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Relationship between Mycoplasma mastitis, antioxidant bio-activities and pesticide residues

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Abstract

The current article deals with inclinations and patterns of pesticides in cow's milk, its impact on dairy animals, factors contributing to pesticide risks and its residues bioaccumulation in milk, in addition to its effect on percent of Mycoplasma mastitis in dairy farms at Qalyoubia Governorate, Egypt.

In this study, by-using California mastitis test, incidence of mastitis was 37.5% and Mycoplasma mastitis showed prevalence of 32.5%. A percentage of 25.6% was identified culturally, biochemically and molecularly as *M. bovis*. 16SrRNA gene of *M. bovis* showed band of amplicon size at 360bp.

In mastitic milk, significant drop in calcium, phosphorus, magnesium and potassium levels combined with significant rise in total protein, albumin, sodium and chloride levels in relation to stages or scores of mastitis was observed. Otherwise, vitamin A and leptin did not alter. Regarding antioxidant activity, it is observed direct significant decrease with degree of mastitis stages in GSH, SOD, Catalase and T. antioxidant while, MDA showed significant increase in mastitic milk.

The obtained milk specimens were contaminated by detectable levels of OCPs. Udder is considered as a bio-magnification organ to OCPs leading to diminish udder immunity and this is promoting factor increase the percent of mastitis in herds. Pyrethroid pesticides were not detected in examined samples indicating good pesticide control in Egyptian fields.

In conclusion, this work spots a light on the importance to monitor trends of milk contaminants in the environment to restrict and prohibit the deleterious effect on milk risk hazard to human consumption and animal health. These results will help in a scientific assessment of the implications of pesticide residues with regards to human risks in Egypt.

Keywords: *Antioxidant bioactivities, mastitis, Mycoplasma bovis, pesticide residues.*

Introduction

Mastitis is a common problem in lactating cows decreases in milk production and quality **(Philpot and Nickerson, 1991)**. Mastitis is still the most economically important illness leading to severe milk losses, drug cost, veterinary cost, increased labor, milk suppression after treatment, reduced genetic improvement **(Leslie and Dingwell, 2000, Wielgosz-Groth and Groth, 2003)** and decreased reproductive performance **(Schrick *et al*., 2001).** Somatic cell count (SCC) associated with an inflammatory state is generally employed for diagnosing subclinical mastitis **(Katsoulos *et al*., 2010)**. Somatic cell count (SCC) has long been viewed as a key factor in the quality assessment of raw milk in the international dairy industry **(Schukken *et al*., 2003).**

Pesticide contamination as a predisposing factor to this condition. The neuroendocrine system, immune system and mineral metabolism interact to coordinate the physiological responses to infection and inflammation **(Ma *et al*., 2006** and **Gera *et al*., 2011)**. Also, the inflammatory products tend to damage udder parenchyma which results in release of intra-

cellular enzyme activity **(Babaei *et al*., 2007)**.
Milk minerals provide the primary mineral requirements of the neonate calf during the critical period after birth when it cannot forage for itself (**Anderson and Sheffield 1988)**. Generally, Milk whey from healthy cows contained more potassium and calcium, and less sodium, than that from cows with evidence of clinical mastitis (**Buriana *et al*., 1981).** Otherwise, study effect of Mycoplasma mastitis on milk electrolytes not well documented.
Fresh milk has the higher antioxidant power (**Păduraru *et al*., 2018**). So, any alter in its compartment especially with presence of organochlorine pesticide could have a great risk to consumer. Malondialdehyde (MDA), is usually used as an indicator of the lipid peroxidation process. In milk, MDA concentrations were measured to evaluate the peroxidation levels when milk was kept under different conditions **(Cesa, 2004 and Miranda *et al*., 2004)**. Some of the most dramatic changes in milk composition have been attributed to bacterial infections of the udder. Mastitis has depressed milk yield, altered the amount and composition of milk proteins, decreased lactose, raised pH, and altered the mineral (sodium, potassium, calcium, magnesium, and chloride) composition of milk **(Batavani *et al.*, 2007)**.
Pesticides are synthetic organic compounds that played imperative role in agriculture and animal pests control throughout the world **(Mitra *et al.,* 2011)**. It is considered a major source of animal health hazards in field. In addition, many studies reported that agricultural and animal origin food is responsible for 90% of pesticides entry in the human body **(Tecles *et al*., 2013).** Organochlorine compounds are highly lipophilic and can accumulate in fat-rich food such as meat and milk **(Hernandez *et al*., 1994**). Pesticides are introduced into cattle mainly through fodder or contaminated water used for household and public purposes. However, organochlorines have been banned in United States and Europe since 1972 **(Jennifer, 2017** and **Daphnis, 2013)**. It is officially prohibited from agriculture in Egypt since 1980 **(Dogheim *et al*., 1996)**. In Africa OCPs banned for agriculture for long time ago, but it still used for malaria control however, DDT is still being used in some countries in Africa **(Sereda *et al.*, 2005)**. Otherwise, it is still detectable in milk and its products in many countries; in Jordan at 2009 **(Nida *et al*., 2009)**, in Bundelkhand region of India at 2008 **(Subir and Mukesh, 2008(** and Uganda in Africa **(Ssebugere *et al*., 2009** and **Kampire *et al*., 2011).**
OCPs are highly toxic and potentially carcinogenic **(Reigart and Robert, 1999** and **USEPA, 1980).** Taking in consideration the bio-magnification property and accumulation observed in water and sediments as well as milk **(Malik et al. 2009)** otherwise, from toxicological point of view, even one molecule reach to target site give a carcinogenic effect.
There is an assumption that pyrethroid pesticides are converted to non-toxic metabolites by hydrolysis in mammals. However, some recent works have shown its bioaccumulation in milk from areas where pyrethroids have been extensively used for agriculture **(Cayo *et al*., 2012)**. In addition to this, pyrethroids pesticide is directly sprayed to the animal accommodation to infest the pest **(Stefanelli *et al*., 2009)**.
The aim of this work was to investigate the presence of Organochlorines and pyrethroids in cow's milk from dairy milk farms at Qalyoubia Governorate. Detection percent of mycoplasma mastitis among mastitic cows. Study the effect of mycoplasma mastitis on milk electrolytes and role of pesticides in mycoplasma mastitis as its effect on antioxidant bioactivities.

