Animal Health Research Journal Vol. 7, No. 4, November 2019 pp. 988-1006

ISSN: 2356-7767

Comprehensive Phenotypic, genotypic, and immunogenic perspective of *Mycoplasma* and *Brucella* mixed infection in a dairy cattle farm at El-Fayoum governorate

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Received in 1/10/2019
Accepted in 1/11/2019

Abstract

A total of 70 serum, 50 milk samples and 20 nasal swabs were collected from 70 animals; over 6 months of age(50 lactating cows,7 dry cow, 12 heifers, and one bull) from *Brucella* unvaccinated private cattle farm located at Tamiya, El-Fayoum with a case history of late-stage abortion and mastitis. Upon recent replacement of newly purchased animals from local markets without previous examination to both *Brucella* and *Mycoplasma* infections. Molecular typing of *Mycoplasma* in clinical samples (50 milk samples and 20 nasal swabs) from the 70 animals were investigated using four groups of primers sets for conventional PCR assays (previously published). 14.3%(10/70) of extracted DNA samples were positive for Mollicutes giving specific band at 280 bp, 12.8%(9/70) were positive for *Mycoplasma* of ruminants giving specific band at 1000bp, 8.5%(6/70) were positive for *Mycoplasma bovis* giving specific band at 360bp, By using primers targeting Variable Surface Protein (VSP) gene for *Mycoplasma bovis* one of the DNA samples give specific band at about200 bp. By phenotypic characterization; *Mycoplasma bovis* was isolated from six clinical samples and three un-typed glucose positive *Mycoplasmas* were isolated also, one *Acholeplasma* strain was isolated. The serological diagnosis of Brucellosis resulted in 13% (9 out of 70) serum samples positivite for both Rose Bengal plate (RBPT), and micro-agglutination tests (MAT). Rivanol test (Riv.T), complement fixation test (CFT), and competitive ELISA gave positive results of 10% (7 out of 70).The estimated apparent prevalence of Brucellosis was 10% in the private farm under the study. Phenotypic characterization for *Brucella* from the milk samples resulted in the recovery of two *Brucella* isolates identified as *Brucella melitensis* biovar 3, Molecular typing of the *Brucella* isolates using AMOS-PCR illustrates PCR band of 731 bp in size-specific for *B. melitensis*. All serum samples were divided into four groups, normal group (G 1), *Brucella* infected group (G 2), *Mycoplasma*- infected group (G 3) and *Brucella- Mycoplasma* infected group (G 4). Then the immune status of the groups was studied by applying the serum nitric oxide (NO) assay, plate lysozyme activity, total proteins, and polyacrylamide gel electrophoresis. The results revealed a significant increase in the nitric oxide and lysozyme levels in *Mycoplasma* infected group compared to the other groups. Also; there were significant differences in the concentration of the total proteins and protein fractions between different groups. The difference in the studied immune parameters may be due to the difference in the severity and stage of infection of each pathogen in the different groups which reflected on the immune response of the animals. **The authors concluded that,** careful selection of replacement animals either purchased or produced from existing stock, should originate from *Brucella*-free and *Mycoplasma* free herds or flocks. Also concluded, the pre-purchase tests are necessary unless the replacements are from populations known to be free of both diseases. Also, the authors recommended isolation of purchased replacements for at least 30 days, besides a serological test before commingling is necessary. Molecular typing of *Mycoplasma* and *Brucella* in dairy herds is a rapid screening test to avoid *Mycoplasma* mastitis and *Brucella* latent infection.

Keywords: *Mycoplasma bovis, Brucella melitensis, AMOS-PCR, El-Fayoum, Nitric oxide, Lysozyme activity.*

Introduction

Mastitis in dairy cattle continues to be an economically important disease. Brucellosis has been proved to induce subclinical mastitis in infected cattle and can affect the composition and manufacturing properties of milk **(Khan, *et al.*, 2014).** Despite extensive management practices including antibiotic treatments, mastitis is estimated to cause annual losses for dairy farmers and industry **(Sordillo and Streicher, 2002)**. These losses arise from reduction in milk yield, waste of milk unfit for consumption, antibiotic use and premature culling. The disease can be caused by a wide range of Gram negative coliform and Gram positive bacteria, as well as yeast, fungi and *Mycoplasma* **(Fox and Gay, 1993).**

There is a worldwide problem of disease caused by *Mycoplasma bovi*s in cattle; it has a significant detrimental economic and animal welfare impact on cattle rearing. Infection can manifest as a plethora of clinical signs including mastitis, pneumonia, arthritis, keratoconjunctivitis, otitis media and genital disorders that may result in infertility and abortion **(Calcut *et al.,* 2018)**

Mycoplasma bovis was isolated for the first time from the abomasal content of an aborted bovine foetus in the UK from a small dairy herd **Byrne *et. al.* (1999)**.Clinical experience has shown that, *Mycoplasma* outbreaks occur most frequently during times of stress, such as calving, extreme weather shifts, and periods of commingling with new animals **(Hanson, 2001)**. He also mentioned that, most *Mycoplasma* infections can be traced to newly arrived heifers or cows on the dairy. Once placed in the milking herd, the highly contagious *Mycoplasma* readily transfer from infected cows to non-infected cows during the milking process on the milking equipment, common towels used for washing or dry udders or milkers'hands. Cows in any stage of lactation – dry or milking – can become infected.

Chopra-Dewasthaly *et al.* (2017) explained that; the Mycoplasmas can propagate under ideal conditions in labs and don't have to expect an immune response. This means that there is no immediate need to respond to the inactivation of the phase variation." In an infected host, the Mycoplasmas as pathogens must become active immediately once they are recognized and attacked by the immune system if they want to survive. The fact that, pathogenic Mycoplasmas can compensate the inactivation of the phase variation in their natural environment in a host organism and how this is done can only be detected and analyzed in a living animal.

Brucellosis is the common name used for the animal and human infections triggered by several species of the genus *Brucella* (**OIE, 2018**).

