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SOME STUDIES ON *PASTEURELLA MULTOCIDA* AS A CAUSATIVE AGENT OF MASTITIS IN DAIRY COWS AND EWES

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ABSTERACT

A total of 233 mastitic milk samples (151 cows and 82 ewes) and 90 mammary gland tissue samples (56 cows and 34 ewes) were collected from different Egyptian Governorates for *P. multocida* investigation as one of mastitis causing pathogens and its effect on the mammary gland tissues. The isolated *P. multocida* from clinical mastitic milk was slightly higher than that from subclinical form in both cows and ewes (15.3% versus 13.1% and 27.3% versus 26.7%, respectively) and it was isolated from the udder tissues of both cows and ewes with percentages of (17.85%) and (23.52%), respectively. Enzyme linked immunosorbent assay (ELISA) was used as confirmatory, rapid and reliable test for detection of *P. multocida* antibodies in the tested milk samples and they were detected in mastitic cow's and ewe's milk whey in (17.2%) and (34.2%), respectively. The antibiogram profile of *P. multocida* was studied for detection of its susceptibility to 15 different antibiotics to detect the drug of choice for its treatment. *P. multocida* isolated from cow's milk showed more resistance to various antibiotics than that isolated from ewe's milk. The DNA integrity of mammary gland tissue cells was detected using comet assay and the percentage of DNA damage was significantly elevated in case of *P. multocida* infected mammary gland ($P<0.05$). In addition, the histopathological findings of *P. multocida* infected udder showed focal and/or diffuse chronic lymphocytic mastitis with an extensive degeneration and necrosis of the alveolar epithelium as well as interstitial tissue. Most of the mammary alveoli were filled with basophilic bacterial colonies with bipolar bodies positively stained by methylene blue and Giemsa stains. Cytological evaluation was conducted on all udder tissue samples and 16/18 positive cases (88.8%) were correlated with their histopathological examination. Histochemically, tissue sections from infected udder showed weak or no alkaline phosphatase activity and density of protein staining. It was concluded that *P. multocida* should be considered as an important sharing etiological agent of mastitis in both cows and ewes (especially in ewes) and associated with significant histopathological alterations in the glandular tissue structure. ELISA was considered as a quick and reliable technique for detection of *P. multocida* infection in the mammary gland especially in the un-vaccinated farms beside the traditional cultural method. The cytological interpretation was quiet helpful in rapid screening of the mammary gland affections.

Key words: *Pasteurella multocida, cows, ewes, mastitic milk, mammary gland tissues, ELISA, comet assay, antibiogram, histopathology, histochemical studies, cytological smear.*

INTRODUCTION

Mastitis is a common disease of ruminants accompanied with physical, chemical, bacteriological changes in milk and pathological alterations in glandular tissues (Abba *et al*., 2014). It is still one of the most important diseases with serious and heavy economic losses because it can reduce milk quality, production, suitability for human consumption and eventually limit the profitability of dairy farmers (Owens *et al*., 1997).

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Ovine mastitis is a widespread disease of dairy ewes with significant adverse production effects and is mainly caused by bacteria that invade and multiply in the mammary gland tissue. It has not been studied as extensively as that in cows; consequently mechanisms of its pathogenesis have not been clarified (Mavrogianni *et al*., 2006). The annual incidence of clinical mastitis in small ruminants was generally lower than 5%, but this incidence can increase sporadically (Ahmadi *et al*., 2014).

The family *Pasteurellaceae* contains Gram-negative, facultative anaerobic and fermentative bacteria of the genera Pasteurella, Haemophilus, and Actinobacillus. Approximately 20 different species of the genus Pasteurella have been identified using phenotypic and genetic analyses but *P. multocida* and *P. haemolytica*

are the most prominent pathogens in domestic animals causing severe diseases and major economic losses in the cattle, swine, sheep, and poultry industries (Confer, 1993). *P. multocida* is a non-spore forming, non-motile, small Gram-negative rods or coccobacilli and it is an important animal pathogen which causes mastitis, pneumonic pasteurellosis. Mastitis was severe, resulted in abnormal secretion, atrophy, and fibrosis of the affected quarter. The general herd management was below average, the environment insanitary and the cattle in poor physical condition (Rimler and Rhoades, 1989).

P. multocida and *M. haemolytica* can cause mastitis in sheep more commonly than in goats (Ajuwape *et al*., 2005). The source of these organisms is usually the upper respiratory system of nursing kids. These pathogens were most often associated with per-acute mastitis and were more common cause of gangrenous mastitis than *S.aureus* in sheep under some management conditions (Eugene, 2007).

P. multocida causes haemorrhagic septicaemia (HS) in Asian and African countries which is a major epizootic disease of cattle with heavy morbidity and mortality. Being simple, rapid, inexpensive and easy for automation, ELISA has emerged as an important tool for diagnosis as well as monitoring the immune status of animals vaccinated against HS in laboratories (Kharb, 2015).

Diagnostic cytology is a quick, inexpensive and easily repeatable technique requiring minimal of sophisticated instruments (Cohen *et al*., 2003) and it has recently been employed for diagnosis of some diseases, when applied to mammary gland lesions, the method showed good diagnostic accuracy (Sangha *et al*., 2011).

Very little recorded researches on the role of *P. multocida* as mastitis pathogen and its pathological effect on the mammary gland, so the aims of this study were to: 1) investigate the incidence of *P. multocida* in mastitic milk and mammary gland tissues in cows and ewes by traditional culture methods and by using ELISA as a rapid and less expensive technique for detection of its antibodies in milk whey of mastitic un-vaccinated animals. 2) Detect the antimicrobial effect of several antibiotics on the isolated strains of *P. multocida*. 3) Detect the effect of *P. multocida* on the infected mammary gland cell's DNA integrity. 4) Spotlight on the diagnosis of *P. multocida* mammary gland lesions on the basis of histopathological picture and to compare with the relative efficacy of diagnostic cytology as well as histochemical examination.

MATERIALS AND METHODS

1. Collection of samples:

1.1. Milk samples: A total number of 233 composite milk samples were collected from different Egyptian Governorates (Giza, El-Behira, Alexandria, El-Gharbia, Kafr El-Sheikh, El-Sharkia, Port Saied and Beni-Suief); from dairy cows and ewes (151 and 82 samples, respectively) using California mastitis test (CMT) for detection of subclinical mastitis (SCM) and physical examination for detection of clinical mastitis. The samples were collected under complete aseptic conditions. The milk sample was pre-incubated at 37°C /12-18 h then 20 µl of the incubated sample was streaked onto brain heart infusion agar, blood agar (with 7% sheep blood), MacConkey agar, Mannitol salt agar and Edward's media (Himedia, Mumbai, India) for detection of the most common bacteria present in milk under study. Bacterial colonies were described for their morphological characteristic appearance and hemolytic activity, followed by Gram staining and motility test then were subjected to further identifications according to Quinn *et al*. (2011). Suspected colonies of *P. multocida* from the initial cultures were re-cultured on brain heart infusion agar and blood agar (round, smooth, mucoid and non-hemolytic colonies were observed) and re-incubated at 37 °C for 48 h, then stained films with Leishman's stain showed bipolar coccoid organisms. Growth test on MacConkey agar medium was negative. Further biochemical identifications for *P. multocida* were done.