Materials and Methods

Chemicals and reagents

Organochlorine and pyrethroid certified reference materials were obtained with purity higher than 98% from Dr. Ehrenstorfer (Augsburg, Germany). Acetonitrile, hexane, acetone and acetic acid (glacial), analytical grade, were purchased from Scharlau (Barcelona, Spain). Primary secondary amine (PSA, 40 µm Bondesil) sorbent was purchased from Supelco (Supelco, Bellefonte, USA). Magnesium sulfate anhydrous, sodium chloride, sodium citrate and sodium hydrogen citrate (sesquihydrate) of analytical grade were purchased from Merck (Darmstadt, Germany). Ultra-pure water was prepared by the Millipore system. Acetonitrile acidified with acetic acid

(1%): 1.0 ml glacial acetic acid was added to 100 ml acetonitrile.

Preparation of Standard:

Stock solution preparation, 100 µg/ml reference standard solutions of analyzed pesticides were prepared in 100 ml hexane in a volumetric flask. Stock solutions were kept in refrigerator at 4°C. Individual standards of 10 µg/ml of pesticides were prepared by diluting 5.0 ml of stock solution in 50 ml hexane. Intermediate solution was kept in refrigerator at 4°C. Calibration standards of organochlorine mixture were prepared by dissolving appropriate amounts of intermediate solutions in hexane and matrix extract.

Sample collection

Aseptically drawn 100 milk samples were taken from healthy dairy cow and 60 mastitic milk samples were taken from cows suffering from mastitis in Qalyoubia Governorate immediately prior to routine daily milking. The udder of each cow was washed with a diluted solution of Bovadine (West Agro-Chemical, Inc.) in warm water, dried with paper towels, and teats were swabbed with 70% ethyl alcohol and allowed to air dry for 1min. Milk samples (ca. 150 ml) were drawn aseptically from each quarter directly into labeled Whirl-pak bags (28g, Van Waters and Rogers Scientific) and placed in iced styrofoam chests. Samples were examined for mycoplasma by traditional golden isolation method and PCR test **(Wegner and Stull, 1978).**

For chemical and pesticide analysis, samples were supplied to laboratory packaged in glass vessels. All samples were kept at -10°C until analyzed.

California Mastitis Test (CMT):

The test was carried out according to **Schalm and Noorlander (1957**). Results were interpreted referring to the change in color and grade of gel formation as negative, trace, 1+, 2+, and 3+ as described by **Schalm *et al.* (1971).**

Isolation and Biochemical Characterization:

Milk samples were cultured by inoculation on PPLO broth medium then plated on PPLO agar medium **(Sabry and Ahmed, 1975)** then, incubated at 37°C for 3-7 days with 24-48h observation interval for fried egg colonies.

Biochemical characterization of the isolated strains was carried out according to **Watson *et al.* (1988)**. Digitonin sensitivity, glucose fermentation, arginine deamination and film & spot formation tests were applied as mentioned by **Erno and Stipkovits (1973)** and **Razin *et al.* (1998).**

Polymerase Chain Reaction (PCR):

DNA extraction: Extraction of DNA using Thermo genomic DNA extraction kit, Cat. No. k0721, Lithuania, was used as described by the manufacturer.