Brucellae show a wide range of host preference. Currently, twelve *Brucella* species exist (**Scholz *et al.,* 2016**) including three that have been reported in Egypt (**Menshawy *et al.,* 2014; Abdel-Hamid *et al.,* 2017**), viz. *B. abortus, B. melitensis,* and *B. suis. B. melitensis* infection of small ruminants is relatively similar in both pathological and epidemiological viewpoints to *B. abortus* infection of cattle. *Brucella melitensis biovar 3* is the most predominate *Brucella* species in Egypt followed by *B. abortus* (**Wareth *et al.,* 2017**). The main symptoms of Brucellosis in ruminants are reproductive disorders in form of abortion or birth of weak off-springs that do not survive, low milk yield (20-25% reduction), orchitis, epididymitis, and less commonly arthritis may occur. Which makes the matter worse is that *B. melitensis* causes no abortion storms in pregnant cattle (**Godfroid, 2017 and Hussein *et al.,* 2019**). Moreover, Brucellosis is famous for its latent infection which hinders any control programs (**Corbel, 2006)**. In Egypt they found a significant correlation between clinical and subclinical mastitis and *Brucella* isolation in infected cows **(Zakaria *et al.*, 2016).**

The host immune response can be functionally classified into innate and adaptive immunity. The innate response is the first line of defense against invading pathogens that occurs during the early stages of pathogen invasion. Its elements include physical barriers, humoral components such as complement, lysozyme and nitric oxide can remove microorganisms by opsonization and bactericidal actions. **(Green *et al.,* 1990, Parkin and Cohen, 2001** and **Ku-**

bota, 2010). While the adaptive immunity is antigen specific effect and immunological memory can eliminate pathogens rapidly and effectively. The adaptive immunity consists of T helper cells, cytotoxic T cells and antigen specific antibody producing B cells which produce antigen-specific antibodies that neutralize or opsonize the pathogen **(Golding *et al.*, 2001).**

Nitric oxide (NO) is a part of the innate immune response. It is a free radical molecule generated by phagocyte as a proinflammatory cytotoxic mediator that defends hosts against infectious agents and tumor cells **(Green *et al.*, 1990).** It synthesized intracellular from the terminal guanido-nitrogen atom of L-arginine by inducible nitric oxide synthase (iNOS). **(Moncada, 1991**). Lysozymes are vital constituents in the innate immune system. Which known as, 1,4,b-N-acetyl muramidase that degrades a glycosidic linkage between C-1 of N-acetyl muramic acid and C-4 of N-acetylglucosamine in the peptidoglycan of bacterial cell wall. Its serum concentration serves as an indirect marker of primitive unspecific defense mechanism associated with the monocyte macrophage system **(Sotirov, *et al.*, 2005).**

Serum protein electrophoresis is a useful laboratory separation technique for fractionation and quantification of serum proteins. Changes in serum proteins can be indicative of many health problems and may provide information on chronic or acute inflammatory disorders **(Yang *et al*., 2012)**. Normal bovine serum protein pattern following electrophoresis can be separated into five fractions, comprising albumin, α1-, α2-, β-, and γ-globulins **(Piccione *et al.*, 2012a).** Whereas **Nagy *et al.*, (2015**) described six fractions comprising albumin, α-1 and α-2, β-1 and β-2, and γ-globulins. Each band is made up of a group of individual proteins, each of which is characterized by independent metabolic properties.

The aim of this study is to investigate the causes stand behind the abortion and mastitis emerged in a private farm located at Tamiya, El-Fayoum besides the epidemiological situation as well as the immunological status of the animals inside the private farm.

Materials and Methods

I-Animals and sampling

A total of 70 serum and50 milk samples and 20 nasal swabs were collected from 70 animals over 6 months of age from *Brucella* unvaccinated private cattle farm located at Tamiya, El-Fayoum with a case history of late-stage abortion and mastitis upon recent replacement of newly purchased animals from local markets without previous examination to both *Brucella* and *Mycoplasma bovis* infections.

II-*Mycoplasma* isolation and identification:

1- Liquid and solid media were used for the isolation and propagation of *Mycoplasma* were prepared as described by **Sabry and Ahmed (1975).**

2-Digitonin sensitivity test was done for the obtained isolates according to **Freundt *et al.*, (1973)** to differentiate between *Mycoplasma* and *Acholeplasma* species.

3-Biochemical characterization was carried out by glucose fermentation, arginine deamination and film and spot formation tests as described by **Erno and Stipkovits (1973).**

4-Serological identification was conducted by growth inhibition test as described by **Clyde (1964).**

III- Detection of *Mycoplasma* using polymerase chain reaction (PCR):

DNA from a *Mycoplasma* suspension and from different samples was extracted using a DNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions.

Four sets of primers were used:1- A set of Primers used to detect members of the Class Molecules 2-one set of Primers used for detection of 16S ribosomal RNA for ruminant *Mycoplasma* and 3- one specific for *Mycoplasma bovis* used for the identification of *M. bovis* isolates.4- One specific for *Mycoplasma bovis* used for the detection of MB virulence gene VSP.

Table (1). Sequences and characteristics of the primers used for *Mycoplasma* Detection:

Oligonucleotide primers used in this study. According to	Sequence	Designation	Species
van Kuppeveld *et al.*, (1994)	5'-GGGAGCAAACAGGATrAGATACC CT-3' 5'-TGCACCATCTG TCACTCTGTTAACCTC-3'	GPO MGSO	Detect members of the Class Mollicutes Gives specific band at about280bp
Alberto *et al.*, (2006)	5'- AGA CTC CTA CGG GAG GCA GCA -3' 5-ACT AGC GAT TCC GAC TTC ATG -3'	MunivF MunivR	Sequence of 16S common gene for ruminant *Mycoplasma* Gives specific band at about1000bp
Yleana *et al.*, (1995)	5'-CCT TTT AGA TTG GGA TAG CGG ATG-3' 5'-CCG TCA AGG TAG CGT CAT TTC CTA C-3'	MboF MboR	*M. bovis*Gives specific band at about 360bp
Alberto *et al.*, (2006)	5'-CTTGGATCAGTGGCTTCATT-AGC-3' 5'-GTCATCATGCGGAATTCTT-GGGT-3'	MYBF MYBREV	VSP Gives variable band at about 400bp

IV-Serological tests for *Brucella*:

1. Serum samples were serologically examined against brucellosis using 1. Screening test, namely Rose Bengal plate test (RBPT) 2. Supplementary tests, namely Riv. T and MAT 3. Confirmatory tests, specifically CFT and CELISA.

2. RBPT and rivanol antigens were purchased from (NVSL/DBL, USDA, USA). Riv. T and RBPT were performed according to **Alton *et al.* (1988).**

3. Antigen for the CFT was imported from NVSL / DBL, USDA, USA. Complement, hemolysin, and antigen were prepared and preserved according to **Alton *et al.* (1988).** Sheep RBCs were collected on Alsever's solution from an adult healthy ram serologically negative to brucellosis. The sheep RBCs standardized to 3% suspension in veronal buffer diluent. A warm fixation of complement was adopted. The test was performed according to **Alton *et al.* (1988).**

4. The antigen for micro agglutination test (MAT) was locally produced by the Veterinary Serum and Vaccine Research Institute, Abbasseya, Cairo. It was standardized to give 50% agglutination at 1/500 of the OIEISS. The MAT was performed according to **Alton *et al.*, (1975).**

5. Multispecies competitive ELISA kit (SVANOVIR® *Brucella*-Ab C-ELISA), was produced by Svanova Biotech AB, Uppsala, Sweden. This kit uses *Brucella abortus* smooth lipopolysaccharide antigen, horseradish peroxidase-conjugated goat anti-mouse IgG monoclonal antibodies and tetramethylbenzidine in

substrate buffer containing H2O2. Validation of the kit was done according to the kit instructions, the validation guidelines of the (ISO/IEC 17025, 2005). The test was performed according to the kit instructions and the percent inhibition (PI) was estimated for competitive ELISA kit from the formula: PI = 100 – [(Mean OD samples×100)/ (Mean OD Conjugate control)]. The status of test results was determined as follows: PI Status ≥ 30% was considered positive. If < 30%, the test was negative.