1.2. Mammary gland tissue samples:

Ninety tissue samples from mammary glands (56 cows and 34 ewes) were collected from adult slaughtered animals, from Cairo and Giza abattoirs, Egypt. The udder tissue samples were randomly collected among the slaughtered animals and were visually examined for gross lesions. Then each tissue sample was divided into four parts, one part was put in a small polyethylene bag in an ice box under aseptic conditions for bacteriological examination. The second part was immersed in 10% neutral formalin solution for histopathological evaluation and the third one kept for cytological smear preparation. The last one was kept for detection of mammary gland cell's DNA integrity using comet assay.

2. Detection of *P. multocida* antibodies in milk whey using ELISA.

Indirect ELISA was used for detection of *P. multocida* antibodies in the tested milk whey samples of both cows and ewes.

2.1. Preparation of sonicated *P. multocida* antigen: A confirmed *P. multocida* isolate was used for preparation of the antigen according to Afzal *et al*. (1992) and the protein content in the extract was measured (Henery, 1974).

2.2. Enzyme-linked immunosorbent assay: An indirect ELISA was performed using prepared diluted antigen (560 µg /ml) in sodium carbonate coating buffer as described previously by Muneer and Afzal (1989) to detect the antibodies against *P. multocida*

with some modifications. The conjugate used was protein A-peroxidase from *S.aureus* (1:80,000 in PBS-T, Sigma, Germany) due to unavailability of anti-ovine IgG. Optical densities were recorded using ELISA reader at 492 nm. Control negative samples were used to detect the cutoff point. The ELISA reading that ≥double figure of the reading of control negative was considered as positive result.

3. Nitric oxide level. The measurement of NO was assessed according to the assay described by Rajarman *et al*. (1998).

4. Lysozyme activity in milk whey. Lysozyme activity was measured according to Schultz (1987).

5. Antibiotic sensitivity testing of the isolates: antimicrobial susceptibility of 23 *P. multocida* strains (15 cow's and 8 ewe's strains) to 15 antibiotics using disk diffusion technique was performed according to the National Committee for Clinical Laboratory Standards (NCCLS, 2008) on Mueller Hinton agar using commercially available antimicrobial test discs [ciprofloxacin; CIP (5µg), norfloxacin; NOR (10µg), levofloxacin; LEV(5µg), ofloxacin; OFX (5µg), chloramphinicol; C (30µg), amoxicillin-clavulanic acid; AMC (30µg), amoxicillin; AMX (25 µg), ampicillin; AM (10µg), penicillin; P (10 U), cefoperazone-sulbactam; CES (10/10µg), tetracycline; TE (30µg), neomycin; N (30µg), gentamycin; CN (10µg), streptomycin; S (10µg) and cefoquinom; CEQ (30µg)]. Results were recorded by measuring the inhibition zones and scored as sensitive, intermediate susceptibility and resistant according to the NCCLS recommendations.

6. Detection of mammary gland cell's DNA integrity using comet assay. Mammary gland cell's DNA damage was determined according to Singh *et al*. (1988). Out of 100 randomly selected nuclei were photographed and scanned for detection of tail length, the percentage of DNA in the tail and the tail moment.

7. Histopathological examination of mammary gland tissues.

7.1. Tissue preparation for histopathological examination: Each tissue sample was fixed in 10% neutral formalin solution. The fixed specimens were trimmed, washed, dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin. The embedded samples were sectioned at 3-5 µm thickness, stained with H&E stain as well as Masson's Trichrome, methylene blue and Giemsa were used as special stains according to Suvarna *et al*. (2013).

7.2. Cytological smear preparation:
It was applied on 90 freshly mammary gland tissue samples of cows and ewes according to Pawar *et al*. (2015). The collected tissues were cut to get a fresh uncontaminated surface and then blotted many times to make it dry. To minimize microbial contamination, the area of the lesion was cleaned with sterile cotton swab moistened with saline solution. Do not use water to prevent osmosis that induced cell rupture. A clean glass slide was pressed against the dried tissue surface and the cellular smears were spread then stained with Leishman stain.

7.3. Histochemical Investigations: Alkaline phosphatase activity (AP) and density of protein staining (mercury–bromophenol blue techniques) in mammary epithelial cells were determined according to Hussain *et al*. (2013).

8. Statistical Analysis: Data were subjected to statistical analysis according to Sendecor and Cochran (1989) by one way ANOVA employing a completely randomized design at $P< 0.05$.

RESULTS

Table 1: Incidence of *P. multocida* infection in cow's and ewe's milk samples by culture method

Isolated bacteria	Cow's milk (151)		Ewe's milk (82)	
	Clinical mastitic milk (59)	Subclinical mastitic milk (92)	Clinical mastitic milk (22)	Subclinical mastitic milk (60)
Single infection of *P. multocida*	**2 (3.4%)**	**-**	**3 (13.6%)**	**4 (6.7%)**
Mixed infection				
P. multocida* +*S.aureus	**-**	**2 (2.2%)**	**-**	**2 (3.3%)**
P. multocida* +*S.aureus* + *E.coli	**5 (8.5%)**	**6 (6.5%)**	**-**	**-**
***P. multocida* +*S.aureus* + environmental streptococci**	**2 (3.4%)**	**1 (1.1%)**	**-**	**-**
***P. multocida* + CNS**	**-**	**-**	**3 (13.6%)**	**5 (8.3%)**
P. multocida* + CNS + *E.coli	**-**	**3 (3.3%)**	**-**	**5 (8.3%)**
Total no. of mixed infection (%)	**7 (11.9%)**	**12 (13.1%)**	**3 (13.6%)**	**12 (20%)**
Total no. (%)	**9 (15.3%)**	**12 (13.1%)**	**6 (27.3%)**	**16 (26.7%)**
	21 (13.9%)		**22 (26.8%)**	

% was calculated according to the type of mastitic milk and the total % was calculated according to the total no. of milk samples of each animal species.

