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INFLUENCE OF SOLAR SIMULATOR, GAMMA IRRADIATION AND LASER RAYS ON THE GROWTH AND AFLATOXIN PRODUCTION OF *ASPERGILLUS FLAVUS* AND *ASPERGILLUS PARASITICUS*

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ABSTRACT

One hundred samples of processed animal feeds, 50 milk samples from cases of mastitis and 30 vaginal swabs from cases of abortion in sheep and cattle were collected from farms at Giza Governorates. The samples were collected from diseased animals showing different clinical manifestation including diarrhea and pneumonia in calves, cattle and sheep; mastitis and some cases of abortion. The mycological examination of these samples revealed the isolation of fungi belonging to 7 genera of moulds and 2 genera of yeasts. The rates of isolation of ***Aspergillus flavus*** from animal feeds, mastitic milk and vaginal swabs were (80%, 50% and 50%), respectively, while the rates of isolation for ***Aspergillus parasiticus*** *were* (35%, 24% and 10%), respectively. Aflatoxins were detected in 60% and 40% of feed and mastitic milk, with the mean levels of (110±3.5 and 10±0.2 ppb), respectively. The isolated strains of ***A. flavus*** and ***A. parasiticus*** were screened for AFB_1 production before and after exposure to doses of gamma radiation and photodynamic inactivation for evaluation of their effect on fungal growth and toxin production. The doses 4 kg of gamma radiation were effective to prevent spore germination and mycelium growth of both *A.* ***parasiticus and A. flavus***. Whereas, AFB_1 production was inhibited at a dose of 2 and 3kGy, respectively. Whenever, the rays of solar simulator and light emitting diodes (LED) in the presence of phloxine B as photosensitizer caused complete inhibition of mycelium growth and AFB_1 production at a dose level of 2.0 mg% phloxine B in case of solar simulator. On the other hand, the application of LED resulted in complete inhibition of mycelium growth and AFB_1 production at a dose level of 1 and 2 mg% phloxine B. The economical and health significance of the present results were fully discussed.

Key words: Solar simulator; gamma irradiation; laser rays; AFB1; A. flavus

INTRODUCTION

Mycotoxicosis due to environmental pollution of feeds and water by toxigenic fungi constitute a serious animal health and public health hazards. The most important mycotoxigenic fungi are those producing aflatoxins, which received greater attention than any of the other mycotoxins because of their demonstrated carcinogenic effects in susceptible animals and their acute toxigenic effects in human and also they are unique in being resistant to degradation under normal food processing conditions **(Ciegler and Vesonder, 1983)**. The correlation between the environmental factors, mycosis and mycotoxicosis in animals and its role in initiation of food born infections had been reported by **Hassan**, (**2003); Hassan and Mogeda 2003, 2004, 2007, 2008 and 2009; Abo-Al-Yazeed et al. (2008)** .Several different physical and chemical approaches have been tried to detoxify mycotoxins from l feeds.

The adverse side effects of chemical compound direct the searches to find other safe natural products to control mycotoxicosis (**Hassan, 2003; Hassan et al., 2007**; **Sayed El Ahl et al., 2006*)***. This makes the selection of proper decontamination methods that will effectively decompose aflatoxins, while retaining the nutritive quality and palatability of the treated food a continuous challenge. One of possible approach is to use photodynamic therapy (PDT), which is a novel and promising biophotonic technology. Photodynamic therapy is an entirely new modality and its development can likened to that of the discovery of antibiotics **(McCaughan, 1999)**. It is important to make an effort to develop safe and practical detoxification methods using different radiation types, as gamma rays, laser and solar simulator. So, the aim of the present work was to study the effect of gamma irradiation and photodynamic inactivation (PDI) on fungal growth, aflatoxin B_1 production of *Aspergillus flavus* and *Aspergillus parasiticus*.

MATERIAL AND METHODS

Materials

Samples One hundred samples of processed animal feeds, 50 milk samples from cases of mastitis in sheep and cattle and 30 vaginal swabs from cases of abortion were collected from farms at Giza Governorates in

which diseased animals suffering from different clinical manifestations including diarrhea and pneumonia in calves, cattle and sheep; mastitis and some cases of abortion.. The collected samples were transported to laboratory of Animal Health Research Institute in clean sterile plastic bags .The samples were examined mycologically and the recovered isolates of *A.flavus* and *A. parasiticus* were used in this study.

Standard aflatoxins: Standards of AFB_1, B_2, G_1 and G_2 were purchased from Sigma (USA).

Photo sensitizer: Phloxine B (D & C No. 28) photo sensitizer with absorption spectrum 537 nm was purchased from Sigma (USA).

Source of Gamma radiation: Cobalt 60 gamma cell (Gamma chamber 4000A) located at the National Center for Radiation Research and Technology (NCRRT), Naser city, Cairo, Egypt

ORIEL Solar simulator: Located at National Institute of Laser Enhanced Science, Cairo University, Egypt (NILES) was used for artificial light exposure**.**

Green light emitting diodes (LED): Located at NILES, Cairo University, Egypt, the wave length used in this study was 530 nm and exposure time was 15 min.

Methods

Isolation and identification of moulds

Each sample of feed, milk and vaginal swabs was subjected for isolation and identification of fungi according to **Conner et al. (1992).**

Production and estimation of aflatoxins (Gabal et al., 1994)

The isolated fungi (*A.flavus and A. parasiticus)* were inoculated into flasks containing 50 ml of sterile yeast extract solution (2%) containing 20% sucrose (YES). Inoculated flasks were incubated at 25ºC for 10-15 days. At the end of the incubation period**,** extraction and detection of produced aflatoxins was estimated by fluorometric method as recommended by **(Hansen, 1993).**

Detection of aflatoxin B1 in animal and poultry feeds

Twenty five grams of the ground feed samples were subjected for extraction and purification of toxins using immunoaffinity column and

quantitatively estimated by fluorometric method according to **AOAC, (1990) and Hansen (1993**).

Evaluation of the effect of different types of radiation on growth and aflatoxin B_1 production of *A. flavus* and *A. parasiticus* :

Preparation of spore suspension of *A. flavus* and *A. parasiticus*:

Aflatoxin $\mathbf{B_1}$ producer strains of *A. flavus* and *A. parasiticus* were grown on potato dextrose agar slants for 10 days at 28ºC. Spores were harvested in sterile 0.1% Tween 80 solution, filtered through four layers of sterile gauze. Collected spores centrifuged at 3000 x g for 5 minutes, washed three times with sterilized distilled water and then re-suspended in sterilized tween 80 solutions. The number of spores was estimated by haemocytometer and the suspension was adjusted to contain approximately 10^6 spores/ ml.

Effects of gamma radiation and PDI using solar simulator or LED on aflatoxin B1 production and mycelium dry weight

The prepared spore suspensions (10^6spores/ml) were distributed into several sterile test tubes. Each containing 5 ml and then irradiated in a CO60 irradiator at a dose level of 0, 1, 2, 3 and 4 KGy. Whereas, in case of PDI using solar simulator or LED, 100 µl of the prepared spore suspension were mixed in tissue plates with 100 µl of different concentration of phloxine B photosensitizer (0 mg%, 0.5 mg%, 1 mg% and 2.0 mg%) **(Shahin and Aziz, 1997; Aziz and Youssef, 2002 ; Abou Srea , 2005):.**Then incubated for 3 hours in dark. After that, the content of the plates were irradiated while mixing with magnetic stirrer (to obtain homogenous distribution of light) using solar simulator at fluency rate of 400 W/m^2 or at 530 nm wave length for 15 min in case of LED. Three wells of each concentration of photosensitized spore suspension remained without irradiations and were considered as negative control **(Abou Srea, 2005)**.

The non-irradiated and irradiated spores suspension (200µl) were inoculated into 100 ml Erlenmeyer flasks having 25 ml of sterile yeast extract sucrose (2% yeast extract and 15% sucrose) and supplemented with 0.019% P-cresol. Inoculated flasks were incubated in the dark for 20 days. At the end of incubation period, YES medium was filtered through

a Buchner funnel fitted with pre-weighted Whattman number 1 filter paper. The mycelium was washed with 10 ml distilled water and then the filter paper with the mycelium was dried at 100ºC for 48 hrs, dried in desiccators and weighed. The aflatoxin B_1 concentration of the culture filtrate was determined as described before.

Statistical analysis

The obtained date were computerized and analyzed for significance.. Calculation of standard error and variance according to **SPSS 14 (2006**).

RESULTS AND DISCUSSION

In the last decades the mycologists and epidemiologists throw the light on mycotoxins elaborated by wide variety of fungal species during their growth and it's contamination of feeds. This directs the attention of researches towards studying various methods to detect, prevent and control mould growth and mycotoxin production in feeds **(Dalcero et al., 1997; Hassan, 1998, 2003; Hassan et al., 2004, 2007, 2009; Abo- Al-Yazeed et al., 2008)**. The collected processed animal feed samples from farms suffering from cases of diarrhea and pneumonia in calves and mastitis in cattle and sheep were screened for fungal contamination and detection of aflatoxin B_1.

The results revealed the isolation of fungi belonging to 7 genera of moulds and 2 genera of yeasts from feeds. The most predominant rate of isolation of moulds were belonged to genus Aspergillus *(85%),* particularly*,* *A. flavus* (80 %) and A. *parasiticus (35%).* Followed by *Penicillium (76%), Mucor (60%), Rhizopus (24%) and Cladosporium (20%*). The yeast cultures were identified as *Candida albicans* (6 %) and *Rhodotorula* species (20%) (Table1).

On the other hand, the isolated fungi from vaginal swabs of aborted animals and milk of mastitic animals yielded nearly the same orders of frequency, where, members of *Aspergillus species* were also at the top incidence of other isolated moulds (60% in both). Moreover, *C.albicanse and Rhodotorula sp*. were recovered at a rate of (66.6%) and (40%) from

samples of vaginal swabs of aborted animals and (73.8%) and (50%) from milk samples of mastitic animals, respectively. These differences in the level of contamination may be due to the exposure of the examined samples to different climatic condition either during preparation or transportation or storage. The yeast growth required more moisture content in the surrounding environment, so their incidence in samples of vaginal swabs and milk were relatively higher than in case of feed samples.

Table (1): Prevalence rate of fungi in feeds, milk and vaginal swabs collected from diseased animals suffering from different clinical manifestations.

Fungal species	Incidence of fungi in samples o:f					
	Vaginal swabs of aborted animals (30)		Milk of mastitic animals (50)		Processed animal feeds (100)	
	No. of (+ve)	%	No. of (+ve)	%	No. of (+ve)	%
Aspergillus(A) species:	18	60	30	60	85	85
1- A.flavus	15	50	25	50	80	80
2-A. parasiticus	3	10	12	24	35	35
3- A. niger	20	66.6	8	16	15	15
4- A.fumigatus	10	33.3	9	18	20	20
5- A.ochraceus	8	26.6	10	20	8	8
Penicillim spp.	9	30	9	18	76	76
Fusarium spp.	4	13.3	5	10	8	8
Mucor spp.	6	20	15	30	60	60
Rhizopus spp.	3	10	10	20	24	24
Cladosporium spp.	1	3.3	6	12	20	20
Alternaria spp.	1	3.3	4	8	0	0
Scopulariopsis spp.	2	6.6	2	4	20	20
Candida albicans	20	66.6	22	73.8	6	6
Rhodotorula spp.	12	40	15	50	20	20

***** % : Were calculated according to the No. of examined examined samples.

Aflatoxin B_1 received greater attention than other mycotoxins because of its demonstrable carcinogenic effect in susceptible animals and its acute toxic effect in human **(Wogan, 1973; Bressac et al., 1991).**Therefore**,** the samples of processed feeds (50) and milk (25) of mastitic animals were evaluated for aflatoxin B_1 contamination. The results showed that AFB_1 was detected in 30 samples of feeds (60%) with the maximum level of (1800 ppb) and minimum level of (15 ppb) with a mean level of (110±3.5). Whereas, in the samples of milk of mastitic animals AFB_1 was detected in 10 samples (40%) with the maximum level of (15 ppb) and minimum level of (3 ppb) with a mean level of (10±0.2)(Table, 2). Whenever, the maximal level allowed by Food and Drug Administration (FDA) is (20 ppb) for all feeds and foods and (0.5 ppb) for fluid milk **(Schuller et al., 1983).** The detected levels of AFB1 in the present work were significantly hazard for human and animal health.

Table (2): Detection of Aflatoxin B1 in samples of feed and mastitic milk samples.

Examined samples	Prevalence of aflatoxins in examined samples(PPB)				
	Incidence		Levels of aflatoxin B_1 in +ve samples		
	No. of +ve	%	Max.	Min.	Mean± SE
Processed animal feeds (50)	30	60	1800	15	110±3.5
Mastitic milk (25)	10	40	15	3	10±0.2

On the other hand, the isolates of *A.flavus (100) and A. parasiticus (50)* that recovered from present samples were screened for AFB1 production on synthetic medium of YES . The obtained results yielded that(70%) of *A.flavus and*(40%) of *A. parasiticus* produced significant levels of toxin with a maximum of (235 and 210 ppb), minimum levels of (30 and 10 ppb) with the mean levels of (190± 4.2 and 115±0.5), respectively. These mycotxoins residues in food and feed causes carcinogenic, teratogenic, haemorrhagic and immunosuppression effect to human and

animal health **(Hassan, 1998, 2003; Hassan et al., 2004, 2007, 2008, 2009; Sayed El Ahl et al., 2006).**

Table (3): Aflatoxin B_1 production by isolated strains of *A. flavus* and *A. parasiticus*. On synthetic medium .

Tested isolates	Amount of $AFB_{1\ ug}$ / l of YES broth				
	Incidence		Levels of aflatoxins (ug/l of YES broth)		
	No. of +ve	%	Max.	Min.	Mean± SE
A.flavus (100)	70	70	235	30	190± 4.2
A.parasiticus (50)	10	40	210	10	115±0.5

It is suggested that in all countries especially developing ones, the animal diseases increased due to the increased consumption of contaminated feed with fungal organisms and their toxins **(Hassan et al., 2004, 2007, 2008, 2009; Sayed El Ahl et al., 2006)**. Therefore, the diverse action and diseases resulted from fungal and mycotoxin contamination enforced the continuous trials of scientists to find out a new and safe method for their control. **.**The updated important methods include the application of rays such as gamma radiation to inhibit the growth of toxigenic strains of *A. flavus* and *A. parasiticus* and AFB_1 production in synthetic medium. The obtained results in table (4) revealed that the mycelium dry weight (g/l) for both *A. flavus* and *A. parasiticus* decreased by increasing the dose of gamma radiation and complete inhibition of fungal growth occurred at a dose of 4.0 kGy. Whereas, AFB_1 production decreased by increasing the dose of gamma radiation. The toxin could not be detected at 2 kGy in case of *A. parasiticus* and at 3 kGy in case of *A. flavus*.

The same findings were observed by **Hassan (1994); Refai et al. (1996); Hassan and Aziz (1998); Aziz and Mahrous (2004)** who studied the effect of gamma irradiation on the viability and production of aflatoxin by *Aspergillus flavus* in feed , field- dried hay and green stuff.

There was a good relationship between mycelium dry weight and the concentration of AFB_1 which significantly decreased from (235.0±3.5 µg/l) at 0.0 KGy to (110.3±4.4 µg/l) at 1 KGy and to (15±0.8 µg/l) at 2 KGy, whereas the toxin could not be detected at 3 KGy in case of *A. flavus*. While in case of *A. parasiticus* the concentration of AFB_1 significantly decreased from (210.0 ± 1.8 µg/l) at 0.0 KGy to (44.8 ± 0.5 µg/1) at 1 KGy, whereas the toxin could not be detected at 2 KGy by **Chang and Markis (1982)** who reported that increasing the radiation dose in the range of 0.0 to 4.0 KGy resulted in decreasing aflatoxin formation in barely. Also, **El- Hadi (1986)**; **Hassanien (1987); Hassan and Aziz (1998)** showed that exposure of *A. flavus* to low doses of gamma rays 2.0 KGy resulted in decreased fungal growth and aflatoxin production and complete inhibition occurred at a dose level of 3 KGy.

Table (4): Effect of gamma radiation on growth of toxigenic strains of *A. flavus* and *A. parasiticus* and AFB_1 production in synthetic medium.

Radiation doses kGy	*Aspergillus flavus*		*Aspergillus parasiticus*	
	Mycelium dry weight (g/l of YES broth)	Aflatoxin B1 concentration (ug/l of YES broth)	Mycelium dry weight (g/l of YES broth l)	Aflatoxin B1 centration (ug/l of YES broth)
Before radiation	200.3 ± 0.69	235.0 ±3.5	220.2 ± 1.5	210.0 ± 1.8
1	106.00 ± 0.99	110.3 ± 4.4	120.00 ± 0.95	44.8 ± 0.5
2	80.7 ± 0.20	15.00 ± 0.8	54.2 ± 0.60	0.0
3	30.83 ± 0.48	0.0	30.0 ± 0.5	0.0.
4	0.0.	0.0	0.0	0.0

Table (5) Photodynamic effect of phoxine B on growth toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* and AFB_1 production after exposure to solar simulator.

Conc. of dye (%)	*A. flavus*		*A. Parasiticus*	
	Mycelium dry weight (g/l of YES broth)	Aflatoxin B1 concentration (ug/l of YES broth)	Mycelium dry weight (g/l of YES broth	Aflatoxin B1 concentration (ug/l of YES broth)
Before add of dye.	200.3 ± 0.69	235.0 ±3.5	220.2 ± 1.5	210.0 ± 1.8
0.5	112..0± 3.00	80.83± 1.96	130.3± 3.7	98.6± 4.7
1	60.3± 1.93	27.33± 1.88	96.0± 2.92	20.2± 2.1
2.0	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00

Table (6): Photodynamic effect of Phloxine B on growth of toxigenic strains of *A. flavus* and *A. parasitcus* and aflatoxin B_1 production after exposure to LED.

Conc. of dye (mg%)	*Aspsegillus flavus*		*Aspsegillus parasiticus*	
	mycelium dry weight (g/l of YES broth)	Aflatoxin B_1 concentration (µg/l of YES broth)	mycelium dry weight (g/l of YES broth)	Aflatoxin B_1 concentration (µg/l of YES broth)
Before add of dye.	200.3 ± 0.69	235.0 ±3.5	220.2 ± 1.5	210.0 ± 1.8
0.10	148.00 ± 2.00	132.7 ±3.5	167.57 ± 2.62	125.3 ± 2.35
0.50	55.00 ± 1.6	65.2 ± 2.3	130.40 ± 3.10	70.10 ± 1.50
1.00	0.0.	0.0	50.00. ± 2.32	30.2± 1.10
2.0	0.0	0.0	0.0	0.0

On the other hand, the evaluation of photodynamic effect of phloxine B on growth of *A. flavus* and *A. parasiticus* and AFB_1 production after exposure to solar simulator, reported that the mycelium dry weight (g/l) for both *A. flavus* and *A. parasiticus* decreased by increasing the concentration of phloxine B. At a concentration of (0.5 and 1 mg %) the mycelium dry weight was decreased to (112.0±3.00 g/l) and (60.3±1.93 g/l) for *A. flavus* and decreased to (130.3±3.7 g/1) and (96.0±2.92 g/1) for A. *parasiticus*, respectively. The complete inhibition of fungal growth occurred at a concentration of (2.0 mg %) phloxine B. AFB1 production decreased by increasing the concentration of phloxine B, at (1 mg%) concentration the AFB1 production was (27.33 µg/l) and (25.27 µg/l)for *A. flavus* and *A. parasiticus*, respectively. Whereas the AFB1 could not be detected at (2.0 mg%) phloxine B concentration for both *A. flavus* and *A. parasiticus* (Table, 4).However, photodynamic affect of phloxine B on growth of toxigenic strains of *A. flavus* and *A. parasiticus* and AFB_1 production after exposure to LED was studied.

The results revealed that the mycelium dry weight (g/l) and AFB_1 (µg/l) production for both *A. flavus* and *A. parasiticus* decreased by increasing the concentration of phloxine B. At a concentration of (0.5 and 1 mg%) the mycelium dry weight was decreased to (55.00 ± 1.6 g/l) and (50.00. ± 2.32 g/l) for *A. flavus*,and A.parasiticus, respectively. Whereas, complete inhibition of fungal growth occurred at a concentration of (1 and 2.0 mg%) phloxine B respectively. Also, AFB_1 production decreased by increasing the concentration of phloxine B, at (0.1 mg%) concentration. The AFB_1 production was (132.7 ±3.5 µg/l) and (125.3 ± 2.35 µg/l) for *A. flavus* and *A. parasiticus*, respectively.

Whereas, the AFB_1 could not be detected at (2.0 mg%) phloxine B concentration for both *A. flavus* and *A. parasiticus* (Table, 5).These findings agree with the finding of **Friedberg et al. (2001)** who tested the fungicidal activity of the photosensitizers Green 2 w activated with 630 nm light against *A. fumigatus*. He found that the fungicidal activity of dye was both inoculums and light dose dependent. However **El- Adly (2002)** tested the photodynamic inactivation of seven isolates of dermatoplytes by different concentration of hematopropryin derivatives (HPD), methylene blue (MB) and toluidine blue O (TBO) after exposure to either solar simulator or natural sunlight.

The result showed significant growth inhibition when the solar simulator light was applied at rate of (400 w/m^2). **Luksiene et al. (2005)** found that there is a clear correlation between the efficiency of inhibition of germination and the amount of photosensitizer accumulated by the fungus (dose dependent).The decrease in AFB_1 production may be due to either the fungicidal activity of phloxine B, which resulted in decreasing of fungal growth (mycelium weight) as there was a good correlation between mycelium weight and AFB_1 production or direct photo dynamic inactivation of AFB_1 by phloxine B or both. Also, **Wilson and Mia (1993); Ouf and Abd Elhady (1999)** found that incubation of *C. albicans* with toluidine blue or methylene blue as photosensitizer was necessary to render it susceptible to killing by laser light.

Also, **Abou Srea (2005)** found that He- Ne laser induced effects ranged from death of tested fungi to partial inhibition depending on the dye (crystal violet) concentration and the fungus under the test. The differences in responses to laser irradiation is usually attributed to specific pigmentation of irradiated propagates **(Antibus, 1989).** Therefore, it is believed that the difference in susceptibility of tested *Aspergillus* may be attributed to difference in melanin content, which may act as endogenous photosensitizer. This assumption is coupled with suggestion of **(Daub et al. (1995)** who stated that the difference in susceptibility of tested dermatophytes to photosensitization processes may be attributed to specific dark pigmentation, which may act as endogenous photosensitizer.

CONCLUSION

The presence of fungi and their toxins in feed and food reflected unhygienic measures during cultivation, irrigation harvesting transportation, handling, storage and processing of feed and food. Therefore, frequent testing programs of food during different stages of production must be monitored before given to animals or human for consumption. The fungal inhibitors may be added if the level of contamination over the limited level. Therefore, continuous investigations for finding new safe methods for controlling the growth of fungi and mycotoxins production are critical demand. The different methods of radiations particularly photodynamic inactivation is more applicable

method at large scale for degradation of AFB1 and control of fungal growth especially with solar simulator. All ways for increasing the quality of human health and animal's wealth.

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تاثير اشعة الشمس واشعة جاما واشعة الليزر على نمو فطريات الاسبرجيللس فلافس والاسبرجيللس باراسيتكس وافرازها سموم الافلاتوكسين.

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قد اشتملت هذه الدراسة على مائة (100) عينة من علائق الحيوان المصنعة و50عينة لبن من حالات التهاب الضرع و30عينة مسحات مهبلية من حالات اجهاض الاغنام والابقار وقد جُمعت هذه العينات من مزارع بمحافظة الجيزة من حيوانات مريضة تعانى من اعراض مرضية مختلفة تشمل الاسهال والاتهاب الرئوى فى العجول والابقار والاغنام والتهاب الضرع وبعض حالات الاجهاض .

وبإجراء الفحص الفط رى لهذه العينات افادت النتائج بعزل فطريات تنتمى ل 7 اجناس من الاعفان وجنسين من الخمائر. وكان معدل عزل الاسبرجللس فلافس من اغذية الحيوانات والبان التهاب الضرع والمسحات المهبلية (٨٠%و٥٠%و٥٠%) على التوالى بينما كان معدل عزل الاسبرجللس باراستيكس (35% و24% و١٠ %) على التوالى.

وقد أكتشفت سموم الافلاتوكسين فى 60% و40% من عينات العلائق والبان التهاب الضرع بمتوسط (110±3.5 و10±0.2 جزء فى البليون) على التوالى. وقد تم در اسة تاثير تعرض العترات المعزولة من الاسبرجللس فلافس والاسبرجللس باراسيتيكس وافراز سموم الافلاتوكسين ب$_1$ قبل وبعد التعرض لجرعات اشعة جاما والحث الضوئى لتقييم تأثيرها على نموالفطريات وانتاج السموم . وقد اظهرت النتائج ان الجرعة 4 كيلوجراى من اشعة جاما كانت فعالة لمنح استنبات الجراثيم الفطرية والنمو الفطرى لكلا من الاسبرجيللس باراستيكس والاسبرجيللس فلافس . حيث هبط انتاج سم الافلاتوكسين ب 1 عند الجرعات 2-3 كيلوجراى على التوالى. بينما كان استخدام ضوء يحاكى اشعة الشمس وضوء يحاكى اشعة الليزر فى وجود مستحث ضوئى فلوكسين ب يؤدى الى تثبيط كامل لنمو الفطريات وانتاج سم الافلاتوكسين ب 1 عند جرعة 2 مجم % فلوكسين ب فى حالة اشعة الشمس . على الجانب الاخرقد اتضح ان استخدام الليزر نتج عنه تثبيط كامل لنمو الفطريات وانتاج الافلاتوكسين ب 1عند جرعات 1-2 مجم % فلوكسين ب . وقد نوقشت الاهمية الاقتصادية والصحية للنتائج الحالية.

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LONG-TERM EFFECTS OF USING VARIOUS ENRICHMENT OBJECTS ON MULTIPLE MEASURES OF WELFARE IN SINGLY-HOUSED RATS

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ABSTRACT

Single housing of laboratory rat may be recommended in some situations such as toxicological and nutritional studies and also to prevent the spread of infectious diseases. However, as single housing of laboratory rat has been shown to be stressful, modification of the housing environment are needed to improve the welfare of these animals. The aim of this experiment was to investigate how long-term enrichment of laboratory cages of singly-housed rats using multiple physical items affects various measures of welfare such as behavioural, weight changes and the weight of internal organs. 24 rats were housed singly in either enriched or unenriched cages. Behaviour was sampled every week and so was body weight and weight gain over a six week experimental period. Behaviours of the rats in the elevated plus-maze were recorded in the seventh week whereas, weight of internal organs were recorded post-mortem. Long-term single housing of rats in super-enriched cages increased levels of indicators of good welfare including sleep, exploration and feeding behaviour, body weights, weight gains and the relative weights of thymus gland and spleen, and decreased levels of indicators of poor welfare such as stationary behaviour and relative weight of adrenal glands. Thus, enriching conventional cages of singly-housed rats with multiple physical structures appeared to improve their ability to control the environment and to promote their species-specific behaviour; potentials that can ultimately result in good welfare.

Key words: Laboratory Rats, Multiple Enrichment, Single Housing, Welfare.

INTRODACTION

Laboratory rodents spend a major proportion of their life span in the laboratory cage, and therefore improving this environment may not only improve their overall well-being **(Rodent Refinement Working Party, 1998)** by improving their ability to cope with the environment, but also

the accuracy of experimental results **(Sherwin, 2004)**. This is in turn likely to provide a valid animal model for research **(Poole, 1997)** and can ultimately result in a reduction in the number of animals used.

Environmental enrichment defined as "the modifications of the environment resulting in an improvement in the biological functioning of the captive animals **(Newberry, 1995)** is an important tool of improving housing conditions of laboratory rodents. Experiments have demonstrated wide beneficial effects of environmental enrichment on group-housed laboratory rodents **(Chamove, 1989; Tsai et al., 2003).** However, despite this consensus over the effects of environmental enrichment in rats, very few studies have considered how enriching laboratory cages of singly-housed rats by adding multiple physical structures may affect their welfare.

Although group housing is the recommended housing situation for laboratory rats (Patterson-Kane et al., 2002), it may not, under certain circumstances, be achievable. For example, in nutritional (metabolism and digestibility) and toxicological studies in which researchers need to know how much animals eat, metabolise and excrete, single housing of the subjects may be necessary. Furthermore, it is sometimes the case that social housing could escalate aggression to the extent that injuries or wounds may occur, and that in turn makes the full time social housing of the injured individuals ubiquitously unimplemented. For large animals such as farm animals, primates and zoo animals, to prevent the spread of infectious disease single housing is also recommended. Moreover, some animals are normally solitary and can only be housed singly, such as laboratory hamsters. It could therefore be interesting to look at the effect of enriching housing conditions for singly-housed animals.

There have already been some studies that looked at the effect of environmental enrichment on some behaviours of singly-housed rat such as exploratory behaviour and general activity (**Denny, 1975)**, on the interest of the rats towards enrichment items **(Townsend, 1997)** and also on the development of their brains in the enriched environment **(Bennett et al., 1969).** However, the fact that none of these studies looked at how long-term enrichment can affect the ability of the singly-housed animals

to cope with their environment by looking at their behavioural, physiological, pathological and psychological responses to the housing condition highlights the need for more research. In addition, most of experiments that looked at the effect of environmental enrichment on behaviour of laboratory rodent relied on supplying cages with a single physical item. There is evidence from research that increasing the extent of enrichment by increasing the number of items supplied to the cages of group-housed animals may augment the effect of enrichment **(Marashi et al., 2004; Abou-Ismail, 2010).** It may thus be worth studying how much environmental enrichment can do in improving the welfare of singly housed animals particularly in laboratory rats.

This experiment was therefore carried out to study the overall long-term effects of enriching cages of singly-housed rats, by adding various physical structures that are thought to stimulate rats' specific behaviours, on multiple measures of welfare such as behaviour, physiology, psychology and pathology.

MATERIALS AND METHODS

Animals

This experiment was carried out in the Department of Hygiene and Preventive Medicine, Faculty of Veterinary Medicine, Kafrelsheikh University. The experiment was conducted in a standardized laboratory animal room. The room was maintained under a 12:12 h light:dark schedule with the white light on between 0200 and 1400 and continuous dim red light (two 60 Watt bulbs, Serma Electrical, Egypt) enabling observation during the dark period, at a constant temperature (20±1 ^{o}C).

The experiment was carried out with two batches of rats in which each experimental treatment (see later) was replicated six times within each batch. The subject animals were 24 newly weaned male rats, 35- 50 g weight at arrival, of the Wistar (outbred) strain (Al-Alamia, El-Gharbia, Egypt). The rats were four weeks of age on arrival and were fed on pelleted food and tap watered (two bottles fitted in each cage) ad-libitum.

Rats were housed singly in cages supplied with sawdust as bedding and a handful of shredded paper as a nesting material. Cages were cleaned once

a week in which rats were re-housed in clean cages with new bedding and nesting material.

Housing conditions

Rats were arbitrarily housed in one of the following two conditions:

1) "Enriched cages" (EC): standard polypropylene cages (48 cm length × 30 cm width × 21 cm height) that were supplied with retreats (20.5 cm L × 15.7 cm W × 11.5 cm H Guinea pig huts, red-tinted, Lillico, UK), nylabone (Regular size, original flavour, (36g), Lillico, UK), crawl ball (115 mm, with 3 × 58 mm holes, red-tinted polycarbonate, Lillico, UK), ladders (9 step wooden ladder 35.5 cm, local pet store, El-Gharbia, Egypt) and nestlets (5 cm × 5 cm sterilized cotton fibre pads, Lillico, UK) **(Abou-Ismail et al., 2010)**.

2) "Unenriched cages" (UC): standard polypropylene cages (48.5 cm length × 33 cm width × 21 cm height) that were not supplied with any additional cage structures.

Behavioural assessment

Ethogram

The observer entered the experimental room 10 minutes before the scheduled start of the observation to allow the rats to habituate to his presence **(Hurst et al., 1999).** Observation was carried out every week in two sessions per day (representing one observation week) for the two housing conditions. The first session took place during the light phase (white light was on); starting at 1230 hr and ending at 1330 hr. The second session was carried out while the white light was off (during the dark phase of the day); starting at 1400 hr and ending at 1500 hr.

Behaviour of the rats in each of the 12 cages, in each batch, was recorded in real time using instantaneous sampling method with 4-s intervals between each consecutive focal animal. Each sample interval was prompted by an audio cue via headphones, and the behaviour recorded onto a check sheet. Each session therefore yielded 75 scans per rat. This meant a total of 150 scans per rat per day (observation week), and a total of 900 scans per rat over the entire experimental period (six observation weeks). The behaviour of each individual rat was sampled and its

position within the cage (underneath food hopper or in the open part of the cage) and state (contacting or away from enrichment) was also recorded **(Abou-Ismail et al., 2010).**

Fear and anxiety measurements (emotional behaviours)

At the seventh week and after behavioural observations were finished, a 5-min elevated plus-maze (EPM) test was conducted for each animal of the two housing conditions. EPM test is widely used in pharmacological research to analyze the level of anxiety in laboratory rodents, and is based on the natural conflict between the tendency of the animal to explore a novel environment and the aversive properties of a brightly lit open area (Menzaghi et al., 1996). The maze had 2 open arms and 2 closed arms (115 × 10 cm). The closed arms had 50 cm high walls. The plus-maze was elevated 100 cm above the floor. The maze was made of wood and was arranged in a manner such that arms of the same type were opposite each other, connected by a central area (15 cm × 15 cm). In order to keep the rats from falling over, the open arms were surrounded by a 0.5 cm high edge. All rats were tested individually in the light phase of the light/dark cycle in the same day between 0900 and 1200. The order of testing was counterbalanced between the two housing conditions to control for possible effects of time of the day on behaviour. Each rat was placed in the middle of the apparatus with its head facing an open arm, and its behaviour was video recorded for 5 min **(Kaliste et al., 2006).** The arms of the plus-maze were wiped with ethyl alcohol (Pharma One, Cairo, Egypt) after each individual rat was tested. The total numbers and durations of entries into closed and open arms, latency to the 1st entry into closed and open arms (seconds), frequency of rearing and grooming behaviours, and the number of head dip was recorded. Analysis was done by an experienced observer who was unaware of which housing conditions each animal belonged to.

Table 1- Ethogram for behavioural elements recorded (**Hurst et al., 1999; Meddis, 1975).**

Behavioural category	Behavioural component	Description
A- General activities	1- Feeding	Eating food from food hopper
	2- Drinking	Drinking water from waterspouts
	3- Non-intake maintenance	Self-grooming and pandiculation (stretching and yawning)
	4- Movement activities	Movement and/or climbing the cage lid
	5- Exploratory behaviour	Sniffing cage wall, cage top and sniffing air outside the cage
	6- Bedding-directed behaviours	Sniffing bedding, eating bedding, bedding manipulation and burrowing
B- Sleep	1- Sleep	Lying unalert with both eyes closed-apparently asleep
C- Other behaviour:	1- Awake non-active	Stationary
D- Enrichment-directed:	1- Enrichment-directed	Sniffing, chewing, climbing, and manipulating the enrichment objects.
E- Position in the cage	1- Underneath hopper	When the whole body of the rat, excluding its tail, is entirely underneath the food hopper or waterspouts at the moment of the scan
	2- In- the-cage	When the whole body of the rat, including its tail, is entirely in the open part of the cage

Weight changes and weight of internal organs

Throughout the six week experimental period rats were weighed weekly. Rats were picked from their cage and weighed using equilibrated scales (Sartorius, AG, Gottingen, Germany). At the end of the 7th week of the housing period rats were euthanized by cervical dislocation. Immediately after euthanasia the weight (in g) of each individual rat was recorded using a digital scale (Oertling, OB033, UK). Each rat was then dissected and selected internal organs, including the thymus gland, spleen and adrenal glands were removed and stored on ice in sterile balanced salt solution. They were subsequently dried, trimmed and weighed (in g).

Statistical analyses

Behavioural and weight changes data

We used a repeated measures General Linear Model (GLM) with week (week 1-6) and session (session 1-2) as within subject factors because the behavioural (ethogram) and physiological data (body weight and weight

gain) had been collected from the same cages at two different time points every week. Treatment (EC and UC) was included as a between subjects factor. SPSS (version 12.0 for windows) was used for all statistical analyses. The average % of scans spent in performing each behaviour was calculated by dividing the total number of scans for each behaviour variable by the total number of scans for each individual rat in each session (75 scans), and each figure was then multiplied by 100.

The relative weight gain (%) was determined by dividing the value of the absolute weight gain by the value of the body weight in the previous week, and then the resultant figure was multiplied by 100. Data were checked for normality and homogeneity of variances to test for the suitability of using parametric tests. Data of organ weight showed normality whereas behavioural data showed normality after square root transformation. All data are presented as EMM ± SE.

2.4.2. Elevated plus maze and weight of internal organ data

Data met the assumptions of parametric statistics (normality, homogeneity of variance, linearity). Relative durations of time spent in open (open/total × 100) and closed arms (close/total × 100), and latency to the 1st entry to open and closed arms were determined for each housing condition. Relative frequency of entries into opens (entries to open arms/total arm entries × 100) and closed (entries to closed arms/total arm entries × 100) arms, and frequency of rearing and grooming behaviours and head dip were also recorded for each group. The organ weights were expressed as a ratio of the body weight (relative weight for each organ). Differences between the rats of the two housing conditions in behaviours of the EPM test, final body weight and the relative weight of internal organs were tested using an independent *t*-test.

RESULT AND DISCUSSION

Behaviour

Main effects

Several behaviours showed an effect of housing conditions, average % scan: sleep ($F_{1,21}$=6.81, P<0.05); stationary ($F_{1,21}$=38.64, P<0.001) (see figure one); moving ($F_{1,21}$=9.62, P<0.05); bedding-directed behaviour ($F_{1,21}$=26.24, P<0.001) (see figure 2); under hopper ($F_{1,21}$=691.57, P<0.001) and in-the-cage ($F_{1,21}$=691.57, P<0.001) (see figure 3). The

values of sleep behaviour, movement activities and being in-the-cage were higher in the EC whereas those of stationary, bedding-directed behaviour and being under hopper were higher in the UC.

Interactions

Average % scan non-intake maintenance behaviour (self-grooming) showed a significant treatment*session ($F_{1,21}$=5.41, P<0.05), increasing significantly in the light phase in the EC; and both average % scan feeding ($F_{1,21}$=5.37, P<0.05) and exploration ($F_{1,21}$=5.43, P<0.01), increasing significantly in the dark phase in the EC (see figure 4).

Elevated plus maze:

Housing rats in enriched versus unenriched cages had a significant effect on their behaviours in the EPM including: relative time spent in open arms (sec) (t_{22}= 3.71, P<0.001); relative time spent in closed arms (sec) (t_{22}= -3.71, P<0.001) (see figure 5); relative open arm entry (t_{22}= 3.45, P<0.001); relative closed arm entry (t_{22}= -3.45, P<0.001) (see figure 6); and latency (sec) to 1st entry to open (t_{22}= -2.78, P<0.01) and closed arm (t_{22}= 5.99, P<0.001).

Weight changes and weight of internal organs

The output of the repeated measures-GLM showed that housing laboratory rats in enriched versus unenriched cages significantly changed weight changes parameters measured in this study, including: body weight (g) ($F_{1,21}$=111.68, P<0.001) and weight gain (g) ($F_{1,21}$=25.98, P<0.01) (see figure 8), with the rats in the EC weighing heavier and gaining more weights every week than rats in the UC.

Similarly, housing rats in enriched versus unenriched cages had a significant effect on the weight of internal organs recorded in this study including: final weight (g) (t_{22}= 5.20, P<0.001); relative adrenal weight (g) (t_{22}= -3.14, P<0.05); relative thymus weight (g) (t_{22}= 3.50, P<0.01); relative spleen weight (g) (t_{22}= 3.41, P<0.05) with the rats housed in the EC weighing more, having heavier thymus and spleen but lighter adrenal than rats housed in the UC (see figure 9).

