Egyptian Journal of Animal Health

P-ISSN: 2735-4938 On Line-ISSN: 2735-4946
Journal homepage: https://ejah.journals.ekb.eg/

Effect of pesticide contamination on mastitic syndrome: a realistic field study

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Received in 6/11/2022
Received in revised from 23/11/2022
Accepted in 20/12/2022

Keywords:

mastitis, pesticide, antibiotic resistance, cow, buffalo, milk, fodder

ABSTRACT

This work was carried out to answer a question of multidrug resistance and fodder pesticide residue relations to increasing mastitis syndrome in cattle and buffalos in Egypt. For this, Milk of cattle and buffalos and fodder samples were collected from 3 different localities in El-Fayoum province.

Pesticide residues analysis was carried out using the modified QuEChERS method followed by liquid chromatography coupled to triple quadrupole tandem mass spectrometry (LC-MS/MS). The residue analysis revealed that about 85% of alfalfa fodder samples and 58% of milk samples were contaminated with pesticides in the valley applied pesticide in alfalfa fodder. The pesticides found in this study were chlorpyrifos, cyhalothrin, lufenuron, and malathion. Identification of bacterial isolates was carried out using conventional PCR. Their prevalence rates were as follows: *Escherichia coli* 4, 11, 15 (5, 10, 13.64%) in milk from the farm, non-pesticide applied, and pesticide applied villages, respectively. For the other microorganisms; *Klebsiella* 0, 2, 5 (0, 1.8, 4.5%), *Staphylococcus aureus* 6, 8, 18 (7.5, 7.3, 16.4%), and *Streptococcus agalactiae* 3, 7, 17 (3.75, 6.4, 15.5%). The isolation rates of these identified bacteria from each sampling point were found to be statisti-

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DOI: 10.21608/EJAH.2023.287818

cally significant using the Fischer Exact Probability test ($P < 0.05$). The results also showed that there was a multidrug resistance to nearly all tested antibiotics in bacteria isolated from milk collected from Ezbet Furqan. The antibiotic sensitivity pattern of bacterial isolates in milk samples collected from a dairy cow's farm in Fayoum province was higher when compared to that of Ezbet Barghout cows. In conclusion, there was a significant correlation between pesticide residues and multidrug resistance.

INTRODUCTION

This research was conducted after observations of veterinarians about increased mastitis cases in Ezbet Furqan, Tamiya city, Fayoum Province Pesticides are widely used in the agriculture sector to avoid or diminish losses from pests. Consequently, this can improve agriculture yield and the quality of the crops, which is important to consumers (Cooper and Dobson, 2007). These pesticides are applied preharvest, which can transfer to animals and accumulate in milk (Shazia and Karam, 2017). The organophosphate insecticide has been reported to bind with human or bovine serum albumin (Ying *et al.*, 2014).

Generally, the source of pesticide residues in milk could be the ambient such as water, soil, and air or fodder or treatment of the animals against disease vectors like ticks, mites, and insects or direct uncontrolled contamination sources such as the dairy utensils that are used during milking or storing it (Özkara et al., 2016). Nevertheless, indirect contamination is a more important way comprising the medication and/or administration of pesticides orally, cutaneous, or via inhalation to the milk-producing animals in closed barns. Whatever the reason does not matter, the active component of the pesticides can be taken into the body, then be metabolized, and finally eliminated into the animal's milk (Fischer *et al.*, 2015).

The mammary gland inflammation is diagnosed as Mastitis and its characteristic is an increase of somatic cell amount in the milk due to the pathology formed in the mammary tissue. Bacteria, mycoplasmas, and fungi are well -known mastitis-causing microorganisms. These can be classified as specific udder pathogens, contagious pathogens, and environmental pathogens.

A well-known problem is that bacteria are gaining antibiotic resistance, and there are several reports related to pesticide residues in the fodders and the antibiotic resistance to cure infectious diseases in domestic animals (Getahun et al., 2007; Fisher et al., 2015 and Wrzecińska et al 2021).

In the light of the limited literature shown above; the presented study aimed to:

determine the microorganisms that caused mastitis in cows and buffalos,

determine the pesticide residues in both milk and fodders

find out any relationship between pesticide residue and antibiotic resistance of mastitis-causing microorganisms.

Material and Methods:

1. Study area:

Fayoum province is located southwest of Cairo with an area of 1,827 km² (CAPMAS 2018). Fayoum is an agricultural province with numerous people in rural dwellings who keep livestock at home. Such a deteriorating situation correlates to some sociodemographic aspects such as high illiteracy rates, poverty, and strong traditional beliefs related to the rural community (HDR 2008; Figure 1).

2. Experimental design:

This study was carried out between October 2021 to April 2022. A cross-sectional study was carried out in two villages (Ezbet Barghout and Ezbet Furqan) and a dairy farm in Taymiyyah city, Fayoum province, Egypt. The samples from dairy farms that grow Egyptian buffalos and local crossbred cows were also collected.

In villages, since there was a lack of recorded formal data; the information about the studied animals was collected from the locals.

The owners of the studied animals are individual farmers and every farmer had only 2 to 3 cows and/or buffalos. These farmers are mainly keeping their animals in the backyard of their house. In Fayoum, the diet of the animals (home-prepared concentrates) is not formulated according to physiological needs. At home, the concentrates (1-3 kg/animal) are provided once daily, in addition to dry wheat hay. The animals are taken to the field early morning after milking every day for feeding by green ration and return home just before sunset. In recent years, some farmers spray different pesticides onto their crops which are used to feed their animals. The samples of both milk and grass (Alfalfa fodder) were collected and analyzed between October 2021 and April 2022.