Table (1) illustrated that *P. multocida* was isolated as single and mixed infection (mainly with *S.aureus* followed by *E.coli* then environmental streptococci) with a percentage of 3.4% (2 samples) and 11.9% (7 samples) in clinical mastitic cows' milk, respectively. While it was isolated from 12 subclinical mastitic cows' milk samples in a mixed form only (13.1%). Moreover, *P. multocida* was isolated from ewes' clinical mastitic milk as single infection (3/22) with % of (13.6%) and 3/22 in a mixed form with CNS (13.6%). It was also isolated from subclinical mastitic ewes' milk in single infection (4/60; 6.7%) and as mixed infection with CNS, *E.coli* and *S.aureus* with total % of 20% (12/60). Generally, it was noticed that *P. multocida* was isolated from clinical mastitis slightly higher than that from subclinical form in both cows and ewes (15.3% versus 13.1% and 27.3% versus 26.7%, respectively). It was announced that *P. multocida* totally isolated from ewe's mastitic milk with a higher rate than that of cows (26.8% versus 13.9%, respectively).

Table 2: Detection of *P. multocida* antibodies in cow's and ewe's milk samples using ELISA

ELISA	Cow's milk (151)		Ewe's milk (82)	
	Clinical mastitic milk (59)	Subclinical mastitic milk (92)	Clinical mastitic milk (22)	Subclinical mastitic milk (60)
Positive	12 (20.3%)	14 (15.2%)	8 (36.4%)	20 (33.3%)
Total	26 (17.2%)		28 (34.2%)	

Tables (2) showed that *P. multocida* antibodies were detected in mastitic cow's milk in 26 out of 151 of the samples (17.2%), while higher % was detected in ewe's milk (28/82; 34.2%).

Table 3: Comparison between incidence of *P. multocida* in milk samples by cultural method and ELISA in diagnosis

Animal species	No. of examined Milk samples	Cultural method		ELISA	
		No.	%	No.	%
Cows	(151)	21	(13.9%)	26	(17.2%)
ewes	(82)	22	(26.8%)	28	(34.2%)

Table (3) showed that incidence of *P. multocida* in milk samples by cultural method was lower than that of ELISA technique in diagnosis; (13.9%) versus (17. 2%) in cow's milk and (26.8%) versus (34.2%) in ewe's milk.

Table 4: Nitric oxide level and lysozyme activity in cow's and ewe's mastitic milk samples positive for *P. multocida* infection (Mean±SE).

Animal species	Grade of mastitis			
	Clinical mastitis		subclinical mastitis	
	NO(umol/ml)	lysozyme(ug/ml)	NO	lysozyme
Cow	274±25.9***	205.5±25***	105.3±10.1	89.6±5.8
Ewe	242.8±18.8***	175.1±17.9**	101.9± 12.4	97.9± 5.78

Table (4) illustrated that both NO and lysozyme were significantly increased according to the grade of mastitis (higher in clinical form than subclinical form) in both cows and ewes.

Table 5: Incidence of *P. multocida* infection in cow's and ewe's mammary gland tissue samples.

Isolated bacteria	Cow's mammary gland tissue samples (56)	Ewe's mammary gland tissue samples (34)
Single infection of *P. multocida*	**4 (7.14%)**	**4 (11.76%)**
Mixed infection		
P. multocida* +*S .aureus	**6 (10.71%)**	**4 (11.76%)**
Total	**10 (17.85%)**	**8 (23.52%)**

Table (5) showed that *P. multocida* was isolated from mammary gland tissues of both cows and ewes with % of (17.85%) and (23.52%), respectively, also the % of isolation in ewes was higher than that of cow's udder tissues.

Table 6: The effect of *P. multocida* natural infection on mammary gland tissue integrity using comet assay in comparison to the un-infected one.

Type of mammary tissue	% of damage	Tail Length (px)	% DNA in Tail	Tail Moment
Un-infected udder tissue	**11.2±0.120**	**2.71±0.505**	**16.9±1.32**	**0.531±0.152**
Mastitic udder tissue	**21.5±1.44****	**4.6±0.353***	**27.4±1.09****	**1.68±0.143****

Table (6) declared that the percentage of damage and DNA % in comet tail was significantly elevated in case of *P. multocida* infected mammary gland when compared with the uninfected one, ($P<0.05$).

The antibiogram profile of 15 representative *P. multocida* strains isolated from infected cows' milk showed that, all of them were highly susceptible to CIP, NOR, LEV, OFX and C (100%). In declining order only 5 out of 15 strains (33.3%) were susceptible to CEQ, CEX, AMC, TE, CN and S, while about 10/ 15 (66.7%) were resistant to CEQ, CEX, AMC, TE, CN and S. On the other hand all the tested strains showed complete resistance to AMX, AM, P and N.

Antibiotic susceptibility results of 8 representative *P. multocida* strains isolated from ewes' milk showed that they were highly susceptible to CIP, NOR, LEV, OFX, AMC, AMX, TE, and C (100%). 4 out of 8 (50%) strains were susceptible only to CEX, CN and AM. On the other hand all the tested strains showed complete resistance to CEQ, P, S and N. From the previous results, it was noticed that, *P. multocida* isolated from cows' milk showed more resistance to various antibiotics than that isolated from ewes' milk.

Histopathological examination was applied on all collected tissue samples. Macroscopically, most of the mammary glands were apparently normal while some cases were pale and fibrosed.

The prominent feature of microscopic examination of the mammary glands of 4 cows and 2 ewes that revealed single *P. multocida* isolates was focal and /or diffuses chronic lymphocytic mastitis in which there was proliferation of intralobular and interlobular fibrous connective tissue accompanied with marked infiltrations of lymphocytes, macrophages and plasma cells (fig. 5). Atrophy and fragmentation of alveoli were observed as well as the alveolar epithelium appeared flattened and granular (fig. 6a&b). While other cases showed epithelial vaculations with cystic dilatation of acini. The damaged alveoli were empty or contained mononuclear inflammatory cells and few neutrophils together with desquamated epithelium and fibrin network. Most of the mammary alveoli were diffusely filled with basophilic bipolar coccobacilli of *P. multocida* positively stained by methylene blue and Giemsa stains (fig. 7a) or pink color by H&E (fig. 7b). Focal epithelial necrosis observed in tissues in most of cases. Corpora amylacea were detected inside acini. The increase of the interstitium was of variable intensity and that confirmed with Masson's Trichrome stain (fig. 8). Moderate hyperplasia of epithelial lining of lactiferous ducts was detected and associated with subepithelial mononuclear cell aggregations as well as lymphocytic exocytosis. Interlobular connective tissue was mild to moderately expanded by edema. Blood vessels showed thickening of their tunica media with vasculitis.