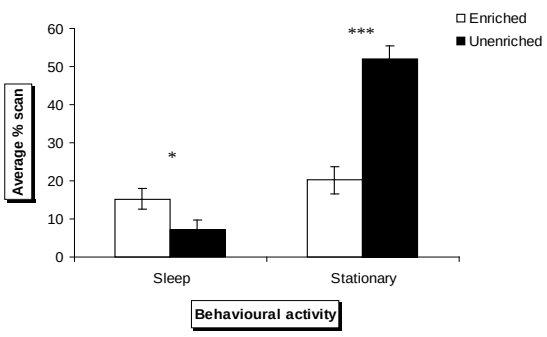

Figure 5: EMM ± SE 'Average % of time spent in the open and closed arm of the elevated plus maze' by the rats in the two housing conditions. *** P <0.001

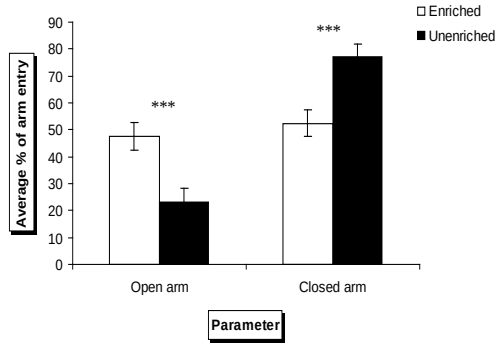

Figure 6: EMM ± SE 'Average % of open and closed arm entry of the elevated plus maze' by the rats in the two housing conditions. *** P <0.001

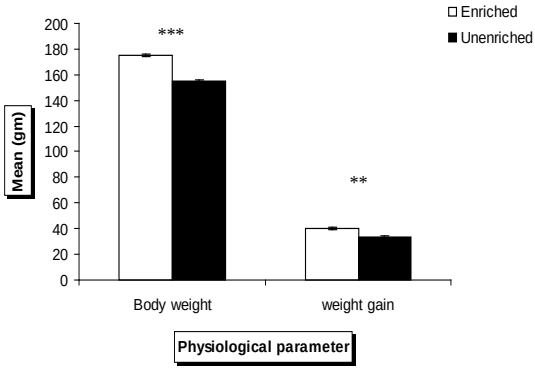

Figure 3: EMM ± SE 'Average % scan under hopper and in-the-cage' by the rats in the two housing conditions. *** P <0.001

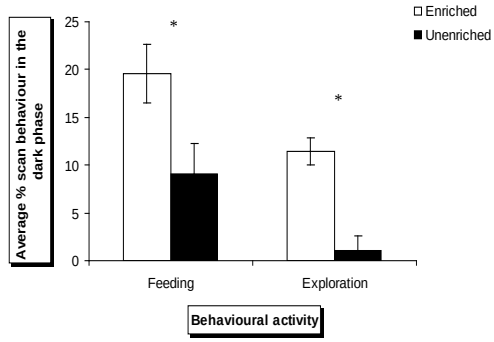

Figure 4: EMM ± SE 'Average % scan feeding and exploration' by the rats in the two housing conditions. * P <0.05

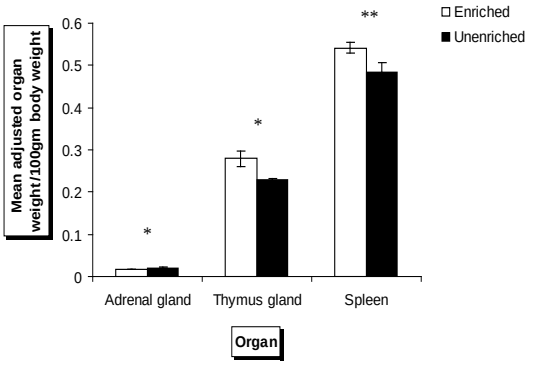

Figure 5: EMM ± SE 'Average % of time spent in the open and closed arm of the elevated plus maze' by the rats in the two housing conditions. *** P <0.001

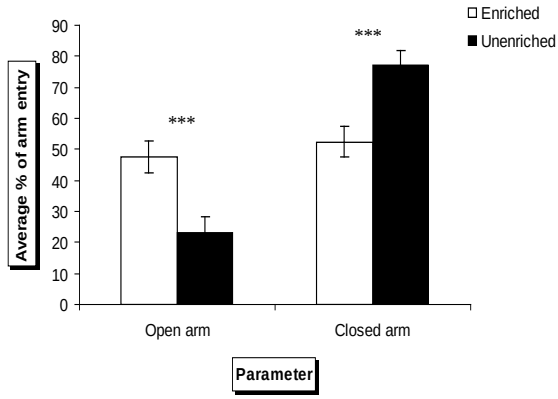

Figure 6: EMM ± SE 'Average % of open and closed arm entry of the elevated plus maze' by the rats in the two housing conditions. *** P <0.001

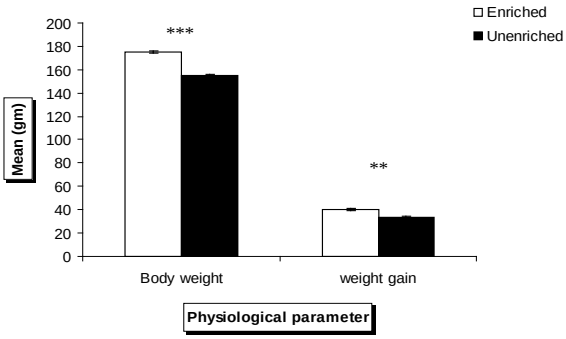

Figure 7: EMM ± SE 'Body weight and weight gain (g)' by the rats in the two housing conditions. ** P <0.05 *** P <0.001

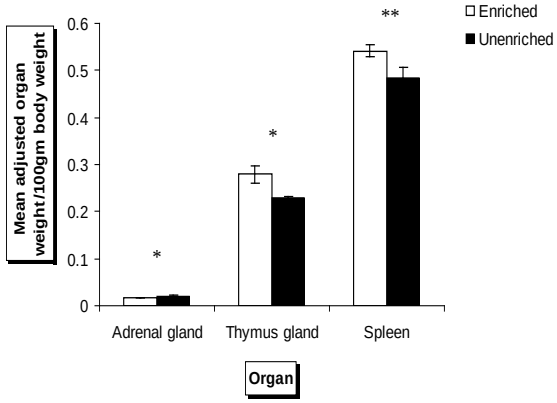

Figure 8: EMM ± SE 'Average relative adrenal, thymus and spleen weight (g)' by the rats in the two housing conditions. * P <0.05 ** P <0.001

DISCUSSION

Behaviour

Our results demonstrate clear differences between rats in the different housing conditions. Rats housed in the enriched conditions displayed higher levels of sleep, movement activities, exploration and both intake (feeding) and non-intake maintenance (self-grooming) behaviours and lower levels of stationary and bedding-directed activity as compared to rats in the unenriched conditions. Moreover, rats in the enriched cages were found to be in-the-cage (in the open part of the cage) more frequently and under hopper less frequently as compared to rats in the unenriched cages. These findings demonstrate that housing laboratory rats in conventional laboratory cages (standard unfurnished cages) is stressful compared to housing them in cages enriched with multiple physical structures.

High levels of sleep behaviour have been shown to indicate good welfare in laboratory rats **(Abou-Ismail et al., 2007)**. Research on both humans and laboratory rats has shown that chronic stress can affect both sleep quantity and quality. In humans, the activity of HPA axis has been shown to influence some features of sleep patterns, and that this may be due to the increased level of CRF, ACTH and cortisol. HPA axis hyperactivity (as in chronic stress, aging and depression) reduces sleep quality and causes sleep disturbances **(Bradbury et al., 1998).** Similarly, in laboratory rats, typically, in a chronic stressful situation, total sleeping frequency and duration decrease, with sleep liable to have more interruptions (**Knat et al., 1995)**. It has been shown that physical stress reduces both sleep quality and quantity (Bradbury et al., 1998). Chronic psychological stress (e.g. subordination) also appears to reduce sleep quantity **(Hurst et al., 1999).**

This high level of sleep displayed by rats in the EC could be due to the increased level of their movement and exploration but also due to the increased activity directed towards the enrichment objects. It could also be due to the ability of rats in the EC to control their environment by avoiding the disruptive effect of the white light. It was shown that unavoidable light constitutes a stressful condition for a nocturnal animal (laboratory rats) and that it resulted in a marked decrement of both types of sleep (rapid eye movement sleep and short wave sleep) **(Fishman and**

Roffwarg, 1972). The provision of multiple physical structures to the cage may have allowed the rats to use some of these structures (such as the retreat and crawl ball) to hide from the direct effect of the white light.

Laboratory rats are well known as thigmotactic (edge-users) preferring to spend most of their resting and sleeping time in contact with the surrounding walls of their environment **(Anzaldo et al., 1994, 1995).** Adding multiple physical structures to the conventional cages might have increased the walls and edges in the cage therefore improving the rats' ability to display more natural behaviour such as sleep. Similar finding of increased sleep behaviour in rats housed in enriched laboratory cages, but in groups, has been reported by **Orok-Edem and Key (1994).**

Rats in the EC displayed also higher levels of exploration and movement activities as compared to rats in the UC. Research work has shown that chronic stress decreases general activity levels and locomotor behaviour (Blanchard et al., 2001), and exploration **(Menzaghi et al., 1996).** These higher levels of movement and exploration by the enriched housed rats could be due to the increased complexity of their environment. **Denny et al. (1975)** illustrated that, when given the choice, rats prefer high complexity in their environment and that they spend more time active (moving and exploring) in the complex environment. This finding of increased levels of movement and exploration by the rats in the EC are in accord with those of **Townsend (1997) and Marashi et al. (2004).**

Rats in the EC displayed higher levels of both intake and non-intake maintenance behaviour as compared to rats in the UC. Research work has reported an inhibition or a reduction in the self-grooming time after chronic stress; a repeated social defeat **(Van De Poll et al., 1982),** chronic stress by anxiogenic drugs **(Maldonado and Navarro, 2001),** chronic psychological stress (predatory stress) **(Blanchard et al., 1998)** and also in the subordinate animals after long period of grouping **(Hurst et al., 1996).** Similarly, a reduction in food intake has been found after chronic stress **(Blanchard et al., 2001).** This high level of self-grooming activity in the enriched housed rats may be due to the higher amount of sleep in these animals. Self-grooming was reported to be the second activity of the laboratory rat that occupies the longest duration of their

time budget after sleep. Indeed, it is the most time consuming activity of the laboratory rat's awake time **(Saibaba et al., 1996).** Self-grooming was reported to be concentrated around sleeping time. It takes place after sleeping, but also occurs when the animal prepares for sleep. However, the high level of feeding displayed by the enriched housed rats could be due to the higher activity levels performed by these animals.

Rats in the EC exhibited lower levels of bedding-directed behaviours than rats housed in the UC. This relative increase in the level of bedding-directed behaviours in the UC could be due to the fact that rats in these cages had no enough cage structures (objects) to interact with. The only available cage structure in these cages was the bedding substrate; thereby these conventional cages limit the available options of the rats for interaction. On the other hand, rats in the EC may have performed bedding-directed behaviours less because they spent time interacting with the various different enrichment objects in their environment. Similar finding of reduced bedding-directed behaviours in groups of rats housed in enriched cages was reported by **Orok-Edem and Key (1994).**

The finding that rats in the EC were present more frequently in-the-open part of the cage and less frequently underneath-hopper compared to rats in the UC could be due to the increased compartmentalization of the EC by the provision of multiple physical structures into them. This might have provided various resources for the rats to hide from the disruptive effect of the white light, particularly in the light phase of the dark/light cycle, and intensified their thigmotactic nature. This might, in turn, have improved the ability of these animals to exert better control over their environments compared to their counterparts in the UC. Good ability of animals to cope with, and to control, the environment is a necessary requirement for good welfare **(Wiepkema and Koolhaas, 1993).**

Rats in the EC directed various behaviours towards the enrichment objects used in the study. The provision of enrichment objects appeared to have fulfilled the animals' "needs" including the choice to rest or sleeps in the open part of the cage during a certain time of the day (e.g. the light phase of the light/dark cycle), seek a refuge, forage and gnaw. "Needs" are requirements that are fundamental to the biology of an

animal e.g. to obtain a particular resource, respond to a particular environmental, or bodily stimulus (**Broom and Johnson, 1993).** In addition, the type of cage modification implemented in this study was of affordable cost, practical to use, clean and easy to replace, did not compromise the physical health of the rats, nor did it prevent ease of checking the animals.

Elevated plus maze:

Our results showed that rats experienced the EC explored the open arms of the maze for longer time and the closed arms of the maze for shorter time as compared to those experienced the UC. The EC rats also entered the open arms more frequently and the closed arms less frequently, and showed short latency to open arm entry as compared to the UC rats. Taken together, the results of the EPM indicate that increasing the extent of enrichment of conventional cages of laboratory rats appeared to decrease the level of stress they experience.

Tests for measuring anxiety, such as elevated plus-maze are generally accepted as a reproducible measure of anxiety in laboratory rodents **(Kantor et al., 2000)**. It has been shown that anxious animals are found to prefer, and are more active in, the closed arms over the less secure open arms; such behaviour which is indicated by less time spent on and low frequency of entries to the open arms as well as low latency to open arm entry **(Menzaghi et al., 1996).**

It appears therefore that long-term housing of laboratory rats in standard unfurnished cages is stressful. In accordance with our results, Batchelor, (1993) has mentioned that laboratory rats housed in conventional laboratory cages are ethologically, physiologically and psychologically aberrant and cannot be considered as normal animals. More importantly, **Sherwin, (2004)** showed that reduced external validity of the research and therefore the benefit gained from the research has been shown to arise when laboratory rodents are housed in standard laboratory cages.

Single housing of laboratory rat has been shown to be stressful **(Dronjak et al., 2004)**. Experiments have pointed out that individual housing

enhances anxiety-like behaviour **(Jankowska et al., 1991).** However, there are also data that have indicated that individual housing perse did not increase the anxiety-like behaviour **(Nakayasu and Ishii, 2008)**. Thus, simply, individual housing perse of laboratory rat may not be stressful **(Arakawa, 2003)** but housing them in standard laboratory cages for long term may be stressful.

Weight changes and weight of internal organs:

Our results showed that rats in the EC had higher weights and weight gains compared to rats in the UC. Moreover, the EC rats had higher relative weight of spleens, thymuses and lower relative weights of adrenal glands as compared to the UC rats. The increased weights and weight gains in the EC rats could be due to their increased feeding, but could also be due to their increased sleep behaviour. One of the many theories that have been proposed for the function of sleep is the protective theory that is: the function of sleep is to protect the organism from excessive wear and tear **(Everson et al., 1989).** This finding indicates that long-term housing of juvenile laboratory rats in conventional laboratory cages appears to be stressful. Body weight and weight gain have been reported to decrease after chronic physical and social stress **(Hurst et al., 1996; Stefanski et al., 2001).**

In accordance with the direction of the data of behaviour, weight changes and elevated plus maze, the findings of the changes in the weights of the internal organs could also indicate that long-term housing of rats in the UC appeared to be stressful. The increase in the weight of the adrenal gland (adrenal hypertrophy) is generally thought to result due to the increased activity of the gland particularly the cortex (cortical hypertrophy) **(Manser, 1992).** This increase in the adrenal cortex weight has been suggested to happen under the frequent stimulation and the increased activity of the adrenocorticotrophic function of the pituitary gland which results from the stimulation of the HPA axis during chronic stress **(Christian, 1955).** Similarly, stress can decrease the weight (reduce the lymphatic tissue mass) of lymphoid organs such as thymus (thymus atrophy) and spleen **(Blanchard et al., 1995).**

The type of cage modification implemented in this study was of affordable cost, practical to use, clean and easy to replace, did not compromise the physical health of the rats, nor did it prevent ease of

checking the animals. The modification regimen provided all the required physical features of enrichment items suggested by previous studies **(Van de Weerd and Baumans, 1995; Pritchett and Corning, 2003).** Importantly, this particular type of cage modification provided the rats with ample opportunities to cope with and to exert control over their environment; characteristics that resulted in improved welfare in the animals experiencing it. It has been suggested that for an efficient environmental enrichment program to improve the welfare of the animals experiencing it, the enrichment should enhance the expression of desirable behaviours such as species-specific behaviours, decrease undesirable behaviours such as abnormal behaviour, or do both **(Kitchen and Martin, 1995; Van de Weerd and Baumans, 1995).** As, adding some physical structures to the laboratory cages should not be considered enrichment until it produces good long-lasting changes in welfare **(Line and Morgan, 1991),** the regimen used in this study appeared to have met this requirement and can therefore be called enriching.

CONCLUSION

Long-term enrichment of conventional cages of newly weaned laboratory rats with multiple physical structures appeared to improve the ability of these animals to control their environment and to promote their species-specific behaviour; potentials that can ultimately result in good welfare. Long-term single housing of rats in super-enriched cages increased levels of indicators of good welfare and decreased levels of indicators of poor welfare. The findings of this experiment showed that laboratory rats housed in enriched cages demonstrated improved welfare and were less stressed compared to those animals housed in conventional laboratory cages. The results, more importantly, demonstrated that when single housing of laboratory rats is necessitated their laboratory cages should be enriched with multiple physical structures in order to improve their welfare. These findings thus strongly support the need of the current conventional housing systems of laboratory rats, particularly singly-housed rats, for re-evaluation to help provide better environment for the animals that can in turn result in an improvement in their welfare.

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التأثيرات طويلة المدي لإستخدام أدوات دعم متعددة علي القياسات المختلفة لمستويات الإراحة في الجرذان منفردة المسكن

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يوصي بالإسكان المنفرد للجرذان المعملية في بعض الأحيان كما في دراسات السموم و التغذية و أيضا لمنع إنتشار الأمراض المعدية. وحيث أن الإسكان المنفرد للجرذان المعملية مجهد لها فإنه يتطلب تعديل بيئة الإسكان لتحسين مستويات الإراحة في تلك الحيوانات.

تم إجراء هذه التجربة لدراسة التأثيرات طويلة المدي لدعم الأقفاص المعملية وذلك بإضافة أدوات عديدة في الأقفاص علي القياس المختلفة لمستويات الإراحة مثل السلوكيات، التغيرات في وزن الجسم وفي أوزان الأعضاء الداخلية للجرذان منفردة المسكن . تم إ سكان الجرذان المستخدمة في هذه التجربة وعددها 24 جرذ منفردا في أقفاص إما 'مدعمة' أو 'عادية'. تم تسجيل السلوكيات وأوزان ومعدلات نمو الجرذان كل أسبوع خلال فترة الأسابيع الستة للتجربة . تم تسجيل سلوكيات الجرذان في المتاهة المتعامدة المرتفعة في الأسبوع السابع في حين تم تسجيل أوزان الأعضاء الداخلية بعد إماتة الجرذان.

كشفت النتائج أن الإسكان المنفرد طويل المدي للجرذان في أقفاص فائقة الدعم أدي إلي زيادة في مستوي مؤشرات الرفاهية الجيدة كسلوكيات النوم والإستكشاف والتغذية، أوزان الجسم ومعدلات النمو والوزن النسبي للغدة التيموسية والطحال ، وإلي إنخفاض في مستوي مؤشرات الرفاهية السيئة كسلوكيات الثبات والوزن النسبي للغدة الكظرية . وبالتالي، فإن دعم الأقفاص الم عملية التقليدية للجرذان منفردة المسكن بإستخدام أدوات عديدة يمكن أن يؤدي إلى تحسين في قدرتها علي السيطرة علي البيئة وإلي تعزيز سلوكياتها الخاصة ، الإمكانيات التي يمكن أن تؤدي إلي تحسين مستويات الرفاهية.

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RUMEN LIQUOR PHYSICAL, CILIATES AND BIOCHEMICAL COMPOSITION IN HOLSTEIN-FRIESIAN DAIRY CATTLE FED ON CORN SILAGE IN EGYPT

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ABSTRACT

Strained rumen liquor (SRL) examination in 37 dairy Holstein–Fresian cattle, at El-Gharbia Governorate, fed on corn silage; for physical characteristics showed that pH level was 6.64 ± 0.086, with green to olive green color, slimy consistency and aromatic odor; total protozoa count, *Entodinium*, *Diplodinium*, *Epidinium*, *Holotricha* and *Ophryoscolex* percentages were 9.3 ± 1.87×10^4, 89.09 ± 1.92, 4.62 ± 1.32, 0.37 ± 0.03, 4.55 ± 1.33 and 1.38 ± 1.38% respectively. Mean levels of ammonia concentration, total volatile fatty acids (TVFAs), total protein, calcium, phosphorus, copper and zinc were 364.66 ± 23.08 mmol/L, 85.10 ± 14.69 mmol/L, 10.51 ± 1.47 g/L, 1.11 ± 0.69 mmol/L, 2.01 ± 1.06 mmol/L, 4.31 ± 1.78 µmol/L and 16.59 ± 2.52 µmol/L respectively. Compared with 19 Holstein-Friesian dairy cattle from selected farms fed on traditional feed stuffs, significant changes were found for total protein, phosphorus, copper concentration and *Epidinium* % ($p < 0.001$); zinc concentration, Diplodinium %, TVFAs concentration, pH, total protozoa count and calcium concentration ($p < 0.01$) and ammonia nitrogen concentration ($p<0.05$). The microscopic examination of stained SRL samples in both groups revealed the identification of 4 families, 3 subfamilies, 12 genera, 39 species and 9 forma with significant variations in the percentages of each family composition. Two new genera (*Buetschlia* and *Ophryoscolex*), and 20 new species belonging to 7 genera (one species in genus *Buetschlia*, 9 in *Entodinium*, 2 in *Diplodinium*, 3 in *Metadinium*, 2 in *Epidinium*, 1 in *Elytroplastron* and 2 in *Ophryoscolex*) were recorded in dairy cattle in Egypt. All genera were demonstrated in figures and their dimensions were measured. These results should be put in consideration during the physical, ciliates and biochemical examination and evaluation of SRL status in dairy cattle.

Keywords: Dairy cattle, rumen, ciliates, biochemical constituents.

INTRODACTION

Silage is a method of forage preservation through stabilizing fermentation process by decreasing the pH within minimum fermentation period. In silage, lack of oxygen and the accumulation of lactic acid inhibit its microbial metabolism and preserves nutrients (Ranjit and Kung 2000). It is essential to investigate the effect of corn silage on rumen physical status, biochemical constituents (Zehra and KILIÇ 2009); and ciliates

composition (Baraka 2006). Importance of rumen ciliates is referred to that; they constitute about 50% of rumen biological population, represent about 20% of gained protein by host with digestibility at abomasum of 91%, detoxify toxins of poisonous plants and eliminate some toxins out of the digestive tract, stabilize number of Streptococci to reduce harmful lactic acid, and *Entodinium* types of ciliate protozoa digest starch and protein to produce amino acids which are essential for bacteria and protozoa. Until now, no sufficient comprehensive recording of rumen ciliates in different ruminants in Egypt was established. This work was carried out to investigate the changes in rumen liquor physical, ciliates and biochemical composition in Holstein-Friesian dairy cattle fed on corn silage in Egypt; and to be put in consideration during the physical, ciliates and biochemical examination and evaluation of rumen liquor status in dairy cattle.

MATERIALS AND METHODS

Thirty seven Holstein-Friesian dairy cattle; belonging to a private milk production farm, at El-Gharbia Governorate, fed on corn silage (Approved by Central Laboratory of Ministry of Agriculture –Egypt)) were used in this study to investigate the physical, ciliates and biochemical constituents in their rumen. Another 19 Holstein-Friesian dairy cattle from different farms fed on traditional feed stuffs were used as control group for comparison. From each cow 50ml of rumen juice were collected using a rubber stomach tube connected to a suction pump and wooden mouth gag. Samples were examined at Laboratory of Rumenology, Department of Medicine and Infectious Diseases, Faculty of Veterinary Medicine, Cairo University. Samples were examined immedietly for pH (using SMP1 pH-meter), color, odor and consistency; then divided and stored for determination of total protozoal count according to the method described by Dehority 1984, generic protozoal composition according to Dehority 1993, ammonia concentration according to Zapletal 1967 and volatile fatty acids concentration according to Cottyn and Boucque 1968.

The identification and description of rumen ciliate protozoa were applied according to Nassar 1971, Dehority 1974, Dehority 1979, Ogimoto and Imai 1981, Dehority1984, Norman 1985, Williams 1986, Sakr 1988; Dehority 1993; Akira et al. 1994; Selim et al 1996; Selim et al 1999;

Bayram 2000; Bayram et al 2001; Baraka and Dehority 2003; Mermer et al 2003; Baraka , et al 2005; Bayram and Karaoglu 2005; Baraka 2006 (1); Baraka 2006 (2); Bayram and Sezgen 2006. Ciliates dimensions were measured and identified using research microscope (Boeco-Germany), micrometer eye piece (MOB-1-16$^{\times}$) and digital camera (Canon A650 IS). The biochemical constituents (total protein, calcium, phosphorus, copper and zinc) were analyzed in the supernatant of centrifuged strained liquor using Apel PD-303S spectrophotometer and the specific chemical kits. The obtained data were statistically analyzed using the SPSS Statistical Computer Software. Copyright (c) SPSS Inc., 2007 version 16.0.

RESULTS

Physical examination of strained rumen liquor samples in dairy cattle fed on corn silage revealed that rumen juice color ranged between green to olive green with slimy consistency and aromatic odor; on the other hand in dairy cattle fed on traditional feed stuffs the color varied according to the type of given rations, with slimy consistency and aromatic odor.

Table 1: Rumen physical, ciliates and biochemical constituents in Holstein-Friesian dairy cattle fed on silage and others fed on different traditional rations:

Parameters	Cattle fed on silage (37)	Control cattle (19)
pH	6.64±0.086 ^{b}	7.27±0.19
Total protozoa count (×10^4)	9.3±1.87 ^{b}	13.08±1.71
Entodinium (%)	89.09±1.92	89.52±10.73
Diplodinium (%)	4.62±1.32 ^{b}	2.08±1.46
Epidinium (%)	0.37±0.03 ^{a}	3.17±4.01
Holotricha (%)	4.55±1.33	6.10±3.18
Ophryoscolex (%)	1.38±1.38 ^{a}	0.15±1.87
Amm. Conc.* (mmol/L)	364.66±23.08 ^{c}	269.85±24.63
Volatile fatty acids (mmol/L)	85.10±14.69 ^{b}	59.89±5.87
Total protein (g/L)	10.51±1.47 ^{a}	6.29±0.396
Calcium (mmol/L)	1.11±0.69 ^{b}	5.36±0.80
Phosphorus (mmol/L)	2.01±1.06 ^{a}	10.01±1.14
Copper (µmol/L)	4.31±1.78 ^{a}	11.8±1.85
Zinc (µmol/L)	16.59±2.52 ^{b}	10.75±1.35

*Amm. Conc.: Ammonia concentration

a: $p<0.001$ b: $p<0.01$ c: $p<0.05$

Significant changes (Table 1) were found in total protein, phosphorus, copper concentration and *Epidinium* % ($p < 0.001$); zinc concentration,

Diplodinium %, TVFAs concentration, pH, total protozoa count and calcium concentration ($p < 0.01$) and ammonia nitrogen concentration ($p < 0.05$); which can be explained by the interaction between these constituents (Table 2).

Table 2: Correlation between physical, cellular and biochemical rumen constituents in Holstein-Friesian dairy cattle fed on corn silage:

	Tprz.	Ent.	Dpl.	Holo.	Ophr.	Am.	VFA.	Tprt.	Ca.	Phos.	Cu.	Zn.
pH	-0.05	0.16	0.37	-0.05	-0.73	0.34	0.08	-0.21	-0.50	0.55	-0.76	0.34
Tprz.		0.16	-0.68	-0.58	0.73	0.61	-0.71	0.82	-0.76	-0.24	-0.50	0.61
Ent.			-0.68	-0.89	0.00	0.55	0.29	0.41	-0.29	0.76	-0.13	0.55
Dpl.				0.89	-0.73	-0.55	0.24	-0.82	0.39	-0.13	0.03	-0.56
Holo.					-0.36	-0.71	0.08	-0.72	0.55	-0.50	0.29	-0.71
Ophr.						0.18	-0.54	0.71	-0.18	-0.54	0.18	0.18
Amm							0.08	0.31	-0.50	0.55	-0.76	0.34
VFA.								-0.72	0.68	0.68	0.16	-0.58
Tprt.									-0.72	0.21	-0.15	0.82
Ca.										-0.05	0.68	-0.89
Phos.											-0.37	0.16
Cu.												-0.37

Tprz: Total protozoa count.
Ent.: *Entodinium.*
Dpl.: *Diplodinium.*
Holo.: *Holotricha.*
Amm.: Ammonia concentration.
VFA.: Volatile fatty acids.
Tprt.: Total protein.
Ca.: calcium.
Phos.: Phosphorus.
Cu.: Cupper.
Zn.: Zinc.

Table 3: Total ciliates concentrations and distribution of total number of genera, species and forms of rumen ciliates in cattle at various localities around the world:

Locality	Total protozoa count $\times 10^4$/ml	Total no. of genera	Total no. of species	Total no. of forms	Number of animals and breeds	References [a]
Brazil	26.4±17.7	14	55	4	4 Zebu cattle	(1)
Canada	6.9 [b]	12	28	-[b]	11 H. F.*	(2)
China	30 [b]	17	20	6	45 Chinese cattle	(3)
Egypt	45 [b]	10	28	11	7 H. F.	(4)
Iran	29±18.2	5	10	-[b]	37 cattle	(5)
Japan	40.3±1.9	15	48	25	125 H. F.	(6)
Kenya	15.1 [b]	13	51	19	13 Zebu cattle	(7)
Libya	81 [b]	9	27	6	9 H. F.	(8)
Mexico	8.3 [b]	13	38	15	10 Hereford cattle	(9)
Philippine	15.8 [b]	10	26	3	70 H. F.	(10)
Sri Lanka	2.9±4.9	16	53	19	20 Zebu cattle	(11)
Tanzania	22.2 [b]	15	46	-[b]	10 Tanzanian cattle	(12)
Thailand	7.1±2.8	17	56	4	46 Zebu cattle	(13)
Turkey	52.4±20.7	13	52	36	28 Domestic cattle	(14)
Egypt	9.3±1.87	12	39	9	37 H. F.	Present study
	13.08±1.71	12	39	9	19 H. F.	

[a] (1) Dehority 1986a, (2) Imai, et al. 1989, (3) Rong and Imai 2002, (4) Selim, et al. 1996, (5) Talari, et al. 2004, (6) Ito , et al. 1994, (7) Imai 1988, (8) Selim, et al. 1999, (9) Imai and Kinoshita 1997, (10) Shimizu et al. 1983, (11) Imai 1986, (12) Mishima, et al. 2009, (13) Imai and Ogomoto1984, (14) Bayram, et al. 2003.

[b] Data not reported. * H. F.: Holstein-Friesian cattle.

The total protozoa count, number of genera, species and forms recorded in both groups (Table 3) were compared with data in other countries (Shimizu et al. 1983, Imai and Ogomoto1984, Dehority 1986a, Imai 1986, Imai 1988, Imai, et al. 1989, Ito, et al. 1994, Selim, et al. 1996, Imai and Kinoshita 1997, Selim, et al. 1999, Rong and Imai 2002, Bayram, et al. 2003, Talari, et al. 2004, Mishima, et al. 2009) .

Although the microscopic examination of stained strained rumen liquor samples in both groups revealed identification of 4 families, 3 subfamilies, 12 genera, 39 species and 9 forma (Fig. I); marked variations in the percentages of each family composition were obvious. This is the

first illustration of identified and measured species and forma of rumen ciliates in strained rumen liquor of dairy cattle in Egypt:

ORDER: PROSTOMA

FAMILY: ISOTRICHIDAE

GENUS: *ISOTRICHA*

1. *Isotricha prostoma:*
The body is oval and measures 80-100 × 50-120 µm. Cilia uniformly covers the body which is tapered at the level of cytostome which is sub-terminal. Macronucleus is kidney shape and connected with micronucleus by fibrils forming the karyophore. The mouth is located at the end opposite the leading or anterior end. This location has elicited speculation as to what is actually the anterior end.

2. *Isotricha intestinalis:*
The body is oval and measures 90-200 × 45-150 µm. Macronucleus is kidney shape. The cytostome is more sub-terminal at the level of macronucleus. The mouth is on one side of the cell equidistant between the posterior end and the middle.

GENUS: *DASYTRICHA*

3. *Dasytricha ruminatum:*

Body is oval, flattened and measures 45-100 × 25-50 µm. It is smaller than the isotricha and commonly occurs in greater numbers in the rumen. The mouth is at the posterior end. Elliptical macronucleus is in middle or posterior third of the body. Cilia are in spiral longitudinal rows. There are no contractile vacuoles.

FAMILY: BUETSCHLIIDAE
GENUS: *BUETSCHLIA*
4. *Buetschlia polymorphella bovis:*
Body is generally ovoid with anterior one third tapered like a flask and measures 25-40 × 20-25 µm. Uniform cilia are present in two ciliary zone; the large one on the tapered anterior one third area and the smaller one is a small tuft consists of a few cilia near a cytoproct on the posterior end of the body. Macronucleus is sub-spherical and situated at central part of the body. Spherical micronucleus is near the margin of

macronucleus. Contractile vacuole is at posterior end of body and concretion vacuole close to body surface at middle of the body.

FAMILY: BLEPHAROCORYTHIDAE
GENUS: *CHARONINA*
5. *Charonina ventricularis:*
The body is cylindrical and wide at anterior end with two ciliary tufts near the posterior end. The body measures 24-36 × 12-15 µm. The esophagus is very long and directed to the macronucleus, which is spherical to globular and located in middle to posterior part of the body.

ORDER: ENTODINOMORPHIDA
FAMILY: OPHRYOSCOLECIDAE
SUBFAMILY: ENOTODININAE
GENUS: *ENTODINIUM*
6. *Entodinium caudatum f. caudatum:*
The body is truncated anteriorly and measures 25-70 × 25-50 µm with single adoral zone. Macronucleus is cylindrical to wedge shaped and is nearly ½ of body length broader anterior than in posterior with a contractile vacuole at anterior pole. Pointed to slight rounded lobes are on both upper and lower posterior left side.
7. *Entodinium cauadatum f. lobospinosum:*
The truncated body measures 30-70 × 30-60 µm. Macronucleus is nearly ½ of body length broader anterior than in posterior with a contractile vacuole at anterior pole. Pointed to slight rounded lobe is on posterior left side.
8. *Entodinium williamsi f. turcicum:*
The body is ovoid or quadric-angular to ellipsoid and measures 35-65 × 28-50µm and generally wider at mid-point. There are two spines and a spinated lobe at the posterior end of the body. Micronucleus is usually ellipsoid or ovoid in shape and situated in left ventral posterior edge of the macronucleus. The contractile vacuole lies to the ventral side and to the left of the macronucleus.
9. *Entodinium caudatum f. dubardi:*
Oval body truncated anteriorly, the body measures 25-45 × 25-35µm. Contractile vacuole at edge of triangular macronucleus. Anus is on right side of small posterior left lobe.
10. *Entodinium longinucleatum:*
Body is ellipsoid, flattened and measures 45-110 × 25-80 µm. Macronucleus is as long as body length. The contractile vacuole is close to upper side of macronucleus.

11. ***Entodinium longinucleatum f. spinolobum:***
Body is ellipsoid in shape and measures 45-60 µm × 30-40µm. there are two spines and one lobe at posterior end of body. One spine on right side and the second at upper left side, while the lobe is at lower left side. Right spine is longer than left one. Esophagus is relatively short. Macronucleus is very long and extends along right body side. Micronucleus is ellipsoid and present at upper third of macronucleus. Contractile vacuoles are at upper left side of macronucleus.

12. ***Entodinium yunnense f. yunnense:***
The body is ellipsoidal and measures 40-60 × 28-40 µm. the macronucleus extends along the right side of the body; from near rectum to anterior end at anterior one sixth of its length. Micronucleus is ellipsoid and lies on the left side of the anterior third of macronucleus. One contractile vacuole is at left upper side of macronucleus.

13. ***Entodinium yunnense f. spinonucleatum:***
Body is ellipsoidal in side view, measuring 26-41 × 24-33µm, both sides are convex, widest part is at middle of cell. Left body side extends at the end with single sharp spine; and may curve slightly to the right. Posterior right side ranges from smooth rounded lobe to sharp spine. Short esophagus of funnel shape is at a distance from mid of macronucleus. Macronucleus extents along entire right side near the anterior end at the rectum. Micronucleus is ellipsoid and located at left of macronucleus and at anterior 1/3 of it. Contractile vacuole lies to the left of upper surface of macronucleus, just anterior to micronucleus.

14. ***Entodinium nanellum:***
The body is ovoid, flattened. The body measures 22-32 × 12-18µm. Thin macronucleus of wedge-shape and longer than ½ of body length. Esophagus curves to the macronucleus.

15. ***Entodinium constrictum:***
The body is ellipsoid or ovoid in side view. The body measures 30-40 × 20-30 µm and has a convex right side. Left side has indentation at the level of base of adoral membranelle zone. macronucleus is occasionally spherical and lies on the right side in the middle to posterior half of the cell.

16. ***Entodinium bovis:***
The body is ellipsoid in side view, measuring 24-44 × 18-33µm, both sides are convex. Small left lobe is present. Adoral membranelle zone slants away from the macronucleus and the esophagus is bending sharply

to the right, terminating posterior to the micronucleus. Macronucleus is triangular to club shaped, lies on right side, its anterior part bends to left. Micronucleus is small ellipsoidal lies to the left of macronucleus below the level of adoral zone. Contractile vacuole lies at left upper part of macronucleus just anterior to the micronucleus.

17. ***Entodinium bursa:***

Flattened body measuring 80-120 × 75-100 µm, macronucleus is 4/5 of body length; dense granular cytoplasm, contractile vacuole is anterior, body surface has longitudinal striation.

18. ***Entodinium exiguum:***

Elongated oval body measures 20-35 × 15-25 µm, straight esophagus, parallel with long body axis, macronucleus irregular shaped (short and thick) shorter than ½ of body length and generally lies in middle third of body.

19. ***Entodinium imaii*****:**

The body is ovoid and widest at 1/4 of the body level. The body measures are 20-35 × 20-25 µm; dorsal side is convex and humpbacked anteriorly. The ventral side is almost straight but slightly depressed on the mid-surface. In the posterior part of the body, there is one dorsal spine, extending outwardly but sometimes bending dorsally and towards the anterior part. Another triangular spine and a back-shaped lobe on the right side are also present. The right lobe is shorter than the secondary spine on the left. Macronucleus, which is bean-shaped, mostly concave on the ventral side and convex on the dorsal side, is located at almost the anterior tip of the body. The micronucleus is ellipsoidal and is situated close to the left posterior of the macronucleus. The contractile vacuole lies left posterior or left of the macronucleus.

20. ***Entodinium oktemae:***

The body is ovoid-ellipsoidal and is widest at the midpoint. The body measures 50-75 × 35-46 µm. A spine and two matching spinated lobes are present at the posterior end. The spine is on the dorsal side, whereas the lobes are located ventrally on both sides. The dorsal spine is bending sometimes dorsally or towards the left side of the body. The macro- and micronuclei are spherical and the micronucleus is situated posterior or anteriorly on the left of the macronucleus, generally in its vicinity. The contractile vacuole is located before or at the level of macronucleus on its ventral side.

SUBFAMILY: DIPLODININAE
GENUS: *DIPLODINIUM*
21. *Diplodinium anisacanthum:*
The body is oval to triangular and measures 150-210 × 90-120 µm with posterior oblique and truncated end. Macronucleus is sausage in shape with anterior curved end toward the ventral aspect.
22. *Diplodinium monocanthum:*
The body is oval to triangular and measures 60-90 × 40-60 µm; with posterior oblique and truncated end. Only single posterior spine is present. Macronucleus is sausage in shape with anterior curved end toward the ventral aspect.
23. *Diplodinium tetracanthum:*
The body is oval to triangular and measures 60-80 × 40-60 µm; ends posteriorly with four spines.
24. *Diplodinium dentatum:*
The body measures 60-80 × 50-65 µm with six heavy incurved posterior spines. Spine on right side is the longest one. The macronucleus is long; it is heavy and rod like. The anterior end is curved. Two contractile vacuoles are on left side.
25. *Diplodinium lobatum:*
The body measures 40-60 × 24-40 µm. Three prominent lobes are on left side of the macronucleus. Two contractile vacuoles are at both poles of the macronucleus.

GENUS: *EUDIPLODINIUM*
26. *Eudiplodinium magii:*
The body measures 110-220× 75-150 µm. One narrow skeletal plate extends downward near the posterior end of macronucleus. Macronucleus is hook or pistol like. Two contractile vacuoles. The rectum is large.

GENUS: *OSTRCODINIUM*
27. *Ostracodinium clipeolum:*
The body is ellipsoidal and measures 60-130 × 40-70 µm with one large skeletal plate board shaped and three contractile vacuoles between macronucleus and left body wall. Macronucleus has two left lobes. The body ends with large lobe.

GENUS: *METADINIUM*
28. *Metadinium banksi:*
Body is ellipsoid, measuring 118-162 × 75-118 µm, both body sides are slighty convex; posterior end issmoothly rounded. Two skeletal plates on

upper side generally fused posteriorly, the plates are not parallel. Rectun is wide and lined with longitudinal fibrils; anus is on upper side, slightly to right of main bodyaxis. Macronucleus consists of 3 lobes; while micronucleus is ellipsoid and lies in a depression anterior to middle lobe of macronucleus. Two contractile vacuoles between macronucleus and lower left body margin, one anterior to micronucleus and one in the depression between middle and posterior lobes.

29. ***Metadinium esalqum:***

Flattened ellipsoidal body measures 70-100 × 50-70 µm, left side is convex while right one is nearly flat. Two skeletal plates fused posteriorly at posterior three fourth of the macronucleus; which consists of main two lobes and two contractile vacuoles. The rectum is large.

30. ***Metadinium medium:***

The body measures 150-250 × 90-175 µm. two skeletal plates, fused at posterior end of macronucleus which consists of three lobes. The body ends with large rectum. ELYTROPLASTRON:

GENUS: ***ELYTROPLASTRON***

31. ***Elytroplastron bubali:***

The body measures 110-165 × 65-100 µm. Two medium width skeletal plates are on upper side, long skeletal plate on lower side and small plate on right side; while four contractile vacuoles between left edge of macronucleus and left side of body.

SUBFAMILY: OPHRYOSCOLECINAE

GENUS: ***EPIDINIUM***

32. ***Epidinium caudatum:***

Elongated twisted body with Left ciliary zone below anterior end of the cell and not parallel with adoral zone. Body around the main axis measures 80-140 × 35-55 µm. Macronucleus is club shaped. The body ends with one caudal spine.