Table (1). Primers sequences used for molecular identification of *Mycoplasma* spp. and *M. bovis*

***Mycoplasma* spp.**	**Target gene**	**Primer sequence (5′→3′)**	**Amplicon Size (bp)**	**Reference**
***Mycoplasma* spp.**	*16S-23SrRNA*	GGT GAA TAC GTT CTC GGG TCT TGT ACA CAC CTT TTC ACC TTT CCC TCA CGG TAC	600-1000	**Volokhov *et al.*, (2006)**
M. bovis	*16SrRNA*	CCT TTT AGA TTG GGA TAG CGG ATG CCG TCA AGG TAG CAT CAT TTC CTA T	360	**Yleana *et al.*, (1995)**

PCR reactions were performed in a Gradient Thermal cycler 1000S (Bio – RAD, USA). The reaction mixture (total volume of 50µl) was 25µl Dream green PCR Mix (Dream Taq Green PCR Master Mix (2X) Thermo Scientific Company, Lithuania), 5µl target DNA, 2µl of each primers (containing 10pmole/µl) and the mixture was completed by RNAse DNAse free sterile distilled water to 50µl.

Table (2). PCR amplification for common gene of *Mycoplasma* spp. and specific gene of *M. bovis* isolates

Thermal profile	16S-23SrRNA gene of *Mycoplasma*	*16SrRNA* gene of *M. bovis*
Initial denaturation	95ºC for 15 min	
Denaturation	94ºC for 30 sec	94ºC for 45 sec
Annealing	60ºC for 30 sec	60ºC for 1 min
Extension	72ºC for 2 min	72ºC for 2 min
Final extension	72ºC for 5 min	72ºC for 3 min
Amplification	35 cycles	35 cycles
References	**Volokhov *et al*., (2006)**	**Yleana *et al*., (1995)**

The presence of amplified PCR products was confirmed by using a 1.5% agarose gel, followed by UV visualization after ethidium bromide staining.

Biochemical milk studies:

The concentration of sodium, potassium, calcium and magnesium in milk samples was determined with an Atomic Absorption Spectrophotometer (AAS). Milk samples were prepared according to the procedures described in the technical manual of the AAS. The data obtained were expressed as mg/dl. Milk phosphorus determination was performed according to the technique of **Varley (1963)**. Milk chloride was performed according to **George (1939)**.

Analysis of vitamin A, leptin and MDA were undertaken using ELISA technique (ELISA kit, Cusabio-Chma). Determination of GSH concentration was determined by the modified method of **Beutler (1971)** and enzymatic activities of catalase according to **Aebi (1983)** and total antioxidant according to method described by **Yagi (1984).**

Determination of total protein and albumin in milk were performed according to method described by **Buzanovskii (2017)**. SOD activity was measured following the method proposed by **Beyer and Fridovich (1987)**.

Pesticide residual analysis:

Extraction procedure (Martins *et al*., 2013):

Milk samples were thoroughly homogenized and 5 ml of homogenate was measured into 50 ml PFTE tube containing 10 ml of deionized water and 10 ml of acidified acetonitrile with acetic acid. The content was shaken manually and 4.0 g of anhydrous magnesium sulphate, 1.0 g sodium chloride, 1.0 g sodium citrate and 0.5 g sodium hydrogen citrate sesquihydrate were added. Then mixture was hand-shaken for 1 min and subsequently centrifuged at 4000 RPM for 3 min. Thereafter, 1 ml of the upper clear solution was transferred into 15 ml of polyethylene tube containing 25 mg PSA sorbent and 150 mg anhydrous magnesium sulphate. Then tube was shaken vigorously for 1 min. and centrifuged for 1 min. at 5000 RPM. Lastly, 0.5 ml of supernatant was taken into a glass vial, evaporated to dryness and redissolved in 0.5 ml n-hexane for GC-µECD analysis.

Gas chromatographic analysis

Organochlorine pesticides were analyzed using Agilent 7890, gas chromatography, equipped with electron capture detector (GC-µECD). GC analysis conducted using HP-5 MS capillary column of 30 m, 0.25 mm Id. and 0.25 µm film thicknesses. Oven temperature was programmed from an initial temperature of 80°C for 1 min, increasing to 30°C /min up to 160 (2 min hold) then increasing to 260°C at a rate of 3°C/min. This was maintained at 260°C for 12 min. Temperature of injector and detector were maintained at 300 and 320°C, respectively. Nitrogen was used as a carrier at flow rate of 3 ml/min. Each set of samples was analyzed against, a solvent blank; a standard mixture and a procedural blank were run in sequence to check for contamination, peak identification

and quantification. Selected samples were analyzed by full scan GC–MS to confirm the GC-μECD results. An Agilent System 6890 series Plus gas chromatograph (USA), equipped with an Agilent 5973 mass selective detector, an Agilent 7683 series Auto sampler and a split/ splitless capillary injector port, was employed. The column used was HP-5 MS (Agilent, Folsom, CA) capillary column of 30 m, 0.25 mm id., 0.25μm film thickness. Helium was the carrier gas at flow rate of 0.5 ml/min. Injection volume was 1μl and inlet temperature was fixed at 225°C. The oven temperature programme was adopted to 60°C (2 min) followed by gradual increase 10°C/min till reach to 160° C and finally rise 3°C /min till reach to 260°C (20 min). The helium carrier gas flow rate was kept in a constant flow mode 1.0 ml/min. Sample injection was carried out splitless at 240°C with 1 min purge off. GC–MS interface was 280°C. Chemistation software was used for instrument control and data analysis.