Two cases of single *P. multocida* mastitic ewes showed acute suppurative mastitis that revealed an extensive infiltration of neutrophils and macrophages

within the alveolar lumen and interstitial connective tissue as well as vacuolar degeneration, desquamation and necrosis of the alveolar epithelium (fig. 9). Most of the mammary alveoli were devoid of milk and filled with bacterial colonies, fibrin network and caseated milk. Focal areas of interstitial haemorrhage were observed. Vasculitis with edema and dilated lymph ducts were detected. While mixed infection (*P. multocida +S .aureus*) was detected in 10 cases (6 cows and 4 ewes) that revealed marked chronic mastitis with highly proliferated connective tissue, inflammatory cell infiltrations and hyperplasia of ductular epithelium.

Cytological smear evaluation of inflamed mammary glands revealed bacteria, inflammatory and tissue cells. Mammary epithelial cells showed granular feature. Fourteen cases (10 cows and 4 ewes) as single and mixed infection showed chronic inflammatory character in which there were macrophages, lymphocytes and few neutrophils appeared in the cytological smears. Two cases of ewes revealed dominant degenerative neutrophils; this was considered as an acute suppurative response. Also, the intra and extra-cytoplasmic bipolar coccobacillus bacteria were demonstrated (fig. 10). The correlation between cytological and histopathological results was also determined. Out of 18 positive *P. multocida* mastitic cases, 16 (88.8%) tissue samples showed changes by cytological smear evaluation and that came in accordance with their histopathological picture.

The activity of alkaline phosphatase in tissue sections of healthy animals was apparent on the outer boundary of alveolar secretory cells indicating the high activity of mammary gland. While in tissue sections from Pasteurella mastitic animals, few alveoli indicated weak activity of alkaline phosphatase and the most of the alveoli showed disappearance of this enzyme reflecting no activity (fig. 11a &b).

Similarly, the greatest density of protein staining was found in the thick-walled mammary alveoli with larger cells in normal non mastitic mammary tissues. On the other side, alveoli showed weak or no staining for protein and the connective tissue was increased concomitantly with the regression of the alveoli into the smallest size and reduced frequency of secretory cells in *P. multocida* mastitic cases (fig. 12a & b).

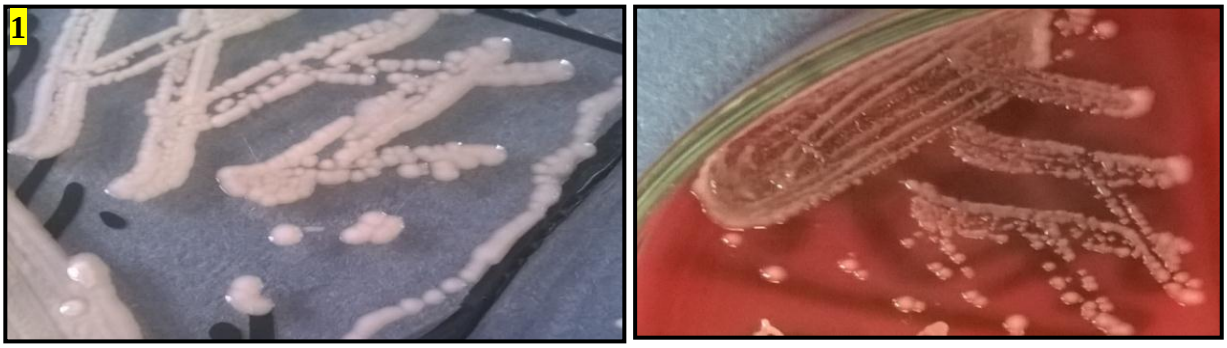


Fig. 1: Colonial morphology of *P. multocida* on brain heart infusion agar (mucoid colonies) and blood agar (non-hemolytic).

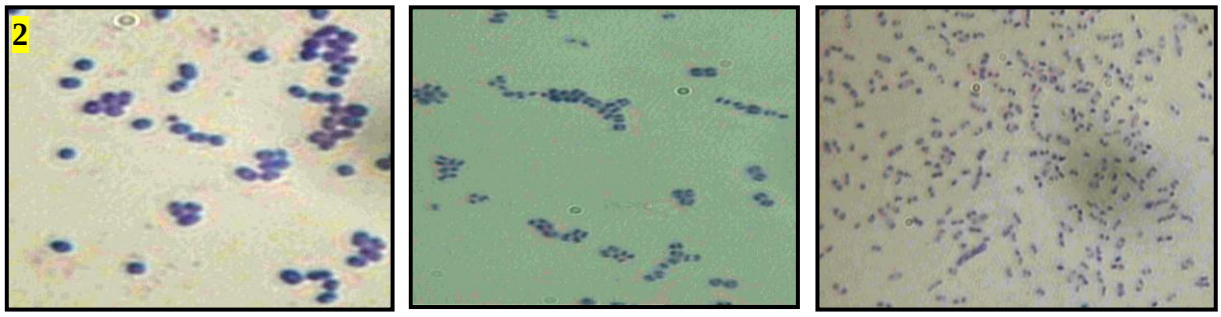


Fig. 2: Microscopical examination of *P. multocida* isolated from mammary gland tissue and milk samples showed bipolar cocobacillus microorganisms stained with Leishman's stain.

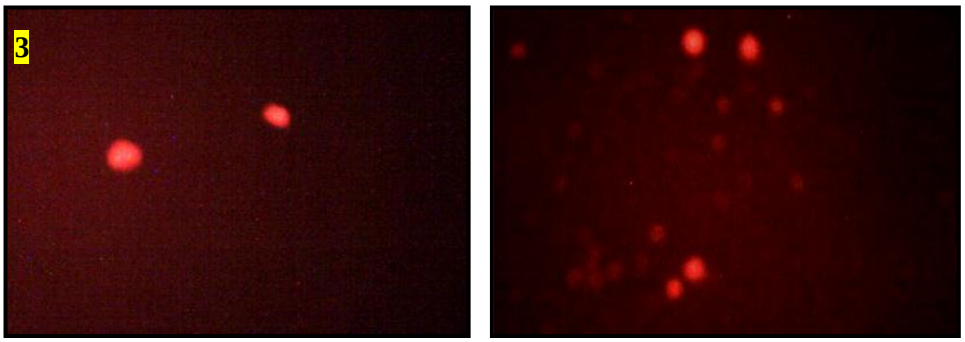


Fig. 3: Undamaged mammary gland cell's DNA in clinically healthy cow measured by the comet assay where DNA remains within the core. The DNA is tightly compressed and maintained the circular disposition of the normal nucleus.