33. ***Epidinium bicaudatum:***

Elongated twisted body around the main axis measures 80-140 × 35-55 µm. Macronucleus is club shaped. The body ends with two caudal spines.

34. ***Epidinium graini f. graini:***

The body is elongated and measures 70-125 × 35-50 µm. There are two transverse periplastic pellicle foldings resembling coronets. Skeletal plate complex is composed of three plates lying close together from left ventral edge of adoral zone to the end of cytoprocalt tube.

35. ***Epidinium graini f. caudatricoronatum:***
There are three transverse periplastic pellicle foldings resembling coronets.

36. ***Epidinium ecaudatum:***
It has an elongated twisted body around the main axis measures 100-150 × 35-60 µm. Macronucleus is club shaped. The body ends without caudal spine.

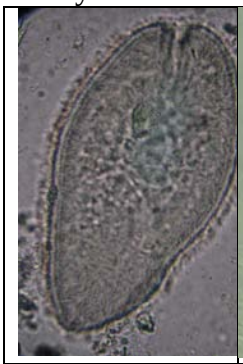
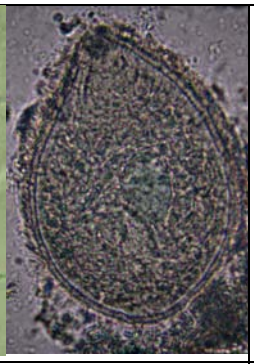
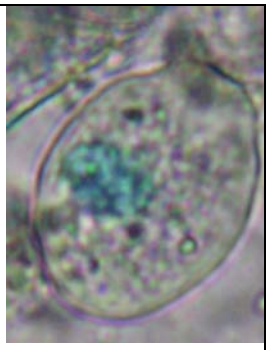
37. ***Epidinium ecaudatum f. cattanei:***
Body is elongated and twisted around the main axis measures 80-120 × 40-70 µm. Macronucleus is club shaped. The body ends with five caudal spines one on the right side, two on the left and one each on upper and lower side, body is relatively short.

GENUS: ***OPHRYOSCOLEX***

38. ***Ophryoscoles caudatus:***
The body is large measures 140-160 × 80-100 µm; and characterized by complicated spination and the long caudal spine which is nearly half length of the body.

39. ***Ophryoscolex purkynje***
The body measures 140-220 × 70-150 µm; and characterized by the long caudal spination which may be in two or three groups.

Fig. I: Photographic illustration of the identified species of rumen ciliates in dairy cattle: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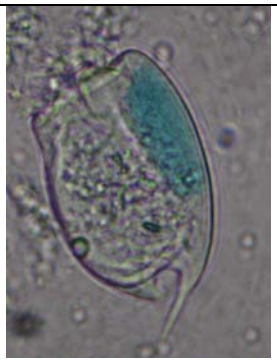
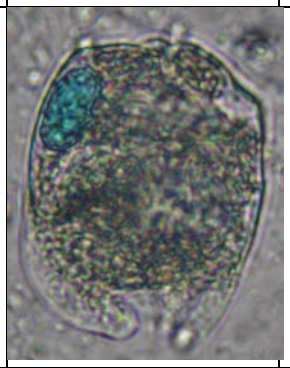
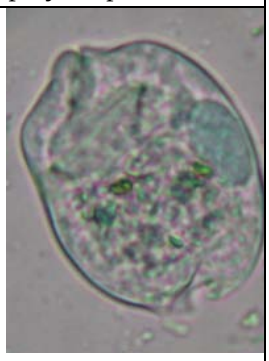
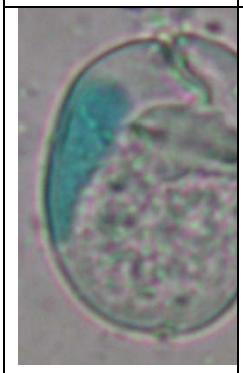
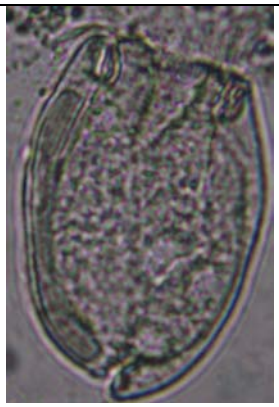
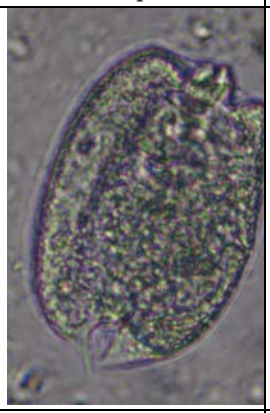
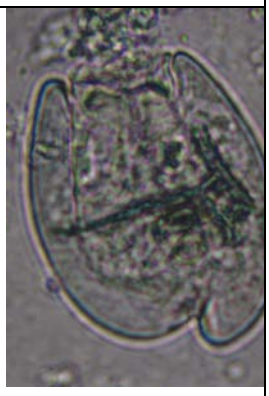
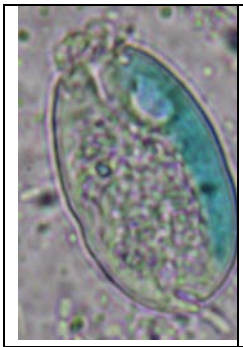
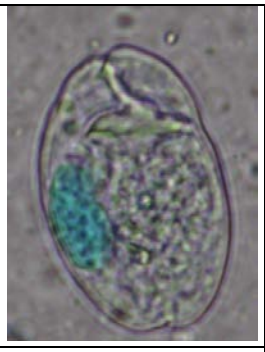
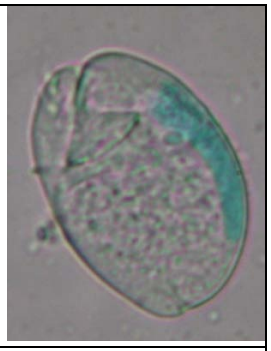
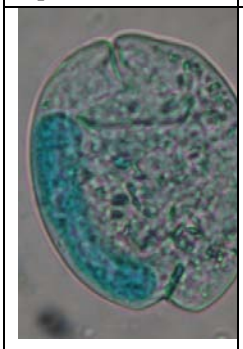
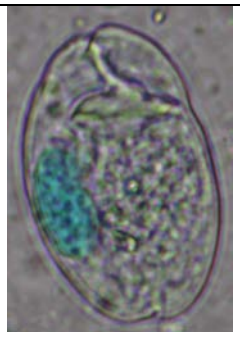
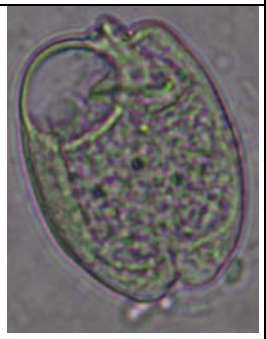
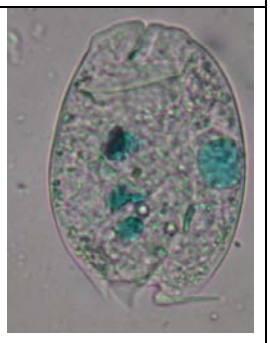
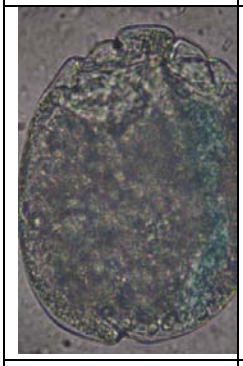
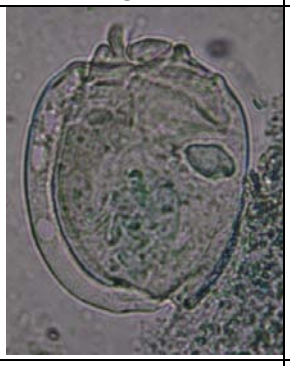
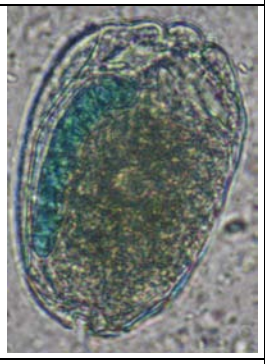
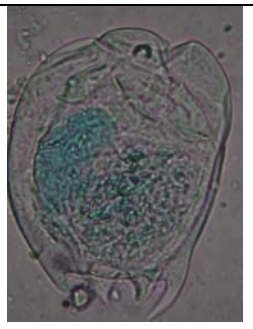
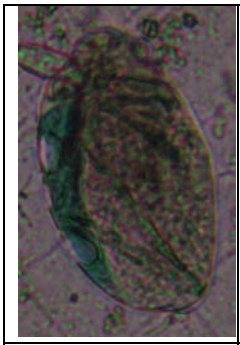
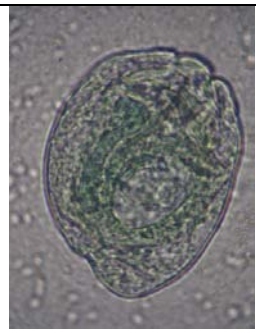
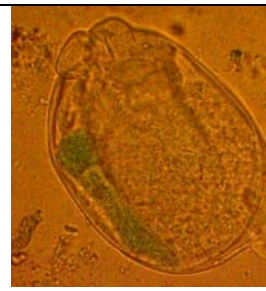
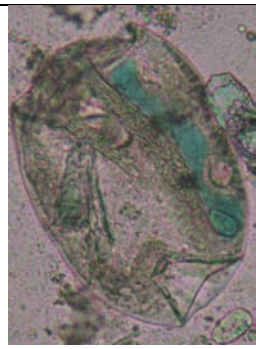
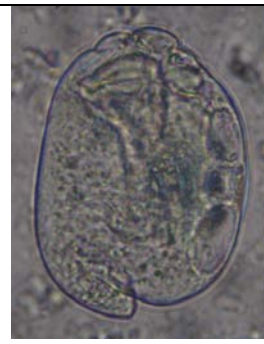
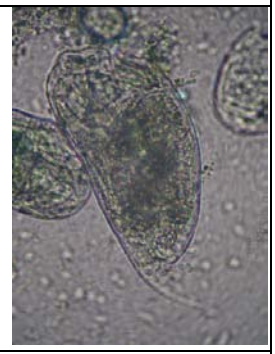
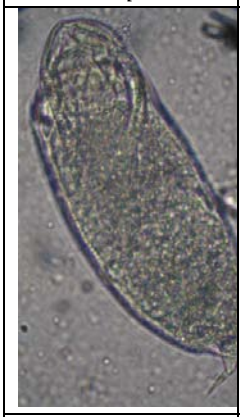
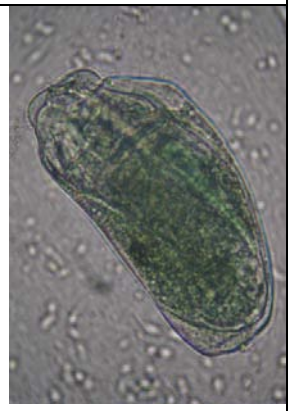
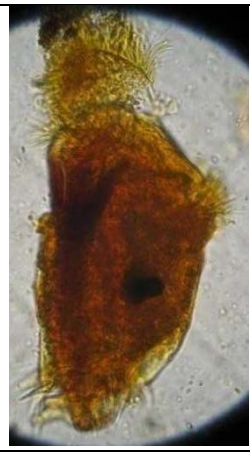
			
5.Charonina ventricularis	*6. Entodinium caudatum f. caudatum*	*7. Entodinium caudatum f. lobospin.*	*8. Entodinium williamsi f. turcicum*
			
9. Entodinium caudatum f. dubardi	*10. Entodinium Longinucleatum f. Longinucleatum*	*11. Entodinium Longinucleatum. f. spinolobum*	*12. Entodinium Yunnense f. yunnense*

			
13. Ent. Yunnense f. spinonucleatum	*14. Entodinium nanellum*	*15. Entodinium constrictum*	*16. Entodinium bovis*
			
17. Entodinium bursa	*18. Entodinium exiguum*	*19. Entodinium Imaii*	*20. Entodinium oktemae*
			
21. Diplodinium anisacanthum	*22. Diplodinium monocanthum*	*23. Diplodinium tetracanthum*	*24. Diplodinium dentatum*

			
25. Diplodinium lobatum	*26. Eudiplodinium magii*	*27. Ostrachodinium clipeolum*	*28. Metadinium banksi*
			
29. Metadinium esalqum	*30. Metadinium medium*	*31. Elytroplastron bubali*	*32. Epidinium caudatum*
			
33. Epidinium bicaudatum	*34. Epidinium ecaudatum*	*35. Epidinium ecaudatum f. cattanei*	*36. Epidinium graini f. graini*

		
37. Epidinium graini f. caudatricoronatum	*38. Ophryoscolex caudatus*	*39. Ophryoscolex purkynjie*

DISCUSSION

The available previous papers dealing with the rumen function were mainly focusing on the evaluation of pH, ammonia nitrogen concentration, volatile fatty acids percentages and fermentation indexes (Insung, et al. 1998, Bosi, et al. 2002, Melendez, et al. 2004 and Chung, et al. 2009). In comparison between SRL constituents of the dairy cattle fed on corn silage and other group fed on traditional rations (Table 1); the significant decrease in pH ($p<0.01$) was within the levels recorded by Insung, et al. 1998, Bosi, et al. 2002, Melendez, et al. 2004, Arelovich, et al. 2008 and Khampa, et al. 2009. The rumen ammonia nitrogen concentration showed a significant increase ($p<0.05$) with higher levels than that recorded by Yang, et al. 2001 and Masoera, et al. 2006. The significant increase in the level of total volatile fatty acids ($p<0.01$) was lesser than the levels mentioned by Yang, et al. 2001, Melendez, et al. 2004, Laugalis, et al. 2007 and Chung, et al. 2009. The significant increase in the level of rumen total protein ($p<0.001$), both of zinc and *Diplodinium* percentage ($p<0.01$); on the other hand the significant decrease ($p<0.001$) in the levels of phosphorus, copper and *Epidinium* percentage; with the significant increase in both of total protozoa count and calcium level ($p<0.01$) can be explained on the basis of interaction between these constituents (Krzywiecki et al 2006).

High negative correlation was recorded between pH and all of *Ophryoscolex*, calcium and cupper levels (Table 3); with high positive

correlation with phosphorus. The total volatile fatty acids showed high negative correlation with total protozoa count, which highly negative correlated with *Diplodinium*, *Holotricha*, calcium and copper levels and positively with *Ophryoscolex*, ammonia, total protein and zinc. In SRL biochemical constituents, high positive correlations between zinc, total protein and between calcium and copper were recorded; while high negative correlation between calcium and total protein and between calcium and zinc were obtained. It was interesting to find that in both groups the ratio between calcium and phosphorus levels was 1:2.

Rumen ciliates showed high negative correlation between total protozoa count and *Diplodinium* and *Holotricha* levels. High negative correlation between *Entodinium*, *Diplodinium* and *Holotricha* and inter-between *Diplodinium* and *Ophryoscolex* was present. The only high positive correlation was recorded between *Ophryoscolex* and total protozoa count. These correlations were in agreement with that recorded by Baraka and Dehority 2003 and Krzywiecki et al 2006.

Total protozoa count in dairy cattle fed on silage was nearly in the ranges recorded in Canada, Mexico and Thailand; while higher number was recorded in Egypt by Selim, et al. 1996. The number of genera and species was in agreement with that in Brazilian, Canadian, Kenyan and Turkish cattle (Table3). Even though, Genus *Polyplastron multivesiculatum* was not recorded and 16 species belonging to 6 genera were absent in all samples; It was interesting to record two new genera (*Buetschlia* and *Ophryoscolex*), and 20 new species belonging to 7 genera (one species in genus *Buetschlia*, 9 in *Entodinium*, 2 in *Diplodinium*, 3 in *Metadinium*, 2 in *Epidinium*, 1 in *Elytroplastron* and 2 in *Ophryoscolex*) in dairy cattle in Egypt.

CONCLUSION

The feeding of dairy cattle on corn silage reduced significantly total number of protozoa ($p<0.01$), increased significantly total protein level ($p<0.001$), both of total volatile fatty acids and zinc levels ($p<0.01$) and ammonia concentration ($p<0.05$) in rumen liquor; and highlighted the importance of investigating its effect on the blood biochemical constituents. Two new genera (*Buetschlia* and *Ophryoscolex*), and 20 new species belonging to 7 genera (one species in genus *Buetschlia*, 9 in *Entodinium*, 2 in *Diplodinium*, 3 in *Metadinium*, 2 in *Epidinium*, 1 in

Elytroplastron and 2 in *Ophryoscolex*) were recorded in dairy cattle in Egypt. All genera were demonstrated in figures and their dimensions were measured to be used in the investigation and banking of cattle ciliates. These data should be put in consideration during the physical, ciliates and biochemical examination and evaluation of rumen liquor status in dairy cattle.

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ANTIMICROBIAL ACTIVITY OF SOME HERBAL PLANTS IN EL-JABAL AL AKHDAR AREA AND THEIR PRESERVATIVE EFFECTS AGAINST MICROBIAL LOADS IN MEAT SLICES DURING COLD STORAGE

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ABSTRACT

This study was carried out to investigate the antimicrobial activity of some herbs against some microorganisms (*Staph. aureus*, *B. subtilis*, *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Candida albican*, *Aspergillus flavu*s and *penicillium chrysogenum*) which play an important role in spoilage of food and sometimes incriminated in food poisoning using the Disc Assay Procedure. In addition evaluation the effect of using of the powder of these plants as additives on the keeping quality of meat stored at refrigerator (microbiological and sensory properties of beef slices). It was found that the powders of these plants as well as their extracts possess an antimicrobial activity; therefore, they can be used in biotechnological fields as natural preservative ingredients in food which could prolong their shelf- life and improve their organoleptic characters.

Keywords: *Staph. Aureus,* food poisoning, Disc Assay, organoleptic .

INTRODUCTION

Meat is a highly favorable medium for growth of microorganisms, and it keeps well for only a short time at refrigeration temperature. The short shelf–life of meat is attributed to its perishable nature, sanitation, practices during handling and time and temperature of storage. Zheng et.al. (2005) reported that the growth of microorganisms on meat is one of the main factors that causes discoloration and spoilage, especially, in an environment of high relative humidity and

insufficient air exchange some microorganisms give rise to disagreeable odors and slime formation.

Furthermore, some microorganisms cause protein and fat degradation, changes in pigmentation and in turn reduce shelf- life of meat at refrigeration temperature. Among meat microbial flora are *E. coli*, *staphylococcus aureus*, Salmonella and yeasts and moulds (Buchanan and Gibbons, 1975). Antimicrobial agents including food preservatives have been used to inhibit food borne bacteria, fungi and extend the shelf life of processed food. Many naturally occurring extracts like essential oils from edible and medicinal plants, herbs and spices have been shown to possess antimicrobial functions and could serve as a source for antimicrobial agents against food spoilage and pathogens (Oussallah et al., 2006).

Spices and their essential oils are the most efficient natural antioxidants and antimicrobial agents which have long been used to preserve food (Mahmoud et al., 2006). The leafy part of plants such as Greek sage *Salvia fruticosa* (Family Lamiaceae), *Ocimum basilicum* (Family Lamiaceae) , *Pelargonium graveolans*(Family Geraniaceae) have been added to meat, poultry products , fish and food products for years. Being natural foodstuffs, they appeal to consumers who tend to question the safety of synthetic additives. It has been suggested that some synthetic chemicals convert some ingested materials into toxic substances or carcinogens by increasing the activity of microsomal enzymes (Farag, et al., 1989).

In restaurants, cafeterias and other food market shops, meat is handled in a couple of ways. The first way is to store meat in the freezer; then, thawed and refreeze for several times; which would destroy its nutritional and quality attributes. The other way of handling meat is to store it in the refrigerator for two or three days until consumed (This allows increasing microbial load which may lead to food poisoning). Hence, it is important to apply some treatments to refrigerated meat in order to inhibit growth; and in the same time , improve the flavor and other quality attributes of meat. El-Jabal AlAkhdar has a high

diversity of plant species that show both economic and medicinal importance. More than 100 species extensively used by Bedouins in folk medicine. Also many local plants are edible for human besides their traditional medicinal uses. El-Jabal Al Akhdar area possesses unique physiographic and climatic conditions that provides an excellent ecological niche and contributed to the restriction of many plant species.Among the most widely used Libyan plants are *Arbutus pavarii* [family Ericaceae], followed by *Pistacia lentiscus* [family Anacardiaceae] and *Myrtus communes* [family Myrtaceae]. There are apparently lack of published studies focusing on their chemistry and activities . The fruits of these plants are commonly considered as an edible fruits for human and animals.

So, the objective of this study was to investigate the antimicrobial activity of Greek sage, Basil, Pelargonium, Myrtle, Arbutus and Mastic against some microorganisms (*Staph. aureus*, *B.subtilis*, *E. Coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Candida albican*, *Aspergillus flavu*s and *penicillium chrysogenum*) which play an important role in spoilage of food and food poisoning using the Disc Assay Procedure. Also evaluation the effect of using of these plants as additives on the keeping quality of meat stored at refrigerator (microbiological and sensory properties of beef slices) .

MATERIALS AND METHODS

1. Preparation of microbial cultures

Eight microorganisms composed of bacteria , moulds and yeast used as test organisms. These microorganisms includes *Staph. aureus*, *B.subtilis*, *E. Coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Candida albican*, *Aspergillus flavu*s and *penicillium chrysogenum* . They obtained from food control department of Omar El-Mukhtar University. Stock culture of bacterial species were grown on blood sheep agar at 37^0C for 24h Nebahat et al. (2008). Yeast strain grown on PDA (Potato Dextrose Agar) for 3days at 25^0C while mould at 25 ^{0}C for 5 -7 days. Final cell concentration ranged from 10^6-10^7 cfu/ml (APHA,1992).

2. Preparation of plant materials

The leaves and stems of the six tested plants were collected from Al – Jabal Al Akhdar (El – Bieda city – Libya during 2010) .The plant materials were kindly identified by Prof. Dr. Mahmoud Ali Hassanan , Professor of medicinal and aromatic plants, Faculty of Agriculture, Omar EL-Mukhtar University, El Bieda. Leaves and stems of the plants under investigation were separately air-dried, powdered and kept in tightly closed amber colored containers .

3. Preparation of total alcohol extracts:

Thirtygrams of the air dried aerial parts (leaves and stems) of each studied plant were separately extracted with alcohol 90% using soxhlet apparatus till exhaustion. Each of the resulted extract was concentrated under vacuum by rotary evaporator. The residues left after distillation of solvent were weighed and kept in a desiccators (AOAC,1980).The percentages of alcohol extract was calculated and recorded in table (1).

Table (1): Percentage of alcohol extracts obtained from the studied leaves

Extract	Total alcohol
Myrtus communus	20.76 %
Pistacia lenticulus	45.00 %
Arbutus pavarii	83.73 %
Ocimum basillicum	16.30 %
Pelargonium graveolans	15.30 %
Salvia fruticosa	47.60 %

4. Preparation of beef slices

Fresh lean beef was purchased from the butcher shop at El – Bieda city. It was transferred to the laboratory in an ice box. The lean beef was boneless and trimmed of fat and connective tissues. Then ,the

beef was cutted into slices (weighing about 50 g with a size of about 5× 5 × 2 cm for each slice). The beef slices were covered by herbal additives powder as designed in table (2). Five slices of each treatment were packaged in polyethylene bags and were stored in the refrigerator (4 ± 1 ^{0}C). The polyethylene bags were held in single layers to ensure that each bag had similar exposure to surrounding air and light. Examination of beef slices were carried out at zero, 3rd, 6th, and 9th day of refrigerated storage.

Table (2): Formulations of herbal additives.

herbal additives	Constituents of herbal additives
1st treatment	Free from any herbal additives (control).
2nd treatment	5g onion+ 0.1 g black pepper+ 5 g Myrtus communus.
3rd treatment	5g onion+ 0.1g black pepper + 5 g Arbutus pavarii.
4th treatment	5g onion+ 0.1 g black pepper +5 g Pistacia lenticulus.
5th treatment	5g onion+ 0.1g black pepper +5 g Ocimum basillicum.
6th treatment	5g onion+ 0.1g black pepper +5 g Pelargonium graveolans.
7th treatment	5g onion+ 0.1 g black pepper +5 g Salvia fruticosa.

5- Paper Disc plate method

1ml of the bacterial and fungal culture was inoculated into 15 ml of sterile nutrient agar and Potato dextrose agar medium respectively. The inoculated agar was poured aseptically into sterile Petri plates. The agar was allowed to solidify. A sterile filter paper of 0.5 cm diameter was saturated with 0.6 µl of each alcohol extract solution and then it placed in the center of each Petri plates containing the inoculated specific agar. The plates were incubated at 37^{0}C/ 24hr while in fungi at 25^{0}C for 5 days according to (Lorian , 1980 ; Collins et al., 1989). The diameter of each inhibition zone was determined in mm. Diameter less than 5 mm. indicates no effect. Disc impregnated with alcohol is used as a negative control as well as discs of Cefotrioxan, and Nystatin were used as positive control for each microorganism.

6 -Microbial load

Aerobic plate count (APC), *Staph. aureus*, Enterobacteriaceae, Psychrophilic and mould & yeast counts of beef slices were determined

by using Nutrient agar , Baird Parker, Violet Red Bile Glucose, Crystal Violet Tetrazolium, Potato Dextrose agar media, respectively according to the procedures described by (APHA, 1992).Incubation were carried out at 32^0C/48hr for TPC; at 37^0C/24hr for *Staph. aureus*, Enterobacteriaceae; at 7^0C/10 day for Psychrophilic and 25^0C/ 5 day for yeasts and moulds counts.

7- Organoleptic evaluation

Organoleptic evaluation of cooked beef slices was carried out according to Watts et al. (1989)

Table (3): Antimicrobial activity of different extracts prepared from the six plant species under *investigation:*

Microorganism / Total Alcohol extract	*Staph. aureus*	*B. subtilis*	*E. Coli*	*P. aeruginosa*	*K. pneumoniae*	*C. albicans*	*A. flavus*	*p. chrysogenum*
Myrtus communus	**18**	**16**	**21**	**25**	**6**	**13**	**15**	**25**
Pistacia lenticulus	**18**	**17**	**15**	**5**	**19**	**16**	**--**	**12**
Arbutus pavarii	**15**	**20**	**6**	**25**	**--**	**6**	**--**	**13**
Ocimum basillicum	**--**	**6**	**13**	**16**	**--**	**6**	**--**	**19**
Pelargonium graveolans	**20**	**17**	**9**	**13**	**16**	**11**	**15**	**11**
Salvia fruticosa	**10**	**12**	**13**	**15**	**--**	**12**	**--**	**7**
Ceftrioxan	**7**	**13**	**30**	**23**	**26**	**--**	**--**	**--**
Nystatin	**--**	**--**	**--**	**--**	**--**	**21**	**13**	**22**

Inhibition zones = <5 [-(negative)] , 6-15 [+(weak)], 16-25 [++(moderate)], >25 [+++(high)]

Data in table (3) concerning the antibacterial and antifungal effect of Ethanol extract prepared from the six studied plants showed that these extracts possessed a broad spectrum effect against both the tested Gram positive and Gram negative bacteria , in addition to their

moderate and high inhibitory effect against both tested fungal strains *C. albican , A. flavus* and *P. chrysogenum*.Ethanolic extract obtained from *Myrtus communus* showed the least antimicrobial activity against *Klebsiella pneumoniae* with the high activity against the other tested Gram negative bacteria. At the same time this extract showed a moderate activity against the tested Gram positive organisms. In addition to its moderately high antifungal effect against *C. albicans* and *A. flavus* and highly antifungal effect against *P. chrysogenum*.

Ethanolic extract obtained from *Pistacia lentiscus* showed moderate antibacterial activity against most of the tested Gram positive bacteria , with the low activity against *P. areugenosa*. Moreover, this extract showed a moderately high activity against the tested Gram negative organisms. In addition to , the negative effect on *A. flavus.* and moderately high activity against the tested *C. albicans.* Ethanolic extract obtained from *Arbutus pavarii* showed the least antibacterial activity against most of the tested Gram negative bacteria [*Klebsiella pneumonia* and *E. coli*] with the high activity against *P. areugenosa*. Otherwise this extract showed a moderately high activity against the tested Gram positive organisms with a low antifungal effects.

Ethanol extract obtained from *Ocimum basillicum* showed the least antibacterial activity against Gram positive bacteria strains, while that of *pelargonium graveolans* and *Salvia fruticosa* possess higher inhibitory effects against *Bacillus subtilis* with a moderate effect on *Staphylococcus aureus* . Ethanolic extract obtained from *Pelargonium graveolans* showed the highest antibacterial activity against the Gram negative bacteria .Moreover, both extracts obtained from *Ocimum basillicum* and *Salvia fruticosa* showed a moderately high activity against *E. Coli* and *Pseudomonas aeruginosa* .In addition to ,their negative effect on *Klebsiella pneumonia*. Ethanolic extract obtained from *Pelargonium graveolans* showed the highest antifungal activity against the three tested strains, while the *Ocimum basillicum* extract demonstrated a highly inhibitory activity against the *P. chrysogenum* while a moderate inhibitoryactivity against *Candida albicans* only with no effect on *Asparagillus flavus*.

The total ethanol extracts obtained from *six plants species under investigation* exhibited a significant anti-bacterial effect against the examined Gram positive and Gram negative bacteria under investigation in comparison with the standard antibiotic Ceftrioxan and moderate antifungal effects in comparison with the standard anti fungal Nystatin. These results agreed with what is mentioned in the previous studies dealing with both the antibacterial and antifungal activities (Shin, 2003; Adam et al., 1998; Burt, 2004; Pepeljnjak et al., 2005; Oussallah et al., 2006; El Akrem et al., 2008; Nebahat et al., 2008 ; Oral et al., 2008).

From table (4) it could be concluded that, the lowest aerobic plate count (APC) was recorded at zero time of first treatment and at 3rd day of storage of 6th and 7th treatments (*Pelargonium graveolans* and *Salvia fruticosa*) .On the other hand APC increase during refrigerated storage by using 5th treatment (*Ocimum basillicum*).The psychrophilic bacterial count tend to be low for 3rd , 4th ,6th and 7th treatments by prolonged refrigeration , while the count increased in case of 5th treatment .

Staphylococcus *aureus* count was lowest for 4th , 6th and 7th treatments at the end of cold storage (9th day) . Enterobacteriaceae counts was lowest for 4th , 6th and 7th treatments while increased in 5th treatment .From the observed results it was found that the highest growth of mould and yeast occurred at 3rd treatment. Many studies have been conducted on the antimicrobial properties of herbs , spices and their derivatives such as essential oils and extracts. For instance, El Akrem et al. (2008) recorded that the antibacterial activities of essential oils in minced beef meat stored at 4-7 ^{0}c for 15 days were clearly evident.

Table (4): Bacterial growth in meat slices during refrigerated storage.

Treatments / Storage period	APC (cfu/g)	Psychrophilic Bacteria counts (cfu/g)	*Staph. aureus* (cfu/g)	Enterobacteriaceae counts (cfu/g)	mould & yeast counts (cfu/g)
zero time	2.1×10^2	8.0×10^2	2.0×10^2	3.5×10^2	1.1×10^2
1st treatment					
3 day	7.5×10^3	3.0×10^3	3.5×10^2	6.2×10^3	4.3×10^2
6 day	2.1×10^4	2.0×10^4	5.1×10^3	7.3×10^3	1.5×10^3
9 day	2.2×10^5	3.1×10^5	6.2×10^3	2.1×10^4	3.4×10^3
2nd treatment					
3 day	3.1×10^2	1.7×10^2	1.5×10^2	3.6×10^2	2.3×10^2
6 day	3.1×10^2	2.0×10^2	1.6×10^2	3.8×10^2	1.8×10^2
9 day	3.1×10^2	2.0×10^2	1.4×10^2	4.0×10^2	1.8×10^2
3rd treatment					
3 day	4.0×10^2	$<10^2$	2.0×10^2	1.9×10^2	2.5×10^2
6 day	3.2×10^2	$<10^2$	1.8×10^2	1.5×10^2	3.1×10^3
9 day	3.2×10^2	$<10^2$	2.0×10^2	1.5×10^2	2.4×10^3
4th treatment					
3 day	3.4×10^2	$<10^2$	$<10^2$	$<10^2$	5.1×10^2
6 day	3.5×10^2	$<10^2$	$<10^2$	$<10^2$	5.0×10^2
9 day	5.0×10^2	$<10^2$	$<10^2$	$<10^2$	5.0×10^2
5th treatment					
3 day	2.0×10^3	2.5×10^3	9.0×10^2	8.2×10^2	3.0×10^2
6 day	2.3×10^3	2.4×10^3	1.5×10^2	1.0×10^3	3.1×10^2
9 day	2.3×10^3	2.8×10^3	1.6×10^2	1.0×10^3	3.1×10^2
6th treatment					
3 day	$<10^2$	$<10^2$	$<10^2$	$<10^2$	1.3×10^2
6 day	$<10^2$	$<10^2$	$<10^2$	$<10^2$	1.2×10^2
9 day	$<10^2$	$<10^2$	$<10^2$	$<10^2$	1.2×10^2
7th treatment					
3 day	$<10^2$	$<10^2$	$<10^2$	$<10^2$	5.1×10^2
6 day	$<10^2$	$<10^2$	$<10^2$	$<10^2$	2.5×10^2
9 day	$<10^2$	$<10^2$	$<10^2$	$<10^2$	3.0×10^2

Recently, there has been much research into the health benefits conferred by the essential oils found in basil. Scientific studies have established that compounds in basil oil have potent antioxidant, anti-

cancer, antiviral, and anti-microbial properties (Bozin et al., 2006 ; Benedec et al., 2007). In addition, basil has been shown to decrease the occurrence of platelet aggregation and experimental thrombus in mice. It is traditionally used for supplementary treatment of stress, asthma and diabetes in India. The Chinese also use fresh or dried basils in soups and other foods. In Taiwan, people add fresh basil leaves to thick soups They also eat fried chicken with deep-fried basil leaves. Basil is commonly steeped in cream (Suppakul et al., 2003) .

Salvia *fruticosa* (Greek sage) family Lamiaceae is a perennial herb or sub-shrub native to the eastern Mediterranean, southern Italy, Israel, the Canary Islands, and North Africa. It has a long tradition in Greece-valued for its beauty, medicinal value, and culinary use, along with its sweet nectar and pollen. In Western cooking, it is used for flavoring fatty meats, cheeses and some drinks. In the United States, Britain sage is used with onion for poultry or pork stuffing and also in sauces. In French cuisine sage is used for cooking white meat and in vegetable soups. Germans often use it in sausage dishes, and sage forms the dominant flavoring in the English Lincolnshire sausage. Sage is also common in Italian cooking. Caution is indicated when used in conjunction with central nervous system stimulants or depressants(En.Wikipedia) .

There are ± 220 species within the genus *Pelargonium*, *Pelargonium graveolens* is a species in the *Pelargonium* genus, which is indigenous to various parts of southern Africa, and in particular South Africa. It is often called geranium This specific species has great importance in the perfume industry. It is cultivated on a large scale and its foliage is distilled for its scent. *P. graveolens* cultivars have a wide variety of smells, including rose, citrus, mint, coconut and nutmeg, as well as various fruits. Anti Bacterial & Anti Microbial: This property does not let bacteria or microbes develop on wounds and otherwise and keeps you safe from infections. The leaves are aromatic, balsamic, haemostatic and tonic. Recent research has revealed a substance in the plant that has an antibiotic action.

Myrtus (myrtle) leaves are aromatic, balsamic, haemostatic and tonic Recent research has revealed a substance in the plant that has an antibiotic action (Aidi Wannes et al., 2010). *Pistacia lentiscus* (Mastic) A 1985 study by the University of Thessaloniki and by the Meikai University discovered that mastic can reduce bacterial plaque in the mouth by 41.5 percent. A 1998 study by the University of Athens found that mastic oil has antibacterial and anti-fungal properties Alem et al. (2008). Another 1998 University of Nottingham study, published in the *New England Journal of Medicine*, claims that mastic can heal peptic ulcers by killing *Helicobacter pylori*, which causes peptic ulcers, gastritis, and duodenitis. Some *in vivo* studies have shown that mastic gum has no effect on *Helicobacter pylori* when taken for short periods of time **Al-Said et al. (1986) and Al-Saimary et al. (2002)** . However a recent and more extensive study showed that mastic gum reduced *Helicobacter pylori* populations after an insoluble and sticky polymer (poly-β-myrcene) constituent of mastic gum was removed and taken for a longer period of time. Further analysis showed the acid fraction was the most active antibacterial extract, and the most active pure compound was isomastic adienolic acid (Afef et al., 2006).

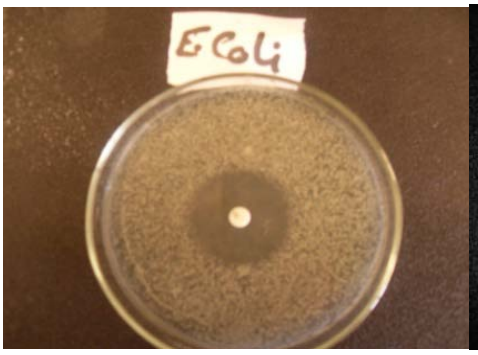
Table (5): Sensory evaluation of meat slices of different treatments at 9th day of refrigeration storage of meat.

Treatments	Evaluation of the sensory properties				
	Taste	texture	odour	colour	Overall acceptability
1st treatment	Appearance signs of decomposition (slimness, abnormal odour, proteolysis)				
2nd treatment	6.50	6.67	6.67	8.25	7.02
3rd treatment	8.92	8.75	6.75	8.42	8.21
4th treatment	4.42	4.17	6.08	8.25	5.73
5th treatment	6.58	6.58	6.25	8.25	6.92
6th treatment	8.50	8.92	9.92	8.17	8.88
7th treatment	8.83	8.75	6.50	8.67	8.19

From data recorded in table (5) it could be observed that sensory properties of beef slices (taste, odour, texture and overall acceptability) were significantly affected by constituents of each herbal. Hence, *Arbutus pavarii* ,*Pelargonium graveolans* and *Salvia fruticosa* had the best taste ,odour ,texture and colour. Treatment by *Pistacia lenticulus* came in the third order after *Myrtus communes & Ocimum basillicum*. Furthermore, control sample had signs of decomposition (slimness, abnormal odour, proteolysis).

Pelargonium graveolans herb

Salvia fruticosa herb



The inhibitory effects of standard antibiotic

The inhibitory effects of the alcohol extracts obtained from studied plant

CONCLUSION

It can be concluded that, the powdered plants or their extracts possess antimicrobial activity, and therefore, they can be used in biotechnological fields as natural preservative ingredients in food which prolong their shelf life and improve their Organoleptic characters.

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EPIDEMIOLOGICAL, GENETIC AND THERAPEUTICAL STUDIES ON *OTODECTES CYNOTIS* INFESTATION IN CATS AND DOGS

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ABSTRACT

Otodectes *cynotis* mite is a common parasite of cats and dogs, survives in the ear canal and causes otitis externa, itching and severe complications. The microscopic examination of ear swabs, skin scraps and faecal samples of 289 cats and 223 dogs revealed that mono-specific and mixed infestations of *Otodectes cynotis* in cats were (24.56%) and(6.57%) while in dogs were (7.17%) and (4.48%) respectively. The highest rate of infestation was in young cats and the lowest was in elder dogs. The mixed infestations were found in combination with *Sarcoptes, Demodex, Dermatophytes, Ticks, Fleas, Ascarids, Dipylidium* and *Isospora.* The RAPD-PCR proved the genetic divergence between cat and dog isolates whereas they are morphologically similar. Selamectin-pour on , Doramectin-subcutaneous injection and Ivermectin-Ear drops were evaluated two weeks post treatment. The rate of success in cats were (96.66%) ,(90.00%) and (83.33%) and in dogs were (77.77%), (75.00%) and (66.66%) respectively. It is concluded that Selamectin pour on is the best acaricide against *Otodectes cynotis* in both cats and dogs. It is also needed to prepare a vaccine in the future to prevent the infestation with *Otodectes cynotis* and its complications.

KEYWORDS: Otodectes, epidemiology, genetic divergence, acaricides.

INTRODACTION

Otodectes *cynotis* mites are non burrowing, white and very active parasites. They infest several species of animals including cats and dogs (**Scott et al., 2001**). It causes severe mechanical irritation due to the presence of the mite inside the ear leading to a higher activity of ceruminal glands and, consequently, the establishment of a favorable environment for secondary infections by bacteria or fungi (**August, 1988**). The infestation with *Otodectes cynotis* mite is termed otodectic mange (**Sweatman, 1958**). It is clinically observed as the infested

animals show discomfort, intense itch, excessive waxy material or pus and even audition interference, depending on the level of parasitism (**Gotthelf, 2000**). Although the importance of mites in cats and dogs as it causes external otitis, information regarding their prevalence and the factors influencing their survival is lacking (**Gram et al., 1994 ; Sotiraki et al., 2001**). The rates of infestation of *Otodectes cynotis* were studied in cats and dogs at different age groups. The morphological and genetic divergences between cat isolates and dog isolates of *Otodectes cynotis* were also studied. Finally, the complications of *Otodectes cynotis* infestations were recorded and the three acaricides were also evaluated in cats and dogs for treatment of *Otodectes cynotis* infestations.