Method of Validation:

The validation technique was carried out according to the SANCO document 10684/2009 (SANCO/10684/2009). Firstly, linearity was evaluated by constructing matrix matched calibration curves in the range of 0.1–20μg/l for GC-μECD. Sensitivity and recovery were determined using spiked samples with the tested pesticides at 3 different levels. The average recovery percentages for fortified samples were determined. Limits of detection (LOD) and limits of quantification (LOQ) were evaluated according to pesticide concentration produced a peak (S/N) signal-to-noise ratio of 3/1 and 10/1, respectively.

Table (3). Percent recovery from fortified milk samples and the minimum detection limits (μg/kg) for various pesticides

Pesticide Name	Recovery	RSD	LOD	LOQ	$_r2$
HCB	87	2.1	0.62	1.43	0.991
a-HCH	101	11	0.003	0.005	0.989
c-HCH	95	9	0.002	0.003	0.994
d-HCH	96	10	0.003	0.005	0.992
Aldrin	87	10	0.002	0.004	0.993
Endrin	105	14	0.001	0.004	0.992
Dieldrin	101	12	0.001	0.005	0.991
Heptachlor	95	10	0.003	0.006	0.990
Hept. Epoxide	101	15	0.001	0.004	0.992
c-Chlordane	91	9	0.003	0.005	0.984
Endosulfan	93	13	0.001	0.006	0.991
p,p0 –DDE	99	9	0.001	0.004	0.992
p,p0 –DDD	92	12	0.002	0.005	0.992
p,p0 –DDT	96	14	0.001	0.006	0.993
Methoxychlor	92	6	0.002	0.004	0.991
Cyhalothrin	101	8	0.003	0.007	0.984
Permethrin	105	7.5	0.009	0.012	0.992
Fenvalerate	102	9	0.006	0.011	0.986

Data analysis:
Data obtained were statistically analyzed using repeated measures ANOVAs. The significant main effects were found using Least Significant Difference (LSD) for multiple comparisons. The level of significance was at $P < 0.005$ using **IBM-SPSS Version 20 (2011)**.

Results
Incidence and biochemical characterization of *Mycoplasma* from mastitic milk samples:
One hundred and sixty milk samples were examined and classified into five groups according to the degree of mastitis on the base of somatic cell count **(California Mastitis Test)**. Sixty milk samples showed mastitis 60/160 (37.5%). Fifty two mastitic milk samples were positive for *Mycoplasma* isolation with incidence 52/160 (32.5%). Forty one samples were identified as *M. bovis*; 41/160 (25.6%) table (4). All *Mycoplasma* isolates produce colonies with dark centers producing typical fried-egg appearance. All isolates were digitonin sensitive. Biochemically; *M. bovis* neither ferment glucose nor hydrolyze arginine but can form film & spot.

Table (4). Incidence of *Mycoplasma* and *M. bovis* in mastitic milk groups

Mastitis group	Incidence related to total samples (n=160)	*M. bovis* incidence related to mastitic quarters (n=60)	*Mycoplasma* spp. incidence related to mastitic quarters (n=60)
Control (–ve)	100 (62.5%)	-	-
Control (+ve)	8 (5%)	-	-
Mastitis (+)	6 (3.75%)	5 (8.33%)	1 (1.66%)
Mastitis (++)	12 (7.5%)	8 (13.3%)	4 (6.66%)
Mastitis (+++)	34 (21.25%)	28 (46.66%)	6 (10%)
Total	160	41(68.33%)	11(18.33%)

*Total number of tested animals = 40 (160 quarter)
Control -ve = mastitis free (somatic cell count < 5 x 10^8/ HPF)
Control +ve = mastitic milk but negative for *Mycoplasma*
Mastitis (+) = Subclinical mastitis (somatic cell count = 5-8 x 10^8/ HPF)
Mastitis (++) = Mastitis (somatic cell count = 9-15 x 10^8 / HPF)
Mastitis (+++) = Mastitis (somatic cell count >15 x 10^8 HPF)
*HPF= High power field

Confirmation of isolated *Mycoplasma* spp. from mastitic cow's milk was applied using molecular technique (PCR). *16S-23SrRNA* gene of *Mycoplasma* isolates showed a characteristic band at 1000bp and *16SrRNA* specific gene of *M. bovis* declared specific band at 360bp as shown in **Figs. (1&2)**.

