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# Effect of spraying chlorpyrifos on green weeds and its relationship to bacterial enteritis in calves: Case Study

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**ABSTRACT**

Three cases of buffaloes and their calves were exposed to acute toxicity after feeding on sprayed alfalfa fodder by chlorpyrifos at Monshaat El-Gammal, Tamiyyah city, El-Fayoum Governorate, Egypt. On treatment of animals with atropine sulphate, adult animals were recovered; two of their calves were recovered within 1 hour, while last one dead. The content of used pesticide active ingredient "chlorpyrifos" and its relevant impurity sulfotep in commercial pesticide formulations (480 g a. i. /L) were estimated by gas chromatography-flame ionization detector (GC-FID) against external standard of high purity. Gas chromatographymass spectrometry method was used for the qualitative and quantitative analyses of chlorpyrifos in the formulated sample and in animal serum, diarrhoeic material as well as milk of exposed animals. The results of analysis showed that the content of chlorpyrifos in the sample is 214.15 g/L which is not comply with the Food and Agricultural Organization (FAO) specifications for the active substance (chlorpyrifos). The content of the relevant impurity sulfotep was estimated as 0.269 % of chlorpyrifos content which comply and close to the corresponding maximum permitted level 3 g/kg of the chlorpyrifos content found. GC-MSMS MRM for chlorpyrifos was 350 m/w for Precursor Ion and Fragment ions were 97, 199 m/z. The recovery percent of chlorpyrifos ranged between 71.8 and 94.65% with relative standard deviation (RSD) below 1.45%. Average calculated residues of chlorpyrifos were 2.6 ppm in milk, 80.8 ppb in diarrheic materials and 37.4 ppm in grass (alfalfa fodder). This disturbance create suitable media for some pathogenic bacteria invasion, Nine bacterial isolates (4 *E. coli,* 2 *Staph. aureus,* 2 *Coliform and* 1 *Klebsiella*) from milk, saliva, nasal and faecal samples of 3 buffaloes and 3 buffalo's calves suffering from severe diarrhoea. In conclusion: This study proved that exposure to chlorpyrifos induced disturbance in the intestinal microflora with stimulated pathogenic bacteria causing enteritis. While true used chlorpyrifos pesticiede did not agree with bottle contents. So, more control over pesticides application is required in villages and Ezabs at rural areas.

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## INTRODUCTION:

**Hafiz et al. (2021)** said that Chlorpyrifos (CPF) is a broad-spectrum chlorinated organophosphate (OP) pesticide used for control a variety of pathogens and insects at homes and other localities in vegetables, fruits and crops**.**

Chlorpyrifos acts on the nervous system of the parasites and so all organophosphate insecticides (but also act on the nervous system of mammals) as inhibitor of acetylcholinesterase enzyme that hydrolyzes acetylcholine which elaborate in the transmission of nervous signals from nerves to muscles and between neurons in the brain **(Trang and Khandhar, 2021).**

Like other organophosphates, signs of its acute toxicity include abdominal pain, bronchospasm, constricted pupils, lacrimation, coughing, decreased heart rate, defecation, difficult breathing, diminished appetite, distress, salivation, and urination **(Santos et al. 2021).**

Milk is a complex frequent lipids and proteins constituents ferquntally contaminate with pesticide residue **(Tripathya et al. 2019).** Several analytical methods for chlorpyrifos have been reported, such as gas chromatography (GC) **(Chandra et al. 2010; Marlena et al. 2016)**, high performance liquid chromatography (HPLC) **(Ata et al. 2013, CIPAC, 2020),** GC–mass spectrometry (GC-MS) **(Cajka et al. 2005)** and liquid chromatography–MS **(Zhang et al. 2015).**

**Joly et al (2015)** reported that total aerobic and anaerobic counts of bacteria in the chlorpyrifos (CPF) groups had significantly higher relative to control animals in the ileum and colon at D21. Exposure to CPF induced by disturbance is often characterized by a decrease in the number of beneficial microorganisms and a simultaneous increase in the number of potentially pathogenic microorganisms leading to dysbiosis (**Xia et al. 2018, Condette et al. 2015**). Diarrhea in ruminants remains the most important cause of death in calves. Various bacterial, viral, and protozoal agents are recognized as causative agents, and failure of transfer of passive immunity is considered an important predisposing factor. Clinical presentation can range from loose stools in an otherwise healthy animal to severe dehydration, coma, and ultimately death.