Animals

Two Hundreds and eighty-nine (289) cats and 223 dogs were examined along two years in the Teaching Hospital of Department of Medicine and Infectious Diseases, Faculty of Veterinary Medicine, Cairo University. Age, breed, sex, clinical signs, complications and history of previous medications were registered for each examined animal.

Samples

Ear swabs and waxy materials from ears, skin scrapings and faecal samples were collected from the examined cats and dogs.

Acaricides

Selamectin pour on (Revolution®, Pfizer company), minimum dose is 6 mg/kg body weight applied topically on skin once, Doramectin injection (Dectomax®, Pfizer company), 1 ml/50 kg body weight injected subcutaneous once, Ivermectin (Iveen®, Adwia company) as ear drops once every 3 days, were evaluated for treatment of cats and dogs infested with *Otodectes cynotis* mites.

Ear swabs

Ear swabs from ear canals of both ears of the examined cats and dogs were collected and examined as described by **Richard and David (2001) ; Stephen and Dwight (2006)** with some modifications. Blackish way material of ear canal was collected by a disposable ear cotton swab. The collected materials were gently mixed with Tap water and examined microscopically under low, medium and high magnification power

lenses. *Otodectes cynotis* mite was counted per slide and their motility was recorded before and after treatment.

Skin scraping

Skin scraping was done in cats and dogs infested with *Otodectes cynotis* mite and suffered from hair loss and skin encrustation. Skin scrapings were done according to **Richard and David (2001)** with some modifications**.** Skin was scraped with a scalpel to collect skin tissue scraps. Skin scraps were gently mixed with sodium hydroxide 10 % solution and examined under a microscope to detect mites or dermatophyte. In few cases flea and ticks were visually detected during collection of skin scraps.

Faecal examination

Faecal smears were collected from cats and dogs infested with *Otodectes cynotis* mite and suffered from diarrhea. Faecal samples were collected and examined as previously described by **Chandler et al. (2004).**

Morphological and genetic divergences of Otodectes cynotis isolates between Cats and Dogs:

Otodectes cynotis mites detected in ear swaps of Cats and Dogs were morphologically studied regarding body and leg sizes as described by **Lohse et al. (2002)**. DNA from ear swaps containing *Otodectes cynotis mites* was extracted and measured. RAPD-PCR using three different primers was done according to (**Hugh and Annette, 1994**). The three primers were H-12 (5`-ACGCGCATGT-3`) (primer-1, P1) , T-20 (5`-GACCAATGCC-3`)(primer-2, P2) and V-07 (5`-GAAGCCAGCC-3`) (primer-3, P3), the primers and RAPD-PCR kits were produced by Gene tech company, Egypt. Three RAPD-PCR reactions were done for cat isolate DNA of Otodectes cynotis and the same reactions were done for dog isolate DNA of Otodectes cynotis using the three primers. One control negative reaction was carried out. Haem-III (DNA marker) was loaded onto gel to know DNA bands molecular weight. Each reaction was represented by small ependolf tube. Then each small ependolf tube containing separated reaction was spun to collect all reagents with each other. A 40 µl of paraffin oil was added to each reaction. Then was incubated and was labeled in small ependolf tubes in a set of PCR with the following program: -

Step-1:	*Intial denaturation* at (94C°/ 3 minutes).
Step-2:	*Denaturation* at (94C°/1 minute), annealing at (27C°/1 minute) and extension at (72C°/1 minute), (repeated 39 cycles).
Step-3:	*Final extension* at (74 C°/10 minute)
Step-4:	*The reaction was preserved* at 4 C°/ overnight
Results of RAPD-PCR reactions were detected by running of RAPD-PCR products with loading buffer in 1.5% agarose gel in 1X TAE buffer.Positive result was seen as bands on gel.	

Evaluation of Acaricides

Each *Otodectes cynotis* mites infested cat and dog was examined before and two weeks post treatment by examination of ear swabs from both ears. Three acaricides, Selamectin pour-on, Doramectin injection and Ivermectin ear drops were evaluated for treatment of *Otodectes cynotis* infestation. The evaluation was depending upon the number of *Otodectes cynotis* mites per swab, the status of mites (either living or dead or absent) and level of improvement of clinical signs especially amount of ear waxy material and degree of itching.

RESULTS

As summarized in tables-1 and 2, and figures 1, 2,3,4,5 and 6; the mono-specific and mixed infestations of *Otodectes cynotis* in cats were (24.56%) and(6.57%) while in dogs were (7.17%) and (4.48%) respectively. The highest rate of infestation was in young cats and the lowest was in elder dogs.

The mixed infestations of *Otodectes cynotis* mites with Sarcoptes, *Demodex, Dermatophytes, Ticks, Fleas, Ascarids, Dipylidium* and *Isospora* were detected. Complications of Otodectes *cynotis* infestations were observed as ear haematoma, in-co-ordination and imbalance, ear scratch and bleeding, deafness, fever and death. The morphological characters of Otodectes cynotis mites from cats and dogs regarding the body and leg sizes were similar. The RAPD-PCR proved the genetic divergence between cat and dog isolates whereas they are morphologically similar. Figure-6 showed that lane-1(Haem-III: DNA

marker), lane-2(cat isolate with primer-1), lane-3 (cat isolate with primer-2), lane-4 (cat isolate with primer-3), lane-5 (control negative), Lane-6(dog isolate with primer-1), lane-7(dog isolate with primer-2), lane-8 (dog isolate with primer-3).

The evaluated Selamectin-pour on , Doramectin-subcutaneous injection and Ivermectin-Ear drops showed variable rates of success for treatment of *Otodectes cynotis* mites two weeks post treatment. The rate of success in cats were (96.66%) ,(90.00%) and (83.33%) and in dogs were (77.77%), (75.00%) and (66.66%) respectively. Causes of treatment failure were recorded as presence of pus, presence of excessive amount of ear waxy material, the eggs of mite resist acaricides, presence of the source of infection such as the place or another in-contact animal.

Table-1: Mono-specific and mixed infestations of *Otodectes cynotis* in Cats and Dogs

An.	Age	*Otodectes cynotis* infested animals						*Otodectes cynotis* Non-infested animals	TOTAL
		Mono-specific infestation with *Otodectes cynotis*		Mixed infestations of *Otodectes cynotis*		TOTAL			
		(No)	(%)	(No)	(%)	(No)	(%)		
Cat	Young	30	10.38	12	4.15	42	14.53	85	127
	Adult	25	8.65	5	1.73	30	10.38	74	104
	Elder	16	5.53	2	0.69	18	6.22	40	58
	Total	71	24.56	19	6.57	90	31.14	199	289
Dog	Young	9	4.03	6	2.69	15	6.72	98	113
	Adult	5	2.24	3	1.34	8	3.58	59	67
	Elder	2	0.89	1	0.44	3	1.34	40	43
	Total	16	7.17	10	4.48	26	11.65	197	223
Total		87	16.99	29	13.00	116	22.65	396	512

Table-2: Evaluation of Acaricides.

Animal	Selamectin-pour on			Doramectin-injection			Ivermectin-ear drops			Total
	No of animals		Rate of success (%)	No of animals		Rate of success (%)	No of animals		Rate of success (%)	
	Treated	Recovered		Treated	Recovered		Treated	Recovered		
Cat	30	29	96.66	30	27	90.00	30	25	83.33	90
Dog	9	7	77.77	8	6	75.00	9	6	66.66	26
Total	39	36	92.30	38	33	86.84	39	31	79.48	116

Figure-1:Four Persian 4 weeks old kittens suffered from *Otodectes cynotis* and Dermatophytosis.

Figure-2:Two German shepherd 2 months old puppies infected with *Otodectes cynotis and Sarcoptes scabiei Var canis*

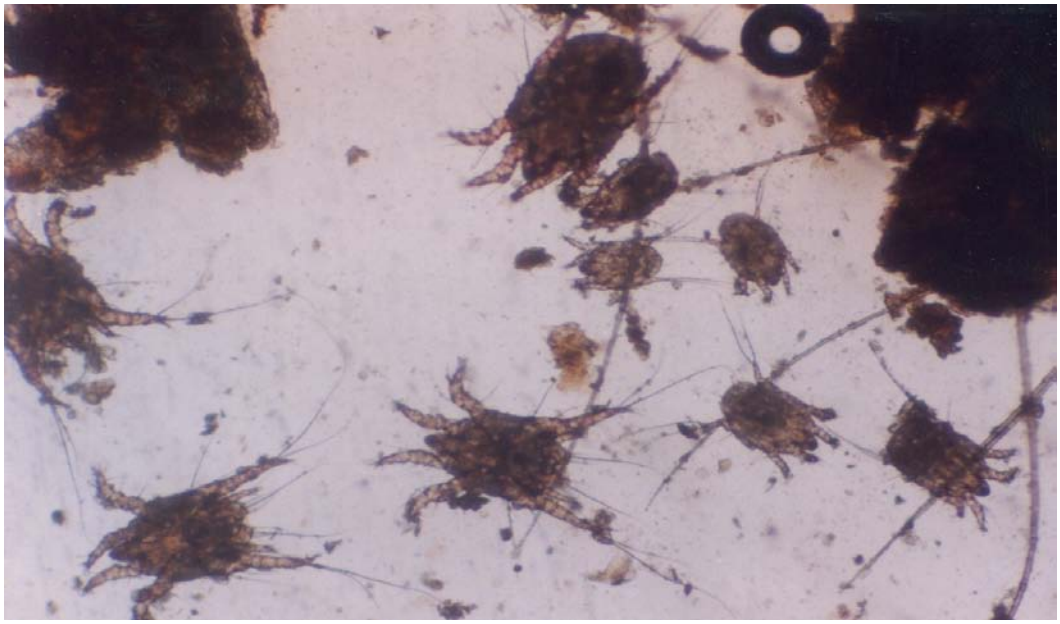

Figure-3: Different stages of *Otodectes cynotis*(40 X).

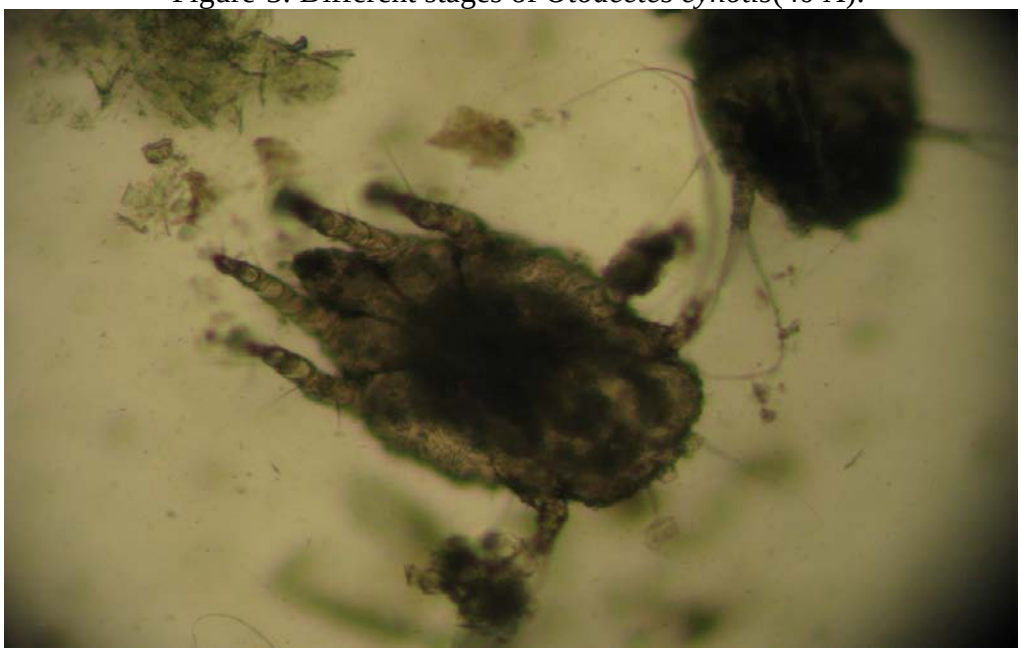

Figure-4: *Otodectes cynotis* adult (100 X) (Ventrodorsal view).

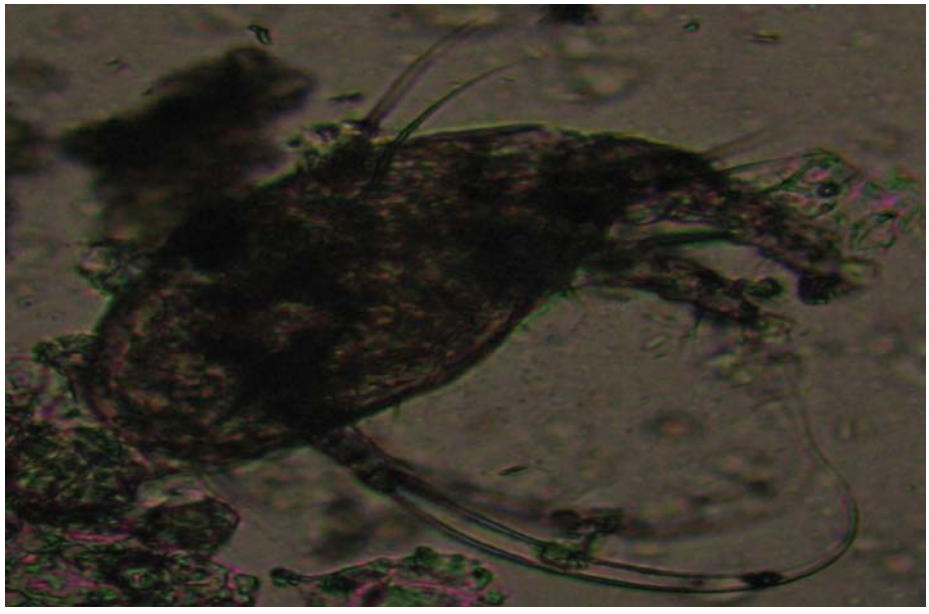

Figure-5: *Otodectes cynotis* adult (100 X) (Lateral view)

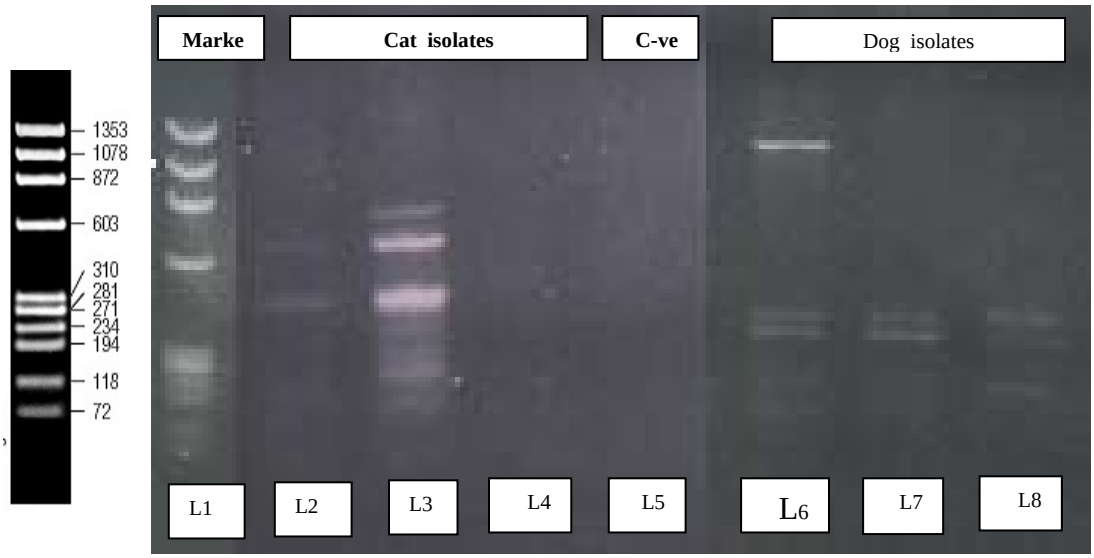

Figure-6: RAPD-PCR results, showing genetic divergences between cat and dog isolates of *Otodectes cynotis*. 8 lanes (L) of the RAPD-PCR products were
L1: DNA Marker (HaeIII Marker) L2: P1-Cat-isolate L3: P2-Cat-Isolate
L4: P3-Cat-Isolate L5: Control negative
L6: P1-Dog-Isolate L7: P2-Dog-Isolate L8: P3-Dog-Isolate

DISCUSSION

Otodectes cynotis mites can survive in ear canal of both cat and dog and as well as surrounding environment. It feeds on epidermal debris inside the ear and is very contagious and, they cause serious ear irritation and damage if not treated (**OIE, 2005**). Rates of mono-specific, mixed and the collective infestations of *Otodectes cynotis* in cats and dogs as illustrated in table-1, shows that the rate of mono-specific infestation with *Otodectes cynotis* was the highest in young cats(10.38%) and the lowest in elder dogs(0.89%). Also the mixed infestation of *Otodectes cynotis* with other parasites and dermatophytosis was the highest in young cats (4.15%) and the lowest in elder dogs(0.44%). The results showed that the mono-specific and mixed infestation of *Otodectes cynotis* in cats were (24.56%) and (6.57%), in dogs were (7.17%) and (4.48%) respectively. It is very clear that the infestation of *Otodectes cynotis* in cats is higher than that in dogs, which may prove the higher susceptibility of cats to *Otodectes cynotis.*

The other parasites infested cat and dog at the same time of *Otodectes cynotis* infestation also dermatophytosis accompanied *Otodectes cynotis* especially in cats. **Xhaxhiu et al. (2009**) found infestation rate of *Otodectes cynotis* in dogs as (6.7%), also they recorded the mixed infestation with three species of ectoparasites as 38.1% of *Otodectes cynotis* infested dogs. Also **Ugbomoiko et al. (2008**) reported the Overall prevalence of ectoparasites was 60.4% and of intestinal helminths 68.4% in Nigerian dogs. **Chee et al. (2008)** found *Otodectes cynotis* infestation as (22.3%) , they reported mono-specific and mixed infestations at the rate of (83.0%) and (17.0%) respectively in *Otodectes cynotis* infested dogs. **Rodriguez-Vivas et al. (2003)** reported the infestation of *Otodectes cynotis* in Mexican dogs was (3.5%).

Although the Cat and Dog isolates of *Otodectes cynotis* were morphologically similar in body and leg sizes , the RAPD-PCR showed the genetic divergence between cat isolates and dog isolates that was proved by presence of different RAPD-PCR bands pattern using three primers as illustrated in figure-6. The genetic divergence may explain the higher infestation rate of *Otodectes cynotis* in cats. We thought that there were two genetically different types of *Otodectes cynotis*, one in cats and another in dogs. That was reported by **Lohse et al. (2002)** who characterized the second internal transcribed spacer (ITS 2) of the rDNA of 16 *Otodectes cynotis* isolates from 11 cats, two dogs, one arctic fox and two ferrets originating from four different continents. In addition, mites from dog, cat and arctic fox were investigated morphologically. Sequence comparisons revealed five different, but closely related genotypes.

Complications of *Otodectes cynotis* were recorded (table-1) as ear haematoma, in-co-ordination and imbalance, ear scratch and bleeding, deafness, alopecia , fever and death. Those increased the importance of early treatment of Otodectes cynotis and change the concept of treatment to be prevention of *Otodectes cynotis* by vaccination. The three different acaricides with different three routes of administration were evaluated in both *Otodectes cynotis* infested cats and dogs as shown in (table-2). Selamectin-pour on , Doramectin-subcutaneous injection and Ivermectin-

Ear drops were evaluated in *Otodectes cynotis* infested animals. The ear swabs were examined in treated cats and dogs before and 2 weeks post treatment and number of *Otodectes cynotis* mites per swab, the status of mite either living or dead or absent, improvement of clinical signs especially amount of ear waxy material and itch were taken in consideration to evaluate acaricides.

The rate of success of Selamectin-pour on , Doramectin-subcutaneous injection and Ivermectin-Ear drops were recorded at the two weeks post treatment (in table-2) in cats as (96.66%) ,(90.00%) and (83.33%) and in dogs as (77.77%), (75.00%) and (66.66%) respectively. The highest rate of success was achieved by Selamectin-pour on in Cats and Dogs while the lowest was reported for Ivermectin-Ear drops in cats and dogs. It can be thought that, the possible causes of treatment failure were presence of pus that might interfere with action of Acaricides so we advise to clean ear canal and evacuate pus to enhance treatment success. Presence of secondary bacterial and/or fungal infection which prolonged the time needed for healing of ear skin . Presence of excessive amount of waxy material of ear that hinder arrival of acaricides to mite. Presence *Otodectes cynotis* eggs that might resist acaricides so newly developed living larva and numph were seen after two weeks post treatment. Presence of the source of infection (place or another animal) which could help in re-infection of already treated Cat or Dog, **(Six et al., 2000; Curtis, 2004; Krieger et al., 2005; Ghubash, 2006; Maggie et al., 2007).**

It can be concluded that, Otodectes *cynotis* is more prevalent in cats than dogs. The genetic divergence is clear between cat's isolates and dogs isolates of *Otodectes cynotis.* The Selamectin-pour on is the best acaricide for treatment *Otodectes cynotis* infestation in both cats and dogs. The future view should be concentrate on the preparation of a vaccine to prevent complications of *Otodectes cynotis* in cats and dogs.

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دراسات عن وبائية والتباين الوراثى ومضادات الحلم للحلم الاذنى فى القطط والكلاب

فايز عوض الله صليب

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يصيب الحلم الاذنى كلا من القطط والكلاب مسببا التهاب فى الاذن الخارجية ومضاعفات اخرى . الفحص الميكروسكوبى لمسحات اذن وقشطات جلد وعينات براز لعدد 289 قطة و223 كلب تبين ان نسبة الاصابة بالحلم الاذنى والاصابة المختلطة للقطط هى 56ر24% و 56ر6% على الترتيب وفى الكلاب 17و 7% و48ر4% على الترتيب وباستخدام اختبار RAPD-PCR تبين وجود تبيان وراثى بين الحلم الاذنى المعزول من القطط والمعزول من الكلاب .بتقيم عدة مضادات حلم لعلاج الحلم الاذنى تبين ان افضلها Selamectin-pour-on حيث ان نسبة نجاحه وصلت 66ر96% فى القطط و77ر77% فى الكلاب.

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PROTECTION AGAINST E.COLI INFECTION IN RABBITS BY CELL WALL EXTRACT VACCINE

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ABSTRACT

Cell wall extract of E.coli was found to be an efficient method for preparation of an effective vaccine against colibacillosis in rabbits. Challenge experiments revealed that this vaccine provided the best protection compared with whole cell formalinized inactivated vaccine. The degree of protection conferred by the vaccine was positively correlated with the results of histopathological examination and (IgA*)* as detected by indirect fluorescent antibody technique.

INTRODUCTION

Prevention of infectious diseases through vaccination is an efficient method of protecting rabbits. Effective prevention can be achieved through proper use of vaccine .Viral diseases such as rabbit hemorrhagic disease virus (RHDV) is prevented by vaccination with inactivated form of virus **(Arguello, 1991).**Bacterial diseases such as rabbit pasteurellosis can be controlled by using the inactivated or attenuated form of bacteria as a vaccine**(Lu and Pakeo, 1981),**

Rabbit colibacillosis is one of the predominant bacterial disease affecting rabbit industry .Enteropathogenic E.coli are only class of pathogenic E.coli responsible of sever enteric diseases in suckling and weaned rabbits, with considerable economical impact in industrial fattening farms **(Boullier et al., 2003).** The disease in suckling rabbits is characterized by yellow diarrhea with soiling of hind quarters, and the mortality reaches 100% while in weaned rabbits, the animal show watery to mucoid diarrhea withough blood. Mortality is mostly between 5%-50%% according to severity of the pathogenic strains, **(Peeters et al., 1988).**

The prevention of Escherichia coli through administration of antimicrobial agents is costly and not always effective method due to

incidence of resistant strains (**Formmer et al., 1994)**. This vaccination could be one of the major important concepts.

The potential success of vaccination against rabbit colibacillosis depend on the antigen used and the method of administration. Two studies demonstrated that immunization can prevent the disease. In the first study, an orally administrated vaccine from formalin killed E.coli protected weanling rabbits against challenge with homologous live organisms **(Camguilhem and Milon, 1990)**, while in the second study,oral administration of live non-pathogenic e.coli protected weanlings against heterologous challenge **(Milon et al., 1989)** It was found that, vaccination with the first vaccine did not completely protect against challenge, while vaccination with latter was not save

The objective of the present study is to evaluate the cell-wall extract of E.coli, as an efficient method for the preparing a new E.coli vaccine of a higher potency against rabbit colibacillosis.

MATERIAL AND METHODS

Bacterial strain

The present work include E.coli serogroups O26/2+ and O128/8+, isolated and identified locally by the aerobic bacterial vaccine department. Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo. These strains was found to be the most common cause of colibacillosis in rabbits .The relative pathogenicity of these strains was re-evaluated in one month old susceptible rabbits before vaccine preparation and pre-challenge

Experimental animals

A total of thirty white New Zealand rabbits to be (one month old) weighing about 350-450 grams were used. All rabbits were confirmed coccidian free. The rabbits were fed a daily maintenance ration and kept under strict hygienic measures in special cages

Vaccine preparation

Preparation of dense culture of E.coli

Each strain of *E.oli* (O26/8+ and O128/2+)was separately seeded into Minica broth medium and incubated aerobically for,24 hours at 37^0C

.The culture of each strain was adjusted at a concentration of $3.8X10^9$ Colony Forming Unite(CFU)/ml. Samples from E.coli cultures were streaked for sterility purity on MacConkey agar media **(Milon et al., 1989) .**

Cell wall Extract vaccine

The vaccine was prepared from the extract of the cell wall of E.coli serotype O26 andO128 According to **Henric (1994)**. The extract was concentrated to 1mg /ml representing the vaccine dose per rabbits.

Inactivated vaccine

A 37% formaldehyde solution was added to bacterial suspension (equal volume of E.coli O26 andO128) to a final concentration of 0.5% according to **Camguilhem and Milon (1990).**

Quality control of the prepared vaccine

The final prepared vaccine in present study was tested for purity, sterility and safety tests according to standard international protocols as described by **British Veterinary Codex (1970) and Code of American Federal Regulation (1985)**

Vaccination

The experimental rabbits were divided into three equal groups (10 animal of each group), the rabbits in the 1st group were orally inoculated on days 0, 7, 14. with (1mg/ml)/animal of cell wall extract E.coli vaccines, while the rabbits in the 2nd group were orally administrated with 2ml /animal with inactivated vaccine at the same intervals The 3ed group was kept as unvaccinated controls.

Evaluation of the immune response of vaccinated rabbits

Flurescent antibody technique

Specimens of ileum, caecum and colon were quick frozen in isopentanein dry ice, and then they were sectioned to a thickness of 6-8 um in a cryostat at -20°C .The sections were stained with labeled fluorescent antibody to secretory IgA.Then it washed with saline and examined under florescent microscope **(Cantey and Blake, 1977)**

Challenge procedure

On day 28 all vaccinated and non-vaccinated rabbits were subdivided into two groups (five for each) and each one were challenged by oral administration of 2ml a fresh broth culture containing $2x10^4$ virulent *E.coli* serotypes O26 and O128.All rabbits were observed for 10 days after challenge for signs of weakness, diarrhea, and death. Each dead animal was autopsied and subjected to postmortem examination for any characteristic lesions**.**

Reisolation

Attempts were made to re-isolate the challenge organisms from caecal contents of freshly dead or scarified diarrheic rabbits.

Histopathological examination

Specimens of ileum, caecum, and colon from both vaccinated and control rabbits were examined for detection of the local mucosal immune response according to the method described by **Culling (1976) .**The results were interpreted according to severity: (+++ =severe diarrhea and sever intestinal lesions, ++ = moderate diarrhea and moderate intestinal lesions, + =slight diarrhea and slight intestinal lesions, - = No diarrhea and no intestinal lesions).

Protective indexes (PIs)

Using the following formula, PIs were assessed according to the incidence of clinical signs (CS), mortality (M), and PM lesions (PML) **(Timms and Marshall, 1989)**.

$$PI = \frac{\%(\text{CS, M OR PML}) \text{ in control} - \% \text{ in vaccinates} \times 100}{\% \text{ in controls}}$$

RESULTS AND DISCUSSION

Vaccination, when available, is undoubtedly the most cost-effective means for preventing and controlling, and even eradicating, viral and bacterial infectious diseases**.** Vaccination of animals serves many different purposes, such as controlling animal infections and infestations, thus improving animal health and animal welfare (**Bernard Vallat, 2007)**

The results of sterility test of the prepared vaccines revealed that these vaccines were free from any contaminants (aerobic, anaerobic bacteria, fungus and mycoplasmas) Concerning safety of the prepared vaccines ,it was found that rabbits vaccinated even with double vaccine did not show any abnormalities or adverse reactions.

Immunity against Rabbit colibacillosis depends largely on activation of cell-mediated responses, and gamma interferon has been shown to play a crucial role in this process in rabbits. Since the intestine is normally the organ in which infection is initiated and is the major site of pathology, immune responses in the intestine play a significant role in restricting initial infection with E.coli. The aim of the present study is to stimulate efficient immunity in the intestine by targeting the gut mucosa (**Mark et al., 2002).**

It can be cleared from the results given in **photo (1)** that conducting of IFA for detection of IgA in the intestinal tissue sections that intensely fluorescent reaction (++++) could be noted in intestine of rabbits vaccinated with cell wall extract vaccine , meanwhile clearly fluorescent reaction (++) was noted in intestinal tissue sections of rabbits vaccinated with inactivated vaccine **(photo 2)** . Negative fluorescent reaction (-) was observed in intestinal tissue sections of control unvaccinated rabbits. These results were explained previously by **O'Hanley and Cantey (1981)** who elicited that The synthesis and secretion of secretory IgA antibody were major components of the immune response of the ileum after infection with an invasive bacterium.

The results of challenge test (table 1) revealed that rabbits vaccinated with cell wall extract vaccine showed a striking reduction in mortality , intestinal lesions with protection of percentage 80% ,while the rabbits vaccinated with inactivated whole culture vaccine showed a relative higher mortality , intestinal lesions with protection of 60%.The control group showed extensive sever intestinal lesions with high mortality . the results indicated that challenge procedure could be considered as a parameter for evaluation of E.coli vaccines a described by **Pangraphy (1983**).

Histopathological examination of duodenum of rabbits vaccinated with cell wall extract vaccine or vaccinated with inactivated vaccine showed an filtration of inflammatory cells, including macrophages, lymphocytes, with hyperplasia in sub mucosal gland and slight edema in sub mucosa (photo 3, 4&5). These results agreed with those reported by **Rott et al. (1996**) who stated that a lymphocyte population that efficiently circulates to sites of mucosal inflammation. The rapid induction of these cells appears to play a crucial role in acquired immunity at mucosal surfaces **(Feng et al., 2000).** The efficiency of cell wall E.coli vaccine in protecting rabbits against challenge suggested that, some of the important immunogenic determinants are expressed. Antibody to these determinants may provide effective protection to vaccinated rabbits, these suggestion agreed with those of **Syuto and Mastumoto (1982)**. In conclusion, locally prepared E.coli extract vaccine elicits a specific protection against rabbit colibacillosis infection with E.coli.

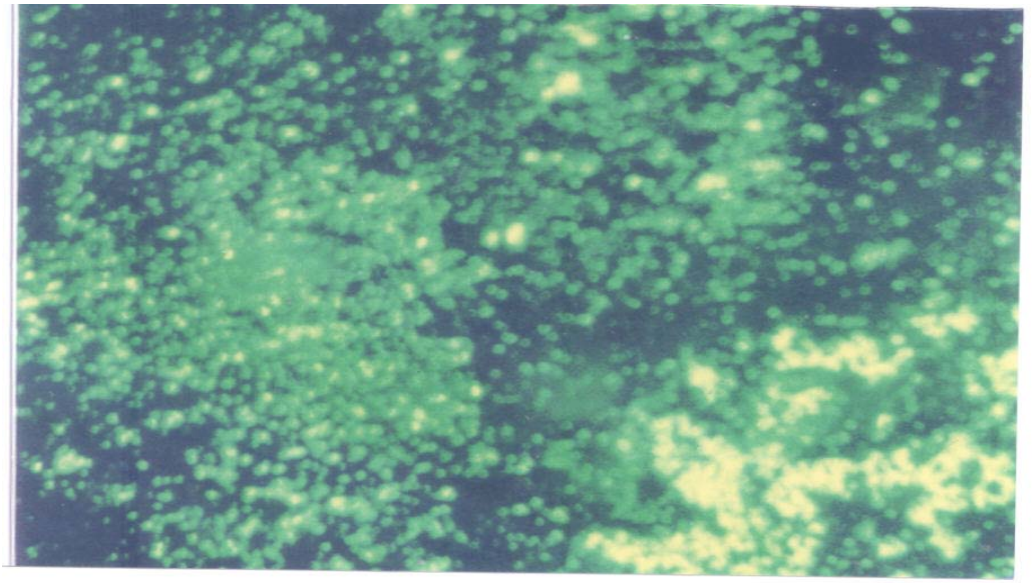

Photo (1) : intensely fluorescent reaction (++++)in intestine of rabbits vaccinated with cell wall extract vaccine

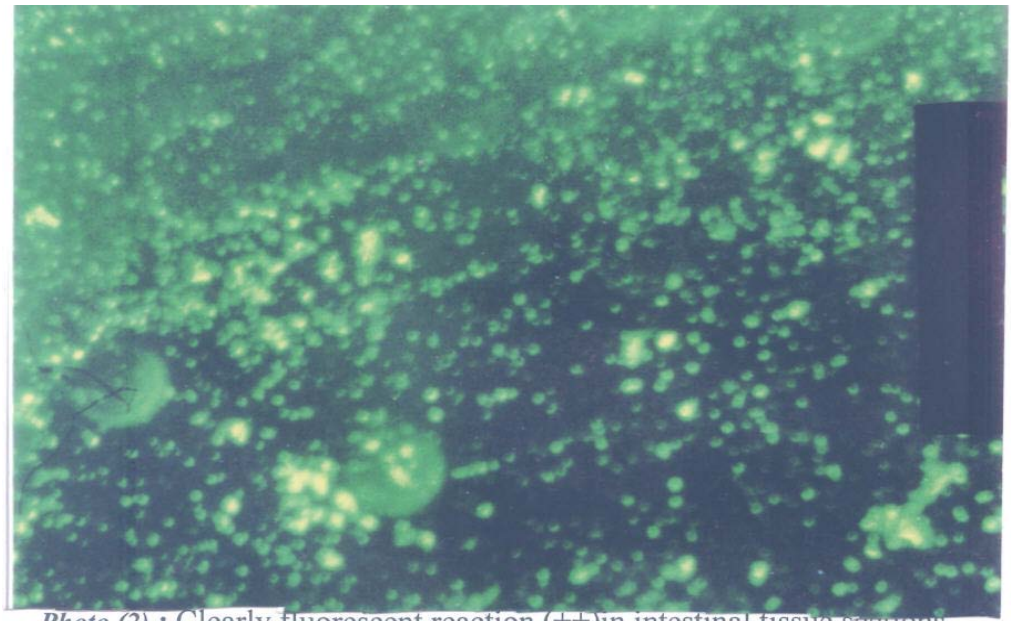

Photo (2) : Clearly fluorescent reaction (++)in intestinal tissue sections rabbits vaccinated with inactivated vaccine (photo 2)

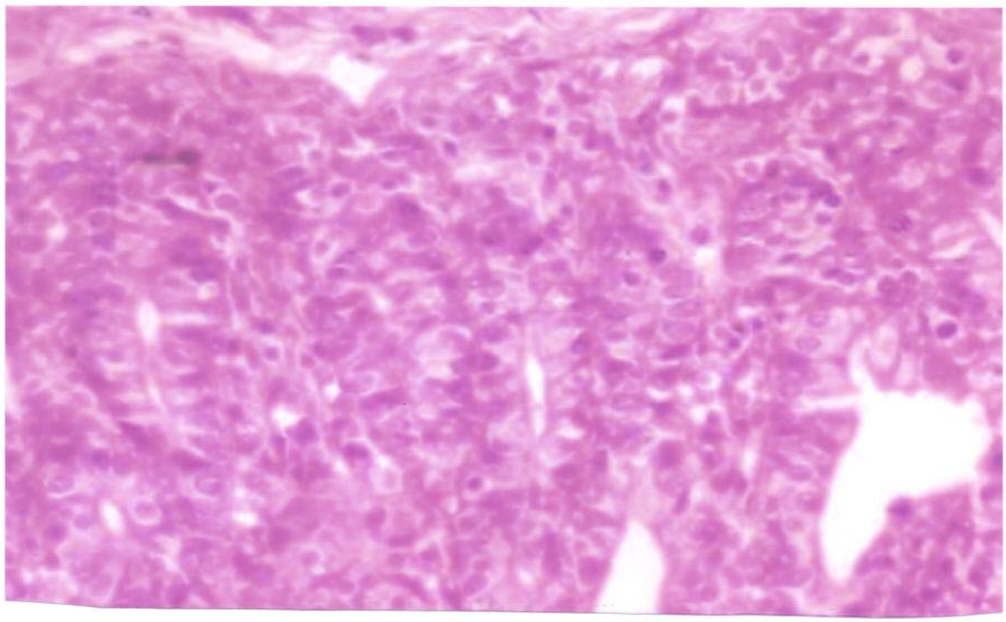

Photo (3): Intestine of rabbit vaccinated with cell wall extract vaccine showing leucocytic cell infiltration in lamina propia (H&E x 200)

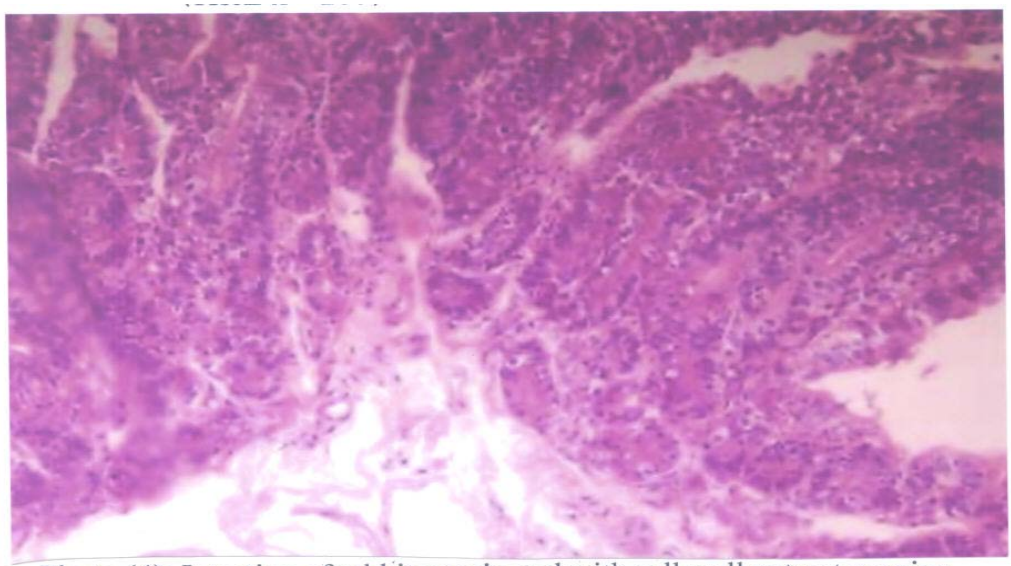

Photo (4): Intestine of rabbit vaccinated with cell wall extract vaccine showing submucosal edema . (H&E x 100)

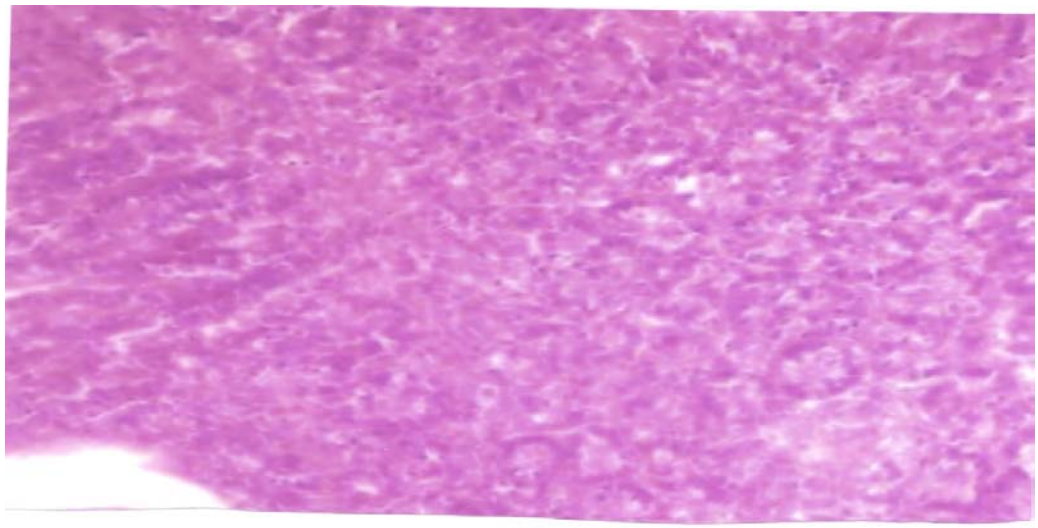

Photo (5): Intestine of rabbit vaccinated with cell wall extract vaccine Showing Hyperplasia of submucosal gland (H&E x 100)

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الحماية ضد عدوى الايشيريشياكولاى في الأرانب باستخدام لقاح مستخلص من الغلاف الخلوي

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تعتبر عدوى الايشيريشياكولاى في الأرانب من أهم المشا كل المرضية و التي تسبب خسائر اقتصادية كبيرة لما تسببه من حالات إسهال و التي يتبعها نسب نفوق عالية في الأرانب الرضيعة , ووقت الفطام .وقد أجريت هذه الدراسة بغرض تحضير ل قاح نوعى ضد هذا المرض باستخلاص الغلاف الخلوي لميكروب الايشيريشياكولاى المسبب للمرض كوسيلة متقدمة للسيطرة على هذه المشكلة المرضية.

