L, Collins JK.(2001):Detection and identification of Mycoplasma from bovine mastitis infections using a nested polymerase chain reaction.J Vet Diagn Invest. Sep;11(5):432-5. Erratum in: J Vet Diagn Invest. Mar;13(2):184

Boothby, J.T.; Jasper, D.E.; Rollinns, M. Hand and Thomas, C.B. (1981): Detection of Mycoplasma bovis specific IgG in bovine serum by enzyme linked immunosorbent assay. Am. J. Vet. Res. 42: 1242-1247.

Busolo, F.; Tonin, E. and Meloni, G.A. (1983): Enzyme-linked immunosorbentassay for serodiagnosis of Mycoplasma pneumoniae infections. J. Clin. Microbiol. 432-435.

Cabaud, Pg. and Worblewski, F. (1958): Colormetric measurement of lactic dehydrogenase activity of body fluid. Am. J. Clin. Path. (30) 234-235.

Cai H.Y.,Bell-Rogers P.,Parker L.,Prescott J.F,(2005): Development of a real-time PCR for detection of Mycoplasma bovis in bovine milk and lung samples.J.Vet.Diagn.Invest.,17(6):537-45.

Calstorn, K.; Balton, N.; Kailner, A. and Kihl, O.R. (1988): Cortisol in assay of reproductive hormones when, why and how. IFCC and forms diagnostic, 23.

Coles, E. H. (1986): Veterinary clinical pathology. 4th Ed. W.B. Saunders company Philadelphia, London and Toronto.

Dacie,J.V. and Lewis, S.M. (1991): Practhaematology.Edinburgh,Churchill livingstone 48.

Davidson, M.K.; Lindsey, J.R.; Broun, M.B.; Schoeb, T.R. and Cassell, G.H. (1981): Comparison of methods for detection of Mycoplasma pulmonis in experimentally and maturally infected rats. J. Clin. Microbiol. 14, 646-655.

Drupt, F. (1974): Calorimetric determination of serum albumin using bromocresol gream at pH 4.2. Pharma. Boil., (9) 77-82.

Eissa,S.I., S.Z. Moussa, Hanaa A. Ahmed and Sahar A.El-

Macdawy (2007): Oxidative stress and immunosuppression induced by Mycoplasma infection in cattle.EJBMB 25,62-77.

El- Sanggary, FHM (1999): Studies on causes, diagnosis biochemical changes and treatment of unthriftiness in sheep Ph.D. Vet. Zagazig Thesis Univ.

El- Shafey, Dina, Y. (2005): Advanced studies on *Mycoplasma bovis* in cattle. Ph.D. Vet. Thesis (infectious diseases) . Cairo University.

EL-Shannawy, M.M; Abd-EL-Karim, S.; Zaghawa, A.; Akl Kh.M.; Metwalli and Abou-EL-Enean, G.E.M. (1993): immunofluorescent studies of clinical and sub clinical Mycoplasma mastitis in Buffaloes. 2nd Sci. Cong. Egyptian Society for Cattle Diseases, 5-7 Dec. Assiut Egypt.

Fan, H.H., S.H. Kleven, And M.W. Jackwood. (1995): Applocation of polymerase chain reaction with arbitrary primers to strain identification of *Mycoplasma gallisepticum* . Av. Dis., 39: 729-735.

Fey, H; Pfister, H.; Messerli., J.; Sturzenegger, N. and Grolimund, F. (1976): Methods of isolation, purification, purification and quantitation of bovine immunoglobulins. Zbl. Vey. Med. B. 23; 269-300.

Gad, A.S.; EL-Balkimi, F. A.; EL-Shennawy, M. M.; Eossa, S. I.; Amal Rashwan and EL-Shabbiny, L.M. (1987): Serological studies on Mycoplasma mastitis. Alex. J. Vet. Sci., 13, No.

Ghadersohi, A.; Coelen, R.J. and Hirst, R.G, (1997): Development of specific DNA probe and PCR for the detection of Mycoplasma bovis. Vet. Microb., 56 (1-2): 87-98.

Ghaderschi,A; Hirst,R.G.; Forbes-Faukener,J;and Coclen,R.J.(2004):preliminary studies in prevalence of Mycoplasmabovis mastitis in dairy cattle in Australia .Vet.Microbiology, 65(3):185-194.

Gonzalez, R.N. and Wilson, D.J.(2003).Mycoplasma mastitis in dairy herd. Vet.clinics of North America food animal practice, 19(1) :199-221.