The present study documents the farmers wrong use the insecticides with insufficient awareness. Farmers spray alfa alfa by chlorpyrifos to control of snails which affect the growth of alfa alfa. Buffaloes feeding this alfa alfa spared suffering from diarrhoea and indigestion and off food while long run exposure cause toxicity by chlorpyrifos and showed salivation, colic, diarrhoea, off food, hypothermia and nervous manifestation.

The aim of this work to know the chlorpyrifos pesticide used as spraying on alfalfa fodder in field as well as pesticide residues in grass and milk from toxicated milking buffaloes and determine bacterial isolation with viable counts of buffaloes rumen microflora during treatment by antidote of chlorpyrifos in this case study. This paper was undergo after recording acute toxicity with chlorpyrifos of three milking buffaloes and death of one buffalo calf.

## MATERIALS AND METHODS:

### Animals:

Three buffaloes and their calves from Monshaat El-Gammal, Tamiyyah cities, El-Fayoum Governorate, Egypt were subjected to acute toxicity with organophosphorus compounds. The signs of toxicity were abdominal distension, bronchospasm, constricted pupils, difficulty breathing, diarrhea, lacrimation, and salivation while tremors and progressive diarrhoea were noticed in buffaloe calve (Photo, 1). This symptoms were disappeared shortly after subcutaneously administration of atropine sulphate by the dose of 0.25 mg/ kg. Animal weight about 400 Kg dosed 30 cm firstly, followed by 10 cm after 2 hours, the repeated till symptoms of toxicity disappeared. One of buffaloe calve did not survive and died after 30 minutes.

Photo (1): One of 3 buffalo calves was dead after 30 minutes of our trial to save it excessive diarrhoeic material.

**Pesticide**:

Sample of pesticide used for spraying alfalfa fodder was taken. The bottle was 1 Litter in size and written on the packaging label that its active ingredient is chlorpyrifos 48% emulsified concentrate, trade name Q-Asia 48% EC.

**Samples:**

Sample of 10 ml were taken from 3 bottles for purity analysis. Three samples were taken from alfalfa fodder in front of animals and diarrhoeic material as well as the milk of toxicated buffaloes for residual analysis.

Total of 21 samples for bacterial examination from animals (3 milk, 6 nasal, 6 salvia and 6 fecal samples) of 3 buffaloes and 3 buffalo's calves suffering from severe diarrhoea and symptoms of toxicity by chlorpyrifos.

Total of 26 samples of the whole rumen content were withdrawn at 08:00 h before the morning meal for three buffaloes and their calves to determine the total viable counts of buffaloes rumen microflora after 1, 3, 5, 7 and 9 day of treatment by antidote (atropine sulphate) of chlorpyrifos

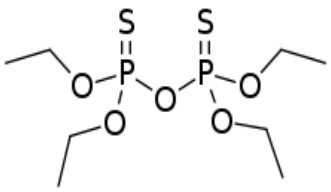
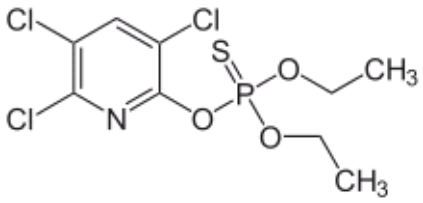
**Reagents and Standards**

All chemicals were of analytical grade. Acetone, hexane, and ethyl acetate were obtained from Merck. Deionized water and sodium sulfate, anhydrous were used.

Analytical standards (Chlorpyrifos and its relevant impurity Sulfotep) of known purity 98 – 99.5% as certified by manufacturer(s) obtained from Research Department of Pesticide Analysis – Central Agricultural Pesticides Laboratory (CAPL). **Table (1)** shows the identity of Chlorpyrifos and its relevant impurity Sulfotep.

The obtained commercial formulations of chlorpyrifos 480 g/l EC (declared concentration) was obtained from farmers used it.