V-Detection of immunoglobulin and *Brucella* micro-organisms in cow milk samples:

Detection of immunoglobulin in milk by INGEZIM® *Brucella* bovine milk indirect ELISA

ELISA was performed on milk samples collected under aseptic conditions from serologically positive animals with the INGEZIM® *Brucella* bovine milk indirect assay (Immunology and Applied Genetics, Madrid, Spain). The assay detects antibodies against *Brucella* spp. in bovine milk. Detection of anti-*Brucella* antibodies was performed and interpreted according to manufacturer's instructions.

VI-Bacteriological examination of *Brucella*:

Milk samples were collected from live serologically positive animals for the isolation and typing of *Brucella* microorganisms. Bacteriological identification at genus (colonial morphology, microscopic appearance, catalase, oxidase and urease), species level (phage lysis) and biovar level (CO2 requirement, H2S production, growth in the presence of thionin and basic fuchsin and agglutination with monospecific antisera) was done according to **Alton *et al.*(1988).**

VII-AMOS-PCR assay for *Brucella*:

DNA was extracted from bacterial culture harvested in Phosphate-*buffered* saline with pH 7.2 and being inactivated at 80ºC for 1 hour using QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany). The PCR assay was done under the following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 56°C for 2 min, and 72°C for 2 min, with a final extension of 70°C for 5 min. AMOS-PCR was performed for typing *Brucella* at the species level and the assay was performed according to **Bricker and Halling (1994) and Bricker *et al.* (2003).** Details about primers' sequences and characteristics were shown in **Table (2).**

Table (2) Sequences and characteristics of the primers used in the AMOS-PCR assay

PCR assay	Primers	Sequences (5´→3´)	DNA targets	DNA products (bp)	Concentration (µM)	Reference
AMOS-PCR	*B.Abortus*	GAC GAA CGG AAT TTT TCC AAT CCC	IS711	498	0.1	Bricker and Halling, (1994) Bricker *et al.,* (2003)
	B,Miletensis	AAA TCG CGT CCT TGC TGG TCT GA		731	0.1	
	IS711	TGC CGA TCA CTT AAG GGC CTT CAT			0.2	

VIII-Measurement of serum nitric oxide and lysozyme in cows serum:

Estimation of the serum nitric oxide and lysozyme activity in all serum samples were carried out according to **Yang *et al.* (2010)** and**Peeters and Vantrapen (1977)** respectively.

IX-Estimation of the serum total protein and electrophoretic pattern:

Total protein and electrophoretic pattern analysis were carried out in all serum samples by polyacrylamide gel technique according to **Sonnenwirth and Jarrett (1980) and Davis (1964),** respectively and calculated according to SyneGeneS. No. 17292* 14518* sme*

mpcs. Also, total of albumens (sum of prealbumen and albumen), **α-** globulins (sum of **α-1 and α-2**), β- globulins (sum of β-1 and β-2), γ-globulins (sum of γ-1 and γ-2), globulins (sum of total **α,** β and γ globulins) and albumens/ globulins ratio (A/G) were calculated.

X-Statistical analysis:

The significance of the data was evaluated by analysis of variance (ANOVA) and LSD at $P<0.05$ to the means by the Statistical Package for Social Science **(SPSS-14, 2006).** Brucellosis apparent prevalence (AP) = Total number of seropositive animals / total number of examined animals (**Thrusfield, 2007**).

Results

I-Results of *Mycoplasma* isolation and identification

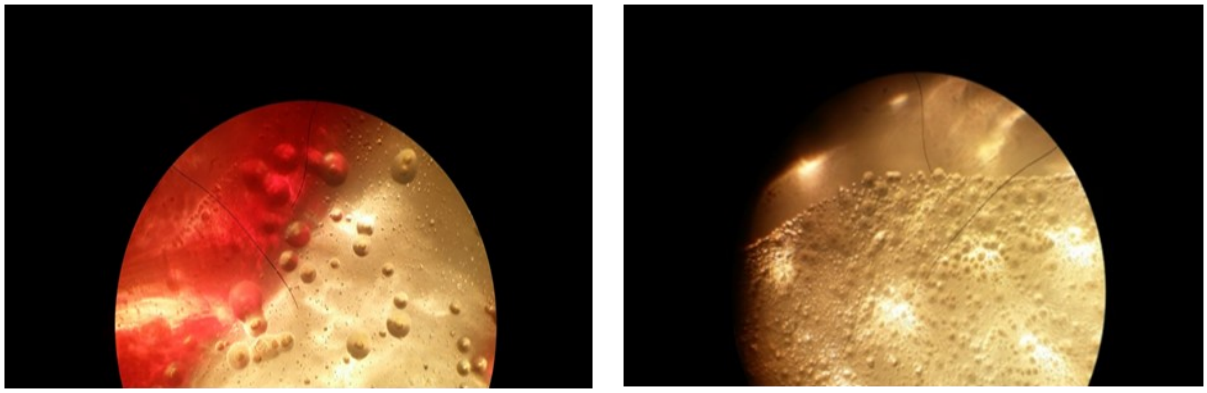


Figure (1). Mixed *Mycoplasma* cultures under stereo microscope showing typical fried egg appearance

Table (3). Identification of the isolates using digitonin sensitivity test:

Type samples	Number	Digitonin sensitive (*Mycoplasma*)		Digitonin resistant (*Acholeplasma*)	
Milk Samples	**No=50**	NO=7	7/50=14%	NO=1	1/50=2%
Nasal swabs	**No=20**	**No=2**	2/20=10%	0	0
Total Number	**No=70**	**No=9**	9/70=12.8%	**No=1**	2%

Table (4a). Biochemical characterization of *Mycoplasma* isolates from cow's milk samples and from nasal swabs:

Samples	No. of *Mycoplasma*	*Mycoplasma bovis* glucose negative, Arginin negative , film and spot positive		Untyped *Mycoplasma* Cultural glucose positive & Arginin negative and negative film and spot	
		No.	%	No.	%
Cow's milk sample	7/50	6	6/50=12%	1	1/50=2%
Nasal swabs	2/20	0	0/20	2	2/20=10%
Total Number	9/70	6	6/70=8.5%	3	3/70=4.2%

II-Results of *Mycoplasma* detection by PCR:

Table (4b). Results of Mycoplasma detection by PCR:

Type of PCR	Positive
PCR primers for Mollicutes give specific band at 280bp	10/70
PCR primers for *Mycoplasma* of ruminant give specific band at1000bp	9/70
PCR primers for *Mycoplasma bovis* give specific band at 360bp	6/9
PCR primers for *Mycoplasma bovis* (VSP gene) give variable band at about200bp	1/6

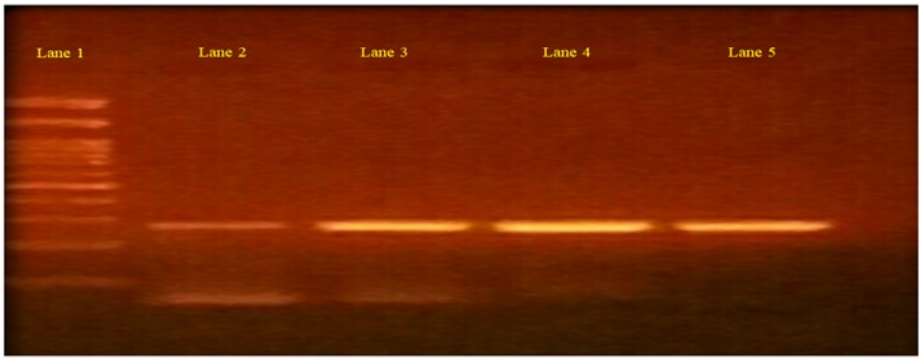


Fig. (2): Results of molecular typing of *Mycoplasma* by PCR1-100bp DNA marker.2-control positive *Mycoplasma* strain 3&4&5-Positive clinical samples

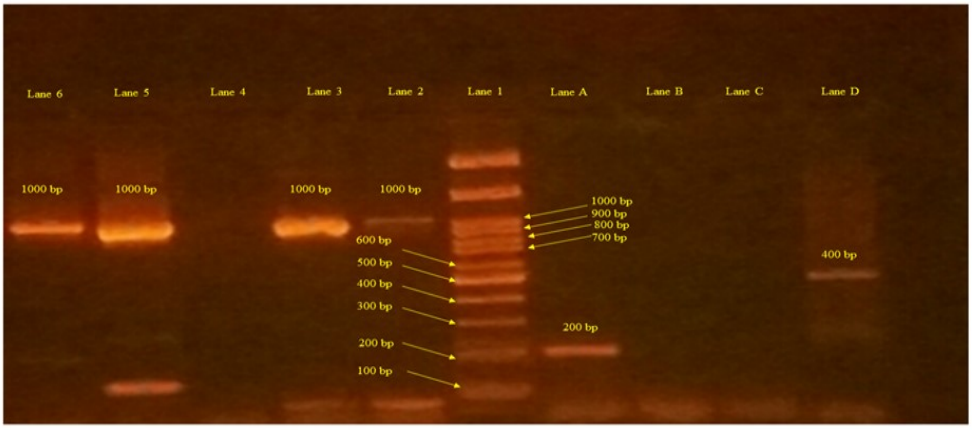


Fig. (3): On the right of the marker: Molecular typing of the *Mycoplasma* using *Mycoplasma* VSP primers giving specific band at 400bp1-100bp DNA marker .2- clinical sample gives variable band at 200bp 3- Negative control. 4- Negative clinical sample 5- Positive control *Mycoplasma bovis* strain at 400bp. On the left of the marker: Molecular typing of the *Mycoplasma* using *Mycoplasma* common ruminant primers giving specific band at 1000bp.1-100bp DNA marker. 2- Positive control *Mycoplasma* strain 3- Positive clinical samples4-Negative clinical sample 5&6-Positive clinical sample

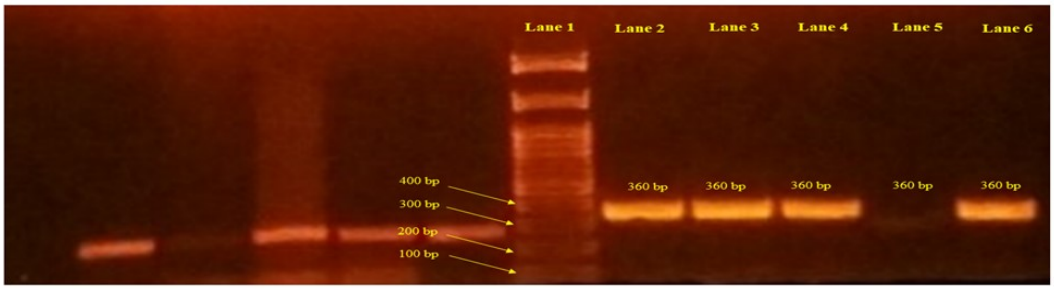


Fig. (4): On the right of the marker: Molecular typing of the *Mycoplasma* using *Mycoplasma bovis* primers giving specific band at 360bp; Lane1(100bp DNA marker). Lane 2&3&4- Positive clinical samples. Lane 5-weak positive clinical sample. Lane 6- Positive control *Mycoplasma bovis*. On the left of the marker: Molecular typing of the *Mycoplasma* using MGPO primers giving specific band at280bp. Lane1-100bp DNA marker. Lane 2&3&4- Positive clinical sample. Lane 5-weak positive clinical sample. Lane6- control positive *Mycoplasma* strain.