Henley,M.K. and Judith,L.V (1985): Hormonal changes associated with changes in body weight .Clin. obesl. And Gynec. 28(3); 615-618.

Hirose K, Kawasaki Y, Kotani K, Tanaka A, Abiko K, Ogawa H.(2001): Detection of mycoplasma in mastitic milk by PCR analysis and culture methodJ Vet Med Sci. Jun;63(6):691-3

Jasper, D.E. (1977): Mycoplasma and Mycoplasma mastitis. J. Vet. Med. ASS., 170-1167.

Jasper, D.E. (1982): The role of Mycoplasma in bovine mastitis J. Am. Vet. Med. Assoc. 181: 158-162.

Jasper, D.E.; Jain, N.C. and Brazil, L.H. (1966): Clinical and Laboratory observation on bovine mastitis due to Mycoplasma. J.A.V.M.A., 148, 9: 1017-1029.

Kaneko,J.J.:Harvey,J.W. and Bruss,M.L (1997):Clinical Bochemistry of domestic animals.5th Ed. Acad. Press. Harcaurt Brace and company San Diago-calif., USA.

Mohsen Dalia M. (2004): Epidemyological and molecular study for diagnosis of Mycoplasma mastitis.Ph.D. Thesis (Infectioous disease) Benisuef, Cairo Uni.

Pal, B.C.; Singh, P.P. and Pathak, (1984): Isolation of Mycoplasma species from mastitic buffaloes. Ind. J. Anim. Sci., 54 (4): 315-318.

Regalla, J. (1995): Complement fixation reaction for the serologic diagnosis of bovine contagious pleuropneumonia: application and interpretation of the results. Rev. Sci. Tech. 14, 63.

Reitman, S. and Frankel, S. (1957): Colorimetric methods for determination of serum AST, ALT. Amir. J. Clin. Path. 28: 56-58.

Richmaund, W. (1973): Enzymatic determination of cholesterol. Clin. Chem., 19: 1350- 1359.

Sabry, M.Z. and Ahmed, A.A. (1986): mycoplasma from buffaloes in Egypt. Egypt J. Vet. Sci., 23: 1-18.

Simecka, J.W.; Davis, J.K.; Davidson,M.K.; Ross, S.E.; Stadtlander, C.T. and Cassell, G.H. (1992): Mycoplsma diseases of animals. In: Maniloff, J. Mc Elhaney, R., Finch, L., Baseman, J. (Eds), Mycoplasmas: Molecular Biology and Pathogenesis. 391-415.

Sonnen wirth A.C. and Jarett, L. (1980): Gradwohl's Chemical Laboratory Methods and diagnosis 8th Ed. Saintlois C.V. Mosby Co.,

Svenstrup H.F, JensenJs, Gevart K,Birkelunds,and Christiansen G.(2006): Identification and characterion of immunogenic proteins of Mycoplasma genitalium.Clin. Vaccine Immunol.Aug;13(8): 913-22.

Thurmond, M.C.; Holmberg, C.A.and Luiz, D.M. (1989): Evaluation of a Digetonin disk assay to discriminate between Acholeplasma and Mycoplasma isolates from bovine milk. Cornell Vet., 79: 71-81.

UIvund M.J.(1990): Changes in blood chemistry. Acta.Vet. Scand.,31:277-286.

Wilson D.J, Skirpstunas R.T,Trujillo J.D, Cavender K.B,Bagley C.V, and Harding R.L(2007): Unusual history and initial clinical signs of Mycoplasma bovis mastitis and arthritis in first- Lactation cows in a closed commercial dairy herd.J.Am.Vet. Med. Assoc. May 15; 230 (10) 1519-23.

Yleana,R. Chave Gonzalez, Carlos Ros Bascunana, Goran Bolske,Jens G. Mattsson, Carmen Fernandez Molina Karl- Erik Johansson.(1995): In vitro amplification of the 16 sRNA genes from Mycoplasma agalactiae by PCR. Vet. Microbiol.,47:183-190.

Zaitoun, A. (1990): Role of some Mycoplasm species in bovine mastitis. Ph.D. Vet., Assiut Uni.

Zaitoun, A.M.; EL-Allawy, T.A.; Abdallah, I.S.; EL-Ebeedy A.A.; Eissa, S.I. and EL-Shabiny, L.M. (1991): Incidence of buffaloes in Upper Egypt Assiut Vet. Med. J. 25, 50: 108-114.