**Table (1)** Identity of chlorpyrifos and its relevant impurity sulfotep.

| Sulfotep | Chlorpyrifos (E-ISO, BSI, ANSI, ESA) | ISO Common name: |
|---|---|---|
| O,O,O',O'-tetraethyldithiodiphosphate | O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate | Chemical name CA: |
| 3689-24-5 | 2912-88-2 | CAS Registry number: |
| EC / List no.: 222-995-2 | 221 EC number: 220-864-4 | CIPAC number: |
|  |  | Structural formula: |
| C8H20O5P2S2 | C9H11Cl3NO3PS | Molecular formula |
| 322.32 | 350.6 | Molecular mass |

**Principles of the analytical procedure:**

Chlorpyrifos and its relevant impurity Sulfotep concentrations were determined using gas chromatography and FID (flame ionization detector) detection, with splitless injection. Nitrogen used as the carrier gas. Quantitation was by peak area measurement using external standard calculations.

**Chlorpyrifos Analytical Standard Preparation**

10 mg of chlorpyrifos analytical standard of known purity was dissolved into a 25 ml grade (A) measuring flask, and completed with methanol. The Chlorpyrifos working solution was prepared at concentration 40 mg pure A.I. / 100 ml methanol.

**Sample Preparation of Chlorpyrifos (480 g/ L) (as declared on the label)**

A specific weight equivalent 10 mg of chlorpyrifos analytical standard (0.0208 g) was taken from the chlorpyrifos formulation and transferred into 25 ml grade (A) measuring flask and completed with methanol.

**Sulfotep Standard Preparation**

10 mg of Sulfotep analytical standard was weighed into a 25 ml grade (A) measuring flask, and completed with methanol.

**Sample Preparation for Sulfotep determination:**

1 g from the chlorpyrifos formulation sample was weighed into a 25 ml grade (A) measuring flask, dissolved and completed with methanol. In all preparations the Ultrasonic bath was used for homogeneity.

**Sample Preparation for residue analysis:**

A modified version of the QuEChERS method for sample preparation of vegetables and milk were used.

**Identification and determination of chlorpyrifos and its relevant impurity Sulfotep**

**Gas Chromatography (FID) Determination**

The procedures were performed using an Agilent 7890B gas chromatograph equipped with a flame ionization detector (GC/FID) for detection. The GC fitted with an auto-injection system, autosampler 7693B and a GC data system (computerized). The GC system used capillary column HP 50+ (30 m x 0.53 mm I.D., 1 µm film thickness) for separation,

injector with splitless mode and carrier gas nitrogen. The injection volume employed was 1 µl.

**GC-MS analysis and determination**

The procedures were performed using GC-MS, model Aglient 7890B gas chromatograph equipped with 5977 A MSD, with a fused silica capillary column HP-5MS (30 m x 0.25 mm x 0.25 µm film thickness). Carrier gas used was helium with 1.0 ml/min pulsed split mode. The injection volume was 1 µl, temperature program was held at 50℃ for 0.5 min, then ramp 10℃ /min to 190℃ for 1 min. followed by ramp 10℃ /min to 300 and held for 2 min (total run time 28.5 min). The injector temperature was set at 280℃. The mass spectra were identified using Wiley mass spectral data base and the National Institute of Standards and Technology (NIST) library.

**Formulation Calculations**

Chlorpyrifos content, percent m/m = (W1 X A2 X P) / (W2 X A1)

Where A1 = peak area of chlorpyrifos in the chromatogram of standard solution. A2 = peak area of chlorpyrifos in the chromatogram of sample solution. W1 = mass in g of standard chlorpyrifos in standard solution. W2 = mass in g of sample taken for test. P = percent purity of chlorpyrifos standard. Sulfotep content was determined according to CIPAC Handbook 1C, 1985.

**Method of Validation for Residue determination:**

Chlorpyrifos was analysed in alfalfa fodder, milk and diarrhoeic material matrix by the QuEChERS and GC-MSMS. Linearity, limit of detection were determined according to guidelines SANCO/12571/2013 (European Commission, 2013 and European Commission, 2018). Limit of detection was estimated at three successive injections of dilute solution to the lowest concentration that resulted in the S/N ratio. Relative standard deviation was determined to entrance precision.

$$\% \text{ RSD} = 100 \text{ X } \frac{\sigma}{x}$$

σ is the standard deviation of replicates, the mean value of the replicates and %RSD is the relative standard deviation percentage.

The accuracy was calculated as recoveries of replicates. Values between 70% and 120% were believed satisfactory. Recoveries were calculated:

$$\% \text{ R} = 100 \text{ X } \frac{x}{\mu}$$

% R is the percentage recovery, X is the experimental concentration of the analyte (mg/kg), µ is the calculated concentration of the analyte (mg/kg).