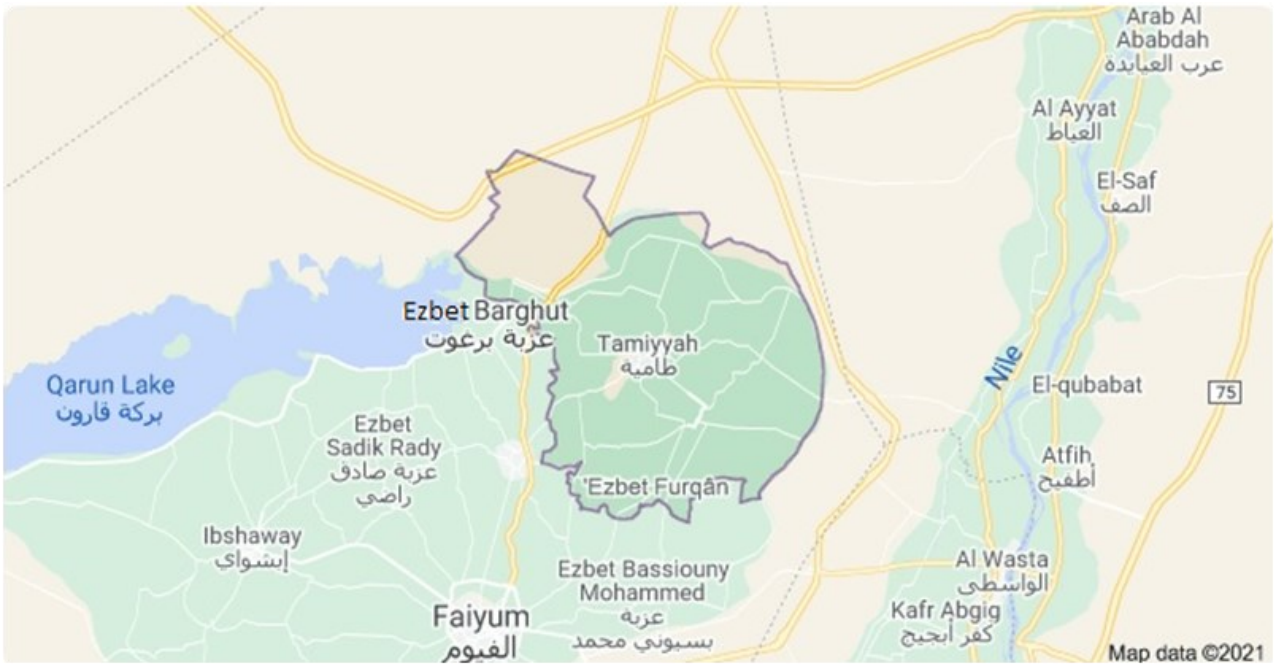

Figure 1: The map of the study area

Animal grouping:

The animals were chosen as fed with pesticide-applied alfalfa fodder and non-pesticide applied alfalfa fodder from dairies located in Ezbet Furqan, and Ezbet Barghout districts.
The study was designed as follows:

a. Both cows and buffalos' milk were tested for subclinical mastitis using the California test. Positive samples were subjected to bacterial isolation and identification and pesticide residue analysis.

b. green grass sample rations were tested for pesticide residue analysis.

c. All mastitis-positive animals' milk samples were then subjected to further examination to determine the bacterial strains by PCR followed by a susceptibility test for different antimicrobial agents as well as pesticide residue analysis.

Table 1. Type and number of samples collected from a dairy farm and two groups from individual farmer in tow valleys.

Species	No of animal	Milk	Grass (Alfalfa)	Total samples
Cow	230	230	35	265
Buffalo	70	70	10	80
Total	300	300	45	345

Grass (Alfalfa fodder) samples were collected from feeds in front of animals under study at 3 studied districts

Chemicals

Chlorpyrifos, Cyhalothrin, Lufenuron, and Malathion reference standards, were purchased from Sigma-Aldrich. Used chemicals were of HPLC grade, acetonitrile, methanol, n-hexane, formic acid, ammonia solution, and glacial acetic acid were obtained from Sigma-Aldrich (USA). QuEChERS Kits 5982-5650 reagent were obtained from Agilent Technologies (USA). Deionized water was produced by the Millipore system.

Sample Extraction and Cleanup:

All the extractions from both fodder and milk samples were carried out by using a modified method of QuEChERS as explained by Lehotay et al., (2005). In the extraction procedure, 10 g of alfalfa fodder, and 2 ml of milk were employed, and the extracts then were subjected to LC-MS/MS analysis.

Preparation of standard solution.

1 mg/ml of stock solution of the analytic standard was dissolved in acetonitrile as solvent. This was used for fortification of the matrices, and a calibration curve was obtained by serial dilutions. All standard solutions were stored at 4°C until use. The standard calibration curve was created by plotting analytic concentrations versus peak area.

LC-MS/MS system:

HPLC (Agilent) 1200 Series instrument coupled to API 4000 Qtrap MS/MS from AB Sciex with electrospray ionization (ESI) interface in the positive mode, source temperature was 400 °C, and ion spray potential was 5500 V. Separation was performed on Agilent C18 column ZORBAX Eclipse XDB 4.6 x 150 mm with 5.0 µm particle size. The injection volume was 10.0 µl.

Table 2 shows the used gradient elution program at a 300 µl/min flow rate. One reservoir contained a mobile phase buffer of 10 mM ammonium formate solution in methanol: water (1:9 v/v) at pH=4 and the other reservoir contained LC-MS grade Methanol. The total run time was 32 minutes.

Table 2. The LC Gradient Elution program.