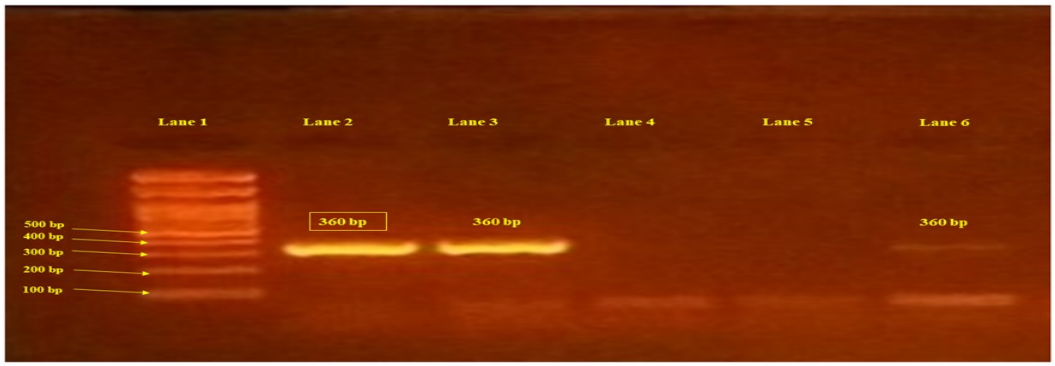


Fig. (5): Molecular typing of the *Mycoplasma* using *Mycoplasma bovis* primers giving specific band at 360bp. Lane1-100bp DNA marker. Lane 2&3- Positive clinical samples.Lane4-negative clinical sample Lane 5-control negative. Lane 6-Positive control *Mycoplasma Bovis*

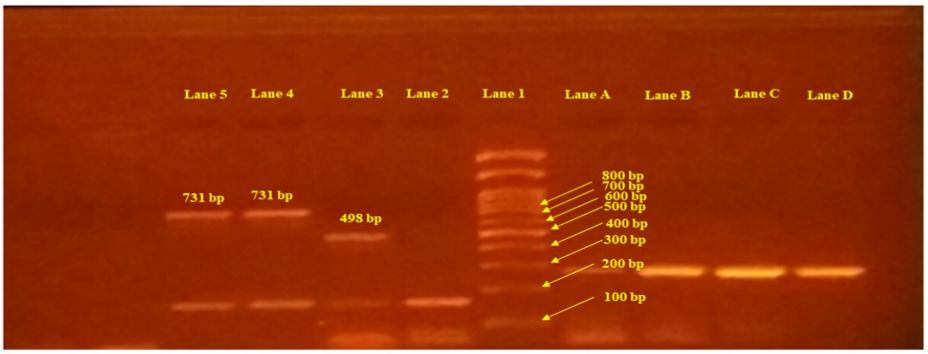


Fig. (6): Illustrates molecular typing of both *B. melitensis* and *Mycoplasma* spp where the right side of the marker showed a Molecular typing of the *Mycoplasma* using MGPO primers giving specific band at 280 bp (Lane A, B, C, and D). On the left side of the marker: Molecular typing of the *Brucella* isolates at species level using AMOS-PCR which illustrates PCR band of 731 bp in size-specific for *B. melitensis* (lane 4; *B. melitensis* reference strain 16M, and Lane5; field strain), a PCR band of 498 bp specific for *B. abortus* reference strain 544 (Lane 3)**,** and *Brucella* negative control represented by lane 2.

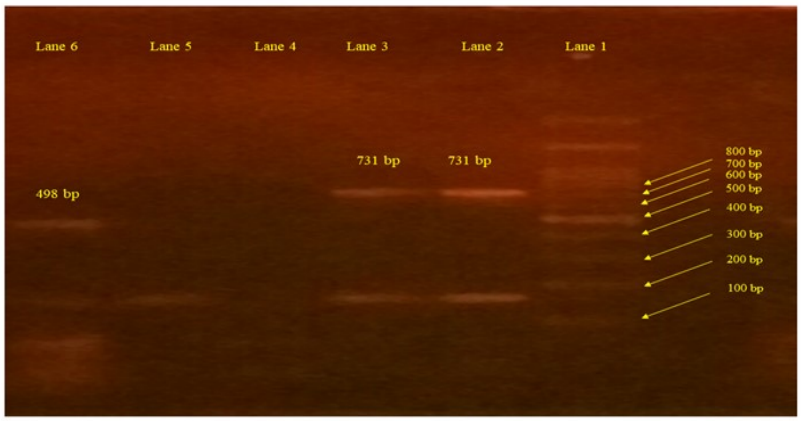
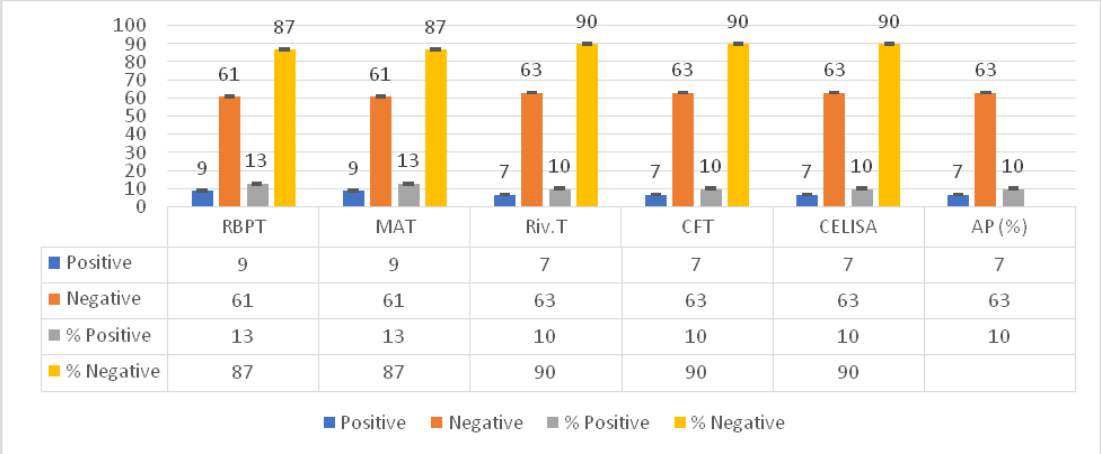


Fig. (7): Illustrates molecular typing of *B. melitensis* using AMOS-PCR where a PCR band of 731 bp (size-specific for *B. melitensis*) is displayed for both *B. melitensis* reference strain 16M (Lane 2), and *B. melitensis* field strain (Lane 3) against 100 bp DNA ladder (Lane 1). A PCR band of 498 bp specific for *B. abortus* reference strain 544 (Lane 6)**,** and *Brucella* negative control (Lane 4 and 5) was exhibited.

III-*Brucella* Results:



	RBPT	MAT	Riv.T	CFT	CELISA	AP (%)
Positive	9	9	7	7	7	7
Negative	61	61	63	63	63	63
% Positive	13	13	10	10	10	10
% Negative	87	87	90	90	90	

Fig. (8): Percent positive of screening, supplementary, and confirmatory immunoassays used in the diagnosis of Brucellosis as well as the estimated apparent prevalence in the private farm

Serological diagnosis:

The collected serum samples were examined in a series manner against brucellosis using a screening test (Rose Bengal plate test), supplementary; Micro-agglutination, and Rivanol plate precipitation tests, and confirmatory tests (Complement fixation test, and competitive ELISA). Where animals considered positive to Brucellosis if their sera gave both positive reactions to screening tests as Rose Bengal plate test (RBPT) followed by either a Complement fixation test (CFT) or competitive ELISA as confirmatory tests.