**Data analysis:**

Data obtained were statistically analyzed using repeated measures for calculation of means and standard error (IBM-SPSS Version 20, 2011).

**Microbiological counts**

**Puppo, et al. (2002)** mentioned that total viable counts were determined according to the 'most probable number' procedure. The rumen samples were immediately treated with a blender-homogenizer and gassed with $CO_2$ (Stomacher, Seward Medical Ltd and UK) to detach bacterial cells from food particles.

In 'brain heart infusion' liquid medium must be incubated at 39ºC for 5 days. The anaerobic technique was used in combination with an anaerobic glove-box (atmosphere: 0.95 $CO_2$ – 0.05 $H_2$).

**Bacteriological examination**

Samples were submitted for isolation and identification of different bacteria (**Quinn et al. 2002; Abera et al. 2010)** by plating on the following plates (Oxoid): Sheep blood agar, MacConkey agar mannitol salt agar, Staph-Strept, media, Aloa agar, XLD, CN Pseudomoas specific media. Morphological characterization of the colonies, the effectiveness of hemolysis on sheep blood agar, microscopic morphology evaluation on Gram Stained samples, and biochemical characterization by oxidase test, catalase test, and Staphtect (Oxoid) and API 20 E biochemical test profile (BioMérieux) was used

**RESULTS**

The results of analysis of the chlorpyrifos formulation 480 g/L EC (declared content) un-

der study showed that the content of chlorpyrifos in the sample is 214.15 g/L.

**Tables (2 & 3)** show the tolerance limit and the measurement concentration of chlorpyrifos and its impurity sulfotep respectively. Tolerance of chlorpyrifos active ingredient was between 5% and 10% should be within 456 to 504 g/L in the sample. (FAO chlorpyrifos specifications, 2020). While, tolerance Limit of chlorpyrifos active ingredient should be within 456 to 504 g/L in the studied sample. (FAO chlorpyrifos specifications, 2020). The content of the relevant impurity sulfotep was estimated as 0.269 % of chlorpyrifos content.

**Table (2)** Tolerance limit of the declared content of chlorpyrifos (FAO-2020).

| Tolerance | Declared content in g/kg or g/l at 20 ± 2°C |
|---|---|
| ± 10% of the declared content | Up to 100 |
| ± 6% of the declared content | above 100 up to 250 |
| ± 5% of the declared content | above 250 up to 500 |
| | Note in each range the upper limit is included |

**Table (3)** Measured Concentration of Chlorpyrifos content and its relevant impurity sulfotep in the EC formulation sample.

| Sulfotep in sample formulation | | | Sulfotep Standard | Chlorpyrifos formulation sample | | | Chlorpyrifos Standard | Injection No. |
|---|---|---|---|---|---|---|---|---|
| Measured Conc. Percent ( % ) | Area of 3 repl. | FAO Max. | Area | Measured Conc. (g/L) | Area of 3 replicates | Declared content | Area | |
| 0.266 | 199.43 | 3 g/kg (0.3%) of the chlorpyrifos content | 3500.39 | 210.63 | 1002.97 | 480 g/L | 2284.43 | 1 |
| 0.268 | 200.68 | | | 213.52 | 1016.20 | | | 2 |
| 0.274 | 205.45 | | | 218.29 | 1038.89 | | | 3 |
| 0.269 | 201.85 | | | 214.15 | 1019.35 | | | Average of the 3 replicates |

Table (4) showed GC-MSMS confirmation parameters for chlorpyrifos residues in tested samples. The recovery % was ranged from 71.83% to 94.65%. RSD% was among between 1.32 - 1.45. Level of detection was 0.009 and level of quantitation was 0.01 ppm.

Table (5) demonstrated MRM of chlorpyrifos, which is 350k/z (Precursor Ion) and 97, 199 k/z (Fragments "Qualifier and quantifier" ions). Table (6) showed slope, intercept and standard error in slope.

Table (7) showed residues of chlorpyrifos in milk, diarrheic materials and grass samples from in front of the animal. Average residues of chlorpyrifos in 3 milk samples was 2.6 ± 0.24 ppm. While, in diarrheic material samples the residue of 4 samples was 19.8 ± 3.5 ppb. Otherwise, in alfalfa fodder chlorpyrifos residues in 4 samples was 37.4 ± 3.5 ppm.