Time (min)	Mobile Phase Buffer %	Methanol %
0	100	0
13	5	95
21	5	95
28	100	0
32	100	0

Internal Quality Control (IQC):

The IQC is an important item in the technical requirements (ISO/IEC 17025, 2005). To assess the extraction efficiency IQC was carried out using spiked blank samples at 0.05 µg/ml. Tables (3) demonstrate the range of recoveries for tested pesticides varied between 75-116 %, which is acceptable according to SANTE and Eurachem guidelines (Magnusson & Ornemark, 2014; SANTE/11945/2015, 2015).

$$\text{Recovery \%} = \frac{\text{µg pesticide found in the spiked sample}}{\text{µg pesticide added in the spiked sample}} \times 100$$

Methods validation:

The method was validated by the conventional validation parameter, including the level of detection (LOD), level of quantitation (LOQ), and accuracy (recovery%) as recommended by SANCO (2013). LOD was calculated considering 3 times the value of background noise obtained for blank samples.

Bacterial Examination:

California Mastitis Test (CMT):

In this test, the method detailed by Leach et al., (2008) was followed. For this, ~ 2 mL of milk sample was collected from each quart in a plastic oar of four shallow cups marked A, B, C, and D. An equal amount of CMT reagent was added to the milk and mixed then the paddle was rotated after ~ 20s, and the score was read. The test was performed daily to support the data obtained by precise somatic cell counting

Microbiological examination:

MacConkey agar *was employed for E. coli* detection after the 0.1 ml milk sample smears on it. After 24h of incubation at 37°C, five lactase-positive colonies were marked and selected. The selected colonies were isolated by subculture on blood agar (BA). After 24 h of incubation, the cultures were tested by their oxidase activity (OXI) (PLIVALachema, Brno, Czech Republic). OXI-negative strains and controls were transferred on Simmons citrate agar and Motility Test Medium and incubated for another 24h at 37°C. After their assessment, biochemical identification was carried out.

0.1ml milk sample inoculum on Mannitol Salt Agar was used in detecting *S. aureus*. After a 36h of incubation at 35°C, typical colonies were subcultured on blood agar media (BA) and incubated for 24h at 37°C, then both catalase and staphytect tests (Oxoid) were determined. Staphytect positive strains were examined by using Voges-Proskauer (VP) test according to the method of Rysanek, *et al. (*2007). 0.05 ml milk sample as inoculum on BA is used to detect the Streptococcus species. After 24-48h of incubation at 37°C, the β- hemolytic colonies were subcultured on BA and incubated at 37°C for 24 h, and a catalase test was carried out. API 20 Strep (Lancefield grouping) was employed in identification as explained by Rysanek, *et al.* (2007).

Bacterial strains and growth conditions.

The bacteria detected in the study were *E. coli, Staphylococcus aureus, Klebsiella,* and *S. agalactiae.* All strains were cultured in Tryptic soy broth (TSB; Difco Laboratories, Detroit, Mich.) at 37°C for ~17 h before DNA extraction. Cell numbers were determined by the preparation of serial dilutions of overnight culture in phosphate-buffered saline (PBS) and plating on blood agar (Columbia agar base supplemented with 5% defibrinated sheep blood).

Procedure for isolation and identification of isolates by using conventional PCR

DNA extraction:

DNA was extracted from the samples by using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH). For this, a 200 µl of sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. Then, 200 µl of absolute ethanol was added to the lysate. The sample was then washed and centrifuged and then nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primer:

Primers used were supplied from Metabion (Germany) and are listed in Table 3.

PCR amplification:

Primers were utilized in a 25 µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 5.5 µl of water, and 5 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

Analysis of the PCR Products:

PCR products were run on 1.5% agarose gel electrophoresis (Applichem, Germany, GmbH) in 1 x TBE buffer at room temperature using gradients of 5V/cm. In the analysis, 15 µl of the products were loaded in each gel slot. A gene ruler 100-1000 bp ladder (Fermentas, Germany) was also used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was evaluated by using computer software.

Table 3. Oligonucleotide primers for identification of *E. coli*, *Klebsiella* pneumoniae, *Staphylococcus aureus and Streptococcus agalactiae*.

Target agent	Target gene	Primers sequences	Amplified segment (bp)	Prim. Den.	Amplification (35 cycles)			Final extension	Reference
					Sec. den.	Ann.	Ext.		
E. coli	*phoA*	CGATTCTG-GAAATGGCAAAA G CGTGATCAGCGG TGACTATGAC	720	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Hu *et al.,* 2011
Klebsiella pneumoniae	*gyrA*	CGC GTA CTA TAC GCC ATG AAC GTA ACC GTT GAT CAC TTC GGT CAG G	441	95˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Brisse and Verhoef, 2001
Staphylococcus aureus	*16S rRNA*	CCTATAA-GACTGGGATAAC TTCGGG CTTTGAG-TTTCAACCTTGCG GTCG	791	95˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Mason *et al.,* 2001
Streptococcus agalactiae	*cfb*	TTTCACCAGCTG-TATTAGAAGTA GTTCCCTGAACAT TATCTTTGAT	153	95˚C 5 min.	94˚C 30 sec.	50˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Konikkara *et al.*, 2014

Antimicrobial susceptibility test of different bacterial isolates:

Four or five typical colonies of similar morphological appearance were transferred to a tube containing 5 ml of Muller-Hinton broth and incubated at 37ºC for 8 hours until its turbidity exceeds that of the standard McFarland 0.5 barium sulphate tube. A sterile cotton swab was dipped into the standardized bacterial suspension. The dried surface of Muller-Hinton plates were streaked by the swab in 3 different planes. The plate lids were replaced and the inoculated plates were allowed to remain on a

flat and level surface undistributed for 3 to 5 min (not more than 15 min.) Then the disks Clarithromycin (CLR 15µg), Gentamicin (CN 10µg), Amikacin (AK 30µg), Ampicillin + Sulbectam (SAM 20µg), Cefotaxime (CTX 30µg), Amoxicillin (AML 10), Cefepime (CFM 30µg) Amoxicillin+clavulenic acid (AMC 30µg), Spiramycin (SP 100µg) Ampicillin (AM 10 µg) and Sulfa/trimethoprim (SXT 25µg) were applied with a fine pointed forceps on the inoculated plates and incubated in 37ºC for 24h. Then measure the sensitivity by measuring the clear zone of inhibition around the disks and the interpretation was applied according to **CLSI (2007).**