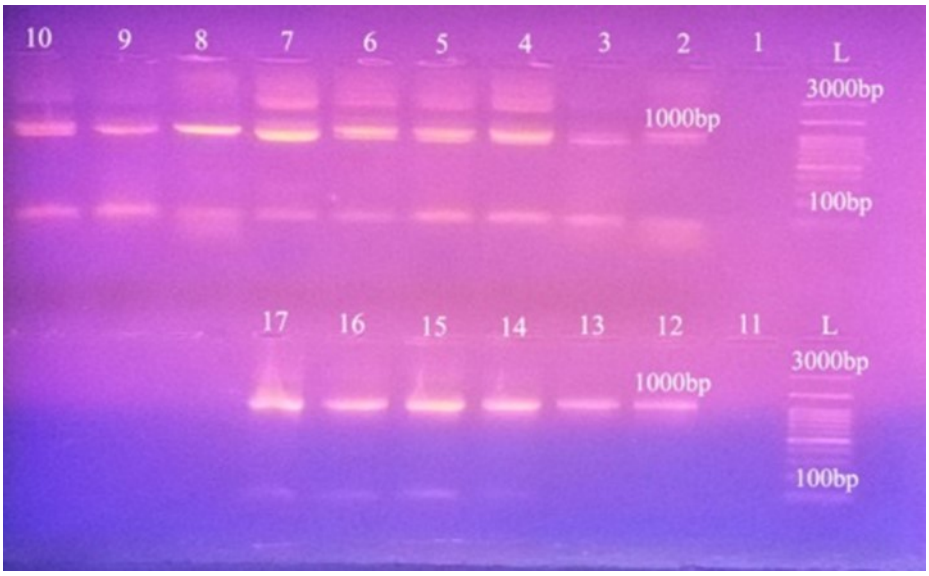

Fig. (1): Agarose gel electrophoretic pattern of *16S-23SrRNA* gene of *Mycoplasma*
L (Lader): 100bp DNA ladder
Lanes (1, 11): Control negative
Lanes (2, 12): Control positive
Lanes (3-10): Positive isolates at 1000bp
Lanes (13-17): Positive isolates at 1000bp

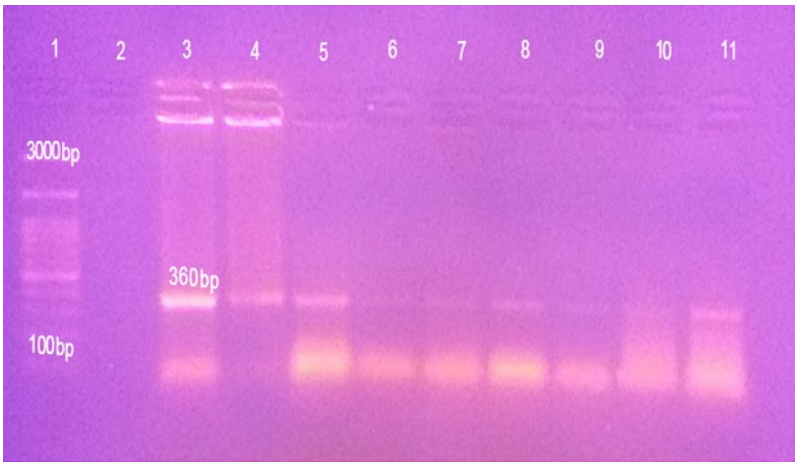

Fig. (2): Agarose gel electrophoretic pattern of *16SrRNA* specific gene of *M. bovis*
Lane (1): 100bp DNA ladder
Lane (2) Control negative
Lane (3): Control positive
Lanes (4-11): Positive isolates at 360bp

Table (5). Electrolytes levels in milk of mastitic and non-mastitic cows (*)

	Ca (mg/dl)	Ph (mg/dl)	Mg (mg/dl)	Na (mg/dl)	K (mg/dl)	Chloride (mg/dl)
-ve control	4.58 ± 0.19A	44.50 ± 0.19A	9.00 ± 0.176A	37.50 ± 0.95A	159.63 ± 1.335A	108.00 ± 3.78A
+ve control	3.83 ± 0.22aB	39.67 ± 0.84aB	7.90 ± 0.228aB	39.67 ± 0.92B	148.83 ± 3.049B	110.33 ± 4.801B
Mastitis +	3.98 ± 0.11aC	37.47 ± 1.106aC	7.94 ± 0.139aC	41.44 ± 1.36aC	142.67 ± 1.130aC	118.11 ± 3.47aC
Mastitis ++	3.73 ± 0.14a	33.50 ± 1.49abc	6.95 ± 0.109abc	57.67 ± 1.48abc	136.33 ± 3.174abc	153.50 ± 5.948abc
Mastitis +++	3.25 ± 0.12abc	31.88 ± 0.64abc	6.15 ± 0.160abc	68.38 ± 1.46abc	139.50 ± 2.57abc	140.63 ± 7.958abc
Sig. level	0.001#	0.001#	0.004	0.001#	0.001#	0.001#

(*) The group size are unequal, Harmonic mean sample size = 9.891
significant using ANOVA test at P < 0.001
Aa, Bb, Cc, Dd Significant difference against capital litter at P< 0.005 using Least Significant Difference "LSD".