**Table (4)** GC-MSMS confirmation parameters for chlorpyrifos residues in tested samples.

| LOQ (ppm) | LOD (ppm) | RSD% | Recovery % | Parameter |
|---|---|---|---|---|
| 0.01 | 0.009 | 1.44 | 92.34 | Milk |
| 0.01 | 0.009 | 1.32 | 71.83 | Diarrheic materials |
| 0.01 | 0.009 | 1.45 | 94.65 | Grass (alfalfa fodder) |

LOD: limit of determination.
LOQ: limit of quantitation.

**Table (5)** GC-MSMS confirmation parameters for chlorpyrifos.

| Fragment (Qualifier and quantifier) ions | Retention Time (min) | Linearity range (mg/mL) | Precursor Ion |
|---|---|---|---|
| 97, 199 | 20.096 | 0.01-1.0 | 350 |

**Table (6)** Accuracy determination using the correlation coefficient of spiked samples at different concentrations with uncertainties parameter (slope, intercept and standard error in slope).

| SES | Intercept | R | Slope | Concentrations (ppb) |
|---|---|---|---|---|
| 0.030 | –0.19779 | 1 | 0.9999 | 0.1, 5, 10, 25, 50, 150 |

r = correlation coefficient SES = Standard error in slope

**Table (7)** Residues of chlorpyrifos in milk (n = 3), diarrheic materials and grass samples (n=4) in front of animal.

| Grass (alfalfa fodder) (ppm) | Diarrheic materials (ppb) | Milk (ppm) | Parameter |
|---|---|---|---|
| 37.4 ± 3.5 | 80.8 ± 3.5 | 2.6 ± 0.24 | Residue |
| 5** | -- | 0.02 *, ** | MRL |

MRL = Maximum Residue Limit
* Codex Alimentarius Commission (FAO/WHO, 2018), is the central part of joint FAO/WHO Food Standards Programme
** European Union (EU) (2015(, Chlorpyrifos, Regulation (EU) 2015/399, Pesticide residue(s) and maximum residue levels (mg/kg).

**Table (8)** Bacterial isolation from three buffalo's cases and their calves suffering from symptoms of toxicity by chlorpyrifos

| **No of buffaloes** | **Bacterial isolates from samples** | | | | **Total isolates** |
|---|---|---|---|---|---|
| | **Milk** | **Saliva** | **Nasal** | **fecal** | |
| **Case 1** | *E.coli* | - ve | - ve | *Staph. aureus* | 2 |
| **Case 2** | -ve | - ve | - ve | *E. coli and Coliform* | 2 |
| **Case 3** | -ve | -ve | -ve | *E. coli* | 1 |
| **Case 4 calve** | NA | -ve | -ve | *Klebsiella pneumonia and E. coli* | 2 |
| **Case 5 calve** | NA | -ve | -ve | *Staph. aureus and Coliform* | 2 |
| **Case 6 calve** | Dead .. Not Examined | | | | |
| **Total isolates** | 1 | 0 | 0 | 8 | 9 |

**Table (9)** Determination the total microflora count of rumen buffaloes after 1, 3, 5, 7 and 9 day from treatment by antidote (atropine sulphate) of chlorpyrifos.

| Total microflora counts×$10^{10}$ cfu/ml | | | | | Animal case |
|---|---|---|---|---|---|
| 9 day | 7 day | 5 day | 3 day | 1 day | |
| 9.9 | 9.1 | 8.2 | 7.1 | 6.86 | **Case 1** |
| 10 | 9.3 | 8.5 | 7.5 | 6.66 | **Case 2** |
| 9.88 | 9.5 | 8.2 | 7.5 | 6.72 | **Case 3** |
| 9.98 | 9.2 | 8.4 | 7.4 | 6.1 | **calve Case 4** |
| 9.9 | 9.1 | 8.2 | 7.3 | 6.2 | **calve Case 5** |
| Dead | Dead | Dead | Dead | 6.0 | **calve Case 6** |

N.B. Calves not survived and dead after 2 days of calving.