RESULTS

As seen in Table 4, the recovery percentage was calculated to be between 81-and 98%. The sensitivity was evaluated by determining LOD and LOQ. The calculated LODs were 5, 3, 5, and 1 ppb, while LOQs were 10. 6, 10, and 2 ppb for Chlorpyrifos, Cyhalothrin, Lufenuron, and malathion, respectively (Table 4).

Table 4. Recovery % for spiking detected pesticide residues by LC-MS/MS.

Compounds	Spiking Recovery %	LOD (ppb)	LOQ (ppb)
Chlorpyrifos	87	5	10
Cyhalothrin	92	3	6
Lufenuron	81	5	10
Malathion	98	1	2

In this study, the pesticide detected in Alfalfa fodder were Chlorpyrifos (4.85±1.23 and 5.71±1.6 ppm) in both non-sprayed and sprayed alfalfa fodder, and Cyhalothrin (3.22 ± 0.79 ppm), Lufenuron (2.76 ± 1.43), and Malathion (1.78 ± 0.58) in sprayed alfalfa fodder (Table 5).

Pesticide residues in milk collected from dairy animals were shown in Table (5). On a dairy cow's farm, pesticide residues were undetectable.

Dairy cows at the valley were fed with non-pesticide applied alfalfa fodder and the detection and incidences of chlorpyrifos and malathion were 4%, 1.33%, and residue levels of 0.017, 0.017 ppm, respectively. All found pesticides were less than the documented MRL. Otherwise in dairy buffalos, only chlorpyrifos was detected in milk by the incidence of 11.4 % and a mean value of 0.01 ppm.

In the valley studied pesticides are used extensively for Agricultural purposes (Table, 6). Since alfalfa was grown intensively and pesticides were used in the region, especially pesticides incidence in milk cows was 57.3, 21.33, 5.33, and 1.33%, as well as detected residues, were 0.037, 0.026, 0.039, 0.019 ppm, for Chlorpyrifos, Cyhalothrin, Lufenuron, and Malathion, respectively.

Buffalos' milk at the same zones were contain higher incidence (60, 51.43, 14.28, and 5.71%) and mean residue levels of 0.041, 0.024, 0.034, 0.017 ppm, for Chlorpyrifos, Cyhalothrin, Lufenuron, and Malathion, respectively.

Table 5. levels of estimated pesticide in Clover bush (Alfalfa fodder) residues comparing with its maximum residue levels.

Group	No of samples	Pesticide (ppm)											
		Chlorpyrifos			Cyhalothrin			Lufenuron			Malathion		
		No	%	Mean ± SE	No.	%	Mean ± SE	No.	%	Mean ± SE	No	%	Mean ± SE
Group 1	20	-	UD	-	-	-	UD	-	UD	-	-	UD	-
Group 2	20	3	15	4.85 ± 1.23 (2)	-		UD	-	UD	-	1	5	-
Group 3	20	17	85	5.71 ± 1.6 (13)	7	35	3.22 ± 0.79 (7)	4	20	2.76 ± 1.43	2	10	1.78 ± 0.58
MRL		5 mg/kg*			1 ppm*, 2 ppm**					-			-

Group1: Alfalfa fodder cultivated in farm
Group 2: Alfalfa fodder cultivated in valley did not use specific pesticide for Alfalfa fodder
Group 3: Alfalfa fodder cultivated in valley use pesticide for Alfalfa fodder.
samples exceeded the permissible limits
*Chlorpyrifos, Regulation (EU) 2015/399, Pesticide residue(s) and maximum residue levels (mg/kg),http://www.fao.org/fao-who-codexalimentarius/codex-texts/dbs/pestres/commodities-detail/en/?lang=en&c_id=20
** Australian MRLs of 1 ppm for green animal feeds and 2 ppm for straw fodder (MacLachlan, 2020).

Table 6. Pesticide residues in milk collected from dairy animals at the three studied zones comparing with MRL.

Group	Species	No of animals	Pesticide (ppm)											
			Chlorpyrifos			Cyhalothrin			Lufenuron			Malathion		
			No.	%	Mean ± SE	No.	%	Mean ± SE	No.	%	Mean ± SE	No.	%	Mean ± SE
Group 1	Cow	80	-		UD	-		UD	-		UD	-		UD
Group 2	Cow	75	3	4	0.017 ± 0.011	-		UD	-		UD	1	1.33	0.017
	Buffalo	35	4	11.4	0.021 ± 0.012	-		UD	-		UD	-		UD
Group 3	Cow	75	43	57.3	0.037 ± 0.014(15)	16	21.33	0.026 ± 0.017	4	5.33	0.039 ± 0.024	1	1.33	0.019
	Buffalo	35	21	60.0	0.041 ± 0.027(21)	18	51.43	0.024 ± 0.012	5	14.28	0.034 ± 0.022	2	5.71	0.017
MRL			0.02 mg/kg*, ** 0.01 mg/kg****					0.2 mg/kg*			0.15 mg/kg*			0.02 mg/kg***

Group1: Dairy cow's farm Group 2: Animal feeding without spray Clover bush Group 3: Animal feeding on sprayed Clover bush
*Codex alimentations, International Food Standards FAO, WHO, Pesticide Database, http://www.fao.org/fao-who-codexalimentarius/codex-texts/dbs/pestres/pesticides/en/
**Australia Codex (MacLachlan, 2020).
***Malathion, Regulation (EU) 2015/399, Pesticide residue(s) and maximum residue levels (mg/kg), https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/mrls/?event=details&pest_res_ids=143&product_ids=&v=1&e=search.pr
****USA Codex (MacLachlan, 2020).