The serological diagnosis of Brucellosis done on the serum samples resulted in 13% (9 out of 70) positivity for both RBPT, and MAT. While Riv. T, CFT, and CELISA gave positive results of 10% (7 out of 70).As shown by **Fig(8).** The estimated apparent prevalence of brucellosis was 10% in the private farm under the study.

2-Phenotypic and genotypic characterization of the *Brucella* isolates:

Bacteriological examination (phenotypic characterization) of the milk samples resulted in the recovery of two *Brucella* isolates. The isolates were subjected to colonial morphology (+ve oxidase, +ve urease in 18-24h, microscopic appearance of convex, circular, translucent, gram -ve coccobacilli, and +ve to catalase test)

for the identification at the genus level. In Phage lysis for speciation, both isolates give complete lysis to Izatnagar phage. Regarding identification at the biovar level, the two strains exhibited a negative H_2S production, growth in the presence of 1/25000 and 1/50000 of thionin and basic fucksin dyes, not required CO_2 for their growth and they were agglutinated with both A and M mono-specific sera. Based on these phenotypic characteristics and phage typing, the field isolates were identified as *Brucella melitensis* biovar 3 (**Table 5**).

Table (5). Detailed identification of *Brucella* isolates at genus, species and biovar levels

Genus Identification	**Microscopic appearance**	**Gram negative coccobacilli, weak acid fast**		
	Colonial morphology	Smooth, convex, honey coloured		
	Oxidase	+ve		
	Urease	+ve within 18-24 h		
	Catalase	+ve		
Species identification	Phage lysis	Tbilisi	Izatnagar	Rough/Canis
		-ve	+ve	-ve
	AMOS-PCR	Band at 731 bp		
Biovar identification	H2S production	-ve		
	CO2 requirements	-ve		
	Growth on dye containing media	Thionin		Basic fucksin
		1/50000		1/25000 1/50000
		+ve		+ve +ve
	agglutination with mono-specific antisera	A	M	R
		+ve	+ve	-ve
Final conclusion		*Brucella melitensis*bv 3		

The results of serum nitric oxide and lysozyme assays:

The results of serum NO assay are presented in table (6) which indicated that, the *Brucella.* -infected group (G2) gave insignificant higher values than the normal group (G1). While the-*Mycoplasma* group (G3) showed the highest NO and gave significant increased than G1 and G4. However the lowest NO level was recorded in *Brucella- Mycoplasma* group (G4). Regarding to the results of the lysozyme assay (table 6), the *Mycoplasma* group gave marked significant increased lysozyme. While theG2 showed the lowest level among all groups. Also, G4 showed decreased value than G1 and G3.

Table (6). Levels of nitric oxide and lysozyme in cow serum from different groups.

Groups / **Parameters**	**G1 normal cows**	**G2 *Brucella*-infected cows**	**G3 *Mycoplasma*-infected cows**	**G4 *Brucella - Mycoplasma* infected cows**
	n=9	**n=4**	**n=3**	**n=3**
Nitric oxide (µmol/ml)	8.57 ± 0.23 A	8.65 ± 0.68	9.92 ± 0.59 Ba	8.39 ± 0.46 b
Lysozyme (µg/ml)	18.23 ± 1.8 A	15.03 ± 3.3 B	197.33 ± 16.95 abC	17.7 ± 1.82 c

Small litters indicate significantly different between groups against their capital litters in the same row. The mean difference is significant at the level, $P<0.05$ .Values represent means ± SE

The results of the total protein and the electrophoretic pattern of cows serum:

The results were illustrated in table (7) which revealed that, the polyacrylamide gel electrophoresis of the cow serum gave 8 fractions (prealbumen, albumen, α-1, α-2, β-1, β-2, γ-1 and γ-2 globulin). And in the *Brucella* group (G2), there were significant decreasing than the normal group in prealbumen, total albumen, total β globulin and total protein. Also, there were insignificant decrease in albumen, α-2, β-1, β-2, γ-2, total globulins and A/G ratio. And insignificant increase in γ-1 and total γ-globulin comparing to the normal group. While in *Mycoplasma* group (G3), there were significant decrease than G1 in prealbumen and insignificant decrease in albumen, total albumen, α-1α-2, total α, β-1, γ-1, γ-2and total γ and total globulin. Also there were significant increase in β-2 and insignificant increase in total β globulin and A/G ratio comparing to the normal group. In *Brucella- Mycoplasma* group (G4), comparing to G1, there were insignificant increase in prealbumen,, α-1,total α, β-1, totalβ, γ-1, total γ, total globulin and total protein. Also, there were insignificant decrease in albumen, total albumen, α-2, β-2, γ-2 and A/G ratio.

Table (7): The concentration of serum total protein and protein fractions (gm/dl) in different groups.

Groups / Parameters	G1 normal cows	G2 *Brucella*-infected cows	G3 *Mycoplasma*-infected cows	G4 *Brucella- Mycoplasma* infected cows
	n=3	n=3	n=3	n=3
Prealbumen	0.59 ± 0.01 A	0.40 ± 0.01 Ba	0.49 ± 0.01 Ca	0.63 ± 0.03 bc
Albumen	1.693 ± 0.049	1.48 ± 0.12	1.63 ± 0.12	1.63 ± 0.07
Total albumen	2.28 ± 0.06 A	1.88 ± 0.03 Ba	2.12 ± 0.03 b	2.26 ± 0.08 b
α1 globulin	0.43 ± 0.05	0.51 ± 0.02	0.38 ± 0.02	0.47 ± 0.03
α2 globulin	1.22 ± 0.36	1.13 ± 0.12	0.898 ±0.06	1.21 ± 0.09
Total α globulin	1.65 ± 0.37	1.64 ± 0.05	1.288 ± 0.03	1.68 ± 0.09
β1 globulin	0.788 ± 0.14	0.41 ± 0.01 A	0.64 ± 0.01	1.010 ± 0.13 a
β2 globulin	0.838 ± 0.15 A	0.76 ± 0.02 B	1.16 ± 0.11 Cab	0.657 ± 0.06 bc
Total β globulin	1.626 ± 0.13 A	1.17 ± 0.4 Ba	1.80 ± 0.17 b	1.667 ± 0.09 b
γ 1 globulin	0.977 ± 0.12	1.153 ± 0.088	0.85 ± 0.173	1.065 ± 0.152
γ 2 globulin	0.694 ± 0.136	0.557 ± 0.015	0.57 ± 0.007	0.628 ± 0.038
Total γ globulin	1.671 ± 0.17	1.71 ± 0.03	1.42 ± 0.08	1.693 ± 0.14
Total globulin	4.947 ± 0.26	4.52 ± 0.58	4.51 ± 0.06	5.04 ± 0.16
Total protein	7.227 ± 0.27 A	6.40 ± 0.12 Ba	6.63 ± 0.12	7.3 ± 0.19 b
A:G ratio	0.46 ± 0.02	0.416 ± 0.01	0.47 ± 0.03	0.448 ±0.03

Small litters indicate significantly different between groups against their capital litters in the same row. The mean difference is significant at the level, $P<0.05$. Values represent means ± SE.