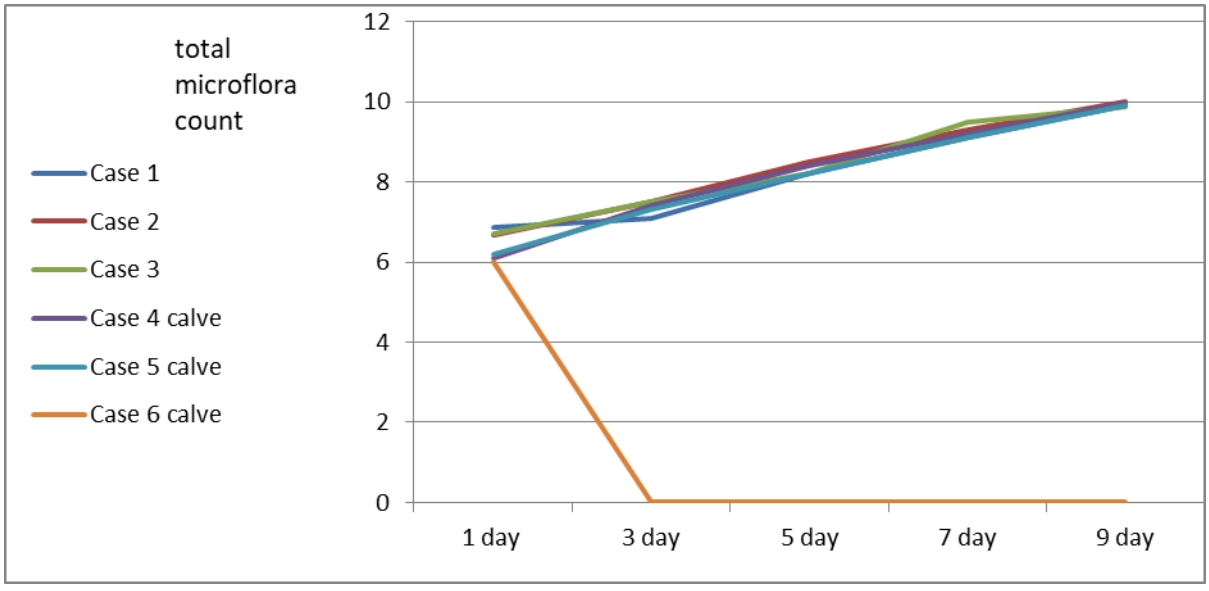



Fig. (1) Demonstration the total microflora count of rumen buffaloes after 1, 3, 5, 7 and 9 day of treatment by antidote (atropine sulphate) of chlorpyrifos

**DISCUSSION:**

Chlorpyrifos have been used as a pesticide since 1965 in agricultural purposes. Although, product adulteration in field was not recorded in inter-country commerce while these products are sold in villages and hamlets, Egypt.

Result of analysis of tested sample (chlorpyrifos formulation) was 214.15 g/L (Tables, 2 & 3). This result be different from FAO/WHO specifications which recorded that the average measured content should not differ than confirmed content above 250 up to 500 g/ L by the tolerance limit ± 5%.

The tolerance limit and the measurement concentration of chlorpyrifos and its impurity sulfotep, respectively were not-conforming to pesticide specifications. Limit of chlorpyrifos active ingredient should be within 456 to 504 g/L in the sample. (FAO chlorpyrifos specifications, 2020).

This results indicated that the used pesticide formulation in that valley is type of an illegal and counterfeit pesticide manufactured from insufficient active ingredient and contaminated with unexpected substances which proved by GC – MS MS analysis.

Samples were analyzed to determine recovery and standard deviation (RSD %) by comparing the peak area acquired from the spiked sample of known concentration with that of working mix standard solution of the same concentration. Chlorpyrifos residues in this study, recovery percent were 92.34, 71.83 and 94.65 in milk, diarrheic materials and alfalfa fodder samples, respectively. RSD% were between 1.32 - 1.45% (Table 4). These results were nearly similar to that of **Singh et al. (2012), Jeong et al. (2012) and Golge et al. (2018)** who reported high recovery% in milk and milk products.

This result is in accordance with the acceptable recovery range in **SANTE (2017)** and **European Commission (2002)** guidelines and falls within the range of 70–120% for recovery and 20% for RSD%. Level of detection and level of quantitation were 0.009 and 0.01 ppm which indicate high sensitivity of the method.