From a microbial presence view, the isolation incidences in milk samples of dairy cows' farms were 5, 7.5, and 3.75% of *E. coli, Staph aureus,* and *Streptococcus agalactiae*, respectively.

In milk samples of both cows and buffalos, the isolated microorganisms were *Klebsiella, Staph aureus, Streptococcus agalactiae,* and *E. col* showing the percentage of isolation of 10, 1.8, 7.3, 6.4%, respectively.

On the other hand, Cows & buffalos milk samples obtained from valley pesticide applied alfalfa showed the incidence of 13.64, 4.5, 16.4, 15.5% of *E. coli, Klebsiella, Staph aureus,* and *Streptococcus agalactiae,* respectively. These results are significantly different with the value of P<0.05 from that obtained in valley non-applied pesticide according to the Fischer Exact Probability Test.

Table 7. Results of isolation of bacteria from milk samples.

Group	Species	No of animal	Isolates from milk sample							
			E. coli (30)		*Klebsiella pneumoniae* (7)		*Staphylococcusaureas* (30)		*Streptococcus agalactia* (27)	
			No	%	No	%	No	%	No	%
Group1	Cow	80	4	5	0	0	6	7.5	3	3.75
	Total	80	4	5a	0	0a	4	5a	3	3.75a
Group2	Cow	75	6	8	1	1.3	6	8	5	6.7
	Buffalo	35	5	14.3	1	2.9	2	5.7	2	5.7
	Total	110	11	10b	2	1.8b	8	7.3b	7	6.4b
Group3	Cow	75	8	10.7	3	4	11	14.7	14	18.7
	Buffalo	35	7	20	2	5.7	7	20	3	2.7
	Total	110	15	13.64c	5	4.5c	18	16.4c	17	15.5c
Total		300	30	10	7	2.33	30	10	27	9

a, b, c significantly difference at P< 0.05 using Fischer Exact Probability test.

Results of PCR bacterial isolates and sensitivity test:
Figures 2-5 show the result of molecular typing of *Klebsiella, E. coli, Streptococcus agalactiae and Staphylococcus aureus* by using PCR following the gel running

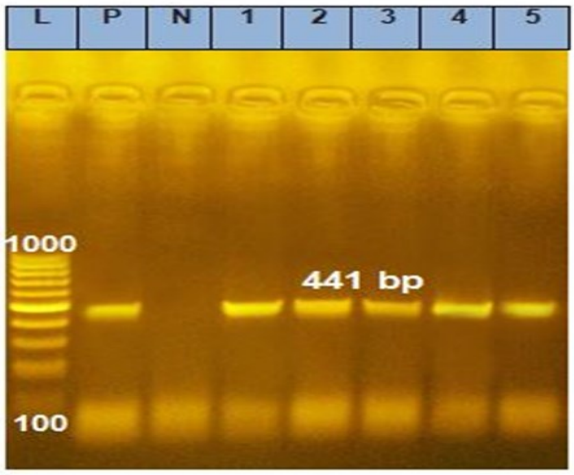

Fig **(2)**: Results of molecular typing of *Klebsiella pneumonia gyrA* gene by PCR. L-100bp DNA marker.P-control positive *Klebsiella pneumonia* strain N-control negative *Klebsiellapneumonia* strain 1 -5 Positive isolates *Klebsiellapneumonia* at 441 bp

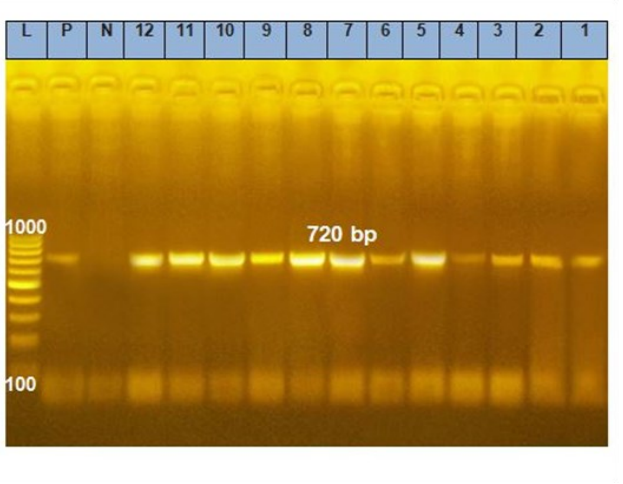

Fig (3): Results of molecular typing of *E. coliphoA* gene by PCR. L-100bp DNA marker.P-control positive *E. coli* strain N-control negative *E. coli*strain 1 -12-Positive isolates of*E. coli* at 720 bp

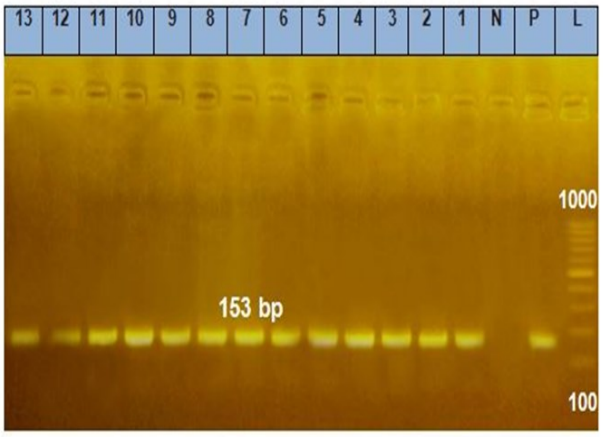

Fig (4): Results of molecular typing of *Strept agalactiae Cfb*genebyPCR. L-100bp DNA marker.P-control positive *Strept agalactiae*strain N-control negative *Strept agalactiae* strain 1 -13-Positive isolates ofrRNAat 153bp