وقد أوضحت الدراسة من خلال اختبار التحدي باستخدام العترة الضارية لميكروب الايشيريشياكولاى في الأرانب المحصنة باللقاح المست خلص من الغلاف الخلوي لميكروب الايشيريشياكولاى قد أعطى حماية عالية مقارنة باللقاح الخلوي المثبط بالفورمالين. جاءت نتائج الفحص النسيج ي لعينات الأمعاء من الأرانب المحصنة بكلا اللقاحين مطابقة لنتائج اختبار التحدي . أمكن باستخدام اختبار الفلوريسين الغير مباشر التعرف على الأجسام المناعية الموضعية IgA وكان التفاعل المناعي أقوى فى عينات الأرانب المحصنة باللقاح المستخلص من الغلاف الخلوي مقارنة بعينات الأرانب المحصنة باللقاح الخلوي المثبط بالفورمالين.

ومن هذا يمكن استنتاج أن اللقاح المحضرمن الغلاف الخلوي لميكروب الايشيريشياكولاى أنة امن في الاستخدام و ذو فاعلية قوية عن اللقاح المثبط بالفورمالين.

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THE PREVALENCE OF *ECHINOCOCCUS GRANULOSUS* INFECTION IN DOGS AND THE PARASITIC RISK IN LIBYA

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ABSTRACT

The parasites causing cystic echinococcosis (CE) are transmitted to man and domestic animals either directly or indirectly from dogs. The role of the dog as a definitive host for a number of zoonotic parasites has been widely studied and recognized as being a significant public health problem worldwide, especially in developing countries and communities that are socioeconomically disadvantaged. In these communities, poor levels of hygiene and overcrowding, together with a lack of veterinary attention and zoonotic awareness, exacerbates the risk of disease transmission. The distribution of *Echinococcus* in 151 dogs was studied in 14 localities that differ in socio-economic status in Libya. One hundred and fifty one stray male and female dogs in different age groups were studied and their small intestines examined for *Echinococcus granulosus*. Forty two (27.8%) harbored several E. *granulosus* in their intestinal content. The prevalence rate was higher in females (29.6%) than in males (25.7%). The prevalence of infestation was generally higher in the coastal area. The infestation was 1 to 200 worms in 24 infested dogs in the areas that were low rated and 201-1000 worms in 10 infested dogs in areas moderately rated while it was over 1000 worms in eight infested dogs in highly rated areas. The maximum recorded numbers of worms was 1282 in a four year-old dogs. We also noticed that the rate of infestation differed with the age of dogs in which it was 12.5 %, 36.6 %, 19.3%, 44.2 % and 14.3. in dogs that aged up to one year, 2 years old , 2 to 3 years old , 3 to 4 years old and over 5 years old, respectively.

INTRODUCTION

Public-health problems caused by the impact of dogs on humans are both direct and indirect, e.g. environmental pollution, contact injuries, and zoonosis **(Baxter, 1984).** Dogs are associated with more than 60 zoonotic diseases **(Dar and Taguri, 1979)** among which, parasitosis and, in particular, helminthosis, can pose serious public-health concerns worldwide **(Rubel and Wisnivesky, 2005).** Many canine gastrointestinal parasites eliminate their dispersion elements (eggs, larvae, oocysts) by the faecal route.

The quantity of canine faeces deposited on public and private property in cities worldwide is both, a perennial nuisance and an important health issue **(Matter and Daniels, 2000).** Public sites such as playgrounds, parks, gardens, public squares, and sandpits may be an important source of human infection **(Rubel and Wisnivesky, 2005).** High levels of human infection have been frequently described in sheep-rearing areas of the world, where the infection cycles between dogs and sheep through the use of working dogs and the feeding of sheep offal to dogs **(Hayward, 2004)**.

Echinococcus granulosus, the Hydatid Tapeworm, is a tiny parasite of the small intestines of dogs, dingoes, foxes, and also wolves, jackals, coyotes, and African Lions **(Thompson, 1982),** causing no disease and no symptoms, even in massive numbers.Each worm produces about 1,000 eggs every two weeks, and dogs can carry up to 300,000 worms, although domestic dogs do not usually carry such large numbers.Dingoes, on the other hand, and wild dogs infected with the "sylvatic strain" of *Echinococcus*, commonly carry heavy burdens **(Thompson et al., 1985; Bryan and Schantz, 1989)**. Passage of large numbers of eggs in the faeces of dogs, especially mobile dogs, results in widespread contamination of pasture, bush land etc. Eggs are spread over wide areas by wind, insects, birds and the like, and a single dog could infect up to 30,000 hectares **(Jenkins and Morris, 1991)**. Eggs are susceptible to desiccation, but are very cold tolerant, and may survive in the field for at least a year **(Dunsmore and Shaw, 2000)**. They are immediately infective. So, the aim of work was to evaluate the prevalence of infection with *Echinococcus* spp. in dogs in 14 areas of Libya and describe the role of dogs as a definitive host for the transmission of the parasite in Libyan society.

MATERIALS AND METHODS

Study area

Fourteen sites in different localities in Libya were chosen for the study from the period of October 2008 to March 2009.

Collection of dogs

Six months or over five years old male and female dogs were killed by shooting or were baited with strychnine. The killed animals were sent to

the laboratory for further processing. The animals were dissected under complete aseptic condition to get the small intestine then the intestine was tied off and removed. The parasite material was removed within 24 hours after the death of the host, cleaned from the host tissue as much as possible, and stored in 70% ethanol.

Parasitological examination

Examination of the intestine and tape worm count**.** Necropsy and examination of the intestines was carried out following strict safety precautions as described by **Deplazes and Eckert (1996)** (e.g. separated laboratory, protective clothes, deepfreezing of intestines at -80oC for at least 4 days). Two techniques were performed. The intestinal scraping technique (IST) was done as described by **Deplazes and Eckert (1996)** using 15 deep mucosal scrapings which were taken from equally distributed sites of the small intestine. The intestinal sedimentation and counting technique (SCT) was performed as described by **Rausch et al. (1990)** with modifications.

Briefly, the small intestine was incised longitudinally and cut into 5 pieces of approximately the same length. These pieces were transferred to a glass bottle containing 1 liter of 0.9% NaCl-solution. After shaking the bottle vigorously for a few seconds, the pieces of intestine were removed and the superficial mucosal layer stripped by means of pressure between thumb and forefinger to dislodge any attached helminths. After sedimentation time of 15 min the supernatant was decanted and the bottle refilled with physiological saline solution. This procedure was repeated 2-6 times until the supernatant was clear. The sediment fraction was examined in small portions of about 5-10 ml in square Petri dishes in transmission light under a stereomicroscope at a magnification of 120 xs.

The whole sediment was checked if up to 100 worms were found; if higher numbers were present the total worm burden was calculated from the count of 1 subsample. A random sample of the total worms collected were mounted on slide for confirmation of their identification .*Taenia* specimens were submitted to Common Wealth Institute of Parasitology if the identification was in doubt.

RESULTS

Out of 151dogs from the 14 localities, only 42 were infected with E. granulosus (27.8 %) (table1). The prevalence of infestation was generally higher in the coastal area. The infestation was 1 to 200 worms in 24 infested dogs in the areas that were low rated and 201-1000 worms in 10 infested dogs in areas moderately rated while it was over 1000 worms in eight infested dogs in highly rated areas. The maximum recorded numbers of worms was 1282 in a four year-old dogs. the rate of infestation differed with the age of dogs in which it was 7.1 %, 9.5 %, 26.2%, 54.8 % and 2.4 in dogs that aged up to one year, 2 years old, 2 to 3 years old, 3 to 4 years old and 5 years old, respectively (table 2). The prevalence rate was higher in females (29.6%) than in males (25.7%).

Table1. The prevalence of E. grsnulosus in the gastro-intestinal tract of the infected dogs.

The locality	Total No. of infected animals	Low prevalence(1-200)	Medium prevalence (201-1000)	High prevalence (more than 1001)
Benghazi	2	-	1	-
Garian	4	3	-	1
Khomes	2	2	-	-
Hon	1	1	-	-
Miarata	4	3	-	1
Naloot	1	1	-	-
Sebha	1	-	-	1
Sirte	3	2	1	1
Tripoli	11	8	2	1
Tubruk	8	1	5	2
Turhuna	5	3	1	1
Gdames	-	-	-	-
Socna	-	-	-	-
Ghat	-	-	-	-
Total	42	24	10	8

Table 2. Water quality measures during the mass mortalities event compared to the average normal values.

Water quality Parameter	Values during the mortalities event	Normal values (Average)
Water temperature	28 ºC	25 ºC
Total Ammonia Nitrogen (TAN)	1.5 PPM	0.3 PPM
Water pH	9.5	8.1
Water dissolved oxygen (DO)	6.00 PPM	9.8 PPM

DISCUSSION

An overall incidence of *E.granulosus* of 27.8 % in dogs in Libya confirms that this disease is a serious problem. The infection rate is much higher than that seen in Egypt where in Cairo, the highest incidence was reported as only 3.4% **(Moch et al., 1974)** while the prevalence rates in different areas of Algeria have been recorded as 6% **(Senvet, 1951)** to 41 % **(Pampiglione, 1965);** and up to 85.8% in Morocco **(Chentoufi, in press).**

The situation in Libya is very serious in some areas as example, Tripoli where 11 out of 42(26.2 %) of the dogs examined were infected. The possible reasons for this level of infection may be due to local animal husbandry practices, inadequate availability and utilization of abattoir facilities, frequent domestic slaughtering of sheep and other intermediate hosts and the feeding of raw offal to dogs. Possible factors responsible for the increasing number of people at risk were use of local people as herders, the existence of community herds, and specific dog management practices. Determinants such as trailing sheep between seasonal pastures, association of sheep men from several counties on winter range, and sheep marketing practices undoubtedly influence distribution of infections in dogs and sheep these data was in accordance with **(Crellin, 1984; Hayward, 2004)**.

The incidence of Echinococcosis in southern areas of the country was quite low, as in Sebha, one out of 42(2.4%) falling to nil in Gdames,

Socna and Ghat. The most significant relevant factors were the very low number of dogs, the neglicable number of the stray dogs, the more efficient use of good abattoir facilities, and the hostile climatic and topographical environments beside that the mainly hot and dry weather in these areas is unsuitable for the long term survival of *Echinococcus* eggs **(Baxter, 1984).**

It is of interest that the Infection rates increased gradually with age in which only three were infected in the youngest group of dogs and 23 of the infected dogs were of three to four years old. This probably indicates a steady challenge of the role played by the environment and the continuous shedding of the eggs of the parasite in dog's feaces together with the lack of immunity in the young age this is in accordance with that reported by **Bryan and Schantz (1989)**; **Matter and Daniels (2000)** and against the result of those recorded by **Ugochukwu and Ejimadu (1985)** who said that Juvenile dogs from three weeks to one month of age were more commonly infested than adult dogs (24 months).

Infection rates were marginally higher in bitches than in male dogs. This point could usefully be investigated in different localities in which significant differences could be related to some factors such as different scavenging and other behavioral changes as guard dogs, and domesticity. This observation is in agreement with that of **Barriga and Al-Khalidi (1991)** who mentioned that the parasites were significantly more numerous in females than in the males

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STUDY ON THE PROPER TIME FOR BEGINNING VACCINE PROGRAM AGAINST FMD USING FMD BIVALENT VACCINE FOR NEWLY BORN CALVES

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ABSTRACT

The present study has been designed to assay the immune response to foot-and-mouth disease vaccines in newly born calves. Maternal antibodies in sera of calves were estimated using serum neutralization test (SNT) and ELISA; the highest level of FMD antibodies was detected in sera taken from new-born calves aged 5-10 days. Calves devoid of maternal antibodies responded satisfactorily to vaccination and the antibody titers at 21 days post-vaccination for the O and A were 2.1 and 1.8 $\log_{10}$ by SNT and in calves from vaccinated dams were 1.2 and 1.1 respectively. A certain degree of suppression for the vaccinal response was observed. Vaccination at age (14-16) week gave the highest antibody titers. Our results suggest vaccination of newly born calves with bivalent FMD vaccine at (4 – 5) months age and re- vaccination 30 may be effective in providing protection against FMD infection.

Keywords: Immune response, ELISA, bivalent vaccine, FMD.

INTRODUCTION

Foot and mouth disease virus (FMDV) is the etiological agent of an acute febrile disease that causes enormous economic losses in many countries of the world. In endemic areas inactivated aqueous (Aq) vaccines with aluminium hydroxide and saponin adjuvant are often used with repeat vaccination at 4-month Intervals **(Inta and Piadc, 1977; Rivenson et al., 1982)**. One of the principal problems in mass immunization against FMD is inducing protection in young calves, since it has been shown that newborn calves with maternal antibodies give very poor or no response to aqueous FMDV vaccines **(Nicholls et al., 1984; Sadir et al., 1988**), and that epidemic waves start in many countries with infection of these unprotected young calves **(Cosalfa, 1981; Ayebazibwe et al., 2010).** In areas of the world where foot and mouth disease (FMD) is controlled by regular vaccination, the incidence of disease is greatest in young stock

under 2 years of age **(Rweyemamu et al., 1982),** suggesting that calves may not respond as well as adults to vaccination. There have been several reports suggesting that maternal antibodies are able to inhibit the calves response to vaccination against FMD **(Graves, 1963; Srubar, 1966; Van Bekkum, 1966; Wisniewsky and Jankowska, 1972; Prudovsky, 1973; Kruglikov et al., 1974; Uppal et al., 1975; Brun et al., 1977; Tekerlekov et al., 1980; Shankar and Uppal, 1982).** The present report describes a series of experiments carried out to examine the effect of maternal antibodies on the primary response of calves and how vaccination regimens could be modified to provide efficient protection of calves from FMD under field conditions.

MATERIAL AND METHODS

Animals

A total of 20 Local breed calves clinically healthy were used in this study.3 calves were free from antibodies against FMD virus and 17 calves showing maternal antibodies as proved by using SNT and ELISA.

FMD viruses O_1/3/93-Egypt Strain and A_1/Egypt/2006 are locally isolated strains of cattle origin. The viruses were typed at Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo and confirmed by Pirbright, International Reference Laboratories, United Kingdom.

FMD vaccine:

Inactivated bivalent FMD vaccine was prepared using the local strains O_1/3/93 Egypt and A_1/Egypt/2006, propagated in BHK-21 cell line. The viruses had a titer of 10^8 $TCID_{50}$ for both and inactivated by Binary Ethylenemine (BEI).

Adjuvant

The inactivated FMD virus's suspension was mixed with 30% Alhydragel solution as adjuvant.

Experimental Design:

In order to determine the proper time for first vaccination and studying effect of maternal antibodies on the calf hood responses to FMD vaccine 2 experiments were carried out. In the first experiment the calves derived from unvaccinated cows and cows which had been vaccinated 4 months before parturition and were vaccinated at 1 week of age. In the second

experiment 14 calves in various ages from 1 - 4 month derived from vaccinated dams (4 month before parturition) .Divided into 7 groups (each group of 2 calves in the same age).all calves were vaccinated with FMD vaccine. One of each group was revaccinated after one month. Blood samples were collected. The immune response was evaluated through the estimation of immune level using SNT and ELISA.

Serum neutralization test (SNT)

It was performed using the technique as described by **Ferreira (1976)**.

Enzyme linked immunosrobent assay (ELISA)

It was carried out according to the method described by **Voller et al., (1976).**

RESULTS

Effect of maternally derived antibodies (MDA) on the primary response of 1-week-old calves

Three calves, were born 40 days after vaccination of their dams, were vaccinated when 1 week old. Twenty-one days later their sera were examined for neutralizing antibodies. A further three calves, from non vaccinated dams devoid of FMD-specific MDA, were also vaccinated when 1-week-old using the same batch of vaccine and serum samples were collected twenty-one days later also. Calves devoid of MDA responded satisfactorily to vaccination and the antibody titers at 21 days post-vaccination for the O and A were 2.1 and 1.8 $\log_{10}$ by SNT respectively and in calves from vaccinated dams were 1.2 and 1.1 respectively**.(Table 1**).

Humoral primary and secondary immune responses of various ages of calves vaccinated with FMD vaccines:

Fourteen calves, born from vaccinated dams were vaccinated when 1-4 months old and serum samples were collected at 30 day later. One calf from each age group was revaccinated at day 30 after primary vaccination and serum samples from all were collected again at day 60 **(Table 2, 3)**.

DISCUSSION

The immune response of newly born calves was born to FMD vaccinated and unvaccinated cows, after vaccination with Bivalent gel FMD vaccine, were studied. The pre vaccination sera of most of the calves (born to FMD vaccinated cows) showed varying levels of maternal antibodies with the SNT ranging from 1.2 to 1.5, while the calves born to unvaccinated cows showed lower antibody levels. Calves of both the groups showed significant rise in SNT antibody titres at 21 days post vaccination however this rise was more appreciable in calves born to unvaccinated cows.

From table (1) the results revealed that calves devoid of MDA responded satisfactorily to vaccination and thire SNT and ELISA titers average antibody at 21 days post-vaccination were higher than that were borne from vaccinated dams, go in hand with the results obtained are consistent with the statement of **Nicholls et al., (1984) ; Ayebazibwe et al., (2010)** who reported that 1-week-old newborn calves responded as well as adult cattle to FMD-vaccines for calves borne from non vaccinated dams .Results were also in agreement with **Francis and Black (1986); Ishikawa and Konishi, (1982); Niedbalski (2003)** who reported the highest level of FMD antibodies in sera of new-born calves aged 5-10 days, and the immune response in calves it is not until around 30 days old that the immune system can respond effectively to most antigens .

The results presented in table (2) for evaluation of Humoral immune response at day 30 using SNT and ELISA of vaccinated calves at various ages, were Supported by **Van Bekkum (1966); Osebold (1982); Brooksby (1974); Sadir et al., (1988)** who mentioned that the maternally-derived antibody (MDA) interferes with the development of active immunity following vaccination.

In table (3) , the obtained results revealed that post vaccination action at day 30 increase the specific antibody titere in the revaccinated calves although there was considerable animal to animal variation in this response.These results supported by **Nicholls et al., (1984) ; Kitching and salt (1995); Pravieux et al., (2007)** who reported that, the

responses to secondary vaccination were more variable than primary responses.

Finally, it can conclude that: vaccination of newly born calves with bivalent FMD vaccine at (4 – 5) month's age and re- vaccination 30 days later is sufficient to provide protection against FMD infection in calves.

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STUDY ON THE PROPER TIME FOR BEGINNING VACCINE PROGRAM AGAINST FMD USING FMD BIVALENT VACCINE FOR NEWLY BORN CALVES

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ABSTRACT

The present study has been designed to assay the immune response to foot-and-mouth disease vaccines in newly born calves. Maternal antibodies in sera of calves were estimated using serum neutralization test (SNT) and ELISA; the highest level of FMD antibodies was detected in sera taken from new-born calves aged 5-10 days. Calves devoid of maternal antibodies responded satisfactorily to vaccination and the antibody titers at 21 days post-vaccination for the O and A were 2.1 and 1.8 $\log_{10}$ by SNT and in calves from vaccinated dams were 1.2 and 1.1 respectively. A certain degree of suppression for the vaccinal response was observed. Vaccination at age (14-16) week gave the highest antibody titers. Our results suggest vaccination of newly born calves with bivalent FMD vaccine at (4 – 5) months age and re- vaccination 30 may be effective in providing protection against FMD infection.

Keywords: Immune response, ELISA, bivalent vaccine, FMD.

INTRODUCTION

Foot and mouth disease virus (FMDV) is the etiological agent of an acute febrile disease that causes enormous economic losses in many countries of the world. In endemic areas inactivated aqueous (Aq) vaccines with aluminium hydroxide and saponin adjuvant are often used with repeat vaccination at 4-month Intervals **(Inta and Piadc, 1977; Rivenson et al., 1982)**. One of the principal problems in mass immunization against FMD is inducing protection in young calves, since it has been shown that newborn calves with maternal antibodies give very poor or no response to aqueous FMDV vaccines **(Nicholls et al., 1984; Sadir et al., 1988**), and that epidemic waves start in many countries with infection of these unprotected young calves **(Cosalfa, 1981; Ayebazibwe et al., 2010).** In areas of the world where foot and mouth disease (FMD) is controlled by regular vaccination, the incidence of disease is greatest in young stock

under 2 years of age **(Rweyemamu et al., 1982),** suggesting that calves may not respond as well as adults to vaccination. There have been several reports suggesting that maternal antibodies are able to inhibit the calves response to vaccination against FMD **(Graves, 1963; Srubar, 1966; Van Bekkum, 1966; Wisniewsky and Jankowska, 1972; Prudovsky, 1973; Kruglikov et al., 1974; Uppal et al., 1975; Brun et al., 1977; Tekerlekov et al., 1980; Shankar and Uppal, 1982).** The present report describes a series of experiments carried out to examine the effect of maternal antibodies on the primary response of calves and how vaccination regimens could be modified to provide efficient protection of calves from FMD under field conditions.

MATERIAL AND METHODS

Animals

A total of 20 Local breed calves clinically healthy were used in this study.3 calves were free from antibodies against FMD virus and 17 calves showing maternal antibodies as proved by using SNT and ELISA.

FMD viruses O_1/3/93-Egypt Strain and A_1/Egypt/2006 are locally isolated strains of cattle origin. The viruses were typed at Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo and confirmed by Pirbright, International Reference Laboratories, United Kingdom.

FMD vaccine:

Inactivated bivalent FMD vaccine was prepared using the local strains O_1/3/93 Egypt and A_1/Egypt/2006, propagated in BHK-21 cell line. The viruses had a titer of 10^8 $TCID_{50}$ for both and inactivated by Binary Ethylenemine (BEI).

Adjuvant

The inactivated FMD virus's suspension was mixed with 30% Alhydragel solution as adjuvant.

Experimental Design:

In order to determine the proper time for first vaccination and studying effect of maternal antibodies on the calf hood responses to FMD vaccine 2 experiments were carried out. In the first experiment the calves derived from unvaccinated cows and cows which had been vaccinated 4 months before parturition and were vaccinated at 1 week of age. In the second

experiment 14 calves in various ages from 1 - 4 month derived from vaccinated dams (4 month before parturition) .Divided into 7 groups (each group of 2 calves in the same age).all calves were vaccinated with FMD vaccine. One of each group was revaccinated after one month. Blood samples were collected. The immune response was evaluated through the estimation of immune level using SNT and ELISA.

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Humoral primary and secondary immune responses of various ages of calves vaccinated with FMD vaccines:

Fourteen calves, born from vaccinated dams were vaccinated when 1-4 months old and serum samples were collected at 30 day later. One calf from each age group was revaccinated at day 30 after primary vaccination and serum samples from all were collected again at day 60 **(Table 2, 3)**.

DISCUSSION

The immune response of newly born calves was born to FMD vaccinated and unvaccinated cows, after vaccination with Bivalent gel FMD vaccine, were studied. The pre vaccination sera of most of the calves (born to FMD vaccinated cows) showed varying levels of maternal antibodies with the SNT ranging from 1.2 to 1.5, while the calves born to unvaccinated cows showed lower antibody levels. Calves of both the groups showed significant rise in SNT antibody titres at 21 days post vaccination however this rise was more appreciable in calves born to unvaccinated cows.

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CLINICAL AND BIOCHEMICAL STUDIES ON HYPOPHOSPHATEMIA (POST-PARTURIENT HAEMOGLOBINURIA) IN CATTLE

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ABSTRACT

This study designed to verify the haematological and biochemical changes that occur in cattle with post-parturient haemoglobinuria (PHU). For this intention, blood and serum samples from 30 PHU-affected and 20 apparently and clinically healthy cattle were collected and analyzed. Mean erythrocyte count, haemoglobin concentration, and haematocrit of the PHU-affected cattle were lower ($P < 0.05$), while their erythrocyte sedimentation rate was higher ($P < 0.05$) in comparison to the healthy cattle. Neutrophils, urea and creatinine concentrations were significantly higher in the PHU-affected cattle, while lymphocytes, erythrocytic glucose-6-phosphate dehydrogenase (G6PD) and glucose were lower than in the healthy cattle. There were significant increase in the levels of GGT and AST with significant decrease in total protein, albumin and globulin in PHU affected cattle in comparison with control group. Serum phosphorus was lower, while calcium was higher in the PHU-affected cattle as compared to those values in the healthy cattle. It was concluded that PHU affected cattle usually suffer from severe anemia and hypophosphataemia, and erythrocytes with significantly reduced G6PD are prone to haemolysis, leading to haemoglobinuria in cattle.

KEY WORDS: Parturient haemoglobinuria, cattle, haematology, biochemistry

INTRODUCTION

The transition from gestation to lactation is a period of great metabolic stress for dairy cows. Homeorhetic mechanisms in early lactation partition nutrients toward the mammary gland to support lactation even at

the expense of other body tissues. At the same time, voluntary DMI is markedly decreased **(Rollin et al., 2010)**

Parturient haemoglobinuria (PHU) is a major disease of dairy animals with detrimental economic consequences **(MacWilliams et al., 1982 ; Chugh et al., 1996).** It is an acute disease of high yielding dairy animals characterised by hypophosphataemia, intravascular haemolysis, haemoglobinuria, and anaemia **(Radostits et al., 2007).** The exact aetiology and pathogenesis of PHU are not known, as a variety of aetiological factors have been reported to be associated with the disease in different parts of the world. Nonetheless, hypophosphataemia is documented consistently in affected animals (**Hussain et al., 1991 ; Chugh et al., 1996).** Dietary phosphorus deficiency and/or rations containing cruciferous plants are suspected causes of severe hypophosphataemia and have been associated with haemolytic anaemia in cows (**MacWilliams et al., 1982**). Copper deficiency is also an aetiological factor of post- PHU, as its deficiency reduces the activity of the copper containing enzyme, superoxide dismutase, which is part of the erythrocyte protection mechanism against oxidative stress **(Smith et al., 1975).**

MATERIALS AND METHODS

Animals

The study included 30 cattle (376 ± 22Kg body weight) in AL-Ahsa, Saudi Arabia, suffering from PHU that were randomly selected from field cases arrived to the veterinary teaching hospital, faculty of veterinary medicine and animal resources, king Faisal university and all of them having a normal labor. The controls were 20 clinically healthy cattle of similar description from the same localities. The study animals were fed on seasonal green fodders, including Trifolium alexandrinum (Berseem) and hay.

Clinical examination

All buffaloes were clinically examined every day until 4 weeks after parturition, according to **Radostits et al. (2007).** The disease was clinically diagnosed on the basis of specific signs, such as haemoglobinuria, anorexia, normal rectal temperature and characteristic straining while defecating during early lactation or advanced pregnancy **(Akamatsu et al., 2007).** Other diseases that cause a reddish

discolouration of urine, like babesiosis, leptospirosis, and bacillary haemoglobinuria, were ruled out through laboratory tests.

Collection and analysis of blood and serum samples

Haematological and biochemical studies Blood samples from were collected from each cow, with and without anticoagulant. Blood samples with anticoagulant were used for the determination of erythrocyte and leukocyte counts (haemocytometer method), haemoglobin concentration (cyanmethaemoglobin method), haematocrit (microhaematocrit method), and erythrocyte sedimentation rate (Westergren tube method), following the techniques described by **Benjamin (1978).**

Differential leukocyte counts were determined by staining the blood smears with Giemsa stain **(Benjamin, 1978).** Serum was separated from blood samples collected without anticoagulant and preserved at -20 C° for further biochemical analysis. Serum urea (Crescent Diagnostics, Jeddah, Saudi Arabia), creatinine (Biocon Diagnostik, Germany), and erythrocytic G6PD (Randox Laboratories Ltd., UK) were estimated spectrophotometrically using the diagnostic kits according to the manufacturer's instructions. Spectrophotometric assays was conducted for colorimetric determination of AST **(Reitman and Frankel, 1957),** GGT **(Yang et al., 1998)** glucose **(Lott, 1975),** phosphorus **(Morinal and Prox, 1973)** and serum calcium **(Glinder and King, 1972).** All steps were performed using a selective chemistry analyzer (Abbott Alcyon 3001, USA).

Statistical analysis

The obtained data of the examined acute phase proteins were compared between groups within different concentrations by using computer package of the statistical analysis system **(SAS 1997).** All data are presented as means ± standard error (S.E.) of the means.

RESULTS

Table 1. The blood picture of control and diseased animals

Variable	Control group	Diseased group
Erythrocyte count (× 10^6 /µl)	8.72 ± 1.32	4.8 ± o.42*
Haemoglobin concentration (g/dl)	12.46 ± 1.32	6.87 ± 0.56*
PCV (%)	37. 23 ± 1.22	18.23 ± 1.11*
Erythrocyte sedimentation rate (mm/1 h)	71.65 ± 12.3	111.45 ± 15.23*
Total leukocyte counts (/µl)	7254 ± 1163.3	10132 ± 1460.3*
Neutrophils (%)	36.24 ± 2.35	45.35 ± 3.45*
Lymphocytes (%)	54. 26 ± 3.56	44.36 ± 4.23*
Monocytes (%)	5.62 ± 0.34	5.53 ± 0.32
Eosinophils (%)	3.2 ± 0.71	3.1 ± 0.62

*Means are significantly different at the level ($p \leq 0.05$).

Table 2. The elemental and biochemical parameters in diseased and control animals

Variable	Control group	Diseased group
Phosphorus (mg/dl)	5.56 ± 0.56	1.8 ± 0.57**
Calcium (mg/dl)	9.92 ± 1.32	10.12 ± 1.22
Erythrocytic G6PD (mU/10^7 TECs)	116.34 ± 14.33	88.43 ± 12.67*
Urea (mg/dl)	25.34 ± 3.54	43.22 ± 8.31*
Creatinine (mg/dl)	1.3 ± 0.22	2.44 ± 0.21*
AST (U/L)	67.65 ± 4.61	99.26 ± 6.67*
GGT (U/L)	7.2 ± 1.54	13.67 ± 1.45**
Glucose (mmol/L)	3.69 ± 0.23	1.66 ± 0.24**

*Means are significantly different at the level ($p \leq 0.05$).
**Means are highly significantly different at the level ($p \leq 0.01$).

DISCUSSION

Hypophosphataemia in PHU-affected animals is consistently documented **(Chugh et al 1996 and Radostits et al., 2007).** In the present study, significantly decreased serum phosphorus in PHU affected cattle was recorded (Table 2) as has been reported previously in PHU-affected cattle **(Stockdale et al., 2005).** Heavy drainage of phosphorus through milk, particularly in high milk yielding animals, leads to hypophosphataemia **(Bhikane et al., 1995).** In advanced gestation, more phosphorus and calcium are required for the developing foetus if supplementary phosphorus is not provided, thereby leading to hypophosphataemia. Moreover, a high calcium to phosphorus ratio results in decreased phosphorus absorption from the intestinal tract and ultimately leads to hypophosphataemia **(Benjamin, 1978).** It is well-known that Berseem as a green fodder is a rich source of calcium. It was concluded that PHU is strongly associated with Berseem feeding in winter season, probably because Berseem has high calcium to phosphorus ratio (>2:1) **(Macwilliams et al., 1982).**

Moreover a significant decrease in erythrocyte count, haemoglobin concentration, and haematocrit in PHU affected cattle (Table 1) indicates severe anaemia. This could be attributed to intravascular haemolysis (**Benjamin, 1978** and **Smith, 1990**) due to an impaired glycolytic pathway and depletion of ATP in erythrocytes, which results from phosphorus deficiency. Subnormal concentration of ATP predisposes red blood cells to alter functions and structure, a loss of normal formability, and an increase in fragility, ultimately leading to haemolysis **(Wang et al., 1985 and Suttle, 1991).**

In the present study, total erythrocyte count was optimistically correlated with hemoglobin concentration and hematocrit in PHU-affected cattle, which were also anaemic. In anaemic cases, total erythrocyte count, haemoglobin concentration, and haematocrit were reported to decrease simultaneously **(Benjamin, 1978).**, indicating a possible positive correlation between total erythrocyte count and both haemoglobin concentration and haematocrit.

The erythrocytic G6PD activity and glucose levels in PHU-affected cattle was significantly lower than that in healthy cattle (Table 2). **Singari et al.**

(1991) suggested that decreased erythrocytic G6PD activity in haemoglobinuric buffaloes may be partially responsible for the decrease in reduced glutathione, thereby causing oxidative stress to erythrocytes, which leads to haemolytic syndrome. Among the 2 major pathways of glucose metabolism in red blood cells, the pentose phosphate pathway (PPP) is of critical significance for normal red cell survival. The first reaction in PPP is the catalytic action of the enzyme G6PD in oxidising glucose-6-phosphate. NADPH generated by the cells PPP has a reducing potential on glutathione, and glutathione maintained in a reduced state protects red cells from oxidative stress; thus, a deficiency of G6DP will result in haemolytic anaemia **(Agar and Board, 1983).**

Deficiency of G6PD, owing to mutation, is the most common enzymatic abnormality in humans and has a high incidence rate, and over 300 genetic variants of the enzyme have been identified; at least 100 million people are deficient in this enzyme owing to these variants **(Agar and Board, 1983).**. G6PD may exist in haemoglobinuric buffaloes, but this needs further exploration. The increasing and decreasing trend in neutrophil and lymphocyte counts, respectively, in PHU-affected cattle could be attributed to the endogenous release of corticosteroids. Increased stress due to PHU (a metabolic disorder) is the source of the release of corticosteroids (**Singari et al., 1991 and Stockdale et al., 2005)** that results in increased neutrophils and depressed lymphocytes. Neutrophils are short-lived and normally the entire neutrophilic population in circulation is replaced 2.5 times daily **(Benjamin, 1978),** therefore, these have to leave circulation rapidly (about 9-10 h), but under disease conditions these are retained in circulation.

Moreover, marginal neutrophils are pooled in the main circulation and increased release of neutrophils from the maturation pool (**(Benjamin, 1978** and **Latimer et al 2003)**.) seems to be the main source of neutrophilia in PHU-affected cattle. According to **Latimer et al. (2003),** recirculating lymphocytes under the influence of corticosteroids remain transiently sequestered in the lymphoid tissues or bone marrow rather than entering efferent lymph and blood, resulting in lymphopenia. Furthermore, lysis of lymphocytes in all tissues and a decline of lymphoid mitosis in lymph nodes, due to corticosteroids, can also lead to lymphopenia. The reduction in glucose level may occur in response to energy restriction in the diet **(Bremmer et al., 2000)** specially at the early

stage of lactation when high rate of glucose utilization in the mammary gland is required **(Nazifi et al., 2008).**

The Erythrocyte sedimentation rate is governed by the balance between pro-sedimentation factors, mainly fibrinogen, and those factors resisting sedimentation, namely the negative charge of the erythrocytes (zeta potential). When an inflammatory process is present, the high proportion of fibrinogen in the blood causes red blood cells to stick to each other. The red cells form stacks called 'rouleaux' which settle faster. In the present study the increased Erythrocyte sedimentation rate is attributed to intravascular haemolysis and the anemic state of the examined cattle (**Benjamin, 1978 and Smith, 1990**).

The presence of AST in many organs of animals makes serum level a good marker of soft tissue damage but preclude its use as an organ-specific enzyme **(Kramer, 1989).** However, determining AST activities in dairy cows is most often connected with fatty liver syndrome **(Cebra et al., 1997).** Moreover, **Steen et al. (1997)** found that AST activity was greater in cows with ketosis (115 U/L) and hepatic lipidosis (252 U/L) than in healthy cows (70 U/L). The infiltration of hepatic cells with fat increases cell membrane permeability with subsequent release of AST enzyme that serves as a good tool for metabolic liver diseases **(Karasai and Schefar, 1984).** Therefore, the increased level of AST and GGT in PHU cattle in our study could be attributed to the fatty liver changes associated with the negative energy balance occurring in the peripartum period. **(Kaneko, 1989).**

Increased blood urea levels in PHU-affected cattle could be attributed to the endogenous release of corticosteroids, starvation, and tubular epithelial necrosis **(Latimer et al., 2003).** Additionally, dehydration usually occurs with PHU, which is a source of decreased renal perfusion, resulting in a reduced glomerular filtration rate and increased blood urea level **(Finco and Duncan, 1976**; **Benjamin, 1978; Latimer et al., 2003**, ; **Stockdale et al., 2005).** Alternatively, increased blood urea could be due to the failure of the urea recycling process through salivary glands and its non-utilisation by microbes in the rumen during digestive disorders. Most of the urea formed by the liver circulates in the circulatory system and remains unutilized (**Stockdale et al., 2005**). In the present study, creatinine was significantly increased in PHU cattle.

In this consider, **Benjamin (1978)** considered that concentrations over 2 mg/dl lead to a reduced glomerular filtration rate, which affects creatinine in a manner similar to that of blood urea **(Latimer et al 2003)**. Both urea and creatinine levels were elevated and positively correlated to each other in PHU-affected cattle. Urea and creatinine are waste products that the kidneys normally filter from the blood, and these are interrelated. If the kidneys are not working properly (**Latimer et al., 2003**), these substances build up in the body, and elevated blood levels of urea and creatinine are indications of pathological kidney function (**Latimer et al., 2003**). It was concluded from the present study that phosphorous deficiency plays a key role in causing haemoglobinuria, anemia disturbed liver and kidney function and reduced G6PD in erythrocytes of affected cattle. Moreover, control of feeding program is very important in the control of such clinical problem.

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دراسات إكلينيكية وبيوكيميائية عن نقص الفسفور في الأبقار بعد الولادة

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أجريت هذه الدراسة بهدف دراسة التغييرات التي تح دث في الدم والتغييرات الكيميائية المصاحبة لحالات نقص الفسفور بعد الولادة في الأبقار . تم إجراء الدراسة علي عدد 30 بقرة مصابة بنقص الفسفور و 20 بقرة سليمة من الناحية الإكلينيكية . وقد أظهرت النتائج وجود انخفاض في العدد الكلي لكرات الدم الحمراء ومستوي الهيموجلو بين مع ارتفاع في نسبة سرعة الترسيب. كما لوحظ ارتفاع في نسبة كريات الدم البيضاء ومستوي إنزيمات الكلي مع انخفاض في مستوي الجلوكوز . كما لوحظ أن هناك ارتفاع ملحوظ في مستوي إنزيمات الكبد وانخفاض ملحوظ في نسبة البروتين الكلي والالبيومين والجلوبيولين G6PD & في الأ بقار المصابة بنقص الفسفور عند مقارنتها بمثيلتها الضابطة . وقد خلصت الدراسة إلي أهمية التغييرات الدموية والكيميائية في تشخيص حالات نقص الفسفور إضافة إلي أهمية هذا المرض كمسبب لحالات الأنيميا في الأبقار.

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CLINICO-EPIDEMIOLOGICAL AND THERAPEUTIC STUDIES ON BOVINE PAPILLOMATOSIS IN NORTHERN OASES

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ABSTRACT
Bovine papillomatosis is a viral disease of cattle characterized clinically by development of multiple benign tumours termed warts. The diagnosis of bovine papillomatosis was confirmed by clinical and pathological examinations of the warts. The prevalence of bovine papillomatosis in Northern Oases was recorded as 4.86%. The prevalence was higher in the females (2.99%) than males (1.87%).The prevalence was the highest in cattle less than one year old (2.99%). The infected cattle were examined for detection of external and internal parasites. The tick infestations were observed in 10 out of totally 13 infected cattle. The Fasciola eggs were also detected and counted in only 4 infected cattle while parasitic gastroenteritis (PGE) nematode eggs were detected and counted in only 5 infected cattle. The correlation between parasitic infestation and number of warts was statistically recorded. It was found that the correlation between number of warts and number of *Fasciola* eggs and number of parasitic gastroenteritis (PGE) nematode eggs was 0.6 and 0.89 respectively. Two therapeutic regimes were evaluated, regime-I and regime-II, all cattle treated were completely recovered in 15 to 115 days post-treatment. We concluded that the regime-I of treatment was better than regime-II depending on mean of days needed for healing and regression of warts that was 42 days for regime-I and 83 days for regime-II.

KEY WORDS: Bovine warts, prevalence, parasite, therapy, Northern Oases**.**

INTRODUCTION
Papilloma viruses are small (55 nm in diameter) non enveloped, icosahedral viruses, containing a double stranded, circular DNA genome about 8000 base pairs long. They are found throughout higher vertebrates, mostly mammals and birds, causing cutaneous and mucosal tumours (William, 2009). Bovine papillomatosis (warts) is a disease caused by host, site and lesion specific papillomaviruses. Bovine papillomavirus (BPV) has six serotypes hitherto **(Olson, 1990**). The

disease is usually spread by direct contact with infected animal where virus penetrates the skin via cutaneous abrasions. It gains its economic importance through interfering with animal sales and shows, and loss of condition in extensive cases especially when the lesions get infected secondarily with bacteria. Teat warts are also interfering with milking process **(Radostitis et al., 2007)**. These warts may spontaneously regress, occasionally persist, and, in presence of additional critical genetic or environmental factors, can progress to cancer **(Campo, 1987**). It is thought to be a multistep affair **(Koller and Olson, 1972 ; Lancaster and Olson, 1982**).