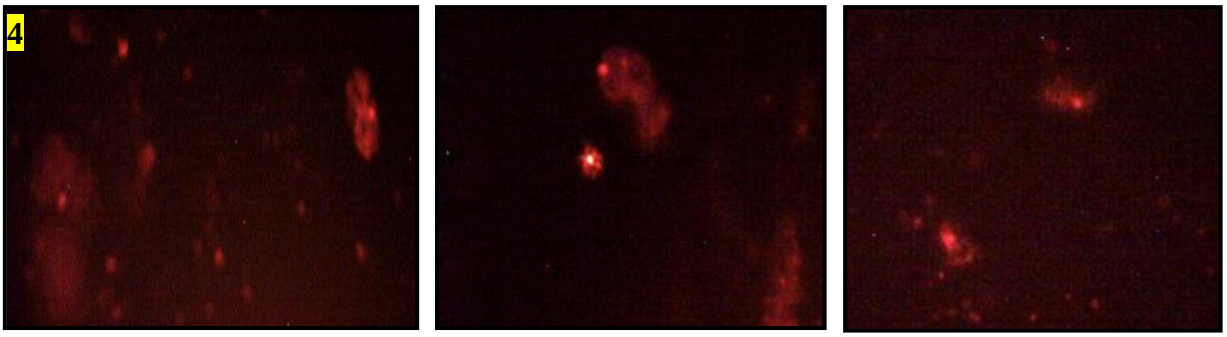
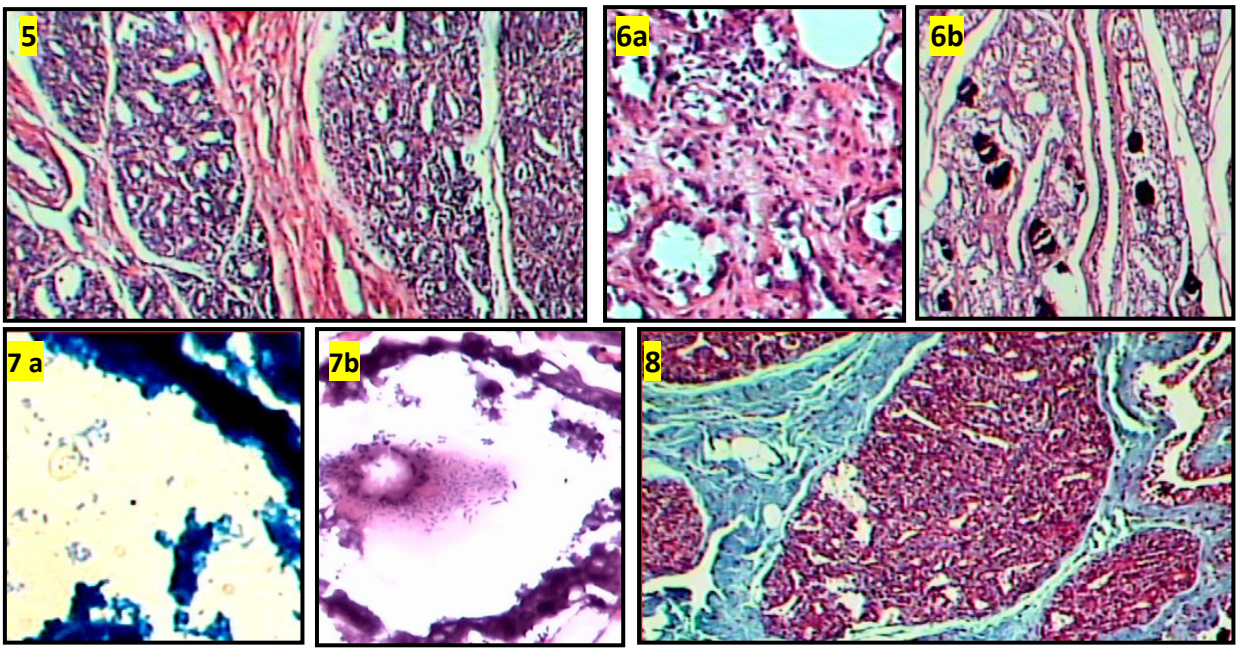


Fig. 4: Severe DNA damage in clinically mastitic mammary gland tissues infected with *P. multocida* measured by the comet assay. The cells were examined by fluorescence microscopy, fluorescent structures corresponding to the stained nuclear DNA. The increase in DNA damage was mostly evidenced by an increase of comet tail.