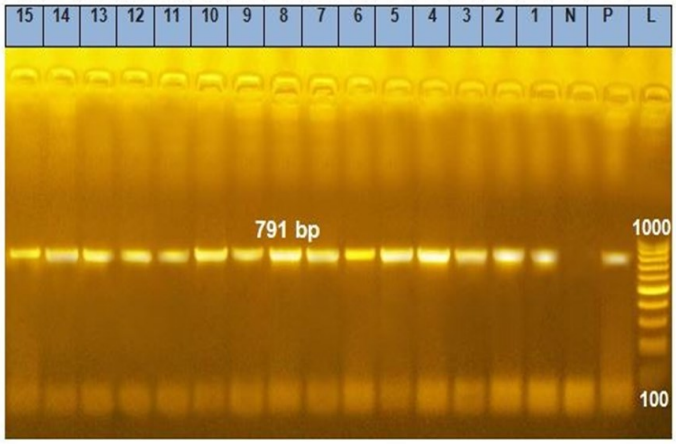

Fig (5): Results of molecular typing of *Staphylococcus aureus16S rRNA* gene by PCR. L-100bp DNA marker. P-control positive *Staphylococcus aureus* strain N-control negative *Staphylococcus aureus* strain 1 -15-Positive isolates of 16SrRNA at 791bp

Tables, 8-11 antibiotic sensitivity pattern of bacterial isolates in milk samples collected from dairy cow's farm, cows & buffalos feeding non-applied clover at Ezbet Barghout as well as cows & buffalos feeding on sprayed Clover bush at Ezbet Furqan, Fayoum show province. These tables show the increased tested bacterial resistance by sequential order

Table 8: Antibiotic sensitivity pattern of bacterial isolates in milk samples collected from Animal feeding without spray Clover bush group 1 (dairy cow's farm) and group 2 (Ezbet Barghout), Fayoum province.

Antimicrobial	*E. coli*(n = 15)			*Klebsiella pneumonia* (n = 2)			*Staphylococcus aureus* (n = 14)			*Streptococcus agalactia* (n = 10)		
	R (%)	I (%)	S (%)	R(%)	I (%)	S (%)	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)
Clarythromycin (CLR 15µg)	4 (26.7)	3(20)	8 (53.3)	0(0)	1 (50)	1(50)	2 (14.3)	5 (35.7)	5 (35.7)	0(0)	2(20)	8(80)
Gentamycin (CN 10 µg)	5 (33.3)	3(20)	7 (46.7)	0(0)	1 (50)	1(50)	2 (14.3)	2 (14.3)	10 (71.4)	2(20)	1(10)	7(70)
Amikacin (AK 30µg)	3(20)	5 (33.3)	7 (46.7)	1(50)	0(0)	1(50)	2 (14.3)	4 (28.6)	8 (57.1)	2(20)	3(30)	5(50)
Ampicil-lin+Sulbactam (SAM20µg)	2 (13.3)	5 (33.3)	8 (53.3)	0(0)	1 (50)	1(50)	0(0)	2 (14.3)	12 (87.5)	2(20)	4(40)	4(40)
Cefotaxim (CTX 30µg)	3(20)	5 (33.3)	7 (46.7)	0(0)	0(0)	2 (100)	1(7.1)	0(0)	13 (87.5)	1(10)	4(40)	5(50)
Amoxicillin (AMX 10µg)	7 (46.7)	5(25)	3(50)	1(50)	0(0)	1(50)	2 (14.3)	4 (28.6)	8 (57.1)	3(30)	3(30)	4(40)
Cefepime (CFM 30 µg)	0(00)	1(6.7)	14 (93.3)	0(0)	0(0)	1(50)	0(0)	2 (14.3)	6 (100)	2(20)	6(60)	2(20)
Amoxicillin +Clavulenic acid (AMC 30µg)	2 (13.3)	1(6.7)	12 (80)	0(0)	1 (50)	1(50)	4 (28.6)	4 (28.6)	6(50)	3(30)	4(40)	3(30)
Spiramycin (SP 100 µg)	3(20)	2 (13.3)	10 (63.6)	1(50)	0(0)	1(50)	3 (21.4)	2 (14.3)	9(75)	4(40)	2(20)	4(40)
Ampicillin (AM 10 µg)	6 (45.4)	5 (33.3)	4 (26.7)	1(50)	0(0)	1(50)	2 (14.3)	4 (28.6)	9 (62.5)	5(50)	4(40)	1(10)
Sulphamethoxazole + Trimethoprim (SXT 25µg)	7 (46.7)	3(20)	5 (33.3)	2 (100)	0(0)	0(0)	3 (21.4)	4 (28.6)	7(50)	4(40)	6(60)	0(0)
Average Resistance	25.45 %			27.27 %			13.63 %			25.45 %		

R=Resistance, I=Intermediate, S=Sensitive, n=number

Table 9: Antibiotic sensitivity pattern of bacterial isolates in milk samples collected from animal feeding on sprayed Clover bush, Ezbet Furqan, Fayoum province.