Papilloma virus infection developed as a result of the virus exposure to single or multiple lesions of the epithelium of the skin. The transformation and multiplication of papillomavirus infected basal cells, lead to wart formation, the most warts are benign and do not proliferate indefinitely causing cancer **(Shah and Howley, 1996).** Different methods have been used to treat bovine papillomas. Formalinized inactivated vaccine of bovine warts proved to be effective treatment and good prophylaxis against bovine papillomatosis **(Barthold et al., 1976; Hunt, 1984; Lesnik et al.,1999; Suveges and Schmidt, 2003)**. Intra-lesional immunotherapy by *Corynebacterium parvum* has also been reported by **Hall et al.(1994)**.

In this study, bovine papillomatosis was suspected in cattle in Northern Oases, 6th October governorate, the developed skin lesions were recorded along with age, sex of affected cattle, number and site of lesions, presence of external and internal parasites. The diagnosis was confirmed by pathological examinations. Therapeutic trials were done for the treatment of affected cattle with two different regimes.

MATERIAL AND METHODS

Clinical and epidemiological examination:

In northern oases, October 6th governorate, Egypt, 267 cattle were examined, general clinical examination was carried out and any skin lesions were recorded and described. Age and sex of infected cattle, sites and numbers of warts were recorded. Suspected infected cattle were visually examined for detection of external parasites and coprologically

examined for detection of internal parasites. The recorded data were statistically analyzed.

Parasitic examination:

Ticks found on the cattle skin were identified macroscopically. *Internal parasites* were diagnosed by faecal examination**.** Faecal matter were collected by back racking from papillomatosis infected cattle and were examined by both concentration flotation and concentration sedimentation techniques according to Denham and Suswillo (1995). The Mc master technique for counting eggs of parasitic gastroenteritis (PGE) nematodes was also done (Dunn, 1969; Georgi, 1980; Whitlock,1948)**,** number of eggs per gram faeces was calculated by multiplying mean number of actually counted eggs per Mac master slide to 100. The Fasciola eggs were counted by using method of Happich and Boray (1969), the actually counted Fasciola eggs were considered eggs per gram faeces.

Pathological examination:

Histopathological and negative staining examinations of warts were done. Histopathological sections were carried out by fixing of excised warts of living animals in 10% neutral buffered formalin solution. The fixed specimens were trimmed, washed and dehydrated in ascending grades of alcohol, cleaned in xylene, embedded in paraffin then sectioned (4-6 micron) and stained with hematoxyline and eosin according to Bancroft et. al., (1996).

Tissue samples were prepared for electron microscopy by the negative staining technique (Nenad et al., 2005), the pelleted viral particles were resuspended in distilled water and a drop of viral suspension was placed on a Petri dish. A Formvar-coated electron microscopy (EM) grid was placed Formvar side down on top of the virus drop for approximately 1-3 minutes. The grid was removed, blotted with filter paper and placed onto a drop of 2.0% phosphotungstic acid (PTA), pH 7.0, for one minute. The excess PTA was removed, and the EM grid was ready for viewing in the electron microscope unites of VACSERA company.

Treatment

The external or internal parasites infested cases were treated by injection of Ivermectin and oral dosing of triclabendazole. The papillomatosis infected cattle were treated with two different regimes and they were evaluated.

Anti-parasitic drugs were given for cattle proved to be infected, Ivermectin (Iveen®, ADWIA company, Egypt) was injected subcutaneous at a dose rate of 200mcg/kg bodyweight (1ml/50kg bodyweight) and triclabendazole was given orally at dose rate of 12 mg/kg bodyweight(Fascinex®, Novartis co.)

Surgical treatment of warts:

Sedation

Cattle was sedated with xylazine 2% solution (xylaject®, Adwia company, Egypt) at a dose rate of 0.1 mg per 1kg bodyweight by intramuscular injection. The animal was well restrained before surgical excision of warts by one of the following regimes:

Regime I*:* *Excision* of large sized warts was performed by sharp scalpel, and hemorrhage was controlled using *electrocautery*.

Regime II*:* *Curetting* to remove overgrowth of warts by a scalpel till blood oozes to allow the reintroduce of virus into blood (Autogenous vaccine).*Trichloro-acetic acid* was applied topically post-curetting to kill and remove the rest warts cells. *Levamizole* was injected subcutaneous at a dose rate of 1 ml/10 kg bodyweight as immune stimulant (Cam et al., 2007).

In both regimes, infected animal was injected with *Multivitamin*® (1 ml/10 kg bodyweight by intramuscular injection, NorBrook company,Egypt). A *Betadine*® spray (Bovidone iodine skin solution, Nile company for pharamaceuticals and chemical industries) was applied topically on the skin wounds to prevent secondary bacterial infection and myiasis.

Statistical analysis

All the obtained data werestatistically analyzed using SPSS (Version 16) program.

RESULTS

Clinical findings, epidemiological data, parasitic infestations, pathological findings, results of treatment including anti-parasitics and surgical treatment of warts using regime-1 or regime-2 and results of statistic analysis were illustrated as in tables-1,2 ,3,4 and 5. And figures 1,2,3,4,5 and 6.

Results of clinical examination

Out of 267clinically examined cattle only thirteen cases of bovine papillomatosis were recorded. The body temperature of infected cattle was normal (38-38.5°C). The appetite of infected cattle was normal. The body condition of heavily infected cattle was poor. The site of warts and the warts count per animal were recorded as shown in table 2. Generalized papillomatosis was observed in one case in which warts distributed on both sides of the body; and the larger sized warts were concentrated in the right side. The largest wart measured 13 cm in width and 20 cm in length, and extended from the base of the ear to reach the edge of the mandible. It was irregular, rough and sessile, with hyperkeratosis. The variable sized warts were observed in other 12 cattle at different sites of the body skin including Back , Chest and neck , Legs, Udder, Face and External genitalia.

Results of the epidemiological study

As presented in table (1), the prevalence of bovine papillomatosis in Northern Oases was recorded as 4.86%. The prevalence was higher in the females (2.99%) than males (1.87%). The prevalence was the highest in cattle less than one year old (2.99%). As illustrated in table (2),the number and percentage of papillomatosis lesions regarding site were recorded as generalized (1, 7.69%), Back (4, 30.76%), Chest and neck (3, 23.07%), legs (2, 15.38), udder (1, 7.69), face (1, 7.69) and external genitalia (1, 7.69%). Spearman rank correlation between number of warts and number of Fasciola eggs per gram faeces was 0.6 while between number of warts and number of PGE eggs per gram faeces was 0.89.

Results of parasitic examination

of 13 papillomavirus infected cattle, 10 infected with ticks (76.92%),4 with fasciola (30.76%) and 5 with PGE (38.46%).

Results of pathology of bovine warts

Histopathologically, there was marked parakeratotic hyperkeratosis with long, thick, hair-like cornified surface projections and papillate epidermal hyperplasia, with patchy areas of erosion, ulceration, and neutrophil infiltration. The underlying dermal papillae had a moderate infiltration of neutrophils, eosinophils, and fewer lymphocytes (Prince Edward, 1994).
Bovine papilloma viruses were identified in the examined tissue samples of skin warts as recorded by Shah and Howley (1996). The virus was small and non-enveloped. The diameter of virus measured approximately 60 nm and it was composed of capsomeres arranged in icosahedral symmetry of the capside.

Results of treatment

All cattle treated were completely recovered in 15 to 115 days post-treatment. The mean period needed for healing and regression of warts was 42 days for regime-I and 83 days for regime-II.

DISCUSSION

Although bovine papillomatosis is a self-limiting disease, the warts in our study need long time to regress and animal to recover. The diagnosis of bovine papillomatosis was confirmed by presence of variable sized cutaneous warts and the histopathology findings. Economical impact of the disease is clearly observed in loss of animal condition, secondary bacterial infection, skin myiasis, interfering with lactation process and lastly reduction of animal price and sometimes hinders the sale.

Out of 13 bovine papillomatosis infected cattle, 5 male and 8 female cattle, were infected. The role of sex in the infection may return to the female cattle usually under stress factors such as gestation, lactation and progression in age. On other hand, male cattle are usually directed to fattening and meat production and are mostly slaughtered at age of 2 years or less of 13 infected cattle, 8 infected cattle were less 1 year of age , 3 infected cattle were less 2 years of age , 2 infected cattle were over 2 years of age. It clear the young ages are more susceptible to the infection than the adult as described by Otter and Leonard(2003)**,** who recorded an outbreak of fibropapillomas in calves. It is thought to be due to ill-

developed immune system, alkaline pH of the skin of young ages; that may facilitate virus infection and also young ages are more susceptible to parasitic infestation and exposure to stress factors.

Ten cases out of 13 papillomatosis infected cattle suffered ticks infestation with a percentage of 76.92 %, so we believe ticks play a role in papillomatosis infection. It is thought that tick has two inducing role for bovine papillomatosis, firstly piercing skin causes skin route for the virus to enter and infect basal keratinocytes, replicating its genome in the differentiating spinous and granular layers causing excessive growth rate forming warts (Radostitis et al., 2007). Secondly, its immune suppressive role, which facilitate virus infection to form warts (Lesnik et al., 1999; Jitka et al.,2004). The tick suck a large volume of host blood where it inserts its hypostome into the skin and secretes a cement from the salivary glands to hold the hypostome in place. Ticks is damaging skin barrier while feeding on host blood; secretes saliva to pierce skin and prevent clotting of blood , tick saliva has immune suppressive effect as recorded by Jitka et al.(2004)**,** they confirmed that Th2 cytokines; IL-6 and IL- 10 were down regulated by salivary gland extract of *Ixodes ricinus.*

The immune suppression enhances papilloma virus infection (Lesnik et al., 1999; Brady et al., 1999; Koski and Scott, 2003). Both PGE nematodes and Fascioliasis play a role as immune suppressive so that they facilitate virus infection. Five out of 13 papillomatosis infected cattle were suffering parasitic gastroenteritis PGE nematodes that have an immune suppressive effect as recorded by (Koski and Scott, 2003), they stated that deficiencies of iron, molybdenum, copper, and zinc, had been associated with higher worm burdens consequently affected immune response. Four out of 13 papillomatosis infected cattle were suffering from Fasciola that have an immune suppressive effect as reported by Brady et al.(1999), they mentioned that Th1 response to *B. pertussis* antigens was markedly suppressed and the bacterial infection was exacerbated following infection with *F. hepatica*. As in table (2), The immune suppressive effect of both parasites was clear where the correlation between number of warts and number of fasciola eggs per gram faeces was 0.6 and the correlation between number of warts and number of PGE eggs per gram faeces was 0.89.

The two therapeutic regimes were evaluated for treatment of bovine papillomatosis, regime-I and regime-II. In regime-I, The surgical excision of large warts was done using a scalpel and bleeding was controlled by electrocautery. The metastatic virus particles may circulate in the blood and act as auto-vaccine. The second regime including curetting the warts aimed to reintroduce the virus to circulation which was considered as autogenous vaccination, topical application of trichloro-acetic acid was done and the injection of immune stimulant (Levamizole) were used for treatment of animals. the regression of warts and time elapsed till recovery of infected animals were taken in consideration. The regression of warts and healing were observed 15-115 days after the beginning of treatment. Regime-I of treatment was better than regime-II depending on mean days required for regression of warts and healing, for regime-I was (42) days while for regime-II was (83) days.

Treatment of bovine papillomatosis with autogenous vaccine produced by grinding and suspending wart indicate variable results. In regime-I, the blood circulating metastatic virus particles stimulate the immune system. In regime-II, curetting of warts aimed to reintroduce papilloma virus into blood (autogenous vaccination) and application of trichloroacetic acid aimed to kill warts cells, papilloma virus, skin bacteria and stop light bleeding. The rate of success in both regimes I and II was 100% that is agree with that was reported that the treatment with autogenous vaccine showed 93.5% success with no difference in the used vaccine after 105 days of vaccination. Autogenous vaccine prepared from sterile homogenized wart and was injected twice; it was proved to prevent new cases and to treat sick animals (Suveges and Schmidt, 2003). Our results disagreed with that recorded by Smith (1990), who found that the treatment with autogenous wart vaccine was failed. Commercial vaccines for cattle rarely seem to effectively promoted regression of existing warts or to prevent malignant progression, although they might be capable of preventing the development of new lesions if the same strain is involved (Smith, 1990; Campo, 1991; Scott and Anderson, 1992).

The role of Levamizole as immune stimulant is mandatory in many infections as reported by Cam et al.(2007), they evaluated it for treatment of bovine cutaneous papillomatosis. Amery and Butterworth (1983)

found that Levamizole had good effect as an immunomodulator for blood disorders, renal failure, vasculitis and photosensitivity, inspite of that, the immune stimulating effect of levamizole against bovine papillomatosis was non-obvious where the levamizole may promote the general immune response but it could not help in eliminating the warts in rapid manner. That was disagree with the past studies of the role of immunomodulator against bovine papillomatosis. As shown in several *in vivo* studies, the stimulation of endogenous, non-antigen related defense mechanisms by parapoxvirus-based immunomodulators opens up new possibilities for the control and treatment of infectious diseases in domestic animals (Strube et al., 1989; Ziebell et al.,1997; Castrucci et al., 1998; Kyriakis et al., 1998; Glitz, 2002). The Inactivated parapox ovis viruses had a complex genetic structure and thereby they were considered as non-specific strong immunomodulator, which induced host immune reaction. There was evidence that such immune reactions resulted in more than elimination of the virus (Fachinger et al., 2000).

The efficacy of bovine papillomatosis treatment with the autogenous vaccine and a parammunity inducer was observed to be useful for the earlier regression of papillomas in the early stage of disease (growing stage of warts). It was believed that a parammunity inducer also shows a beneficial effect in additional treatment of bovine papillomatosis (Nenad et al., 2005)**.** That was disagree with our conclusion, the surgical excision of warts is better than curetting and autogenous vaccine.

Table-1: Epidemiological data of papillomavirus infected cattle

Papillomatosis	**Infected cattle**		**Non infected cattle**	**Total**
	Number	**Prevalence (%)**	**Number**	
Male cattle	5	1.87	108	**113**
Female cattle	8	2.99	146	**154**
Cattle less than 1 year old	8	2.99	76	**84**
Cattle less than 2 years old	3	1.12	57	**60**
Cattle from 2 to 5 years old	1	0.37	63	**64**
Cattle above 5 years old	1	0.37	58	**59**
Total	**13***	**4.86**	**254**	**267**

Table-2: Site and number of warts and the accompanied parasitic infestations.

Animal serial number	animal		Warts		Parasitic infestation		
					Internal		External
	Sex	Age (years)	Site	Number	Fasciola (EPG)*	PGE (EPG)*	Ticks
1	Male	Less than 1	Generalized	113	+(7)	-	+
2	Female	Less than 1	Back	9	-	+ (500)	+
3	Male	Less than 1	Chest and neck	17	+(2)	-	+
4	Female	Less than 2	Legs	11	-	+(700)	+
5	Female	Over 5	Udder	1	+(3)	-	-
6	Female	Less than 1	Face	2	-	-	+
7	Male	Less than 1	External genitalia	2	+(13)	-	+
8	Female	Less than 1	Back	23	-	+(900)	+
9	Female	From 2 to 5	Chest and neck	3	-	+(500)	-
10	Male	Less than 2	Chest and neck	17	-	-	+
11	Female	Less than 2	Back	1	-	+(500)	-
12	Male	Less than 1	Back	15	-	-	+
13	Female	Less than 1	Legs	4	-	-	+

***EPG: eggs per gram faeces.**

Table-3: Comparison of the counts of warts, PGE eggs and fasciola eggs.

Counts	Number of examined animals	Range	Minimum	Maximum	Mean	Std. Error	Std. Deviation
Warts	13	112	1	113	16.77	8.275	29.836
PGE eggs	5	400	500	900	620.00	80.000	178.885
Fasciola eggs	4	11	2	13	6.25	2.496	4.992

Table-4: Results of treatment of internal and external parasites and warts.

Animal serial number	Treatment					
	Anti-parasitic		Treatment of warts			
			Regime I		Regime II	
	Tri-clabendazole	Ivermectin	Animals treated	Days needed for healing and regression of warts	Animals treated	Days needed for healing and regression of warts
1	+	+	+	93	-	-
2	+	+	+	57	-	-
3	+	+	+	68	-	-
4	+	+	-	-	+	75
5	+	-	+	21	-	-
6	-	+	+	18	-	-
7	+	+	+	15	-	-
8	+	+	-	-	+	115
9	+	-	-	-	+	62
10	-	+	-	-	+	71
11	+	-	-	-	+	63
12	-	+	-	-	+	109
13	-	+	+	19	-	-

Table-5: Comparison of Regime-I and II for treatment of warts.

Treatment	Number of treated cattle	Days needed for healing and regression of warts				
		Minimum	Maximum	Sum	Mean	Std.Deviation
Regime-I	7	15	93	291	41.57	31.026
Regime-II	6	62	115	495	82.50	23.441

Figure-1: A calf showing multiple large sized warts distributed all over the skin before treatment (right side).

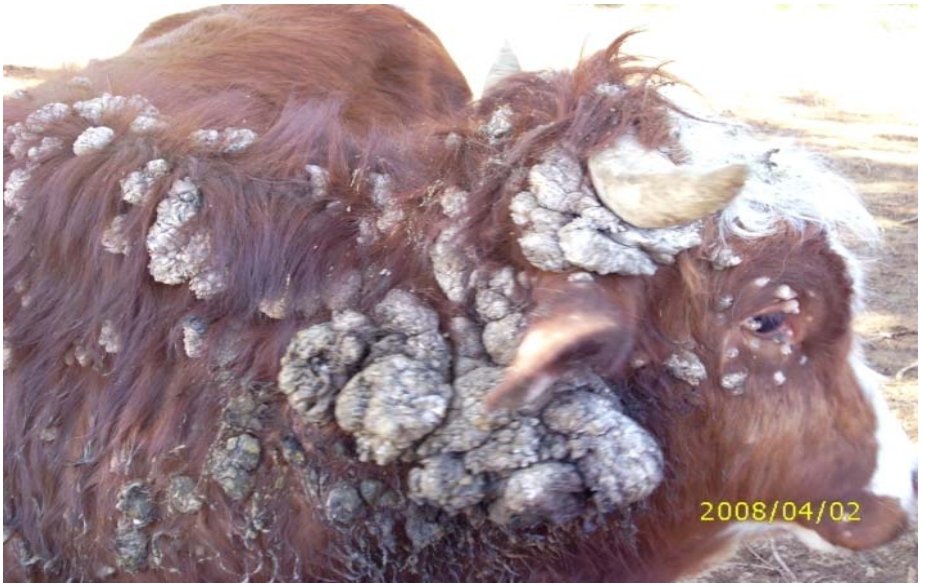

Figure-2: The same calf showing smaller sized warts on the left side of skin.

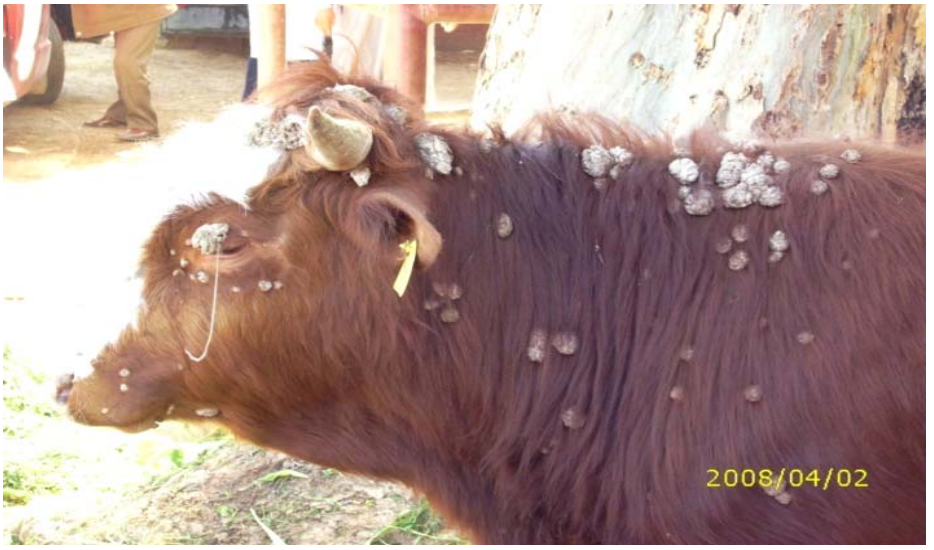

Figure-3: The excised large sized warts.

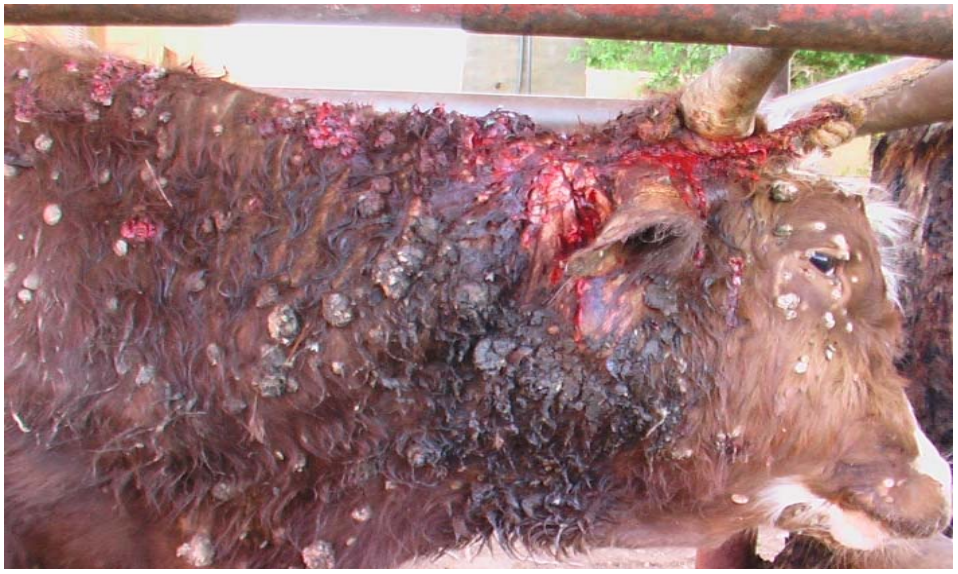

Figure-4: The Calf after excision of large sized warts.

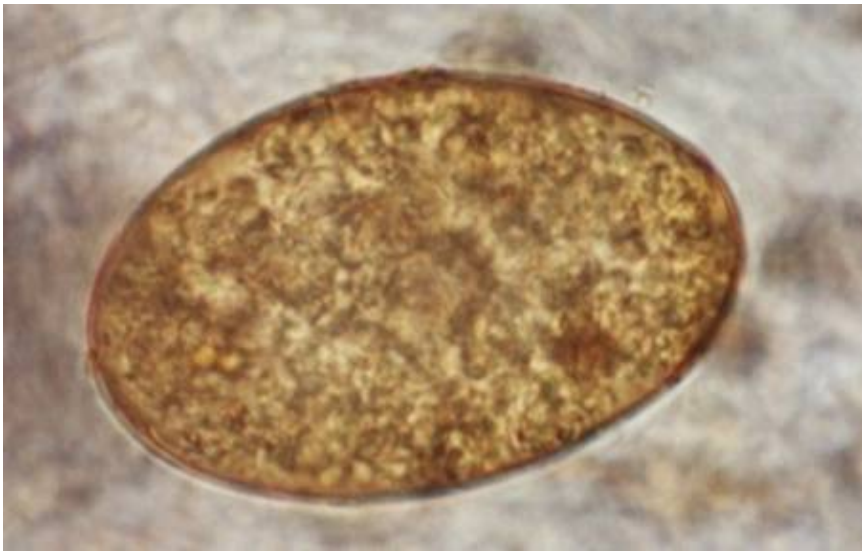

Figure-5: Fasciola egg (X100).

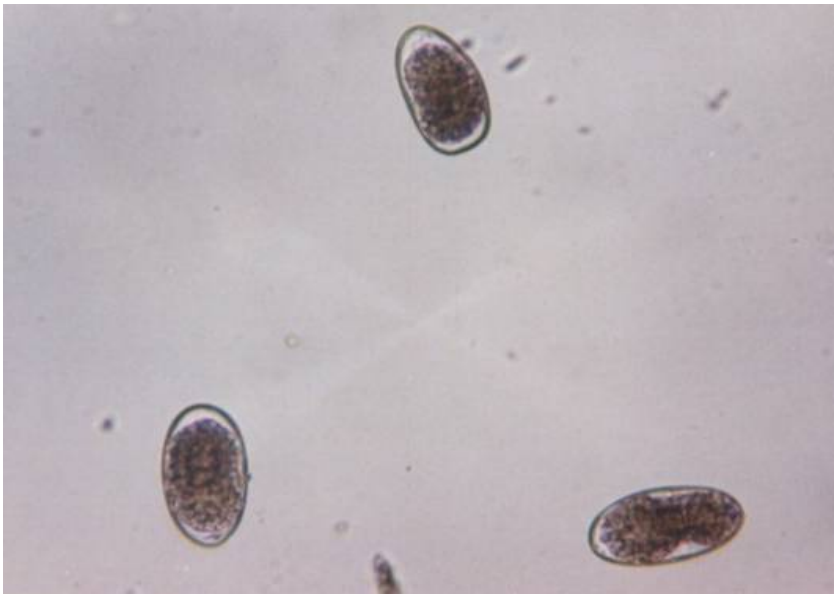

Figure-6: Parasitic gastroenteritis nematodes eggs (X40).

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THE IMPACT OF LAMBING STRESS ON POST-PARTURIENT BEHAVIOUR OF SHEEP WITH CONSEQUENCES ON NEONATAL HOMEOTHERMY AND SURVIVAL

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ABSTRACT

The expression of appropriate behavioural response from both the ewe and the lamb are extremely important to lamb survival. The aim of this study was to show the effect of length and difficulty of the birth process on the expression of maternal and neonatal behaviour with consequences on homeothermy and survival of the neonate lamb. Data were collected from sixty-one Finnish Landrace x Rahmani crossbred (2^{nd} generation) primiparous ewes and their single born lambs. Based on the average length of parturition, the ewes were grouped into short birth (less than 32.5 min) and long birth (equal to or higher than 32.5 min) classes. The data recorded include maternal and neonatal behaviour, lamb body temperature over the first 3 days of life and survival rate of the neonate lamb during the first week after birth. In addition, blood samples were collected from the lamb, pre-suckling and at 24 and 72 h of birth. The obtained sera were assayed for thyroid hormones (T_3 and T_4), known to be involved in heat production. Ewes had prolonged and difficult births did not show as competent maternal behaviour as mothers with short and un-complicated deliveries, as they were slower to begin to groom their lambs after birth, spent less time licking their lambs, made less low-pitched vocalizations and nosing, more likely to show rejection behaviour (10.34 v 5.4 %, $P < 0.05$), and were more likely to move away as the lamb seeks the udder and attempts to suck (acceptance rate, 55.5 v 64.79 %, $P < 0.05$). Similarly, lambs with prolonged birth that had a difficult delivery were significantly less vigorous after birth, as they taken more time to stand, reach the udder and to suck successfully. These lambs had lower serum concentration of T_3 and T_4, and they were also less able to maintain body temperature after birth and this effect persisted over the first 3 days of life associated with higher neonatal mortality in the first week after birth (11.54 %) compared with only (2.86 %, $P < 0.01$) in lambs from short and non stressful birth process. From the present study it can be concluded that, prolonged deliveries with birth difficulty were one of the main causes of death of large, single-born lambs as it causes the expression of inappropriate behavioural responses from both the ewe and neonatal lamb. Thus, interventions designed to reduce the incidence of prolonged parturitions are likely to be associated with better welfare for the ewe and the lamb and consequently improved lamb homeothermy and survival.

Key words: Lamb; Behaviour, Birth, Thyroid hormones, Homeothermy, Survival.

INTRODUCTION

Lamb mortality in both extensive and intensive system is considered as a major constraint to profitable sheep production **(Haughey, 1991 ; Christley et al., 2003)**. Pre-weaning lamb mortality of 15-20% is common in farming system world-wide **(Wassmuth et al., 2001 ; Darwish et al., 2010)**. Lamb deaths are invariably concentrated in the first week of life reflecting the difficulty of the transition from an intra-uterine life to an extra-uterine existence **(Nowak et al., 2000 ; Hatcher et al., 2009)**. Most lamb deaths result from a failure in bonding between the ewe and the lamb **(Kuchel and Lindsay, 1999)**, thus studies in ewe and lamb behaviour could help to improve lamb survival.

Two main factors; dystocia and starvation-mismothering-exposure have been most often implicated with lamb losses **(Kerslake et al., 2005 ; Nowak and Poindron, 2006)**. Dystocia can be a consequence of lamb birth weight, sire breed, dam pelvic conformation **(Fogarty and Thompson, 1974),** malpresentation, maternal over feeding or prolonged parturition **(Sargison, 1997 ; Everett-Hincks et al., 2007)**. Ewe maternal behaviour is known to be affected by a difficult delivery **(Dwyer et al., 2001)**. In addition, Lambs that indure difficult births have trouble to maintain their body temperature and have retarded behaviours in teat searching and suckling **(Eales et al., 1982)**. Such lambs have increases chances of death when subjected to cold stress or malnutrition.

In sheep, vocalizations represent an important element of mother-young interactions **(Vince, 1993 ;Frédéric Sébe et al., 2007).** Sheep utilize auditory cues togehter with olfactory ones to establish a rapid bond between a ewe and her newborn lambs **(Alexander, 1977 ; Shillito-Walser et al.1981).** In addition, vocalizations are used as a long-distance recognition signals particularly between mother and young, although they appear to acts as a secondary signal to visual information **(Shillito-Walser, 1978)**. On the other hand, lamb bleat may be regarded as an essential adaptive mean for attracting maternal attention **(Brunelli et al.1994 ; Weary and Frazer, 1995).**

A large area of skin through which lamb lose heat, a birth coat of poor insulation value, and being born wet-all add togehter to make the newborn lamb highly susceptible to hypothermia due to exposure **(Eales and Small, 1995).** Thus, the newborn lamb must produce as much heat as

it loses to maintain its body temperature. This partly could be supplied by oxidation of fat from brown adipose tissue **(**BAT) by a process under the control of triiodothyronine (T_3) which produced from thyroxin (T_4) in BAT by the enzyme 5-monodeiodinase **(Dauncey, 1990 ; Brent, 1994),** but mainly by promoting early colostrum ingestion, which is extremely important, in addition to its immunoglobulin; colostrum provides the lamb with fuel to maintain body temperature **(Al-Jassim et al., 1999 ; Charismiadou e al., 2000)**. Therefore, an essential priority for homeothermy and survival of the neonate is the early access to the udder **(Coureaud et al., 2002b).** This study aimed to investigate the effect of birth length and difficulty on the expression of maternal and neonatal behaviour and its relationship with homeothermy and survival of the neonate lamb.

MATERIALS AND METHODS

Animals

This study was carried out at Sakha Animal Production Research Station, Animal Production Research Institute, Ministry of Agriculture, Kafr El-Sheikh Governorate, Egypt, during the period between 2007-2008. Sixty-one Finnish Landrace x Rahmani crossbred (2^{nd} generation) primiparous ewes and their single born lambs were used in this study. Oestrus was not synchronized, and the ewes were naturally mated and had an average body weight of 38.59 kg at mating and 42.18 kg at parturition with 1.72 years an average age. Pregnancy diagnosis was confirmed by transabdominal ultrasonic scanning at Day-70 of pregnancy. Based on the average length of parturition which was recorded in the present study that is 32.5 min, the ewes were grouped into short birth (less than 32.5 min) and long birth (equal to or higher than 32.5 min) classes (Asante et al., 1999). Thirty- five of the ewes fell into the short birth class with an average lamb birth weight of 3.34 kg and twenty-six in the long or protracted birth class with an average lamb birth weight of 4.29 kg.

Management

Ewes were given free access to green fodder (*Trifolium Alexandrium*) during the green season, hay in the dry one and fresh drinking water. Concentrate mixture (cotton seed cake, Soya bean meal, yellow corn, limestone and mineral mixture) containing 16.6% crude protein, 12.7 % crude fiber and 73.4 % TDN was provided during pregnancy at a rate of

400 gm daily /ewe.This amount was increased gradually till reach 1000 gm/ewe at the late stage of pregnancy (last 4-6 weeks). Ewes were vaccinated with 2 ml clostridia vaccine (Covexin, Schering-Plough Company) subcutaneously at week 17th of pregnancy. Ewes were housed in semi- covered large pens (6 m ×20 m), in groups of 30 ewes / pen.

Two weeks prior to the expecting lambing time, ewes were transported into well straw-bedded pens (6 m x 9 m), in groups of approximately 10-11 ewes / pen for lambing. Ewes due to lamb, were kept under 24-hour observation for 2 weeks for the exact time of lambing. At lambing, ewes were allowed to give birth without assistance; however, if the ewe is seen to strain for long time without further progress of the lamb, assistance is required. Lambing assistance was provided 1h after the water bag breaks without appearance of any part of the lamb (n=3) and /or 2h after parts of the lamb were seen at the vulva with no further progress being made(n=4) **[Paula Simmons, 1989 ; Dwyer, 2003].**The given assistance involved firstly correcting lamb presentation then manually delivered the lamb. Since assistance was based on time intervals, the interval prior to assistance was accepted as an indication of the true length of parturition **(Cloete et al., 2002**).

Data recording

Once any part of the lamb appears at the vulva, the observation was started immediately by focal observation (Martin and Bateson 1993) using a video camera (Sony, 450X, Japan), and continued for the first 2 h after birth. The data recorded include length of parturition, defined as the interval (in minutes) from the appearance of fluids until the birth of the lamb **(Dwyer, 2003)**, maternal behaviour (latency to groom, time spent grooming, frequencies of low-pitched bleat and nosing, lamb rejection and acceptance of lamb suck attempts), neonatal behavioural progress including latency to first stand, reach the udder and sucking and the average time spent sucking during the first 2h following birth as well as lamb bleating, pre-suckling lamb body temperature and lamb temperature at 24 and 72 h of life, and neonatal mortality over the first week of life.

Blood assay

Sampling

Once the lamb stands successfully, it was caught and a 3-ml blood sample was taken prior to suckling by jugular venipuncture within few minutes

of entering the lambing pen. Rectal temperature was also recorded at this time. Blood samples were then centrifuged (3000 rpm / 20 minutes). The obtained sera were separated and stored frozen at–20^{o}C until assayed for T_3 and T_4. Blood sampling and temperature recording procedures were repeated at 24 and 72 h after birth **(Schermer et al., 1996 ; Dwyer and Morgan, 2006)**.

Analytical procedures

Serum **c**oncentrations of T_3 and T_4 hormones were determined using a solid phase competitive chemiluminescence immuno-assay system (Elecsys 2010, Roche, Diagnostic, Mannheim). Concentrations were determined using kits, controls, mono-clonal mouse antibodies and reagent supplied by Roche, Diagnostic, 2005 .The intra – and inter assay coefficients of variation (C.V. %) were 3.6 and 5.4% for T_3 and 4.7 and 6.9 % for T_4.The minimum detectable levels of the assay were 0.195 ng /ml and 0.42 µg /dl for T_3 and T_4 respectively.

Statistical analysis

Ewe maternal behaviour were compared between the two birth classes using independent *t*-test and Chi-square test. Neonatal lamb behaviour, concentration of T_3 and T_4 and lamb rectal temperature over the first 3d of life were compared between the two birth classes using independent *t*-test. Lamb mortality rate during the first week of life were tested between the two groups using Chi-square test. Statistical analyses were computed using SAS version 12.0 (SAS, 1987). Differences were considered statistically significant at p=0.05 or less. All data are expressed as Means ± S.E. except the rejection behaviour, acceptance of lamb suck attempts and lamb mortality rate which expressed as percentages.

RESULTS AND DISCUSSION

Ewe maternal behaviour

In this study, ewes with prolonged labour and complicated deliveries were fail to show better maternal care and frequently abandon their new born lambs as compared to those with short and unassisted births (Table 1)

In sheep, as in many other species, an intensive period of behavioural interactions between the ewe and her new born lamb are likely to occur after birth. The ewe show intense licking and grooming of the wet lamb

(Dwyer, 2007), and emit frequent low-pitched bleat (care given bleat emitted by the ewe to her newborn lamb to strengthen the bonding with lamb) **(Frédéric Sébe et al., 2007).**These behaviours are of importance to promote the bonding formation between the ewe and her newborn lams and also encourage the early suckling by the lamb. The findings of the present study showed that, these behaviours are likely to be less frequent in ewes with prolonged and difficult births than those with short and un-complicated deliveries. Additionally, the ewe devotes the majority of her grooming time immediately after parturition **(Alexander, 1988),** and as the lamb dries, grooming wanes. The present study revealed that ewes that experienced prolonged and difficult births were significantly slower to begin to groom their lambs after birth as shown previously **(Arnold and Morgan, 1975; Poindron et al., 1984)**. On contrast, the decline in grooming attention with time since the birth of lamb was inversely related to an increase in sniffing or nosing attention to the lamb **(Dwyer and Lawrence, 1998)**. Our data demonstrated that, Lambs with long and complicated births had received less nosing attention than those with short and non stressful birth process. This is likely to have real consequences on survival of lamb and the strength of bond formed between ewe and lamb.

Table (1): Effect of length and difficulty of birth process on maternal behaviour.

Lambing process / Behavioural element	**Short uncomplicated birth**	**Prolonged birth with assistance**	*P* - Value
Latency to groom (sec)	14.286± 0.85	63.5±4.21	< 0.001
Time spent grooming (min)	51.72± 1.53	45.53± 1.26	< 0.01
Low- pitched bleat frequency	381.00±12.75	338.92±15.125	< 0.037
Nosing frequency	49.77±2.08	41.46±2.238	< 0.01
Acceptance of lamb suck attempts (%)	64.79	55.5	< 0.05
Rejection behaviour (%)	5.4	10.34	< 0.05

A poorer quality of maternal care showed by ewes that experienced prolonged and difficult births may be explained on the basis of, a delay or prolong parturition may act as a source of stress for ewes. These ewes

may fail to show better maternal care and frequently abandon their lamb as had been demonstrated previously by Nowak and Poindron, 2006 and also shown in the current study. Moreover, ewes with prolonged labour were more likely to require assistance at the birth of their lambs and this was associated with a delay in the onset of grooming behaviour and is known to inhibit maternal behaviour in several species (Alexander, 1988).

The results of the present study also showed that when the lamb stands and try to find the udder for initial suckling, ewes with short and unassisted deliveries had higher rate of acceptance to lamb sucking attempts (64.79 %) when compared with those of prolonged and difficult births (55.5%), and were less likely to show rejection behaviour towards their neonate (5.4 v 10.34 %, $P < 0.05$, Table 1). This is likely to be attributed to the less stressful birth process of these ewes as a result of increasing in the speed and ease of parturition.

Neonatal lamb behavioural progress:

Our data showed that, birth length and difficulty had a major impact on lamb neonatal behaviour (Table 2). Lambs with short and un-complicated deliveries those requiring no assistance at birth were significantly more active in the first 2h following birth as compared to those with prolonged and difficult births, since they stood and suck quickly after birth, and were more likely to suckle within the first 2h following birth.

Table (2): Effect of length and difficulty of birth process on neonatal behaviour.

Lambing process / Behavioural element	**Short uncomplicated birth**	**Prolonged birth with assistance**	*P*- value
Latency to:			
First stand (min)	15.387±0. 5	18.21±0.65	< 0.001
Reach the udder (min)	23.76±0.7	27.65±0.91	< 0.001
First suck (min)	33.92±0.99	39.01±1.06	< 0.001
Time spent sucking / 2h (min)	13.31±0.73	**10.483±0.78**	< 0.01

Neonatal lambs may experience pain and injury as a result of prolonged and difficult births. Birth injury is reported to present in over 80% of

lambs classified as parturient deaths and up to 57% of lambs dying from starvation-mismothering-exposure (Haughey, 1993). These lambs can suffer a range of injuries; such injuries include brain and liver damage, fractures (jaws, spinal column, ribs and limbs), dislocations, abrasions and bruises (Alexander, 1984 and Henderson, 1990). These injuries cause pain and if they are not immediately fatal, usually impaired sucking and locomotor activities of birth-injured lambs (Haughey, 1980; and Dwyer, 2003) as reported in the present study, thereby interfering with mother-young interactions and other behaviours that promote homeothermy and survival of these lambs.

Lamb bleating activity

In the present study, lambs with prolonged and difficult births took shorter time to vocalize for the first time after birth and tended to bleat frequently during the observation period than those with short and unassisted births (Figure 1). The maternal data of this study showed that these lambs had received delayed and less maternal care. Thus, the changes in lamb bleat rate of the current study were consistent with the vocalizations of young being indicators of need, supporting the view of a link between lamb bleat and the quality of receiving maternal care that had been suggested previously by Garcia-Gonzalez and Goddard, 1998. For that, this may explain the higher frequency of bleats in lambs with long and more complicated deliveries that received low level of maternal care from their mothers. Although, in another view with lamb bleat, Nowak, 1990 has been suggested that a high bleating activity by lambs improves the quality of the mother-young bonding by establishing better communication and improving mother recognition.