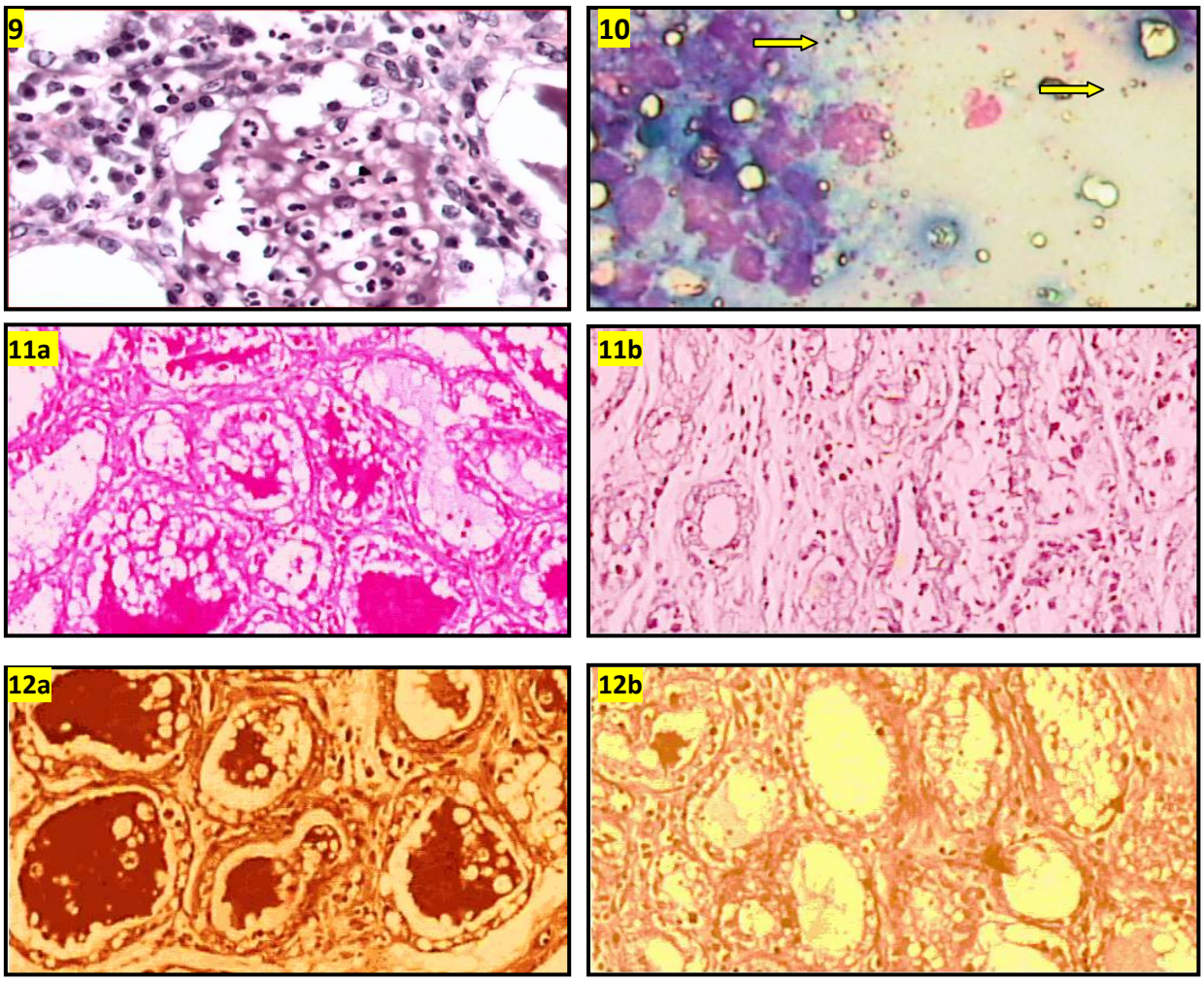


Fig.5: Cow's mammary gland showing chronic lymphocytic mastitis with marked proliferation of fibrous connective tissue (H&E, X40).
Fig.6a: Ewe's mammary gland showing atrophy and damaged alveoli as well as marked fibrous connective proliferation and mononuclear cell infiltrations (H&E, X100).
Fig.6b: Ewe's mammary gland showing chronic mastitis associated with the presence of corpora amylacea (H&E, X40).
Fig.7 a: Cow's mammary gland showing basophilic bipolar coccobacillus bacterial colonies inside damaged alveoli (methylene blue stain, X100).
Fig. 7b: Ewe's mammary gland showing pink bipolar coccobacillus bacterial colonies inside and attached to alveolar epithelium (H&E, X100).
Fig.8: Cow's mammary gland showing marked proliferation of fibrous connective tissue with atrophied lobules (Masson's Trichrome stain, X40).
Fig.9: Ewe's mammary gland showing acute suppurative mastitis with neutrophils and macrophages infiltration in the mammary acini and interstitial connective tissue as well as desquamated epithelium and cellular debris of necrotic alveolar epithelium (H&E, X400).
Fig.10: Cytological smear from ewe's mammary gland with acute mastitis showing granular mammary epithelial cells, neutrophils with intra and extracytoplasmic bipolar bacteria (yellow arrows) (Leishman stain, X 1000).
Fig.11a: Section of mammary tissue from healthy cow showing high level of alkaline phosphatase activity (H&E, X100).
Fig.11b: Section of mammary tissue from *P. multocida* mastitic cow showing no to weak alkaline phosphatase activity (H&E, X100).
Fig.12a: Section of mammary tissue from healthy cow showing high protein staining (H&E, X100).
Fig.12b: Section of mammary tissue from *P. multocida* mastitic cow showing weak protein staining (H&E, X100).

DISCUSSION

Mastitis is a multifactor disorder which increases risks of culling of animals and requires understanding of exact mechanism of its pathogenesis. Even though great technological advances have been made, mastitis continues to be a major economic issue for dairy producers, necessitating researchers and dairy advisors continue to refine the recommended mastitis control program (Hussain *et al*., 2013 and Shaheen *et al*., 2016). Pasteurella mastitis is not in the center of veterinary attention, although Tabatabaei and Firouzi (2002) mentioned that *P. multocida* is an endemic disease in most countries of Africa. However, the presence of a small number of strains in more than one host species suggests that transmission of bacteria between different host species and it considered as a factor in the population biology of *P. multocida* (Davies *et al*., 2004).

So the present study aimed to highlight on *P. multocida* as one of the very little recorded and neglected microorganism that affect the lactating mammary gland and its role as one of bacteria that may be involved as an important mastitis pathogen in cows and ewes.

In the current study, it was noticed that *P. multocida* was isolated as single and mixed infection with a percentage of (3.4%) and (11.9%) in clinical mastitic cows' milk, respectively as well as in mixed forms (13.1%) from subclinical mastitic cows' milk samples. The isolation rates of *P. multocida* varied in many researches. In the far past, Barnum (1954) reported an outbreak of *P. multocida* mastitis with a severe clinical condition involved 14/20 low-producing cows. Also, Ribeiro *et al*. (2010) reported that the uncommon *P. multocida* mastitis in nine dairy cows which milked in the presence of their calves and they noted the low interference of immunosuppressive, predispose factors and absence of pulmonary signs in these animals. Meanwhile, Heleili *et al*. (2012) isolated *P. multocida* at a low frequency (1.51%) from subclinical mastitic cows.

Moreover, *P. multocida* was isolated in this study from ewes' clinical mastitic milk as a single and mixed infection with CNS (13.6% for each of them) as well as a single and mixed infection with other bacteria (6.7% and 20%, respectively) from subclinical mastitic ewes' milk. Dewani *et al*. (2002) determined different bacterial species causing ewe mastitis included *P. multocida* (6.89%) which was closely similar to our results in ewe's subclinical mastitis (6.7%). Also, Al-Majali and Jawabreh (2003) isolated *Pasteurella* spp. in single and mixed forms from suclinical mastitic ewes. Otherwise, Hawari *et al*. (2014) isolated 4 *P. multocida* strains from 40 negative CMT ewe's milk samples. Furthermore, Ahmadi *et al*. (2014) concluded that *P. multocida* should be considered as an etiological agent of acute septic mastitis in goats.

Generally, it was noticed that *P. multocida* was isolated from clinical mastitis slightly higher than that from subclinical type in both cows and ewes (15.3% versus 13.1% and 27.3% versus 26.7%, respectively) as reported previously by Ribeiro *et al*. (2010). Also, *P. multocida* totally isolated from ewe's mastitic milk with a higher % than that of cows (26.8% versus 13.9%) and this may be attributed to a case history of respiratory manifestation in suckling lambs of ewes under this study. This phenomenon was explained by many authors as *P. multocida* is a commensally common organism of the nasopharynx of healthy sheep, goats and calves and it is considered as a primary pathogen, as in case of hemorrhagic septicemia in cattle and a secondary invader in cases with pneumonic lesions (Ewers *et al*., 2004 and Lainson *et al*., 2013). Additionally, the reduced hydration of chapped skin decreases lipid content which contains antibacterial fatty acids, bacteriostatic salts, proteins and immunoglobulins as well as decreasing resistance to bacterial colonization (Mavrogianni *et al*., 2006). Most of the infectious agents can enter the mammary gland in an ascending route via the contaminated skin of the teat or oral cavity of the lambs following sucking (Omalekil *et al*., 2011). It is possible that the causative organism hide in the mammary gland tissue in a latent form and eventually in case of traumatic teat injuries, the bacteria become infectious and produces the functional and structural abnormality in the affected udder resulting in clinical mastitis (Radostits *et al*., 2007). More recently, Ahmadi *et al*. (2014) explained the presence of *P. multocida* in the mammary gland as these bacteria persist either in the environment or within the oral cavity of kids and directly entered the teat canal following sucking or after the entry to the body and the establishment in the target organs being introduced hematogenously to the udder.