Table (6). Total protein, albumin, vitamin A and leptin levels in milk of mastitic and non-mastitic cows (*)

	Total protein (g/dl)	Albumin (g/dl)	Vit A (u/dl)	Leptin (ng/ml)
-ve control	1.53 0.19 ±A	0.18 ±0.04A	43.50 ±1.389A	0.695 ±0.0170A
+ve control	1.86 ±0.14aB	0.27 ±0.029a	48.33 ±1.476a	0.1153 ±0.0259aB
Mastitis +	1.91 ±0.15aC	0.26 ±0.017aC	49.22 ±1.681a	0.884 ±0.0251abC
Mastitis ++	2.15 ±0.17abc	0.24 ±0.021a	44.67 ±2.124a	0.788 ±0.0414ab
Mastitis +++	2.61 ±0.16abc	0.23 ±0.018a	47.00 ±1.890a	0.700 ±0.0456ab
Sig. level	0.001	0.018	0.054	0.001

Table (7). Antioxidant activity levels in milk of mastitic and non-mastitic cows (*)

	MDA (nm/ml)	GSH (nm/ml)	SOD (µ/ml)	Catalase (u/ml)	T antioxidant capacity (µmol/l)
-ve control	0.47 ± 0.013A	13.00 ± 0.378A	2.75 ± 0.094A	5.10 ± 0.151A	716.50 ± 2.457A
+ve control	0.97 ± 0.042aB	9.33 ± 0.211aB	2.03 ± 0.021aB	4.67 ± 0.056aB	580.67 ± 13.901aB
Mastitis +	1.39 ± 0.066abC	7.32 ± 0.359abC	1.77 ± 0.053abC	3.05 ± 0.164abC	508.56 ± 15.903aC
Mastitis ++	1.01 ± 0.041acD	8.83 ± 0.307acD	1.98 ± 0.060acD	4.17 ± 0.053abcD	581.50 ± 10.570abcD
Mastitis +++	1.99 ± 0.055abcd	6.23 ± 0.236abcd	1.53 ± 0.052abcd	3.60 ± 0.113abcd	458.13 ± 14.177abcd
Sig. level	0.001#	0.001#	0.001#	0.001#	0.001#

(*) The group size are unequal, Harmonic mean sample size = 9.891
MDA = Malondialdehyde GSH = glutathione Peroxidase SOD = superoxide dismutase

Table (8). Pesticide residues (µg/liter) determined in milk samples collected from Qalyoubia, Governorate (Egypt)

Pesticide name	Non-mastitic milk samples (n=100)			Mastitic milk samples (n=60)		
	No.	%	Mean ± SE	No.	%	Mean ± SE
HCB	11	11	11.5 ± 0.41	12	20*	9.8 ± 0.34
a-HCH	0	-	ND	0	-	ND
c-HCH	9	9	2.5 ± 0.11	4	6.7	1.7 ± 0.31
d-HCH	0	-	ND	1	1.7	0.11
Aldrin	5	5	8.4 ± 0.31	3	5	5.2 ± 0.42
Endrin	4	4	9.1 ± 0.54	6	10	8.1 ± 0.46
Dieldrin	0	-	ND	2	3.4	2.0
Heptachlor	2	2	5.1	1	1.7	7.8
Hept. epoxide	13	13	9.5 ± 0.83	9	15	11.2 ± 0.88
c-Chlordane	2	2	6.3	3	5	7.2 ± 0.93
Endosulfan	0	-	ND	0	-	ND
p,p′ -DDE	3	3	2.2± 0.18	4	7	2.3± 0.28
p,p′ -DDD	12	12	4.1± 0.33	14	23.33	1.9± 0.38
p,p′ -DDT	1	1	18.0	1	1.7	21.0
Methoxychlor	0	0	ND	0	0	ND
Total OCP	19	19	26.7 ± 19.53	26	43.3*	38.31 ± 24.62
Cyhalothrin	0	0	ND	0	0	ND
Permethrin	0	0	ND	0	0	ND
Fenvalerate	0	0	ND	0	0	ND
Deltamethrin	0	0	ND	0	0	ND

* Significantly different using Fischer Exact probability test.