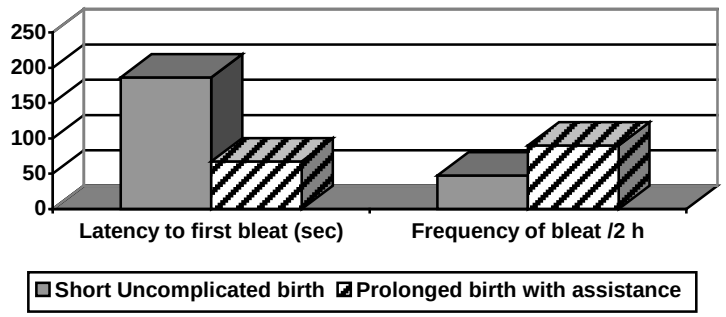

Fig. (1): Effect of length and difficulty of birth process on lamb bleating activity.

*** P<0.001

Lamb homeothermy

In the present study, lambs with short and less stressful birth process had higher circulating concentration of T_3 and T_4 (Table 3), and also had higher rectal temperature over the first 3d of life than lambs with prolonged and difficult births (Figure 2). These significant differences between the two birth classes in lamb temperature and concentration of T_3 and T_4 were markedly observed pre-suckling, although, there was also still a tendency for birth stress to influence these measures at 24 and 72 h after birth.

Table (3): Effect of length and difficulty of birth process on lamb thyroid hormones .

Lambing process / Lamb age	Short uncomplicated birth	Prolonged birth with assistance	*P*-Value
Pre-suckling:			
T_3 (ng /ml)	3.81±0.17	3.1±0.22	0.01
T_4 (µg /dl)	9.587±0.29	8.032±0.37	< 0.001
At 24h of birth:			
T_3 (ng /ml)	4.585±0.23	3.78±0.29	< 0.03
T_4 (µg /dl)	10.59±0.36	9.29±0.37	< 0.01
At 72 h of birth:			
T_3 (ng /ml)	4.97±0.23	4.085±0.30	< 0.02
T_4 (µg /dl)	10.91±0.4	9.44±0.39	< 0.01

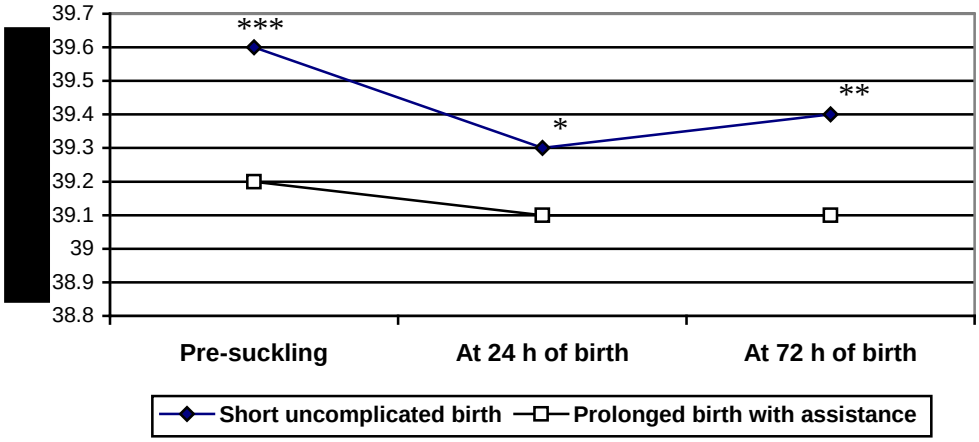

Fig (2): Effect of length and difficulty of birth process on lamb rectal temperature.
*** P<0.001, **P<0.01, *P<0.05

Inactive lamb may experience hunger due to inability to suck sufficient milk; this can lead to an inability to produce sufficient heat and hence to hypothermia (Slee and Springbett, 1986 and Dwyer and Morgan, 2006), where the newborn lamb has limited energy reserves stored in its body and is totally dependent on its mother for its energy supply (Eales and Small, 1995). Likewise, newborn lambs suffering from pain as in case of prolonged and difficult births may fail to suck sufficiently from their mothers (Eales and Small, 1981 and Eales et al., 1982) and will experience hunger associated with impaired thermoregulation in the neonate as shown in this study. This finding was underlined by low concentrations of thyroid hormones (T_3 and T_4) in these lambs which known to be involved in heat production associated with low body temperature over the first 3 d of life. On the other hand, as grooming behaviour has been considered to dry and to prevent heat loss from the newborn lamb (Levy and Poindron, 1987), ewes with prolonged and difficult births that took longer time to lick their newborn lambs after birth as reported in this study, their lambs were more likely to succumb to lower body temperature.

Neonatal lamb mortality

The results of the present study showed that a long duration of birth and a more complicated delivery were associated with a higher rate of lamb mortality during the first week of life (11.54 v 2.86 %, $P<0.01$, Fig. 3). Protracted labour increase the likelihood of suffering birth trauma and fetal hypoxemia associated with impaired heat production in the newborn lamb (Comline and Silver, 1972 and Haughey, 1993) and, if they are not immediately fatal, usually lead to delivery of an injuried lamb. Such lambs in addition to suffering pain as a result of birth trauma are generally less vigorous at birth, slower to stand and suck successfully and may establish a weak bond with the mother (Eales et al., 1982 and Haughey, 1980). Additionally, a difficult lambing was also associated with inappropriate behavioural response from the ewe (Dwyer et al., 2001 and Poindron et al., 1984). For that, these factors may increase the probability of neonatal death in these lambs (Haughey, 1991 and Cloete et al., 1993), since the ewe and lamb behaviour at birth has a large effect on lamb survival (Nowak, 1996; Hinch, 1997 and Darwish et al., 2010).

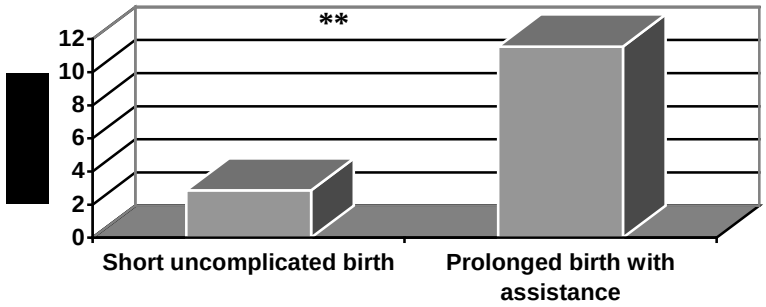

Fig. (3): Effect of length and difficulty of birth process on neonatal lamb mortality. ** P<0.01

CONCLUSION

In conclusion, this study had demonstrated that prolonged lambing and birth difficulty were significant risk factors, affecting the post-parturient behaviour of ewe and neonatal lamb with more severe consequences on homeothermy and survival of the neonate lamb. Thus, selection for an increase in the speed and ease of parturition are more likely to be associated with better lamb survival.

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تأثير مدة وعسر الولادة علي سلوك الأغنام بعد الولادة وعلاقة ذلك بدرجة حرارة ومعدل نفوق الحملان

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تعتبر مشكلة نفوق الحملان أحد أهم المشاكل التي تواجه تربية الأغنام ويرتبط بهذه المشكلة العديد من العوامل التي تؤثر عليها. وقد أجريت هذه الدراسة لبيان تأثير أحد هذه العوامل وهى مدة وعسر الولادة علي سلوك الأغنام بعد الولادة وتأثير ذلك علي درجة حرارة ومعدل نفوق الحملان. أستخدم فى هذه الدراسة عدد (61) أغنام خليط رحماني فنلندي (الجيل الثاني) تم تقسيمها حسب مدة وعسر الولادة الى مجموعتين أحداها ذات مدة ولادة قصيرة (أقل من 32.5 دقيقة) وعددها 35 حيوان ومجموعة آخرى مدة ولادتها طويلة وبعض منها أحتاج مساعدة عند الولادة (32.5 دقيقة أو أكثر) وعددها 26 حيوان. تم تسجيل سلوك النعاج والحملان بعد الولادة ، درجة حرارة الحملان وتركيزهرمونات الغدة الدرقية (T3 and T4) المرتبطة بإنتاج الطاقة خلال الثلاث أيام الأولى ومعدل نفوق الحملان خلال الأسبوع الأول من الولادة. وقد أسفرت النتائج عن الآتي:-

1- **تأثر سلوك النعاج بعد الولادة بمدة وعسر الولادة حيث كان سلوك الأمومة أكثر تحسناً في النعاج ذات مدة الولادة القصيرة عنه في النعاج ذات مدة الولادة الطويلة وظهر ذلك بوضوح في إرتفاع وقت لحس هذه النعاج لحملانها وزيادة معدل الصوت المنخفض المصاحب له وأيضا معدل شم النعاج لهذه الحملان . وهذه السلوكيات ذات أهمية قصوى في إرتباط النعاج بحملانها . أيضا كانت هذه النعاج أكثر استجابة لمحاولات رضاعة الحملان عنه في النعاج ذات مدة الولادة الطويلة.**
2- **بالمثل تأثر التطور السلوكي للحملان بعد الولادة بمدة وعسر الولادة حيث كانت الحملان ذات مدة الولادة القصيرة أكثر نشاطاً بعد الولادة وتمكنت من الوقوف والوصول إلي الضرع أسرع من الحملان ذات مدة الولادة الطويلة ولهذا إستطاعت الرضاعة والحصول علي السرسوب الغني بمكوناته فى وقت أقل.**
3- **إرتفاع تركيزهرمونات الغدة الدرقية ودرجة الحرارة خلال الثلاث أيام الأولى فى الحملان ذات مدة الولادة القصيرة عنه الحملان ذات مدة الولادة الطويلة.**
4- **أدي تحسن سلوك النعاج والحملان بعد الولادة في الأغنام ذات مدة الولادة القصيرة عنه في الأغنام ذات مدة الولادة الطويلة إلي انخفاض معدل نفوق حملانها (2.86% مقابل 11.54%) خلال الأسبوع الأول من الولادة.**

من هذه الدراسة نستنتج أن مدة وعس ر الولادة له تأثير واضح على سلوك النعاج والحملان بعد الولادة وأيضا على درجة حرارة ومعدل نفوق الحملان.

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SEROPREVALENCE OF *NEOSPORA CANINUM IN* CATTLE IN SOME PROVINCES IN IRAQ

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ABSTRACT

A seroepidmiology study of Neospora caninum was conducted in Dawania , Nasseria and Basrah provinces, Iraq on 92 cows by using commercial ElISA kit .The overall seroprevalence of Neospora caninum was 19.56% on provincial basis *Neospora caninum* infection was present in the three provinces Antibodies to N.caninum was found in 13(40.625%) of 32 aborted cows . The prevalence of N.caninum was significantly higher in the aborted cows than in non-aborted cows ($p<0.05$). Comparison of *N.caninum* serological status with age groups (≤ 2, 1year , 2 -4 ≥ 5 years) showed seropostivity prevalence rate 33.33% in 2-4 years age group while greater than 5 years was lowest. Our result indicate that Neospora infection is widespread in Iraq.

Keywords: Neospora caninum, Seroepidemiology; Abortion, ELISA

INTRODUCTION

Neospora caninum is considered as one of most important causes of abortion and infertility in cattle world wide **(Dubey and Lindsay, 1993 ; Anderson et al. ,1995; Barr et al. , 1997 ; Dubey et al. , 2007).** Abortion in some herds up to 88%. of one infected **(Campoero et al. , 1998)**. *Neospora caninum* is obligate intracellular apicomplexa protozoan parasite .

It has been detected in several mammalian species , i.e. , sheep , goat , horses , deer **(Dubey and Lindsay , 1993)** water buffaloes **(Guarino et al. , 2000)** , rhinoceros **(Williams et al. , 2002)** and foxes **(Almeria et al. , 2002)** . Dog were the first definitive host of *N. Caninum* **(Mc Allister et al. , 1998)** also coyotes (Canis Latrans were also demonstrated to be definitive hosts of the parasite **(Gondim et al. , 2004 ; Mc Allister et al. , 2004)** .Other possible hosts of interest are of course , human , sera famers and aborting women have been examined for presence of antibodies but no clearly positive samples were found **(Petersen et al. , 1999; Graham , 2006)** . However a recent study found antibodies to *N. Caninum* in 38% of HIV infected patient **(Lobato et al. , 2006)** . These findings might bring a new concern for the actual role of *N.Caninum* infection in immuno-compromssed patients .

Cattle can be infected with *N. Caninum* in two ways the first by transmission of parasite from the cow to her fetus during gestation , second by transmission of the parasite through faces from definitive host **(Pare et al. , 1997 ; Wouda et al., 1998; Davison et al., 1999)** and In experimental study new born calves were infected through colostrums mixed with tachyzoites **(Venturini et al. , 1999)** . The parasite elicits an antibody response in infected animals , detection of these antibodies is a sign of exposure to the parasite but not necessarily predictive of abortion **(Dubey et al. , 1996) .**

ELISA has become one of the most commonly used assay for the serologic diagnosis of *N. Caninum* infection in cattle **(Anderson et al. , 1995)** . ELISA enables rapid analysis of samples and extremely useful for large-scale screening of cattle herd **(Atkinson et al. ,2000). S**erological diagnosis for *N. Caninum* are needed to obtain information about epidemiology of life cycle differentiate between recent and chronic infection and determine seroprevalence in regions and countries **(Bjorkman and Uggla , 1999) .** They have been no reports of *N.Caninum* infection in Iraq .So, the objective of the present study was determine of seroprevalence of *N.Caninum* antibodies in healthy and aborted cattle with ELISA for the first time .

MARTIAL AND METHOD

Field study area

The study samples were collected from healthy and aborted cows in three Iraqi provinces (Dawania, Nasseria and Basrah) .Table (1), indicate that, the first province was located in the middle of Iraq and the second and third were located in south of Iraq. The climate is moderate and cold winter and very hot summers, the temperature reach 55C at summer.

Serum collection

Ninety two blood samples were taken on Jan. and June 2009 by using disposable needle from jugular vein in sterile tubes .All samples were immediately transported to the diagnostic laboratory. Serum was obtained after centrifugation at 2000 rpm for 15 minutes and stored at -20 C until serological tests were conducted.

Serology

Serum samples were analyzed for antibody activity to *N. caninum* by using the commerically available ELISA kit (IDEXX laboratories) all reagents were in room temperature and vortexed , used diluted samples 1: 100 in phosphat buffered saline solution . Negative and positive controls were dispensed (100 µl) into recorded wells serum samples were in duplicates and incubated for 30 minutes at Room temperature., each well was washed four times, then 100 µl of anti-bovine: HRPO conjugate was dispended into each well and incubated for 30 minutes at room temperature., 100 µl of TMB substrate solution was dispensed into each well and incubated for 15 minutes at room temperature., 100µl of stop solution was dispensed into each well of test plate to stop the reaction. Measures were recorded at absorbance 630 nm. Results were calculated under the following: formula of cut- off

$$s\backslash P = \frac{\text{sample A(360)NCX}^{-}}{\text{PCX}^{-} - \text{NCX}^{-}}$$

PCX^{-}= positive control mean NCX^{-}= Negative control mean

Sera with absorbance values above the cut-off level of 0.20 were considered positive according to manufacture instruction. Two repetitions from each sample were perform.

RESULTS

The results of serological examination by IgG Elisa of *N.caninum* were defected in (18/92) 19.56% . Positive while the negative results were (74/92) 80.43% of cows. Each results were found in three provinces which were Dawania , Nasseria and Baserah , non significant differences ($p>0.05$) . Table.1

Table (1): Seroprevalance of N.caninum in cows in three provinces, Iraq

.

Provinces	Negative	Positive	Total	X2 value p<0.05
Dawania	**25**	**5**	**30**	**Cal.x^2=0.921**
Nasseria	**24**	**8**	**32**	**Tab. X^2 =7.814**
Basrah	**25**	**5**	**30**	**df =3**
Total	**74**	**18**	**92**	**Non significant**

From the 92 cows sampled ,32 had a previous record of abortion of these 18 were seropostive and 74 were seronegative , the seroprevalance of *N.caninum* was significantly differ (p<0.05) of aborted group than in non aborted group .

Table (2) Seroprevalance of *N.caninum* in non- aborted and aborted cows

Provinces	Non aborted cows %	aborted cows %	Seropositive rate in each Provinces	^{2} value
Dawania	**1/20 (5 %)**	**4/10 (40 %)**	**5/30 (16.66 %)**	**Cal.x2=5.802 Tab. X2 =5.991 df = 2 Non significamt p>0.05**
Nasseria	**3/20 (15 %)**	**5/12 (41.66 %)**	**8/32 (25 %)**	
Basrah	**1/20 (5 %)**	**4/10 (40 %)**	**5/30 (5 %)**	
Total	**5/60 (8.3 %)**	**13/32 (40.62 %)**	**18/92 (19.56 %)**	**Cal.x2=21.295 Tab. X2 =3.841 df = 1 significant p<0.05**

Table(3) Showed the distribution of seropostive cows in the different age groups. The result of seropostivitity in the age group were 33.33% and 8% of the age group 2-4 years group and 5-8 years respectively which was significantly differ at **(p<0.05)**

Table (3) Seropostivitity related to age of cows.

Age group	2-4 years Age group %			Above 5 years (5-8) years				X^2 value p<0.05
Age	≤2	3	4	5	6	7	8	Cal.x^2=74.08
Sera test Positive	4/12 33.3 %	4/14 28.5 %	6/16 37.5 0 %	3/23 13.1 %	1/14 7.14	0/8 0 %	0/5 0	Tab. X^2 =11.0705 df = 5 significant
Total Sera test Positive of each groups	14/42 33.33 %			4/50 8.0 %				Cal.x^2 =15.52 Tab. X^2 =3.841 df = 1 significant

DISCUSSION

Neosporosis has been related with epizootic and sporadic abortion in cattle worldwid. Since the discovery of neosporosis some studies have been conducted to assess the prevalence and to identify factors related to the disease . Prevalences have been estimated in ranges between 4.3% and 70% **(Pare et al. , 1995 ; Pare et al. , 1997 ; Thurmond et al. , 1997 ; Waldner et al. , 1998**) . It has been reported in many countries with different prevalence rates since the disease was recognized in 1988 **(Buxtone et al.,1997 ; Campero et al., 1998 ; Cabaj et al.,2000 ; Dijkstra et al., 2001 ; Waldner et al.,2001; Kim et al.,2003).** In the present study, the prevalence was 19.06% . This result is higher than that reported for cattle in Poland (15.6%), Turkey (13.96%) ,Canada(9%),

korea (4.1%),but is lower than reported France (83%) , Spain (58%) , Iran (46%) and Paraguy (29.8%) **(Quintanilla – Genazalo et al. , 1999 ; Ould-Amrouche et al. , 1999 ; Waldner et al. , 2001 ; Osawa et al. , 2002 ; Kim et al. , 2003; Razmi et al. , 2006 ; Vural et al. , 2006).**

The Variation in the percentage of seroprevalance in our area and other parts may be caused by different climatic and geographical conditions , and may be reflected differences in prevalence between countries and also due to the characteristics (sensitivity , specify) of test used **(Tree et al. , 1994 ; Pare et al. , 1997 ; Pare et al. , 1998 ; Perez et al. , 1998 ; Wouda et al. , 1998 ; Barling et al. , 2000 and Bergeron et al. ; 2000) .** On the other hand this might be related to the presence of many dogs definitive host in farms from which the samples has been collected because of it play an important role in introduction and maintenance of the infection in herds **(Dubey , 1999)** .

The association of infection with abortion ,in the present study showed that the prevalence of *N.caninum* was higher in the aborted group 13/32 (40.62%)than non aborted group 5/60 (8.33) which was significant differ ($p<0.05$).Table (2) . A more definitive diagnosis can achieved when the abortion problem is examined on a herd. Serological approaches have been proposed to determine association between infection and abortion (**Thurmond et al. , 1997 ; Jenkin et al. , 2006) .** The result of study was in agreement with studies of **Osawa et al. (2002) ; Razmi et al. (2006)** which showed that abortion rate in total herd 56.7% , 46% respectively and seroprevalence of Abs was 262/879 (29.8%) , 85/170 (50%) , however the probability of abortion in seropositive cattle is twice that in seronegative cattle **(Moen et al. , 1998).** Several studies demonstrate that chronically infected seropositive cows can have more than twofold-increased risk of abortion compared to seronegative dams **(Wouda et al. 1998; Sager et al. 2001; Lo´pez-Gatius et al., 2004).**

Moreover, there are indications that the risk of endogenous abortion is influenced by the parity of the dams **(Lo´pez-Gatius et al., 2005) .**

Thurmond and Hietala (1996) observed a markedly increased abortion risk in congenitally infected heifers during their first gestation but not in later gestations, compared to the abortion risk in seronegative controls

.Seroepidemiological studies have assessed the increased risk for abortion in seropositive cows **(Thurmond and Hietala, 1996; Perez et al., 1998; Waldner et al., 1998;Wouda et al., 1998)** which also have higher risks for stillbirth **(Waldner et al., 1998)**, a seropositive offspring, culling for reproductive reason **(Thurmond and Hietala, 1996; Paré et al., 1997;Waldner et al., 1998)** compared to seronegative ones. It is expected that infected cows have higher odds of subsequent abortions Neosporosis-induced abortions occur year- round. Cows with *N. caninum* antibodies (seropositive) are more likely to abort than seronegative cows and this applies to both dairy and beef cattle **(Dubey , 1999).**

The result of our study is in agreement with studies carried out by **(Osawa et al. , 2002 ; Razmi et al. , 2006)**, that showed the abortion rate in total herd 56.7% , 46% respectively and seroprevalence of Abs was 262/879 (29.8%) , 85/170 (50%) , however the probability of abortion in seropositive cattle is twice that in seronegative cattle **(Moen et al. , 1998)** . The study showed an association between serological status and cow age significantly ($p<0.05$) (Table 3). **Sanderson et al. , (2000)** determined that seropositivity in cows under three years old which is in agreements with our study , also A higher seroprevalence for cows in age group between 1-3 years old was observed by **Dijkstra et al. (2001)** . The differences in seroprevalence by age group might be due to point source of infection as suggested by **McAllister et al. (1998).** The other studies have not showed an association between serological status and cow age **(Vural G., et al. , 2006 ; Waldner et al. , 1998 ; Davison et al. , 1999).**

CONCLUSION

In conclusion this is the first seroprevalence study of bovine neosporosis in Iraq . Results showed the presence of disease ; further epidemiologic studies are needed to provide a better understanding of neosporosis , and determine the incidence of abortion due to *Neospora caninum* in Iraq.

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دراسة مصلية وبائية في انتشار البوغية الكلبية الجديدة في ابقار مدن عراقية (الديوانية ,الناصرية والبصرة)

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الخلاصة

تم اجراء دراسة مصلية وبائية في انتشار البوغية الكلبية الجديدة في مدن عراقية هي الديوانية ,الناصرية والبصرة على 92 بقرة باستخدام اختبار الاليزا التجارية وكانت الاصابة في جميع المحافظات وبنسبة انتشار 19.56 %واظهرت الدراسة ان نسبة الاجسام المضادة في الابقار ال مجهضة هي 40.625 %من32 بقرة مجهضة وبفرق معنوي ذو دلالة احصائيةعن غير المجهضة وبالمقارنة بين العمر ونسبة الاصابة اظهرت الدراسة ان نسبة انتشار المرض 33.33%بين المجموعة العمرية 2-4 سنةاكثر من المجاميع الاخرى.نتائج دراستنا تشير لانتشار المرض في العراق .

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SEROPREVALENCE OF *NEOSPORA CANINUM IN* CATTLE IN SOME PROVINCES IN IRAQ

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ABSTRACT

A seroepidmiology study of Neospora caninum was conducted in Dawania , Nasseria and Basrah provinces, Iraq on 92 cows by using commercial ElISA kit .The overall seroprevalence of Neospora caninum was 19.56% on provincial basis *Neospora caninum* infection was present in the three provinces Antibodies to N.caninum was found in 13(40.625%) of 32 aborted cows . The prevalence of N.caninum was significantly higher in the aborted cows than in non-aborted cows ($p<0.05$). Comparison of *N.caninum* serological status with age groups (≤ 2, 1year , 2 -4 ≥ 5 years) showed seropostivity prevalence rate 33.33% in 2-4 years age group while greater than 5 years was lowest. Our result indicate that Neospora infection is widespread in Iraq.

Keywords: Neospora caninum, Seroepidemiology; Abortion, ELISA

INTRODUCTION

Neospora caninum is considered as one of most important causes of abortion and infertility in cattle world wide **(Dubey and Lindsay, 1993 ; Anderson et al. ,1995; Barr et al. , 1997 ; Dubey et al. , 2007).** Abortion in some herds up to 88%. of one infected **(Campoero et al. , 1998)**. *Neospora caninum* is obligate intracellular apicomplexa protozoan parasite .

It has been detected in several mammalian species , i.e. , sheep , goat , horses , deer **(Dubey and Lindsay , 1993)** water buffaloes **(Guarino et al. , 2000)** , rhinoceros **(Williams et al. , 2002)** and foxes **(Almeria et al. , 2002)** . Dog were the first definitive host of *N. Caninum* **(Mc Allister et al. , 1998)** also coyotes (Canis Latrans were also demonstrated to be definitive hosts of the parasite **(Gondim et al. , 2004 ; Mc Allister et al. , 2004)** .Other possible hosts of interest are of course , human , sera famers and aborting women have been examined for presence of antibodies but no clearly positive samples were found **(Petersen et al. , 1999; Graham , 2006)** . However a recent study found antibodies to *N. Caninum* in 38% of HIV infected patient **(Lobato et al. , 2006)** . These findings might bring a new concern for the actual role of *N.Caninum* infection in immuno-compromssed patients .

Cattle can be infected with *N. Caninum* in two ways the first by transmission of parasite from the cow to her fetus during gestation , second by transmission of the parasite through faces from definitive host **(Pare et al. , 1997 ; Wouda et al., 1998; Davison et al., 1999)** and In experimental study new born calves were infected through colostrums mixed with tachyzoites **(Venturini et al. , 1999)** . The parasite elicits an antibody response in infected animals , detection of these antibodies is a sign of exposure to the parasite but not necessarily predictive of abortion **(Dubey et al. , 1996) .**

ELISA has become one of the most commonly used assay for the serologic diagnosis of *N. Caninum* infection in cattle **(Anderson et al. , 1995)** . ELISA enables rapid analysis of samples and extremely useful for large-scale screening of cattle herd **(Atkinson et al. ,2000). S**erological diagnosis for *N. Caninum* are needed to obtain information about epidemiology of life cycle differentiate between recent and chronic infection and determine seroprevalence in regions and countries **(Bjorkman and Uggla , 1999) .** They have been no reports of *N.Caninum* infection in Iraq .So, the objective of the present study was determine of seroprevalence of *N.Caninum* antibodies in healthy and aborted cattle with ELISA for the first time .

MARTIAL AND METHOD

Field study area

The study samples were collected from healthy and aborted cows in three Iraqi provinces (Dawania, Nasseria and Basrah) .Table (1), indicate that, the first province was located in the middle of Iraq and the second and third were located in south of Iraq. The climate is moderate and cold winter and very hot summers, the temperature reach 55C at summer.

Serum collection

Ninety two blood samples were taken on Jan. and June 2009 by using disposable needle from jugular vein in sterile tubes .All samples were immediately transported to the diagnostic laboratory. Serum was obtained after centrifugation at 2000 rpm for 15 minutes and stored at -20 C until serological tests were conducted.

Serology

Serum samples were analyzed for antibody activity to *N. caninum* by using the commerically available ELISA kit (IDEXX laboratories) all reagents were in room temperature and vortexed , used diluted samples 1: 100 in phosphat buffered saline solution . Negative and positive controls were dispensed (100 µl) into recorded wells serum samples were in duplicates and incubated for 30 minutes at Room temperature., each well was washed four times, then 100 µl of anti-bovine: HRPO conjugate was dispended into each well and incubated for 30 minutes at room temperature., 100 µl of TMB substrate solution was dispensed into each well and incubated for 15 minutes at room temperature., 100µl of stop solution was dispensed into each well of test plate to stop the reaction. Measures were recorded at absorbance 630 nm. Results were calculated under the following: formula of cut- off

$$s\backslash P = \frac{\text{sample A(360)NCX}^{-}}{\text{PCX}^{-} - \text{NCX}^{-}}$$

PCX^{-}= positive control mean NCX^{-}= Negative control mean

Sera with absorbance values above the cut-off level of 0.20 were considered positive according to manufacture instruction. Two repetitions from each sample were perform.

RESULTS

The results of serological examination by IgG Elisa of *N.caninum* were defected in (18/92) 19.56% . Positive while the negative results were (74/92) 80.43% of cows. Each results were found in three provinces which were Dawania , Nasseria and Baserah , non significant differences ($p>0.05$) . Table.1

Table (1): Seroprevalance of N.caninum in cows in three provinces, Iraq

.

Provinces	Negative	Positive	Total	X2 value p<0.05
Dawania	**25**	**5**	**30**	**Cal.x^2=0.921**
Nasseria	**24**	**8**	**32**	**Tab. X^2 =7.814**
Basrah	**25**	**5**	**30**	**df =3**
Total	**74**	**18**	**92**	**Non significant**

From the 92 cows sampled ,32 had a previous record of abortion of these 18 were seropostive and 74 were seronegative , the seroprevalance of *N.caninum* was significantly differ (p<0.05) of aborted group than in non aborted group .

Table (2) Seroprevalance of *N.caninum* in non- aborted and aborted cows

Provinces	Non aborted cows %	aborted cows %	Seropositive rate in each Provinces	^{2} value
Dawania	**1/20 (5 %)**	**4/10 (40 %)**	**5/30 (16.66 %)**	**Cal.x2=5.802 Tab. X2 =5.991 df = 2 Non significamt p>0.05**
Nasseria	**3/20 (15 %)**	**5/12 (41.66 %)**	**8/32 (25 %)**	
Basrah	**1/20 (5 %)**	**4/10 (40 %)**	**5/30 (5 %)**	
Total	**5/60 (8.3 %)**	**13/32 (40.62 %)**	**18/92 (19.56 %)**	**Cal.x2=21.295 Tab. X2 =3.841 df = 1 significant p<0.05**

Table(3) Showed the distribution of seropostive cows in the different age groups. The result of seropostivitity in the age group were 33.33% and 8% of the age group 2-4 years group and 5-8 years respectively which was significantly differ at **(p<0.05)**

Table (3) Seropostivitity related to age of cows.

Age group	2-4 years Age group %			Above 5 years (5-8) years				X^2 value p<0.05
Age	≤2	3	4	5	6	7	8	Cal.x^2=74.08
Sera test Positive	4/12 33.3 %	4/14 28.5 %	6/16 37.5 0 %	3/23 13.1 %	1/14 7.14	0/8 0 %	0/5 0	Tab. X^2 =11.0705 df = 5 significant
Total Sera test Positive of each groups	14/42 33.33 %			4/50 8.0 %				Cal.x^2 =15.52 Tab. X^2 =3.841 df = 1 significant

DISCUSSION

Neosporosis has been related with epizootic and sporadic abortion in cattle worldwid. Since the discovery of neosporosis some studies have been conducted to assess the prevalence and to identify factors related to the disease . Prevalences have been estimated in ranges between 4.3% and 70% **(Pare et al. , 1995 ; Pare et al. , 1997 ; Thurmond et al. , 1997 ; Waldner et al. , 1998**) . It has been reported in many countries with different prevalence rates since the disease was recognized in 1988 **(Buxtone et al.,1997 ; Campero et al., 1998 ; Cabaj et al.,2000 ; Dijkstra et al., 2001 ; Waldner et al.,2001; Kim et al.,2003).** In the present study, the prevalence was 19.06% . This result is higher than that reported for cattle in Poland (15.6%), Turkey (13.96%) ,Canada(9%),

korea (4.1%),but is lower than reported France (83%) , Spain (58%) , Iran (46%) and Paraguy (29.8%) **(Quintanilla – Genazalo et al. , 1999 ; Ould-Amrouche et al. , 1999 ; Waldner et al. , 2001 ; Osawa et al. , 2002 ; Kim et al. , 2003; Razmi et al. , 2006 ; Vural et al. , 2006).**

The Variation in the percentage of seroprevalance in our area and other parts may be caused by different climatic and geographical conditions , and may be reflected differences in prevalence between countries and also due to the characteristics (sensitivity , specify) of test used **(Tree et al. , 1994 ; Pare et al. , 1997 ; Pare et al. , 1998 ; Perez et al. , 1998 ; Wouda et al. , 1998 ; Barling et al. , 2000 and Bergeron et al. ; 2000) .** On the other hand this might be related to the presence of many dogs definitive host in farms from which the samples has been collected because of it play an important role in introduction and maintenance of the infection in herds **(Dubey , 1999)** .

The association of infection with abortion ,in the present study showed that the prevalence of *N.caninum* was higher in the aborted group 13/32 (40.62%)than non aborted group 5/60 (8.33) which was significant differ ($p<0.05$).Table (2) . A more definitive diagnosis can achieved when the abortion problem is examined on a herd. Serological approaches have been proposed to determine association between infection and abortion (**Thurmond et al. , 1997 ; Jenkin et al. , 2006) .** The result of study was in agreement with studies of **Osawa et al. (2002) ; Razmi et al. (2006)** which showed that abortion rate in total herd 56.7% , 46% respectively and seroprevalence of Abs was 262/879 (29.8%) , 85/170 (50%) , however the probability of abortion in seropositive cattle is twice that in seronegative cattle **(Moen et al. , 1998).** Several studies demonstrate that chronically infected seropositive cows can have more than twofold-increased risk of abortion compared to seronegative dams **(Wouda et al. 1998; Sager et al. 2001; Lo´pez-Gatius et al., 2004).**

Moreover, there are indications that the risk of endogenous abortion is influenced by the parity of the dams **(Lo´pez-Gatius et al., 2005) .**

Thurmond and Hietala (1996) observed a markedly increased abortion risk in congenitally infected heifers during their first gestation but not in later gestations, compared to the abortion risk in seronegative controls

.Seroepidemiological studies have assessed the increased risk for abortion in seropositive cows **(Thurmond and Hietala, 1996; Perez et al., 1998; Waldner et al., 1998;Wouda et al., 1998)** which also have higher risks for stillbirth **(Waldner et al., 1998)**, a seropositive offspring, culling for reproductive reason **(Thurmond and Hietala, 1996; Paré et al., 1997;Waldner et al., 1998)** compared to seronegative ones. It is expected that infected cows have higher odds of subsequent abortions Neosporosis-induced abortions occur year- round. Cows with *N. caninum* antibodies (seropositive) are more likely to abort than seronegative cows and this applies to both dairy and beef cattle **(Dubey , 1999).**

The result of our study is in agreement with studies carried out by **(Osawa et al. , 2002 ; Razmi et al. , 2006)**, that showed the abortion rate in total herd 56.7% , 46% respectively and seroprevalence of Abs was 262/879 (29.8%) , 85/170 (50%) , however the probability of abortion in seropositive cattle is twice that in seronegative cattle **(Moen et al. , 1998)** . The study showed an association between serological status and cow age significantly ($p<0.05$) (Table 3). **Sanderson et al. , (2000)** determined that seropositivity in cows under three years old which is in agreements with our study , also A higher seroprevalence for cows in age group between 1-3 years old was observed by **Dijkstra et al. (2001)** . The differences in seroprevalence by age group might be due to point source of infection as suggested by **McAllister et al. (1998).** The other studies have not showed an association between serological status and cow age **(Vural G., et al. , 2006 ; Waldner et al. , 1998 ; Davison et al. , 1999).**

CONCLUSION

In conclusion this is the first seroprevalence study of bovine neosporosis in Iraq . Results showed the presence of disease ; further epidemiologic studies are needed to provide a better understanding of neosporosis , and determine the incidence of abortion due to *Neospora caninum* in Iraq.

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دراسة مصلية وبائية في انتشار البوغية الكلبية الجديدة في ابقار مدن عراقية (الديوانية ,الناصرية والبصرة)

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الخلاصة

تم اجراء دراسة مصلية وبائية في انتشار البوغية الكلبية الجديدة في مدن عراقية هي الديوانية ,الناصرية والبصرة على 92 بقرة باستخدام اختبار الاليزا التجارية وكانت الاصابة في جميع المحافظات وبنسبة انتشار 19.56 %واظهرت الدراسة ان نسبة الاجسام المضادة في الابقار ال مجهضة هي 40.625 %من32 بقرة مجهضة وبفرق معنوي ذو دلالة احصائيةعن غير المجهضة وبالمقارنة بين العمر ونسبة الاصابة اظهرت الدراسة ان نسبة انتشار المرض 33.33%بين المجموعة العمرية 2-4 سنةاكثر من المجاميع الاخرى.نتائج دراستنا تشير لانتشار المرض في العراق .

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BREED AND EXPERIENCE EFFECT ON BUCK SEXUAL BEHAVIOUR OF DAMASCUS AND EGYPTIAN-NUBIAN GOATS

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ABSTRACT

The objectives of this study were to compare the sexual behaviour of bucks from 2 pure breeds of goats named Damascus and Egyptian-Nubian (Zaraibi) and to assess its relation with pregnancy and kidding rates of their inseminated does. Twenty-three bucks (n=12 Damascus and 11 Egyptian-Nubian bucks) were used in this study. According to sexual experience, bucks used in this study were either in their first season of service (n=12) with 1.51 years an average age and a mean body weight of 40.65 kg or previously used in service for several seasons (n= 11, 3.34 years and 54.05 kg an average age and body weight). Buck service behaviour towards estrous doe was continuously recorded from the moment of appearance of doe and continued for 30 min. The results revealed that, Egyptian-Nubian bucks were sexually highly active with estrous does compared with Damascus bucks, togehter with higher pregnancy and kidding rates of their inseminated does. They were likely taken less time to ejaculate for first ($p<0.04$) and second time ($p<0.0002$), and tended to sniff, nudge and vocalize more frequently than Damascus bucks. As well as, they were denoted more ejaculations and higher mating efficiency (27.48 v 10.21 %, $p<0.001$). For experience effect, the data revealed limited influence. No great differences in sexual behaviour were recoded between the age groups of bucks used in the present study. Moreover, no relationship was found between ejaculations number and pregnancy and kidding rates as well as litter size. From these results, it can be concluded that, there was breed differences in sexual behaviour of Damascus and Egyptian-Nubian bucks with limited experience effect in this study. In addition, pregnancy and kidding rates as well as litter size were not affected by total number of ejaculations.

Key words: Goat, Nubian, Buck, Sexual behaviour, Pregnancy, Kidding.

INTRODUCTION

Despite the importance of goats as a potential source of meat and milk has been emphasized, goats still receive less attention than the other

livestock species.Todays, goat breeders throughout the world, are searching the best ways of increasing the efficiency and profitability of their farming enterprise. Reproductive behaviour of goats is a major determinant of productivity and economic viability of commercial goat farm (**Maurice Shelton, 1978; Mellado et al., 2006 ; Katz, 2007)**, since high conception rates and prolificacy are indispensable for the financial sustenance of farming system (**Panagiotis et al., 2006)**. Sexual behaviour is mainly influenced by the reproductive potential of both male and female animal as well as the constraints resulting from husbandry handlings **(Chemineau, 1989; Sambraus, 1991; Fabre Nys et al., 1993 and Delgadillo et al., 2001),** and the net effect of all these influences will determine the level and efficiency of reproduction **(Erasmus and Fourie, 1985; Bocquier et al., 1996 ; Absy et al., 2001).**

Sexual behaviour is manifested by a group of traits. In goat buck, it is expressed in the following sequence: anogenital sniffing, flehmen, nudging, tongue-lapping, vocalization, fore-leg striking, mounting attempt of the estrous doe, mounting and ejaculation **(Ian Gordon 1997 ; Vèliz et al., 2004).** These activities do not necessarily occur sequentially or every time, with the vomeronasal system assuming considerable importance **(Ladewig and Hart, 1980 and Thwaites, 1982**). Considering the number of behavioural components described and the possibility of genetic influences, the present study was undertaken to determine that if breed and experience differences in buck sexual behaviour exist and that if there is relationship between mating frequency and pregnancy and kidding rates.

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MATERIALS AND METHODS

Animals

This study was performed on a private farm located at El-Amriya city, Alexandria Governorate, Egypt. Twenty-three bucks from 2 pure breeds of goats (Damascus and Egyptian-Nubian) were used in the present study to show the breed effect on buck sexual behaviour. Damascus bucks (n= 11) averaged 53.75 kg and 2.46 years an average age were exposed to 24 multiparous does during the breeding season, while Egyptian-Nubian bucks (n= 12) with a mean body weight of 39.55 kg and 2.37 years an average age were exposed to 25 does. Estrus was not synchronized, and the does were mated in August-October, 2008 mating season. Only one

mating season was performed in the farm, and the mating period lasted for 45 days. Based on service season, bucks used in this study were divided into two age groups to investigate the experience effect on buck sexual behaviour. The first one (n=6 Damascus and 6 Egyptian-Nubian bucks) aged on average 1.51 years with mean body weight of 40.65 kg and was in the first season of service. These bucks never previously come in contact with an estrous does and only trained to mount at 10-12 months of age with an estrous-induced doe to check its readiness for mating as a routine practice in farm. The other group (n= 5 Damascus and 6 Egyptian-Nubian bucks) aged on average 3.34 years and of 54.05 kg mean body weight had been used previously in service for several seasons (more than two seasons).