MRM of chlorpyrifos, were 350 m/z (Precursor Ion) and **97**, **199** m/z (Fragments "Qualifier and quantifier" ions). This values was nearly similar to that observed by Elham *et al.* (2016); Song *et al*. (2019).

Concerning sample residue analysis, the main concentration of chlorpyrifos in 3 milk samples was 2.6 ± 0.24 ppm. This data much higher than MRL recorded by Codex Alimentarius Commission (FAO/WHO, 2018) and European Union (2015 which was 0.02 ppm.

Most of previous data were reviewed are very higher than obtained result like those recorded by **Eman and Eman (2015)** in raw buffalo milk from Assuit city, Egypt (1.870–3.514 ppm) and **Bedi *et al*. (2015)** in bovine raw milk samples collected throughout Punjab, India (2.2 ppm). The significant differences observed in this could be regarded to the high dose which find in animal diet (alfalfa fodder) as well as the recorded adulteration in sprayed formulation.

The residue data indicate that chlorpyrifos residue levels in alfalfa fooder alfalfa fodder was 37.4 ppm. This result likely to exceed the MRL (5 ppm) recorded by European Union (2015(.

In diarrheic material samples the residue was 19.8 ppb. With observation that no data were find concerning chlorpyrifos in diarrhoeic material, this result can't compare its concentration in feed this mostly drop by affecting ruminal microflora to non-observed pesticide. Chlorpyrifos causing sever diarrhoea and animal off food so decrease fiber content on rumen and dray mater lead to low microflora in rumen very bad digestion so animal low production of milk and loss weight as photo (1). **Jiyana *et al*. (2021)** mentioned that cows able to yield high rumen total microbial count when they fed high-fibre diets.

It is observed that the total bacterial count of rumen buffaloes microflora very decrease during severe diarrhoea by toxicity of chlorpyrifos and after treatment increase to normal total count during 9 day as showed table (8).

Overall, whether it is through direct or indirect exposure to CPF, there are changes in changes in levels of selected gut bacteria, disruption of body metabolism of lipid and glucose consequently body weight **(Djekkoun et al. 2022)**.

Exposure to CPF induced by disturbance is often characterized by increase in the number of potentially pathogenic microorganisms leading to dysbiosis and that is mainly due to a decrease in the number of beneficial microorganisms (**Xia *et al*., 2018** and **Condette, 2015**). In addition, CPF has been shown to increase intestinal permeability in rats or in vitro **(Réquilé *et al.,* 2018)** inducing a bacterial translocation which corresponds to the passage of viable bacteria of the gastrointestinal flora through the barrier of the intestinal mucosa.

Table (9) and fig. (1) showed the Nine bacterial isolates (4 *E. coli,* 2 *Staph. aureus,* 2 *Coliform* and 1 *Klebsiella pneumonia*) from Milk, Saliva, Nasal and faecal samples of 3 buffaloes and 3 buffalo's calves suffering from severe diarrhoea and symptoms of toxicity by chlorpyrifos. This agree with **Djekkoun et *al.* (2022)** who found that CPF exposure was associated with significant microbial perturbation, showing the influence of CPF exposure on a number of bacteria, with reduced abundance of *Lactobacillus* spp. and *Bifidobacterium* spp., and a higher level of *Enterococcus* spp., *E. coli*, *Staphylococcus* spp. and *Clostridium* spp. in rats treated with CPF. Similar results were shown in previous work and other studies **(Condette et al. 2015** and **Zhao et al. 2016)**.

In Egypt and throughout the world cattle diarrhoea is a major problem in livestock production (**Farid et al. 2001** and **Ibrahim, 2007**). Significant economic losses in Egypt was recorded due to Enteritis which lead to high morbidity and mortality in newborn calves **(Ashraf, 2007**). So we have to improves the health and welfare without exposure animals for stress However, in some cases these measures may not be enough.

In conclusion, the results demonstrate that these substances might lead to intoxication in animals and raise their residues in studied body fluids. The exposure to chlorpyrifos induces disturbance in the microflora with stimulate pathogenic bacteria causing enteritis. This study sheds light on the importance of monitoring uses of pesticides in villages and hamlets in rural areas in Egypt. Taking into account that chlorpyrifos is not recommended for use in alfalfa crop by Agricultural Pesticides Registration Committee in Egypt, the behaviour of its application in agricultural crops should be reviewed.

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