Discussion

As Mycoplasma mastitis is one of the most frequent diseases in dairy cows, the detrimental effects on the animal health and the profitability of the milk industry are well documented **(Ruegg, 2017)**. Many *Mycoplasma* species exist but few are associated with mastitis, with more than 50% of Mycoplasma mastitis cases, caused by *Mycoplasma bovis* **(Wen *et al.*, 2019)**. In the current study, monitoring the prevalence of *M. bovis* in bovine mastitis milk was occurred. This was done through bacteriological and molecular investigation from dairy herds. The main symptoms of mastitis due to *M. bovis* include great alteration in milk consistency; vary from watery to purulent, a rapid decrease of milk yields, quick spread of the infection from one udder quarter to another, and lack of response to any antibiotic treatment. The incidence of *M. bovis* among tested quarters was 41/160 (25.6%) which was lower than that concluded by **Wen *et al.* (2019)** with total average value of 34.2% to 53.0% in China. *M. bovis* prevalence was so higher also than that of **Abd El-Tawab *et al.* (2019)** and **Timonen *et al.* (2016)** which was 8.96% and 17.2%, respectively.

Prevention of mastitis in large dairy farms needs a sensitive, rapid and specific test to identify *Mycoplasma* as one of the important causes of mastitis. Traditional identification methods are labor-intensive and most of them are not designed to identify important veterinary pathogens (**Watts**, **1989**). Thus, PCR test applied on DNA extracted from milk was used to confirm the identification with high sensitivity and accuracy of *Mycoplasma* mastitis so that, two genes were used for *Mycoplasma* identification; 16S-23SrRNA common gene for ruminant *Mycoplasma* **(Volokhov *et al.*, 2006)** showing a characteristic band at 1000bp and 16SrRNA specific genes for *M. bovis* showing specific band at 360bp **(Yleana *et al.*, 1995)**.

Table (5) showed significant drop in calcium, phosphorus, magnesium and potassium levels in mastitic milk compared with non-mastitic milk with increased levels or scores of mastitic stage. Otherwise, Sodium and chloride was significantly increased with rise stage of mastitis. Taking in consideration, differentiation in minerals levels according to beef species, these

findings are similar to those recorded by **Buriana *et al.* (1981)** in milk obtained from Egyptian cows and **El Zubeira *et al.* (2005)** in milk from clinically mastitis, subclinically mastitis infected and healthy Friesian cows at Sudan.

These differences could be regarded to udder bacterial infection resulting in injury of the ductal secretory cells leads to increased permeability of the blood capillaries **(Peaker, 1975)**. Logically, these changes may be due to that the quantity of milk produced during clinical mastitis in dairy cows decreases accordingly, the composition of the milk is altered. As the severity of inflammation associated with mastitis increases, the chemical composition of milk approaches that of blood as a consequence of increase the permeability of blood mammary barrier, or de novo intra-mammary synthesis **(Hogarth *et al.*, 2004)**. This could be a new approach for the diagnosis and treatment of mastitis.

Table (6) showed that mastitis causes significant increase in total protein and albumin correlated with increased level of infection. Otherwise vitamin A and leptin did not show any noticeable changes. This observation was nearly similar to that observed by **Hogarth *et al.* (2004)**. The observed increase in determined total protein in mastitic milk samples could be caused mainly by the increased proportions of blood protein (BSA and Ig) and lactoferrin in total protein **(Urech *et al.*, 1999)**.

Table (7) showed the antioxidant activity levels in milk of mastitic and non-mastitic cows. GSH, SOD, Catalase and T. antioxidant significantly decreased at $P < 0.05$ in mastitic milk compared with non-mastitic milk. Also, it is observed direct significant correlation with degree of mastitis. Vice versa, with the same pattern MDA showed significant increase. This rise could be regarded to increase somatic cell count associated with mastitis in raw milk **(Suriyasathaporn *et al.*, 2006)**.

The body antioxidant defense mechanism is guaranteed by endogenous and exogenous factors including several enzymes, vitamins, protein components, derivates and oligoelements. Milk has been proven to have important and essential antioxidant composition to prevent and protect against different diseases in infants and adults **(Păduraru *et al.*, 2018)**. The observed decrease in antioxidant parameters in milk could be regarded to stress condition in udder due to bacterial infection.

Out of 100 tested non-mastitic milk samples, 19 (19%) were contaminated with OCPs, while, in mastitic milk samples were 26/60 (43.3%). Statistically, mastitic milk samples were significantly higher using Fischer Exact Probability test at $P < 0.05$ for HCB, Heptachlor epioxide and total OCPs residues (Table, 8). Residues of different OCP compounds like DDT, HCH, dieldrin, heptachlor, trichlorophenol (TCP), pentachlorobenzene (PCB), etc., have been reported to be occurring in pork, chicken, sheep, lamb, goat and beef from many places **(Nag and Raikwar, 2008; Avancini *et al.* 2013** and **Witczak *et al.*, 2016)**.