Antimicrobial	*E. coli* (n = 15)			*Kellebsiella pneumoniae* (n = 5)			*Staphylococcus aureus* (n = 18)			*Streptococcus agalactiae* (n = 17)		
	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)
Clarythromycin (CLR 15µg)	9(60)	4 (26.7)	2 (13.4)	2 (40)	2 (40)	1 (20)	13 (72.2)	2 (11.1)	3 (16.7)	11 (64.7)	2 (11.8)	4 (23.5)
Gentamycin (CN 10 µg)	8 (53.4)	2 (13.3)	5 (33.3)	2 (40)	1 (20)	2 (40)	8 (44.4)	2 (11.1)	8 (44.4)	7 (41.2)	2 (11.8)	8 (47.1)
Amikacin (AK 30µg)	7 (46.6)	4 (26.7)	4 (26.7)	3 (60)	1 (20)	1 (20)	5 (27.8)	5 (27.8)	8 (44.4)	6 (35.3)	0(0)	11 (64.7)
Ampicillin +Sulbactam (SAM 20µg)	8 (53.4)	4 (26.7)	3 (23.1)	2 (40)	2 (40)	1 (20)	6 (33.3)	7 (38.9)	5 (27.8)	4 (23.5)	6 (35.3)	7 (41.2)
Cefotaxim (CTX 30µg)	6 (40)	2 (13.3)	7 (46.6)	2 (40)	1 (20)	2 (40)	9 (50)	3 (16.7)	6 (33.3)	8 (47.1)	2 (11.8)	7 (41.2)
Amoxicillin (AMX 10µg)	12 (80)	2 (13.4)	1 (6.7)	3 (60)	1 (20)	1 (20)	14 (77.8)	2 (11.1)	2 (11.1)	9 (52.9)	4 (23.5)	4 (23.5)
Cefepime (CFM 30 µg)	4 (26.7)	3 (23.1)	8 (53.3)	2 (40)	2 (40)	1 (20)	8 (44.4)	3 (16.7)	7 (38.9)	10 (58.8)	3 (17.6)	4 (23.5)
Amoxicillin + Clavulenic acid (AMC 30µg)	7 (46.6)	5 (33.3)	3 (23.1)	0 (0)	2 (40)	3 (60)	7 (38.9)	3 (16.7)	8 (44.4)	6 (35.3)	4 (23.5)	7 (41.2)
Spiramycin (SP 100 µg)	9 (60)	4 (26.7)	2 (13.3)	0 (0)	3 (60)	2 (40)	3 (16.7)	5 (27.8)	8 (44.4)	4 (23.5)	3 (17.6)	10 (58.8)
Ampicillin (AM 10 µg)	12 (80)	3 (20)	0 (0)	4 (80)	1 (20)	0 (0)	12 (66.6)	4 (22.2)	2 (11.1)	10 (58.8)	4 (23.5)	3 (17.6)
Sulphamethoxazole + Trimethoprim (SXT 25µg)	6 (40)	5 (33.3)	4 (26.7)	2 (40)	2 (40)	1 (20)	9 (50)	5 (27.8)	4 (22.2)	11 (64.7)	4 (23.5)	2 (11.8)
Average Resistance	53.33 %			40.0 %			47.47 %			45.99 %		

R=Resistance, I=Intermediate, S=Sensitive, n=number

Table 10: Average percentage of studied antimicrobial resistance for isolated microorganisms.

	E. coli	*Klebsiella pneumoniae*	*Staphylococcus aureus*	*Streptococcus agalactiae*
Animal feeding without spray Clover	25.45 %	27.27 %	13.63 %	25.45 %
Animal feeding on sprayed Clover	53.33 %	40.0 %	47.47 %	45.99 %

a, b, c significantly differences against higher litter using Fischer Exact Probability test at $P < 0.05$.

DISCUSSION

Mastitis is caused mostly by bacteria. There are several different antibiotics for mammary infection therapeutic and preventive applications. These antimicrobial compounds are usually injected into the infected part after the infection happened

In this work, it was to comprehend that pesticide usage in agricultural areas should be considered as they extend to the milk obtained from grazing animals including cows and buffalos. The effect of pesticides on antibiotic sensitivity causes a big problem in the treatment of mastitis and antibacterial resistance is increasing.

The LOD and LOQ values in this study were lower than the MRLs established by Codex (Codex Alimentarius Commission, 2019) and Australian MRLs (MacLachlan, 2020) for milk and alfalfa fodder samples (Tables, 3 through 5), and these results give high precision to our conclusion.

In the valley where extensively, pesticides were applied in agriculture, especially on alfalfa, the detected pesticides were chlorpyrifos, cyhalothrin, lufenuron, and malathion (Table 4). While in non-sprayed alfalfa fodder was Chlorpyrifos only. In contrast, alfalfa fodder cultivated on the dairy farm was free of any pesticides. Data analysis proved that percentage of pesticide residues was higher and higher than MRL in group 3 than that recorded in group 2. This could be because of using pesticides in Alfalfa culture as well as other pesticides used in cultivated crops. This was augmented by alfalfa fodder taken from cultivated farms that did not contain any pesticide residues.

Although the application of pesticides did not recommend for alfalfa in Egypt (Agricultural Pesticide Committee Recommendation), its usage is still a fact in some areas. In addition, no residue data were provided for alfalfa fodder and there is insufficient information to suggest MRLs for alfalfa fodder (FAO/WHO, 2020).

In this work, estimation of pesticide residues in milk is made based on the tendency of pesticides to transfer to milk with expected alfalfa dietary exposure. Otherwise, some pesticides estimated in this study registered for use on other crops than alfalfa but find their ways to alfalfa.