ELISA has emerged as a simple important tool for diagnosis as well as monitoring the immune status of animals vaccinated against hemorrhagic septicemia caused by *P. multocida* in laboratories (Kharb, 2015). We used ELISA as a quick technique for detection of *P. multocida* antibodies in milk whey of un-vaccinated animals under this study. These results revealed that, *P. multocida* detected in milk samples by cultural method was lower than its antibodies in milk whey that detected by ELISA technique as a tool of diagnosis; (13.9%) versus (17. 2%) in cow's milk and (26.8%) versus (34.2%) in ewe's milk. In this aspect, Habashy *et al*. (2009) found nearly the same difference between traditional *P. multocida* isolation and ELISA technique as they identified *P. multocida* in 26.9%, 33.6%, 10% and 50% of blood samples from diseased and apparently healthy sheep, respectively.

It was illustrated that both NO and lysozyme were significantly increased according to the grade of mastitis (higher in clinical form than subclinical form) in both cows and ewes and that was agreed with Osman *et al*. (2010) and Malvisi *et al*. (2016). These results suggested that the useful use of both NO and lysozyme for detection of severity and type of mastitis in ewes as well as in cows.

Our results showed that *P. multocida* was isolated from udder tissues of both cows and ewes with % of (17.85%) and (23.52%) in single and mixed form, respectively, also the rate of isolation in ewes was higher than that of cows tissues. The most recorded published literatures studied the *P. multocida* effect on lung tissues while scanty data was recorded on mammary tissues but recently Ahmadi *et al*. (2014) isolated *P. multocida* from infected goat's mammary gland with an unusual acute septic mastitis.

The antibiogram profile of representative *P. multocida* strains isolated from both cows and ewes under this study was showed different susceptibility and resistance for the used antibiotics. It is known that the occurrence of antimicrobial resistance varies between countries and regions Güler *et al*. (2013). Mevius and Hartman (2000) in agreement with this study recorded no resistance to florfenicol and resistance to TE was reported. Similar observation for CIP sensitivity was noticed by Mohamed *et al*. (2012). Antimicrobial resistance profiles of *P. multocida* isolates obtained by Khamesipour *et al*. (2014) nearly similar to our results, they showed that, all the isolates were susceptible to CIP, enrofloxacin, TE and resistance to AM, P, S and AMX at different frequencies.

From the present results, it was noticed that, *P. multocida* isolated from cow's milk showed more resistance to various antibiotics than that isolated from ewe's milk. In agreement with our results, a study of Güler *et al*. (2013) found that 22% of isolates were resistant at least for one antimicrobial agent. Among these resistant isolates, the majorities were from cattle strains and only one was from sheep strains. That may be related to more antimicrobial use in cattle populations than in sheep populations generally. Moreover, the high resistance rates against some antimicrobial agents may be due to intensive production systems and common use of antimicrobial in these types of productions.

In studying the mammary cell's DNA integrity using comet assay, it was found that the percentage of damage and DNA % in comet tail was significantly elevated in case of *P. multocida* infected mammary gland when compared with the uninfected one, ($P<0.05$). This was agreed with Praveena *et al*. (2010) who detected apoptotic nuclei in splenocytes, hepatocytes and infiltrating leukocytes of the lungs in mice experimentally infected with *P. multocida* serotype A1. On the other side, Preuss *et al*. (2010) showed that toxigenic *P. multocida* strains produce a high mitogenic activity 146 kDa protein toxin (PMT) that blocked apoptosis induced by tumour chemotherapeutic agents in human cancer cell lines, indicated that PMT was a highly potent anti-apoptotic agent, which supported the view of a carcinogenic potential of the toxin.

In the present study, the histopathological features of marked focal and/or diffuse chronic lymphocytic mastitis were described in both cows and ewes which indicated severe tissue damage induced by *P. multocida* as single infection. Mastitis was more severe at the alveolar lumen than in the ducts. These pathological alterations were came in accordance with Hussain *et al*. (2012); Abba *et al*. (2014) and Ibrahim *et al*. (2016) who recorded similar changes in mammary glands of naturally and experimental infected ruminants.

Also, acute mastitis due to *P. multocida* as single infection was described in only two cases of ewes which were characterized by diffuse and severe suppuration with presence of Gram-negative bacteria in the affected udder tissue sections and that came in accordance with results of Ahmadi *et al.* (2014). Additionally, mixed infection (*P. multocida* + *S. aureus*) was detected in 10 cases which revealed severe chronic mastitis in the current study.

The ability of *P. multocida* to cause diseases is mainly due to its specific virulence factors as lipopolysaccharide (LPS), outer membrane proteins (OMP), capsule and adhesions, which allow the bacteria to invade the host cells (Boyce *et al.*, 2010 and Wilson and Ho, 2013). LPS is an integral outer membrane component, with a critical role in the disease by interacting directly with innate host immune defense (Omaleki *et al*., 2011). The OMP enhances the bacterial colonization through its binding to the host extracellular matrix proteins like fibronectin. It also serves as bacterial protective surface antigen and was reported to have a remarkable heterogenicity in different strains isolated from different animal species (Wilson *et al.*, 2011). In *vitro* studies using ovine mammary epithelial cells have demonstrated that adherence and intracellular localization of bacteria occurs within 10 min, an event considered to be an important step in the development of mastitis (Vilela *et al*., 2004).

The movement of neutrophils and macrophages during inflammatory process triggered by pathogens determine the severity of the symptoms. PMNs act by destroying the invading bacteria via intracellular granules resulting in release of enzymes, such as N-acetyl-b-D-glucosaminidase (NAGase) and Lactate Dehydrogenase (LDH) (Hussain *et al*., 2013). The later lead to mammary epithelial damage and thus decrease milk production (Barbano *et al*., 2006).

The results of the present study evaluated the cellular and nuclear details of mammary cytological smears; it was possible to diagnose the nature of inflammatory type. This can help to make a preliminary idea about the mode of treatment within a short period. Our findings were similar to those reported by Sangha *et al*. (2011).