Generally, our result proved that organochlorine compounds still detectable in cow's milk (Table, 8). The most prominent organochlorine compound residues found in cow's milk was hexachlorobenzene (HCB), either in mastitic or in non-mastitic cows. Mostly, percentage of organochlorine pesticides residues in mastitc cows was significantly higher using Fischer Exact probability test comparing with non-mastitic milk. This results by different residue levels nearly similar to that recorded by **Nag and Raikwar, (2008)** in Bundelkhand region of India, **Nida *et al.* (2009),** who found OCPs in milk, butter, cheese, labaneh and yoghurt collected from Jordan and **Kampire *et al.* (2011)** who extract OCPs from fresh and pasteurized milk samples in Kampala markets, Uganda. Also, **Avancini *et al.* (2013)** in Brazil and **Hassan *et al.* (2014)** in Punjab, Pakistan found that, milk samples indicated the highest percentage (44 and 35%) of contamination with aldrin pesticide. Finally, **Witczak *et al.* (2016)** recently evaluated the presence of organochlorine pesticides in milk goat produced in two farms in Poland.

From the toxicological point of view, there is an important question about, why OCPs permanently still detectable in milk up till now, although it is banned from several years ago. Firstly, OCPs consider one of persistent organic pollutants (POPs) due to their high persistence nature for its chemical stability and lipophilic character which accumulate in different environmental compartments and in food chain

thus causing elevated bio-magnification phenomena in the body **(Thiombane *et al*., 2018)**. These residues bio-concentrate in lipid rich tissues according to equilibrium pattern of internal transport and lipid tissue content and decline at a very slow rate even after sources of contamination are eliminated **(Arpana *et al;*., 2015)**. Also, OCPs are considered very thermostable **(Mülow-Stollin *et al*., 2017)** so; it has very slow degradation rate in the soil and keeps in nature for a long time. Additionally, total organic carbon (TOC) in soils has been regarded as a key factor influencing concentrations of OCPs in sediments **(Malik *et al*. 2009)**. Lastly, metabolic process of organochlorine pesticide derivatives could be produce new more persisted products, the half-lives for the transformation of DDT to DDE is highly variable and were estimated to range from 20 to 50yr **(Crowe and Smith, 2007)**. Otherwise, aldrin is removed by oxidation to dieldrin **(WHO, 1989)**. Heptachlor epoxide is formed by biological and chemical transformation of heptachlor in the environment. Half-lives of total heptachlor were longer than heptachlor, because of the more persistent nature of heptachlor epoxide **(Shivankar and Kavadia 1989)**.

Percent of mastitic cows containing OCPs residues was significantly higher than those of non mastitic cows (Table, 8). This could be regarded to the immunotoxicity effect of OCPs **(Kleanthi *et al*., 2008)**. Free-radical mediated oxidative stress is, associated with some of OCPs residues in human breast tumors **(Iscan *et al*., 2002)**.

Neither, cyhalothrin, permethrin, fenvalerate nor deltamethrin were detected in any of the examined samples whether in mastitic or non-mastitic samples (Table, 8). These results are different from those recorded by **Hassan *et al.* (2014)** in Punjab, Pakistan who detected permethrin and deltamethrin with mean residue level of 1.24µg/ml and 0.21µg/ml, respectively. These differences could be due to the complete control in pesticide applications in Egyptian fields as well as the fact that these pyrethroids less persistent in the environment (short half-lives) in field. Where, synthetic pyrethroids in the environment are rapidly degraded in soil and in plants **(WHO, 1990)**. So, it is not detected in this study.

Furthermore, cyhalothrin degraded with a half-life of approximately 9 days **(Hill and Inaba, 1991)**. Permethrin persist in fatty tissues, with half-lives of 4 to 5 days in brain and body fat **(Hayes and Laws, 1990)**. Deltamethrin c degradation of fenvalerate in the environment is rather rapid. Half-lives are 4-15 days in river water, 8-14 days on plants, 1-18 days by photo -degradation on soil and 15 days-3 months in soil.

Conclusion

Mycoplasma is one of the most important pathogens causing mastitis in dairy farms especially *M. bovis* causing severe economic losses. OCPs reduce the animal immunity and increase the probability of mastitis in udders. Although OCPs banned over 50 years ago, it is continues to be present at relatively detectable concentrations in milk. Our study proved that, it is so necessary to continue monitoring organochlorine pesticide residues in milk and its products to detect exposure risks from the standpoint of food safety. Milk electrolytes could be used as new approaches for the diagnosis and treatment of mastitis. Fresh milk has the higher antioxidant power**.** So, any alter in its compartment especially with presence of organochlorine pesticide could be have a great risk to consumer. Organochlorine pesticides is a carcinogenic so, any drop in antioxidant activity in milk as a result of bacterial infection could be predisposing factor to udder cancer or cause cancer to consumers.

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