Pesticide residues in milk show nearly the same pattern in alfalfa fodder (Table, 5). In milking animals fed with pesticide-applied alfalfa fodder (Ezbet Furqan), 4 pesticides were detected in milk such as chlorpyrifos, cyhalothrin, lufenuron, and malathion, by descending order. Milking buffalos in that zones contain higher incidence and pesticide residue levels than milking cows. This observation could be regarded as higher fat percentages in buffalos' milk than that of cow's milk. Also, buffalo's immunity system could be more sensitive to pesticides than cows, this notice needs further studies to prove this phenomenon.

Chlorpyrifos is an organophosphate insecticide used for the control of several insects in harvests. It is enumerated in Egypt on many pastures to control various pests. Generally, it is not grazed or cut for stock food for 2 days after application (MacLachlan, 2020). The maximum transferred factor for cattle feeding at 30 ppm Chlorpyrifos in the feed was 0.007 ppm for milk (JMPR, 2000). Expected Chlorpyrifos residues from feeding alfalfa fod-

der with residues of 5.17 ppm at 100 % of the diet 5.17 ppm ×0.007 = 0.036 ppm, nearly similar to that detected in this study (0.037 & 0.041 in cattle and buffalos, respectively), where these animals depending mainly on alfalfa fodder in their diet during the studied winter season.

Lambda-cyhalothrin is a synthetic pyrethroid used to control various insects in crops. It is registered on pasture for control of many pests in Egypt. It is residues in cattle fat degenerated with a half-life of 7-9 days. The recommendation for its application for stock food is 7-14 days after application. Presume the residues of cyhalothrin in alfalfa fed to animals 3.22 ppm (obtained result, Table, 4), cyhalothrin, residues in milk fat were 0.5 ppm (Cyhalothrin, 1999) and total fat for milk is 0.02 ppm, thus cyhalothrin, anticipated maximum residues in whole milk would be 3.22×0.5×0.02 = 0.032 mg/kg (MacLachlan, 2020).

Regarding Malathion (organophosphorus pesticide) and Lufenuron (agricultural pesticide), insufficient data were located to offer a confidential view of livestock residue risks (MacLachlan, 2020).

As stated by Fisher et al., (2015), pesticide residues in milk might have some potential sources. Whatever the source does not matter and pesticides reach into the milk of the lactating animal.

Out of 300 milk samples of cows and buffalo cows, 4 microorganism isolates could be identified (Table, 6) with different incidences. The isolated microorganisms were *E. coli, Klebsiella, and Staph. aureus* and *Streptococcus agalactia*.

To get reliable results on microbial etiology in mastitic animals PCR confirmation occurred (Figures 1-3). Since cases of mastitis in the current study were sampled under pesticide polluted feed criteria and originated from pesticide applied alfalfa, the relation among them was evaluated. The two most common udder pathogens, *E. coli S. aureus,* and *Streptococcus agalactiae* were almost found in other investigations (Ericsson *et al.*, 2009).

The percentage of microorganisms recovered from dairy animals feeding on sprayed clover bush was significantly higher than that from animals feeding without spray clover bush subsequently higher than milking cows on a closed farm. This could be regarded as restricted biosafety and biosecurity roles applied in farms compared with that in rural animal husbandry. *E. coli* causes mastitis in dairy cows and buffalos around parturition and early during lactation with arresting local and sometimes severe clinical symptoms (Christian *et al.*, 2003).

In this concern, many management factors affect dairy buffalos or cow's conditions during late pregnancy. Pesticides detected in animal feed might be the cause of metabolically immunocompromised buffalos and cows that cause lower resistance to stress-induced by parturition and early lactation, so, animals become highly susceptible to environmental pathogens. There is a tendency to believe that low milk SCC fails to protect the udder from environmental pathogens (Suriyasathaporn *et al.*, 2000). This assumption is based on epidemiological data (Shuster *et al.*, 1996).

Cow and Buffalo mastitic milk (Tables 8-10), showed antimicrobial susceptibility and a resistant pattern of the mastitis-causing organism. Generally, bacterial isolated from pesticide residues milk demonstrated the highest resistance rates to Gentamycin, Amikacin, Ampicillin +Sulbactam, Cefotaxime, Amoxicillin, and Ampicillin. This pattern of resistance could be regarded as excessive use of antibiotic and/or pesticide residues detected in milk. The relevance of pesticides to AMR development is alarming. More evidence of pesticides as agents disturbing bacterial antibiotic susceptibility and generating transient adaptive responses is imperative. The role of pesticides, not only as toxins, but as pathways to AMR must be further evaluated to address the current crisis of antibiotic resistance and to raise awareness of the need for environmental monitoring and regulation (Malagón-Rojas *et al* 2020).

Occurrence of the highest number of *E. coli* and *Staph. aureus* agrees with those reported by Akram et al. who reported a higher isolation rate of E. coli and Staphylococcus in studied milk samples in India (Akram *et al.*, 2007). Higher *E. coli* and *Staphylococcus* incidence in this work could be a reference to the poor hygienic practices in the dairy environment at this valley, while these organisms initiated from the

milking environment and contaminated the udder via the teat canal. In the case of environmental mastitis, the contagion of the ending of the teat is a major predisposing factor (Akram *et al*., 2007, Sarne *et al*., 2018). In addition, these observations might be due to harboring the organism in the skin, udder, and milk of the infected gland which acts as a reservoir.

Taking into consideration that all studied animals were taken from the same city but with different districts, the significant differences in antimicrobial resistance patterns could be regarded as excessive pesticide exposure to dairy animals.

In conclusion, this study proved that pesticide pasture and/or feed contamination cause easier access for environmental pathogens to the udder through their open teats during calving and/or drop-in udder immunity. More studies must be needed to prove the observed correlation between pesticide-contaminated milk and antimicrobial resistance. Despite the great efforts made by the Agricultural Pesticides Committee in Egypt, more control overuse the pesticides in Egyptian villages and hamlets must be applied

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