Alkaline phosphatase (AP), a membrane-associated glycoprotein enzyme, increases hydrolysis of phosphate and is located mainly on the outer cellular membranes of alveolar secretary epithelial cells where it helps in active transport processes (Murray and Ewen, 1992). Tissue sections of mammary glands of mastitic animal revealed low or no activity of alkaline phosphatase. In accordance with our results, Silanikove and Shapiro (2007) and EL sayed *et al*. (2009) have shown that AP is located almost on the mammary epithelial cells apical membrane. This weak activity of AP in mastitic tissues may be related with deactivation of this enzyme owing to negative regulatory process of mammary gland as a result of negative effects of pathogens. The weak activity of alkaline phosphatase enzyme could be due to impaired milk secretory mechanism (Silanikove, 2008).

The weak to negligible activity of protein in tissue sections of mastitic animal in the present study could be due to degenerative changes of mammary epithelium with connective tissue proliferation and impaired activity of endoplasmic reticulum induced by microbial agents. However, different researchers reported that protein staining was decreased (Elsayed *et al*., 2009 and Hussain *et al*., 2012). These degenerated mammary cells encompassing the active cellular protein was substituted by the spread of connective tissue under the effect of *P. multocida* which results in poor biosynthetic capacity of udder and decreases cellular differentiation (Hussain *et al*., 2013).

CONCLUSION

From this study, it was concluded that *P. multocida* should be considered as an important sharing etiological agent of mastitis in both cows and ewes which causes a significant pathological alteration in the glandular structure. ELISA was considered as a quick and reliable technique for detection of *P. multocida* infection in the mammary gland especially in the un-vaccinated farms beside the traditional cultural method. The cytological interpretation is quiet helpful in rapid screening of the mammary gland affections. Histochemical data showed weak alkaline phosphate activity and low protein staining density in tissues sections of mastitic animals that reflecting impaired activity. The DNA integrity of mammary gland cells was declared using comet assay. These observations strongly support the belief that mastitis lead to losses in mammary gland function and that were directly related to disruption of alveolar cell DNA integrity, sloughing of cells, necrosis with a consequent fibrosis and an increase in the inflammatory cells.

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بعض الدراسات علي الباستيريللا مالتوسيدا كمسبب لإلتهاب الضرع في الأبقار والنعاج الحلابه

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تم تجمع عدد ٢٣٣ عينة لبن من حيوانات حلابه مصابه بإلتهاب الضرع الظاهرى والغير ظاهرى من محافظات مختلفة في مصر (١٥١ عينة لبن أبقار و ٨٢ عينة لبن نعاج) و عدد ٩٠ عينة أنسجة ضروع من حيوانات مذبوحه فى المجازر (٥٦ أبقار و ٣٤ نعاج) وذلك للتحقق من الإصابه بميكروب الباستيريللا مالتوسيدا كمسبب من مسببات إلتهاب الضرع مع دراسة تأثيره الهستوباثولوجى على أنسجة الضرع. ولقد تم عزل الباستيريللا مالتوسيدا من عينات اللبن المجمعه من حيونات مصابه بإلتهاب الضرع الظاهرى وكانت أعلى قليلا من تلك المصابه بالتهاب الضرع الغير ظاهرى في كل من الأبقار والنعاج (١٥,٣٪ مقابل ١٣,١٪ و ٢٧,٣٪ مقابل ٢٦,٧٪ على التوالي) وكذلك تم عزل هذا الميكروب من أنسجة الضروع لكل من الأبقار والنعاج بنسبة (١٧,٨٥٪) و (٢٣,٥٢٪) على التوالي. ولوحظ أن النسبة المئوية لعزل الباستيريللا مالتوسيدا من النعاج أعلى من الأبقار في كل من اللبن والأنسجة. أيضا تم إستخدام الإليزا كإختبار تأكيدي سريع ودقيق للكشف عن الأجسام المضادة للباستيريللا مالتوسيدا في عينات اللبن تحت الإختبار والتي تم الكشف عنها في ألبان الأبقار المصابة بإلتهاب الضرع في (١٧,٢٪) من العينات ، في حين كانت نسبتها أعلي في عينات ألبان النعاج (٣٤,٢٪). أيضا تم قياس كل من أكسيد النيتريك والليزوزيم كمؤشرات إضافية لإلتهاب الضرع واللذان أظهرا زيادة كبيرة وفقا لدرجة إلتهاب الضرع (أعلى في إلتهاب الضرع الظاهرى عنه في الغير ظاهرى) في كل من الأبقار والنعاج. كذلك تم دراسة تأثير ١٥ نوع مختلف من المضادات الحيوية علي بعض عترات الباستيريللا مالتوسيدا المعزوله للكشف عن قابليتها للإستجابه لهذه المضادات الحيويه وقد أظهرت العترات التى تم عزلها من الأبقار مقاومه أكثر للمضادات الحيوية المختلفة من العترات التي تم عزلها من النعاج. أما بالنسبة للكشف عن سلامة الحمض النووي لخلايا الغدد اللبنيه باستخدام إختبار المذنب فكانت نسبة التلف في الحمض النووي مرتفعه بشكل ملحوظ في حالات أنسجة الضروع المصابه بالباستيريللا مالتوسيدا ($P < 0.05$) عن مثيلتها الغير مصابه. بالإضافة إلى ما سبق، أظهرت نتائج الفحص الهستوباثولوجي لأنسجة الضرع المصاب بالباستيريللا مالتوسيدا إلتهاب ليمفاوي مزمن محدد أو منتشر مع نخر في بطانة الغدد اللبنية وكذلك الأنسجة الخلالية. وكانت معظم الحويصلات اللبنية مملؤة بالمستعمرات البكتيرية التي أظهرت اللون الأزرق بصبغة الميثيلين الأزرق وصبغة الغيمزا. أيضا تم إجراء التقييم الخلوي على جميع عينات أنسجة الضروع و قد أظهرت ١٦ حاله من ال ١٨ المصابه بالباستيريللا مالتوسيدا (بنسبة ٨٨,٨ ٪) توافقا كاملا مع فحصهم الهستوباثولوجي. أما الفحص الهستوكيميائي للأنسجه فقد أظهر ضعف أو إنعدام لكل من نشاط الفوسفاتيز القلوي وكثافة البروتين في الحالات المصابة بالباستيريللا مالتوسيدا عن مثيلتها الغير مصابه. وقد خلصت هذه الدراسة إلي أن ميكروب الباستيريللا مالتوسيدا يجب إعتباره كأحد المسببات الهامه لإلتهاب الضرع في كل من الأبقار والنعاج على وجه الخصوص مما يؤدي إلى تغيير باثولوجي كبير في بنية أنسجة الغدد اللبنية. وإعتبار الإليزا كإختبار سريع وموثوق به للكشف عن الإصابة بالباستيريللا مالتوسيدا في الألبان وخاصة في المزارع الغير محصنه بجانب طرق العزل التقليديه. كذلك التقييم الخلوي للأنسجة كان مفيد جدا كتشخيص سريع لإلتهاب الضرع.