Discussion

In this study the need for a rapid and sensitive detection method for efficient determination of *Mycoplasma* contamination of apparently normal or sub clinical mastitic milk and interpretation difficulties due to the presence of bacteria observed in some milk samples, so we applied molecular typing technique using four groups of primers sets for conventional PCR assays previously published the first was; 16S rRNA-based Mycoplasma group-specific PCR assay ,this method amplify *Mycoplasma* of ruminant and also all other mollicutes species belonging to the genera *Mycoplasma*, *Acholeplasma*, *Ureaplasma*, and *Spiroplasma* but not amplify sequences of any other prokaryote. (**vanKuppeveld *et al.,* 1994 and Vega-Orellana *et al.* 2017**). The second and the third was according to (**Alberto *et al.,* 2006**) 16sRNA common for Ruminant Mycoplasma and VSP gene specific for variant strains of *Mycoplasma bovis*. The fourth was specific for *Mycoplasma bovis* according to (**Yleana *et al.,* 1995**). Mycoplasma molecular typing could be of great use for screening dairy farms, clinical samples as well as detecting nonculturable, unknown species of mollicutes or mollicutes whose growth is slow or difficult.

Conventional and molecular identification confirmed the presence of nine mycoplasma isolates gave specific band at 280bp (**van Kuppeveld *et al.,* 1994)**, six of them were *Mycoplasma bovis* gave specific band at 360bp (**Yleana *et al.,* 1995**).. Only one of these isolates was containing the VSP gene gave a band at 200bp which indicate the presence of a variant strain (**Alberto *et al.,* 2006**).

Bacteriological isolation and identification of *Brucella* micro-organisms from the suspected animal is still the gold standard diagnostic method that offers conclusive evidence of Brucellosis. Yet, this method has inadequate sensitivity and has, also, a difficulty of being unpractical to apply at a wide scale in control strategies **(Gall and Nielsen, 2004)**

No single serological test is appropriate under all the epidemiological circumstances; all have limitations especially when it comes to screening individual animals (**Godfroid *et al.,* 2002; Moriyon *et al.,* 2004; Corbel, 2006)**. Therefore, samples that are positive in screening tests should be confirmed using an established standard confirmatory test (**European Commission, 2009; OIE, 2018**).

The detection of specific immunoglobulins to *Brucella* in serum or milk samples still the most practical means of the diagnosis. The most proficient and cost-effective method is usually screening all samples using an inexpensive and rapid test which is sensitive enough to detect a high proportion of infected animals like RBPT used in this research. Reactors to screening tests (RBPT) are then confirmed using standard, accurate and specific tests like CFT and cELISA for the final diagnosis to be made **(Corbel, 2006).**

High positive percentage detected by RBPT (13%), the screening test, over the other serological tests (**Fig8**) is matching with its use as a screening test especially for detecting infected herds or for ensuring the absence of the disease in Brucellosis-free herds (**OIE, 2018**).The high positive percentage of MAT under the umbrella of this study may be attributed to the fact that the test (MAT) is performed at a neutral pH and therefore detects IgM, highly agglutinogenic at neutral pH, efficiently and is therefore very sensitive to recent infection (**Alton *et al.,* 1988**). When it comes to confirmatory tests (CFT, Riv.T, and cELISA) they give less positive results (7/70) compared to screening tests (9/70) matching with their higher specificities over screening tests (lower false positives) due to the high affinity of these tests to detect principally IgG1, and to a lesser extent IgG2, (**Alton *et al.,* 1988; Mikolon *et al.,* 1998**).

Conventional and molecular identification resulted in the recognition of two isolates as *Brucella melitensis* biovar 3 from cattle **(Table 5 and Fig 7&8).** *B. melitensis* biovar 3 is the predominate biovar which has been reported over the last 14 years in Egypt (**Sayour, 2004; Afifi *et al.,* 2011; Abdel-Hamid *et al.,* 2012, Abdel-Hamid *et al.,* 2017).** No matter how many *Brucella* isolates were recovered from different animal species since the isolation of micro-organism from a single animal is a de-

finitive proof to establish the infection status of a herd or flock (**Gall and Nielsen, 2004; Elbauomy *et al.,* 2014**) and supporting the serological results. in recent years, this bacterial species has been recognized as a potential source of infection for both cattle and buffaloes (atypical hosts) suggesting cross-species infection (**Corbel, 2006**), particularly in Egypt. Besides, *Brucella* is classified among risk group 3 micro-organisms (high individual risk, low community risk) that required containment in a biosafety level three lab (**WHO, Laboratory biosafety manual, 2004**).

Among 70 tested animals, a high apparent seroprevalence (AP) of Brucellosis was estimated to be 10% in this cattle farm **(Fig 8).** However, A higher seroprevalence was previously reported for cattle (12%) using RBPT as a screening test in Egypt (**Hegazy *et al.,* 2011**).

Interestingly, at the herd level, natural insemination has not been previously reported as a risk factor of the transmission of *Brucella* (**Matope *et al.,* 2011).** This infected farm practiced both (natural and artificial) insemination ignoring the fact that the artificial insemination can transmit the disease and semen must only be collected from Brucellosis free animals. High apparent prevalence may be a result of shedding much greater organisms in unvaccinated than vaccinated ones especially if an abortion occurs as in the case of this private farm. Also, purchasing replacement heifers from local markets especially in endemic countries with the disease or unknown sources will, in turn, increase the seroprevalence. The infection of *Brucella* in the non-preference host is particularly dangerous to humans because of the high virulence of most *B. melitensis* strains and of the large numbers of bacteria that are excreted by cattle **(Corbel, 2006)**.

In order to evaluate the immune status of animals in different groups, the serum NO assay, plate lysozyme activity, total protein and polyacrylamide gel electrophoresis were carried on. In *Brucella* infected group (G 2), the results of serum NO assay revealed that, NO did not show clear variation than the normal group (G 1). This result agreed with **Pancarci *et al.* (2016)** who found that, there were no increase in NO concentrations in serum of patients with brucellosis moreover, there were no relationships among positive *Brucella* antibody titers and serum NO concentrations. Also, **Melek *et al.* (2006)**, demonstrated that *B. melitensis* induces NO production in the early stage of infection, but these levels subsequently decline to basal levels from 45th day in plasma. Moreover, the serum lysozyme activity in *Brucella* group showed slight decrease than the G1. This result come in accordance with **Sung and Yoo (2014)**, who reported that the lytic activity of the lysosome slightly decreased in the serum with high *Brucella* antibody titers as the lysozyme enzyme is one of component of the lysosome which plays an accessory role in the bactericidal actions of antibodies and complement or directly acts through bacteriolysis and opsonization. This weak activity of the lysozyme and NO in G2 may be due to the ability of *Brucella* to reside in tissues of the reticuloendothelial system and survives in macrophages, a feature essential to the establishment, development, and chronicity of the disease through inhibition of fusion between the *Brucella* containing vacuole (BCV) and lysosomes and the alteration of BCV maturation along the endocytic pathway **(Arayan *et al.*, 2018).** The obtained results of protein electrophoretic pattern in *Brucella* group (G2) showed that, the values of most of the proteins fractions were decreased in comparing to the normal group except the**γ-**1 and total**γ-** globulins which were slightly elevated. These results agreed with **Nath *et al.* (2014),** who reported that albumen and A/G ratio were decreased and the γ- globulin was increased in brucellosis cows. It is known that, globulin fraction increased in chronic or subacute bacterial infections, particularly the γ-globulins resulting from production of different immunoglobulins by plasma cells in response to chronic antigenic stimulation **(Morag, 2002).** The results of electrophoresis together with the weak NO and lysozyme in *Brucella* group suggest that it may suffer from subacute or chronic brucellosis.

While the *Mycoplasma* group (G3), showed the highest level of NO and dramatic increased in lysozyme. These results agreed with **Osman *et al.* (2008) and Ramadan *et al.* (2009).** The increased levels of NO and lysozyme in *Myco-*

plasma group may be due to the inflammatory response induced by the *Mycoplasma* infection as a result of the formation of *Mycoplasma* biofilm and secondary metabolites as hydrogen peroxide which increase damage in host tissues leading to attraction of immune cells, releasing lysosomal enzymes, reactive oxygen and nitrogen species **(McAuliffe *et al.,* 2006** and **Hermeyer *et al.,* 2011)**,and secretion of proinflamatorycytokines (TNF-*α*, IL-1*β*, TGF-*α*, IFN-*γ*, IL-12, IL-10, TGF-*β*1, and TGF-*β*2) **(Kauf *et al.,* 2007)**. These increasing levels of NO and lysozyme in *Mycoplasma group* (G3) were accompanied with an increasing in some protein fractions such as β -2 and total β–globulin protein. These findings come in accordance with **Eissa *et al.* (2007)** and **Tothova *et al.* (2017).** The β-globulin fractions include some important proteins such as complement, transferrin, β2-microglobulin, and C-reactive protein. As these proteins are involved in the inflammatory diseases and stress responses **(Bernabucci *et al.,* 2009).** While, the levels of γ-1 globulin, total γ- globulins and total globulins in this group recorded the lowest levels among all groups which supported the finding that G3 was suffering from acute tissue inflammation and mastitis due to *Mycoplasma* infection.

Regarding to the results of NO and lysozyme assays in *Brucella-Mycoplasma* group (G4), there were insignificant difference than normal (G1) and *Brucella* infected (G2) groups, whereas there were significantly decreased values than the mycoplasma group (G3).The controversial results of NO and lysozyme between *Mycoplasma* group (G3) and *Brucella-Mycoplasma* group (G4), may attributed to the effect of *Mycoplasma bovis* infection which was found to evoke suppression as well as stimulation of the host immune system. Immune suppression or immune evasion due to infection with *Mycoplasma* suspected to occur by its secretion of a 26-amino-acid peptide that is 84% homologous to the C-terminal end of the VspL protein (**Vanden Bush** and **Rosenbusch, 2004)**. This peptide appears to take part in the down regulation of lymphocyte proliferation and thereby ameliorates an appropriate immune response by the host. Another mechanism of immune evasion may involve the ability of *Mycoplasma bovis* to inhibit neutrophil oxidative burst by a mechanism that appears to involve protein kinase C signaling **(Thomas *et al.,* 1991).** Also it was found that bovine monocyte infected with *Mycoplasma bovis*, produce anti-inflammatory cytokines such as IL-10 which suppress the pro-inflammatory cytokines such asIFN-γ and TNF-α. IL-10 also impair the antigen-presenting capacity of monocytes and macrophages through decreased expression levels of surface major histocompatibility complex class II and other costimulatory molecules and shifts the immune response toward the T helper cells type 2 (Th2), resulting in strong expression of IgG1, leading to poor opsonization and immunity which prolonged survival and systemic dissemination of *Mycoplasma bovis* in the host **(Mulongo *et al.*, 2014).**

In contrary to the decreased levels of NO and lysozyme in this group (G4), there were increased levels in several miscellaneous protein fractions (prealbumen, α-1, total α, β-1, totalβ, γ-1, total γ, total globulins and total proteins) compared to the normal cows. The α andβ globulin fractions are composed of a large number of acute phase proteins. The α-1globulin include, α-1antitrypsin, α-1 acid glycoprotein, α-1 antichymotrypsin, α-1 fetoprotein, and α-1 lipoprotein, they have specific functions in the regulation of different stages of inflammatory processes and in the restoration of the homeostasis (**O'Connell *et al.*, 2005).** These varied results in this group (G4) as decreased immune and inflammatory responses (decreased levels of serum NO and lysozyme), while increased in the serum protein fractions of inflammatory and immune responses(α-1, total α, β-1, totalβ- globulins), may attributed to the mixed pathogens infection, different stage and different severity of each pathogen infection in this group which affected on the inflammatory and immune responses.

Cattle movements play a significant role in the dissemination of disease **(Robinson and Christley, 2007)**, so, maintenance of a closed herd ranks amongst the most important biosecurity measure in disease prevention **(Mee *et al.,* 2012).** In the OIE Terrestrial Animal Health Code, biosecurity is defined as 'a set of management and physical measures designed

to reduce the risk of introduction, establishment and spread of animal diseases, infections or infestations to, from and within an animal population'.

Conclusion:
Authors concluded that, careful selection of replacement animals either purchased or produced from existing stock, should originate from *Brucella*-free and *Mycoplasma* free herds or flocks. Also concluded, Pre-purchase tests are necessary unless the replacements are from populations known to be free of both diseases. Also, the authors recommended isolation of purchased replacements for at least 30 days. Besides, a serological test before commingling is necessary. Molecular typing of *Mycoplasma* and *Brucella* in dairy herds is a rapid screening test to avoid *Mycoplasma* mastitis and *Brucella* latent infection.

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