Management

Bucks of each breed were housed all together in a semi-covered shed, with visual and auditory contact with the does. Throughout the study period, each buck was given 800-1000 gm of commercial concentrate/day (14.8 % CP), with free access to hay, minerals blocks and fresh drinking water. During mating season, does were checked twice daily at 0800 and 1600 h for estrous behaviour by a group of bucks not used for services. The genitals of each buck of this group were covered before introduction to an estrous female to avoid unwanted intromission (teaser buck). The doe was considered in estrous if she stood immobile when mounted by buck **(Chemineau et al., 1992).**

Data recording

Buck sexual behaviour was assessed individually in 3.5×4m pen with one male and one sexually receptive female to avoid interference from fighting. Buck activity was continuously monitored by focal observation (**Martin and Bateson 1993)** using a video camera (Sony, 450X, Japan) from the first look of estrous female and continued for 30 min **(Bench et al., 2001 ; Ungerfeld et al., 2008).** From these observations, the frequency of anogenital sniffing, nudging, and vocalization and the latency to anogenital sniffing, mounting attempt, mounting and ejaculation were recoded, as well as, the total number of mounts and ejaculations throughout the period of observation. The definitions of behaviour patterns recorded were described in table 1 according to

Bernon and Shrestha (1984). Mating efficiency defined as the ratio of ejaculations to total mounts **(Bench et al., 2001)** was also calculated. All does, were received further insemination after 24h from the first one. Bucks from each breed were used in rotation, in order to avoid the negative effect of doe and buck preferences and assure their cyclic use. The non-pregnant does or the return does were mated again in order to avoid reduce fertility rate. Late pregnancy was diagnosed by abdominal palpation after 100 day of mating. Estrous and natural services dates were recorded for each individual doe during breeding season. Abortions, still births, pregnancy rate (number of does pregnant / does exposed to bucks), kidding rate (number of does kidded / does pregnant) and litter size (number of kids / female) were recorded **(Charring et al., 1992)**.

Table (1): Sexual behaviour component description.

Behaviour	**Description**
Sniffing	Nasal investigation of anogenital region
Nudging	Flank, hip region of ewe physically bumped by head and/or shoulder of ram.
Mount	Attempts to mount or mount without pelvic oscillations.
Ejaculation	Mount accompanied by pelvic oscillations. Usually accompanied by penile insertion.

Statistical analysis

Buck sexual behaviour was compared between the two breeds of goats (Egyptian-Nubian and Damascus) and between the two age groups using independent *t*-test and Chi-square test. The relationship between ejaculations number and pregnancy and kidding rates as well as litter size was carried out by logistic regression coefficient. Statistical analyses were computed using SAS version 12.0 (SAS, 1987). Differences were considered statistically significant at p=0.05 or less. All data are expressed as Means ± S.E. except mating efficiency, still-birth, pregnancy and kidding rates which expressed as percentages.

RESULTS AND DISCUSSION

Breed effect

The results of the present study revealed that the breed of buck influenced the expression of sexual behaviour in goats. Egyptian-Nubian buck showed higher sexual efficiency when compared with Damascus ones. The reaction time, measured from the moment the buck was presented to the estrous female until ejaculation, was shorter in Egyptian-Nubian buck than Damascus buck (1.34 v 3.82 min, $P<0.04$). Moreover, Damascus buck tended to sniff, nudge and vocalize less frequently towards estrous doe as compared to Egyptian-Nubian buck. In addition, the total number of ejaculations throughout the observation period was more clearly higher in Egyptian-Nubian buck than Damascus one togehter with higher mating efficiency (Table 2). This may be due to the reduced sexual interest and libido of Damascus bucks associated with a delay of perception of doe estrous status. These findings are similar to other studies recorded with rams by **Orgeur (1991); Price et al. (1996) ; Panagiotis et al. (2006)** who stated that the number of investigations is associated with high sexual efficiency.

Table (2): Effect of breed on buck sexual behaviour:

Buck breed / Behavioural element	Egyptian-Nubian buck	Damascus buck	*P* - Value
Latency to:			
Anogenital sniffing (sec)	12.4±2.045	18.46±4.86	Ns
First mount attempt (sec)	18.64±2.63	29.08±3.92	0.03
First ejaculation (min)	1.34±0.35	3.82±1.19	0.04
Second ejaculation (min)	4.61±0.89	12.67±1.9	0.0002
Frequency of:			
Anogenital sniffing	44.82±5.99	28.83±5.25	0.05
Nudging	23.65±5.64	11.21±2.12	0.03
Vocalization	33.06±11.09	18.37±5.94	0.02
Mounts / 30min	12.41±1.9	14.94±3.56	Ns
Ejaculations / 30 min	3.41±0.36	1.52±0.14	0.0001
Mating efficiency (%)	27.48	10.21	0.001

Successful reproduction depends on internal fertilization of a female gamete and a male one. To achieve this, a male and female of adequate reproductive status have to come into close contact. This is only possible through a coordinated expression of appropreciate mating response from both male and female animals. Behaviour leading to internal fertilization is obviously very different across species. They vary according to the anatomy of the sexual partner and to the physical and social environment in which this behaviour take place **(Fabre-Nys and Gelez, 2007).**

Breed and individual differences in sexual behaviour have been reported in several studies of goats. In Sudan, the reaction time was calculated in Saanen bucks under tropical climate of about 61.9±7.3 sec **(Ahmed et al., 1997)** in compared with 34.25 ± 2.2 sec for Nubian bucks **(Kamal et al., 2005)**, and this may be attributed to climatic conditions and breed difference. Under Egyptian climatic condition, **Barkawi et al.(2004)** found that Zaraibi bucks took 43.4 ± 1.5 sec with a mean of 1.7 ± 0.04 mounts from the first mount to ejaculation; moreover, the number of mounts per ejaculation did not differ significantly between the first and second ejaculates. On the other hand, Damascus buck showed reaction time of 23.69 ± 2.04 sec during breeding season and 26.25 ± 2.56 sec during non-breeding season **(Ramadan et al., 2009).**

Our results for reaction time were in disagreement with former results, where we recorded longer time (1.34 ± 0.35 and 3.82 ± 1.19 min for Egyptian-Nubian and Damascus bucks respectively) and this may be attributed to the fact that in these studies, calculation of reaction time was occurred during semen collection where estrous does were hold for facilitation of collection, and this tended to reduce this time. Another possible explanation is that, in these studies reaction time was calculated on a basis of time interval between the first mount and ejaculation opposite to our recording from the moment the buck was presented to the estrous female until ejaculation. Also in sheep, **Lindsay (1979)** mentioned that in rams, the ability to perform many completed mounts in a unit time may be innate and within wide limits may not be directly influenced by hormones.

Based on our data, Damascus bucks tended to express reduced rates of sexual interest and libido accompanied by a delay in detecting estrus doe. Coincides with the Damascus goats being seasonal breeder animal **(Al-**

Ghalban et al., 2004 ; Ramadan et al. 2009), it is recommended that hand-mating system that depending firstly on detection of estrous doe by teaser buck then inseminated this doe with a selected active buck being used with Damascus breed to be sure that each doe in estrus will be detected and inseminated with the aim of improving breed fertility. While with respect to Nubian breed, the higher activity of Nubian bucks in perception and mating of estrous does that demonstrated in this study, suggested that there was no risk to miss any doe in estrus if either hand-mating system or to allow bucks to present with a herd of does all time in an insemination groups were applied.

Data presented in table (3) demonstrated that, Nubian goats were expressed higher pregnancy and kidding rates together with higher litter size as compared with Damascus ones. On the other hand, results in table (4) revealed no relation between ejaculations number throughout the recording period and pregnancy and kidding rates as well as litter size as reported previously by **Mellado et al.(2000)** who concluded that neither number of copulations nor number of services from different bucks affected pregnancy or kidding rates. Similarly, in sheep, **Mickelsen et al.(1982)** demonstrated that there were no relationship between conception rate or lambing percentages and number of ejaculations. For that, this requires studying the different risk factors that affecting pregnancy and kidding rates in goats.

For instance, in Mexico, **Mellado et al. (2006)** reported that the most important risk factor affecting pregnancy rate was breed of doe, traditional dairy goats such as Saanen and Toggenburg were nine times less likely to become pregnant compared to Nubian and Granadina goats. Also, in common with other studies with dairy breeds of goats, pregnancy rate was markedly affected by season **(Majid et al., 1993)**.On the other hand, Sexual behaviour and semen quality are the main factors that limit male reproductive efficiency **(Barkawi et al., 2004).** These factors could vary according to the breed, geographical location, season of the year (**Chemineau, 1986; Canedo et al., 1996 ; Karagiannidis et al., 2000),** testicular size **(Dufour et al., 1984 ; Ahmed and Noakes, 1995)** and circulating gonadotrophins **(Perez and Mateos, 1995 ; Kaya et al., 1999).**

Table (3): Abortion, still-birth, pregnancy and kidding rates and litter size of inseminated does.

Doe breed / **Variable**	Egyptian-Nubian does	Damascus does	***P*- value**
Abortion	0	0	-
Still-birth	4.76 %	0	-
Pregnancy rate	90.48 %	81.81 %	0.05
Kidding rate	85.71 %	**81.81 %**	0.05
Litter size	2.11± 0.14	**1.22± 0.1**	0.0001

Table (4): The logistic regression coefficient of total ejaculations number with pregnancy and kidding rates and litter size.

	Pregnancy rate	**Kidding rate**	**Litter size**
Ejaculations number	0.649	- 0.127	1.373
Sig.	0.332 (NS)	0.780 (NS)	0.092 (NS)

(NS) Non significant

Experience effect

Analysis of buck sexual behaviour within breeds in two age groups of this study (Table 5) showed that buck sexual experience only influenced certain aspects of service behaviour. With regard to Nubian bucks, significant difference in sexual behaviour was recoded only in latency to second ejaculate, while for Damascus bucks; the differences were observed in latency to anogenital sniffing and first ejaculate as well as ejaculations number during 30 min-observation session. In several cases, our direct observations revealed that first used bucks were strived to copulate, without exhibiting the rest necessary courtship elements such as anogenital sniffing in a considerable way. On the other hand, in sheep, **Shackleton, 1991** founded that a female which is courted, will stand to mate, while those receiving only forced copulation attempts will run away. Therefore, this may explain the higher latency to ano-genital sniffing and the latency to first ejaculate demonstrated in this study in Damascus bucks that in their first season of service.

Table (5): Effect of experience within breeds on buck sexual behaviour:

	Breeds					
	Egyptian-Nubian buck			Damascus buck		
	Service seasons			Service seasons		
	First season	≥ 2 seasons	*P*-value	First season	≥ 2 seasons	*P*-value
Latency to:						
Anogenital sniffing (sec)	14.00 ± 3.24	10.92 ± 2.61	NS	18.56 ± 6.42	5.36 ± 0.57	0.01
First mount attempt (sec)	19.92 ± 3.00	17.46 ± 4.32	NS	28.8 ± 6.80	29.29 ± 4.86	NS
First ejaculation (min)	1.50 ± 0.46	1.18 ± 0.53	NS	7.18 ± 2.69	1.70 ± 0.37	0.02
Second ejaculation (min)	6.52 ± 1.64	2.98 ± 0.54	0.05	15.79 ± 5.44	11.47 ± 2.07	NS
Frequency of						
Anogenital sniffing	48.87 ± 9.52	41.22 ± 7.87	NS	36.88 ± 9.37	59.7 ± 12.14	NS
Nudging	13.62 ± 3.94	27.37 ± 8.81	NS	11.78 ± 3.28	10.70 ± 2.90	NS
Vocalization	26.37± 14.44	39.00± 17.14	NS	14.44 ± 5.58	20.9 ± 10.27	NS
Total mounts / 30 min	12.12 ± 2.23	12.67 ± 3.14	NS	15.67 ± 6.12	14.4 ± 4.11	NS
Total ejaculation / 30 min	2.62 ± 0.46	4.00 ± 0.55	NS	1.22 ± 0.22	1.90 ± 0.18	0.02

Furthermore, the combined data of both breeds tended to follow the same trend (Table 6). In this study, no great differences in sexual behaviour were recoded between the age groups of bucks. Differences in sexual behaviour were only significant for latency to anogenital sniffing and first ejaculate as well as ejaculations number throughout the period of observation and mating efficiency, whereas, all other parameters of sexual behaviour were non-significant (Table 6).

Table (6): Effect of experience on buck sexual behaviour:

Service seasons / Behavioural element	First season bucks	≥ 2 seasons bucks	*P* - Value
Latency to:			
Anogenital sniffing (sec)	24.14±8.97	8.04±1.38	0.05
First mount attempt (sec)	23.95±3.54	23.59±3.41	Ns
First ejaculation (min)	3.94±1.31	1.44±0.32	0.04
Second ejaculation (min)	8.99±2.07	7.17±1.37	Ns
Frequency of:			
Anogenital sniffing	42.53±6.64	50.95±7.52	Ns
Nudging	12.65±2.47	21.05±5.22	Ns
Vocalization	20.05±7.3	30.00±9.69	Ns
Mounts / 30min	14.18±3.37	13.63±2.55	Ns
Ejaculations / 30 min	1.94±0.27	2.89±0.36	0.04
Mating efficiency (%)	13.69%	21.33%	0.05

The reasons that no great differences existed in sexual behaviour between the two age groups of bucks used in this study were presumably may be attributed to the fact that, first service season group was previously trained to mount at yearling with an estrus-induced female, and also they were sexually fully mature as the old bucks, can to great extent perform complete mating response and to produce good quality semen, where **Skalet et al. (1988)** stated that Nubian bucks started producing good quality semen at 8 months of age. Zaraibi bucks aged 18-19 months were able to produce high semen index and libido especially during summer and autumn **(Barkawi et al., 2004).** This indicates complete physiological maturity of first service season used bucks of the current study associated with appropreciate sexual behavioural response. Similarly, **Chakraborty et al. (1989)** founded that sexual maturity in male Nubian goat was reached at a mean age of 32.4 ± 0.9 weeks at an average body weight of 37.7 ± 3.3 kg with ejaculate volume and sperm concentration at puberty of 0.92 ± 0.07 ml and 1.25 ± 0.37 $\times 10^9$/ml of ejaculate.

The greater male sexual performance depends primarily up on a male having great sexual motivation coupled with vigorous physical ability, while the lesser sexual performance occurs when any combination of

poor sexual motivation, lack of experience, inadequate physical strength, poor coordination, and abnormal conformation occurs **(Price, 1985 ; Katz and McDonald, 1992).** Likewise, in sheep, **Bench et al. (2001)** demonstrated that differences in ram sexual performance appear to be associated with libido and sexual interest rather than the ability to perform efficiently the motor patterns of mounting and copulation. Moreover, Tilbrook et al., 1987 concluded that rams also exhibit mating preferences for particular estrus ewes because the ewes differ in their individual sexual attractiveness. This attractivety is affected by a number of factors, such as breed, age, live-weight, size and general appearance **(Tilbrook and Lindsay, 1987).** Thus, this warrant to show the role of sexual status of doe for sexual stimulation of buck.

CONCLUSION

In conclusion, this study had demonstrated that Egyptian-Nubian buck had more efficient mating response than Damascus bucks. Thus, hand-mating system is recommended for Damascus breed especially with less experienced bucks. Buck experience had little effect on sexual behaviour in this study. Additionally, no relationship was found between ejaculations number and pregnancy and kidding rates as well as litter size. The present work need further study to examine semen characteristics and hormonal status of buck as well as different factors related to both buck and doe that associated with pregnancy and kidding rates.

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تأثير سلالة وخبرة ذكور الماعز علي سلوكها الجنسي في كل من الماعز الدمشقي والنوبي المصرية

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أجريت هذه الدراسة لمقارنة السلوك الجنسي لذكور الماعز الدمشقي والنوبي المصرية وبيان علاقة السلوك الجنسي للذكور بمعدل الحمل والولادة. أستخدم في هذه الدراسة عدد 23 ذكر ماعز (12 دمشقي , 11 نوبي مصري). وعلى حسب خبرة هذه الحيوانات تم تقسيمها إلي مجموعتين. المجموعة الأولي وعددها 12 ذكر ومتوسط عمرها 1.51 سنة و40.65 كجم متوسط وزن الجسم لم تستخدم سابقاً في التلقيح أما المجموعة الأخرى وعددها 11 ذكر بمتوسط عمر 3.34 سنة و54.05 كجم متوسط وزن الجسم قد استخدمت سابقاً في التلقيح لأكثر من موسمين. تم تسجيل السلوك الجنسي لذكور الماعز فردياً من لحظة رؤيتها للإناث فى الشياع وحتى 30 دقيقة. وقد أسفرت النتائج عن الأتي:-

1- وجد اختلافاً واضحاً في السلوك الجنسي لذكور الماعز بين السلالتين. حيث كانت ذكور الماعز النوبي المصرية أكثر نشاطاً عن مثيلتها الدمشقية واستطاعت إجراء القذفة الأولي والثانية في وقت أقل وسجلت عدد أكبر من القذفات خلال مدة الملاحظة . أيضا كان معدل شم الإناث التى فى الشياع والاحتكاك بها وإصدار الأصوات تجاهها أكثر في هذه الذكور عنه في ذكور الماعز الدمشقية.
2 - لم يكن هناك تأثير واضح لعامل الخبرة والعمر علي السلوك الجنسي لذكور الماعز في هذه الدراسة.
3 - كان معدل الحمل والولادة أعلي في اناث الماعز النوبي المصرية عن مثيلتها الدمشقية.
4 - لم يكن هناك علاقة بين عدد القذفات ومعدل الحمل والولادة وأيضا عدد المواليد.

من هذه الدراسة نستنتج الأتي:-
1. وجود تأثير واضح لسلالة ذكور الماعز علي سلوكها الجنسي.
2. وجود تأثير محدود لخبرة وعمر ذكور الماعز في هذه الدراسة علي سلوكها الجنسي.
3. لا يوجد علاقة بين عدد القذفات ومعدل الحمل والولادة وأيضا عدد المواليد.

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MYCOLOGICAL, BIOCHEMICAL AND HISTOPATHOLOGICAL STUDIES ON ACUTE FUSARIOTOXICOSIS IN SHEEP

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ABSTRACT

The present study was conducted on one hundred affected sheep which were collected from desert districts in governorates of (Giza; 6th. October and El-Wadi-El-Gadid). Sixty percent of the collected sheep sera had a mean levels of T-2, zearalenone and fumonisins (2.5±0.2, 4.3±0.5 and 25.0±2.0) respectively. The used feeds and underground water in breeding of this sheep were examined mycologically which revealed that all examined samples gave a variable severity of pollution. Seven genera and 15 species of fungi were recovered from feeds and water. The most predominant isolates belong to members of genus Aspergillus with an avarage of (5-100%), followed by Fusarium spp. (40-90%), Penicillium spp. (10-55%) and Mucor spp. (10-50). The Fusarium toxins were detected in the same feed samples, the highest amount was estimated in crushed yellow corn (60%) namely FB1, T2 and zearalenone with the mean levels of (48.4±1.0; 3.0±0.1 and 0.84±0.03) respectively. The significant high levels of FB1 in the present feed samples and serum of diseased sheep gave a large possibility that FB1 was responsible for this disease outbreak in sheep. On the other hand, the biochemical examination of diseased sheep sera for estimation of toxic effects was based on the assumption that the elevated activities in levels of serum enzymes such as AST, ALT, GGT, LDH and urea (Table, 5). Slightly decrease in ceratinine, calcium and phosphorus levels comparing with the apparently healthy group. The pattern of protein electrophoresis showed a significantly decrease values in were recorded serum total protein, alpha globulin, beta globulin with slightly increase in gamma globulin. The internal organs of dead cases had various significant pathological changes in vital organs including hemorrhagic, alveolar pneumonia and calcification in lung. The liver showed hemorrhage, oedema, vacuolar degeneration and necrosis of hepatocytes with evidence of preneoplastic stage in liver cells. Whereas, the kidney showed vacuolar degenerating changes and necrosis of the tubular epithelium, in addition to glomurular oedema and calcium deposition. Significant dangerous effect of environmental pollutions particularly fusarium species and their toxins were recorded in the study.

Keywords: pollution; biochemical alterations; fusarium

INTRODUCTION

The increased importance of animal production due to progressive elevated requirement of human consumption gave an intensive attention of animal health status. The environmental pollution is considered the essential cause of animal diseases particularly pollution with fungi and their toxins for the used feed and water in animal breading and elsewhere, contamination of human food. Mycotoxins are a group of structurally diverse, mold elaborated compounds that induce diseases known as mycotoxicosis in humans and animals. As much as twenty-five percent of the world's food crops are estimated to be contaminated with mycotoxins. Ingestion of sufficient quantities of mycotoxin-contaminated material leads to acute, and more commonly, chronic intoxication **(Hassan et al., 2003 and 2009).**

The mycotoxins of greatest agricultural and public health significance include aflatoxins, ochratoxins, trichothecenes, fumonisins, zearalenone, and ergot alkaloids (**Hassan et al., 2004, 2008 and 2009).** However**,** the fungi of Fusarium species and their toxins are widely distributed through the world where they occur in soil, on plants, plants debris and similar organic subtracts. They cause significant economic losses in agriculture, morbidity and mortality in animals and immunological compromised humans, where it is capable of killing cells by causing extensive damage to cellular membrane **(Ajello and Hay, 1998 ; Mogda, Mansour, et al., 2002).** On the other hand, epidemiological studies associated with fusarium toxins had a wide range of biological effects, including pulmonary oedema in pigs and ruminants **(Harrison et al., 1990),** nephrotoxicity and liver cancer in rats **(Gelderblom et al., 1996).** Although, its effects on human are difficult to be determined.

Fumonisin B9 had been statistically associated with a high incidence of oesophageal cancer in certain areas of Transkei, South Africa and also in China **(Chu and Li, 1994).** The International Agency for Research on Cancer has declared F. moniliforum form toxins as potentially carcinogenic to human. **Gelderblom et al. (1994)** proposed that FB1 was a tumour promoter at doses not causing significant liver pathology but when given at overtly hepatotoxic dose, it was also a weak initiator. Also, the lymphocytes decreased in response to Zeraralonone especially

for LD50 dose. Many data showed that this mycotoxin induced immunosupression in depressing T or B lymphocyte activity **(Berek et al., 2001).** All the previous literatures recorded that the pollution affect upon the growth rate and health of human being and animals including aneamia , stunted growth , carcinogenic, tremorgenic, haemorrhagic, dermatitic, pulmonary edema, immunosuppressive and hormonal effects **(Hassan, 1998 and 2003 ;and Hassan et al., 2003 ;2004;2008 and 2009)**. Whenever, sheep breeding and their production is the main source of food for human in the desert districts. So, the aim of the present work was to investigate the problem of fungal and fusarium mycotoxins pollution of feed and underground water and its role in the health status of sheep at some deserts Governorates (Giza, El-Wadi El Gadid and 6 th October).

MATERIAL AND METHODS

Material

Samples

Serum, feed and water samples

Blood samples were collected from one hundreds diseased cases of sheep at desert districts in governorates of Giza; 6th October and El-Wadi-El-Gadid were investigated. The cases of sheep suffered from loss of weight gain, low productivity, diarrhea, mastitis, disturbance in fertility and sudden mortality of some cases. From districts of diseased cases, 100 samples of sera, 150 feeds and 20 samples of underground water which used in breeding of diseased sheep were collected. The samples of feed and water were collected in sterile plastic container to prevent any contamination.

Internal organs

From the recently deed cases of animal from disease outbreak, the internal organs were collected and imbedded in 10% formalin solution for further histopathological examination. These organs included liver, kidney, lung, bronchial lymph node and heart.

Mycotoxins standards

Standers and immunoaffinity column of Zearalenon, T2 and FB1, were purchased from Sigma Chemical Company (USA).

Methods

Mycological examination of samples

The samples of feeds and underground water which were consumed by symptomatically affected sheep were subjected for isolation and identification of fungi as recommended by **(Conner et al., 1992).**

Detection of mycotoxins in feed and sera of diseased sheep

Detection of mycotoxins in serum of sheep and feed stuffs by fluerometric methods as described by **Hansen (1993)** using immune-affinity column method**.**

Biochemical investigations of sheep sera:

Blood samples were collected in small labeled dry and clean vials without anticoagulant in centrifuge tube, allowed to clot and then centrifuged at 3000 rpm for 15 minutes for separation of serum which used to assay the biochemical parameters The biochemical assays of serum gamma glutamyle transferase (GGT) and lactic dehydrogenase (LDH) activities were determined according to methods of **(Szase et al., 1976)** ,aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities according to **Reitman and Frankel (1957**), serum urea according to **Wybenga et al. (1971**), serum creatinine level according to ***Henry (1974)***, Estimation of serum total protein and electrophoretic pattern were carried out after **SonnenWirth and Jaret (1980) ; Davis (1964) ,** respectively.

However, measurement of calcium, ph. and Mg. were carried out as the technique described in the references **(Brown et al., 1986 ; Brown and Taylor, 1995)**.

Histopathological studies

Tissue specimens were collected directly from lung, bronchial lymph node, heart, liver, spleen, kidneys and intestine of freshly dead cases for histopathological examination. They were kept in 10% neutral buffered formalin for at least 24 hours, routinely processed by the standard paraffin embedding technique and stained with Hematoxylin and Eosin for routine histopathological investigation. Prussian blue stain for detectimy hemosidrin pigments **(Bancroft et al., 1994).**

Statistical Analysis

The obtained date were computerized and analyzed for significance, Calculation of standard error and variance according to (**SPSS 14, 2006**).

RESULTS AND DISCUSSION

The economical importance of sheep animals in desert districts Governorates were at the top to other part in Egypt, where, peoples in these districts their life depend on its products such as meat, milk, wool and leather obtained from these animals (**Agaoglu, 1991; Camas et al., 1994 ; Hassan et al., 2008**)

In the present study, the current data in table (1) showed that, sera of one hundred cases of diseased sheep outbreaks which suffered from loss of weight gain, low productivity, diarrhea, mastitis, disturbance in fertility and sudden mortality of some cases at desert districts in governorates of Giza; 6thOctober and El-Wadi-El-Gadid, contained significant levels of fusarium toxins. Meanwhile, sixty percent of these sheep had the mean levels of fusarium toxins as T-2, zearalenone and fumonisins (2.5±0.2, 4.3±0.5 and 25.0±2.0) respectively. .The results indicated that serum of diseased sheep contained higher mean significant level of FB1 than other types of fusarium toxins which suggested being the essential cause of disease. Mycotoxins in sera of sheep and cattle in Egypt in association with symptoms of toxicities were previously reported by **Hassan (1994); Hassan et al. (2003; 2004 and 2009).**

Table (1): Determination of fusarium toxins in serum of diseased sheep

Animals	Prevalence of fusarium toxins			Mean levels of fusarium toxins (ppm)		
	No. of tested	No. of +ve	%	Fumonisins	T-2	Zearalenone
Sheep	100	60	60	25.0±2.0	2.5±0.2	4.3±0.5

- Results are expressed as means ± SEM, student 't' test

The effects of fusarium toxins in human and animals were varied from carcinogenic and nephrotoxic and immunosuppressive health effects (**Morriss, et al, 1997)**. Although the main route of human exposure to mycotoxins has been identified as the direct ingestion of contaminated cereals and grains **(Morriss, et al, 1997)**, there are many studies about whether the ingestion of meat, milk, and eggs originating from mycotoxin-exposed food-production animals is a significant pathway for mycotoxins among humans **(Hassan et al., 1997; Wafia, H. Abdallah and Hassan, 2000 ; Hassan et al., 2004 and 2009).** The search focused to recovered the accurate causes and sources of this collected cases, therefore, the direct factors to the animal consumption were examined .The fungal examination for feeds , feedstuffs and underground water (which the only available source of water in these districts), revealed that all examined samples gave a variable rates of pollution. Seven genera and 15 species of fungi were isolated from consumed foods and drinking water. The most predominant isolates belong to members of genus Aspergillus with a range of (5-100%), followed by Fusarium spp. with (40-90%), Penicillium spp. (10-55%) and Mucor spp. (10-50%).

Whereas, the frequency of isolation of other spp. as Rhizopus spp., C.albicanse and Rhodotorula spp. were relatively low. On the other hand, the fungal contamination of underground water was significantly high as compared with standard healthy water which must be free from any signs of pollution (Table, 2). However, F.moniliform, F.oxysporum and F. solani were the most frequent isolated members of Fusarium from feed samples (Table, 3). The fungus of F.moniliform was recovered from all examined feed samples with(20-65%), while, F.oxysporum was isolated from lower examined samples (5-10%) with exception of wheat straw samples .Whereas, the species of F. nival and F. fusaroides were only isolated from (Soya bean meal and crushed yellow corn), respectively with the same rate (5%). It is clear from the result that crushed yellow corn and wheat straw were the most contaminated materials followed by hay, Soya bean and drawa. While, the underground water was the lowest contaminated samples. These differences in the level of contamination may be due to the exposure of the examined samples to different climatic condition either during preparation or transportation or storage. These

findings were in agreement with the results of (**Hassan et al. 2003; 2004; 2008 and 2009)**, who recovered most of these fungi from the examined feed and water samples.

Table (2): Prevalence of fungi in feeds and underground water used for breeding of sheep

Fungal Species	Crushed yellow corn(30)		hay(35)		Wheat straw(20)		Soya bean meal(35)		Drawa (Leaves of yellow corn) (30)		Underground water (20)	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Aspergillus sp.	**20**	**100**	**19**	**95**	**20**	**100**	**15**	**75**	**10**	**50**	**1**	**5**
A. flavus	**18**	**90**	**17**	**85**	**18**	**90**	**7**	**35**	**40**	**20**	**1**	**5**
A. niger	**16**	**80**	**15**	**75**	**15**	**75**	**14**	**70**	**36**	**18**	**10**	**50**
A. candidus	**1**	**5**	--	--	--	--	**2**	**10**	**30**	**15**	**0**	**0**
A. fumigatus	**4**	**20**	**7**	**35**	--	--	**2**	**10**	**20**	**10**	**1**	**5**
A. ochraceus	**5**	**25**	**19**	**5**	**1**	**5**	**1**	**5**	**16**	**8**	**0**	**0**
A. terrus	**5**	**25**	**2**	**10**	**3**	**15**	**3**	**15**	**10**	**5**	**0**	**0**
Fusarium sp.	**10**	**50**	**18**	**90**	**15**	**75**	**8**	**40**	**8**	**40**	**0**	**0**
Penicillim sp.	**7**	**35**	**9**	**45**	**6**	**30**	**10**	**50**	**11**	**55**	**2**	**10**
Mucor sp.	**10**	**50**	**6**	**30**	**2**	**10**	**10**	**50**	**3**	**15**	**0**	**0**
Rhizopus sp.	**1**	**5**	**1**	**5**	**3**	**15**	**4**	**20**	**1**	**5**	**0**	**0**
C.albicanse	**2**	**10**	**0**	**0**	**0**	**0**	**1**	**5**	**2**	**10**	**1**	**5**
Rhodotorula sp	**1**	**5**	**0**	**0**	**1**	**5**	**0**	**0**	**2**	**10**	**2**	**10**

Table(3):Prevalence of fusarium species in feeds of sheep suffering from problems of animal diseases from different districts at el Wadi El Gedid

Fusarium Species	Crushed yellow corn		Hay		Wheat straw		Soya bean meal		Drawa (Leaves of yellow corn)	
	No.	%	No.	%	No.	%	No.	%	No.	%
F.moniliforme	4	20	13	65	8	40	6	30	7	35
F.oxysporum	1	5	1	5	-	-	1	5	2	10
F.solani	1	5	1	5	4	20	-	-	-	-
F.sporotrichoides	1	5	-		1	5	-	-	-	-
F. aquaeductum	1	5	-	-	1	5	-	-	-	-
F. nival	-	-	-	-	-	-	1	5	-	-
F. fusaroides	1	5	-	-	-	-	-	-	-	-
F. equiseti	-	-	-	-	1	5	-	-	-	-
F. tricinctum	1	5	3	15	-	-	-	-	-	-

When, the feed samples which contaminated with fusarium spp. were subjected for detection of Fusarium toxins, the results revealed that the largest amount was detected in crushed yellow corn (60%) namely FB1, T2 and zearalenone with the mean levels of (48.4±1.0; 3.0±0.1 and 0.84±0.03 ppm)**,** respectively.

It was interesting to report here that the samples of wheat straw contained only FB1 at a rate of (70%) with a mean level of (20±0.9 ppm) (Table, 4). The significant levels of FB1 in the present feed samples and serum of diseased sheep gave a large possibility that FB1 was responsible for the disease outbreak in sheep**.** The Food and drug administration has established recommended maximum levels for aflatoxins and fumonisins in animal feed. For swine, ruminants including sheep, and poultry, the recommended maximum levels of total fumonisins in complete feeds are 10, 30, and 50 µg/g, respectively **(FDA, 1994).** Therefore, the detected levels of FB1 were significantly

over the permissible limits in feeds particularly FB1 toxin in examined sheep feed samples which ranged from (15.0±0.2-48.4±1.0 ppm). The same findings were detected by many authors as **(Hassan et al., 2002; 2003;2004; 2008 and 2009) ; El-Hamaky (2001) ; El Ahle et al.(2006)**

Table (4): Detection of fusarium toxins in feeds.

Fusarium Species	Prevalence of fusarium toxins			Mean levels of fusarium toxins (ppm)		
	No. of tested	No. of +ve	%	Fumonisins	T-2	Zearalenone
Crushed yellow corn	10	6	60	48.4±1.0	3.0±0.1	0.84±0.03
Hay	10	5	50	17.0±1.3	-	0.71±0.0
Wheat straw	10	7	70	20±0.9	-	-
Soya bean meal	10	4	40	15.0±0.2	2.0	0.99±0.005
Drawa (Leaves of yellow corn)	10	4	40	27.0±3.22	1.0±0.01	1.50±0.0

- Results are expressed as means ± SEM, student 't' test

On the other hand, the biochemical examination of affected sheep sera for estimation of toxic effects was based on the elevated activities in levels of serum enzymes activites such as (AST, ALT, GGT, LDH) and urea concentration in Table, (5). While, a slightly decreases in ceratinine level compared with the apparently healthy group. These results reflected the damage in the different organs **(Cheng et al., 2001 ; Asrani, et al., 2006).** Increased serum enzymes activity was observed by feeding toxic diets due to hepatic degeneration and subsequent leakage of enzymes into circulation. **(Chen** et **al., 2008 ; Wang et al., 2008).** It is reported that the significant effect of fusarium toxins are the alteration in serum concentration of kidney and liver enzymes ,total protein, albumin, minerals and lipid profiles **(Kubena et al., 1997 ; Mogdaet al., 2002).**

Table (5)*;* Biochemical parameters in serum of diseases sheep cases at desert districts in comparison to healthy cases.

Parameter	Apparently healthy	Diseased group
AST u/l	53.67±4.91	124.9***±7.94
ALT u/l	40.66±2.18	93.6***±5.48
GGT u/l	97.57±1.38	111.56*±5.11
LDH u/l	718.4±22.36	811.0*±24.11
urea mg%	41.11±2.15	53.52**±3.81
Creatinin mg%	1.31±0.07	0.9±0.14*
Uric acid mg%	3.17±0.37	5.1**±0.34
Calcium mg%	9.22±0.33	7.46**±0.41
Phosphorus mg%	6.31±0.32	5.77±0.17

Results are expressed as means ± SEM ,student 't' test

The high concentrations of serum urea in sheep fed on contaminated diet may be leading to increased ammonia absorption caused by altered protein turnover in the rumen micro-flora, or altered protein metabolism in sheep tissues. In ruminants, serum urea levels are affected by protein digestion and metabolism by the rumen biomass. A large portion of dietary protein is hydrolyzed and deaminated by rumen micro-flora, giving rise to peptides and free ammonia in the rumen **(Herdt, 2000).** A part of free ammonia is absorbed and is metabolized to urea in the liver. If microbial protein synthesis in the rumen which is inhibited by mycotoxins, more free ammonia remains in the rumen, is absorbed into the blood, and is metabolized to urea, resulting in elevated blood urea concentrations. **Danicke et al. (2005)** observed that postprandial rumen fluid ammonia concentrations were consistently higher when *Fusarium* mycotoxin-contaminated wheat was fed to sheep**.** Inhibition of protein synthesis results in elevated concentrations of free Amino acid that are used for energy utilization, resulting in increased serum urea. The results of the present study are in agreement with those of **Chowdhury and Smith (2004),** who observed that excessive serum concentrations of uric

acid in laying hens were a result of feeding feedborne *Fusarium* mycotoxins. Moreover, in a subsequent study with laying hens, they found that feeding contaminated grains led to reduced hepatic fractional protein synthesis rates **(Chowdhury and Smith, 2005). Danicke et al. (2006)** also observed a reduction in fractional protein synthesis rates in the kidneys, spleen, and ileum of pigs exposed to DON.

At the same time concentrations of serum calcium and serum phosphorus were decreased due to feeding *Fusarium* mycotoxin-contaminated diets This results were agree with **Díaz and Smith (2006).** *Fusarium* inducing significantly decreased values in serum total protein, alpha globulin, beta globulin and while slightly increase in gamma globulin, these results agree with **(Rotter et al., 1994**).

Table (6)*;* Patterns of protein electrophoresis in serum of diseases sheep cases at desert districts in comparison to healthy cases (mg/dl).

Parameter	Apparently healthy	Diseased group
Alb	2.35±0.12	1.87**±0.07
T.alpha	0.96±0.1	0.87±0.09
Alpha1	0.41±0.03	0.4±0.02
Alpha1	0.55±0.02	0.47*±0.02
t. beta globulin	1.09±0.04	1.02±0.03
Beta1	0.5±0.02	0.55±0.04
Beta2	0.59±0.01	0.47*±0.04
Gamma1	1.59±0.11	1.53±0.05
Gamma2	0.34±0.03	0.520.03
Gamma globulin	1.93±0.15	2.05±0.1
T.globulin	3.98±0.33	3.940.29
A/G ratio	0.59±0.03	0.43**±0.03
T. protein	6.33±0.55	5.81±0.08

The globulin component (Table, 6) showed drop in α1, α2 and β2 globulin in all the experiment while decrease γ1 globulin. This may be attributed to that *Fusarium* fungi cause's hepatotoxic, nephrosis, hemorrhages in (liver and kidneys) **(Tietz, 1996)** *Fusarium* mycotoxins might affect the synthesis of globulins of hepatic origin as well as globulins of lymphoid origin. **Rotter et al. (1994)** suggested that *Fusarium* mycotoxins can directly affect α-globulin synthesis in the liver. In addition, *Fusarium* fungi has immunosuppressive effect inhibit nearly cellular and humeral immunologic reaction have been reported by **Nelson et al. (1994) i**ncluding disruption of normal cell function by inhibiting RNA, DNA, and protein synthesis; inhibition of cell division; stimulation of ribotoxic stress response; and activation of mitogen-activated protein kinases. It has been found that T-2 toxin is a potent member of the trichothecene group of mycotoxins produced by *Fusarium* fungi **(Bamburg et al.,1970).** It has been found that T-2 toxin is a mycotoxin with immunomodulatory activity, where it can stimulate (immune-stimulation) or inhibit (immune-suppression) the activity of the immune system **(Shinozuka et al., 1997; Pestka et al., 2004).**

To give complete idea about the effect of these collected cases, the internal organs of dead cases during disease outbreak in the same desert districts were subjected for histopathological studies. The results revealed that thickening of the pleural membrane with infiltration of mononuclear inflammatory cells, hemorrhage and proliferation of the epithelial cells lining bronchioles. Moreover, in some cases the proliferation was severe and uncontrolled which lead to occluded the bronchial lumen and form nest of epithelial cells with clear eosinophilic cytoplasm giving the feature of preneoplastic stage (Fig. 1, a & b).Some alveoli were filled with red blood cells accompanied with mononuclear inflammatory cells (alveolar pneumonia). Destruction of the wall of some alveoli with infiltration of inflammatory cells (lymphocytes, macrophages and neutrophils) were noticed accompanied with hemorrhage and calcification (Fig. 2, a & b). Severe hemorrhages with infiltration of inflammatory cells with compensatory emphysema (hemorrhagic pneumonia) were seen in some cases.

While, bronchial lymph node showed moderate to severe depletion of lymphoid follicles, where lymphocytes detected inside alveoli and interalveolar septa. The respiratory tract is the primary rout of entry for Fusarium spp. and their toxins based on the sinopulmonary involvement. It has been speculated that the fusarium toxins produced damage the tissues which allowing the fungus to spread more easily **(Ajello and Hay, 1998).** However**, Halloy et al. (2005) and (Haschek et al. (2001)** mentioned that the lung of experimentally fusariotoxicated piglets particularly with FB1 showed a minimal enlargement of the alveolar septa due to an increase in the macrophage, lymphocyte number and develop lethal pulmonary edema within 4-7 days. Whereas, muscles necrosis and oedema were evident in heart in our study. A various degrees of myocardial degeneration with foci or cellular infiltration and fibrosis were observed in rats with several doses of T-2 toxin, a trichothecene metabolite of Fusarium **(Schoental et al., 1979)**.

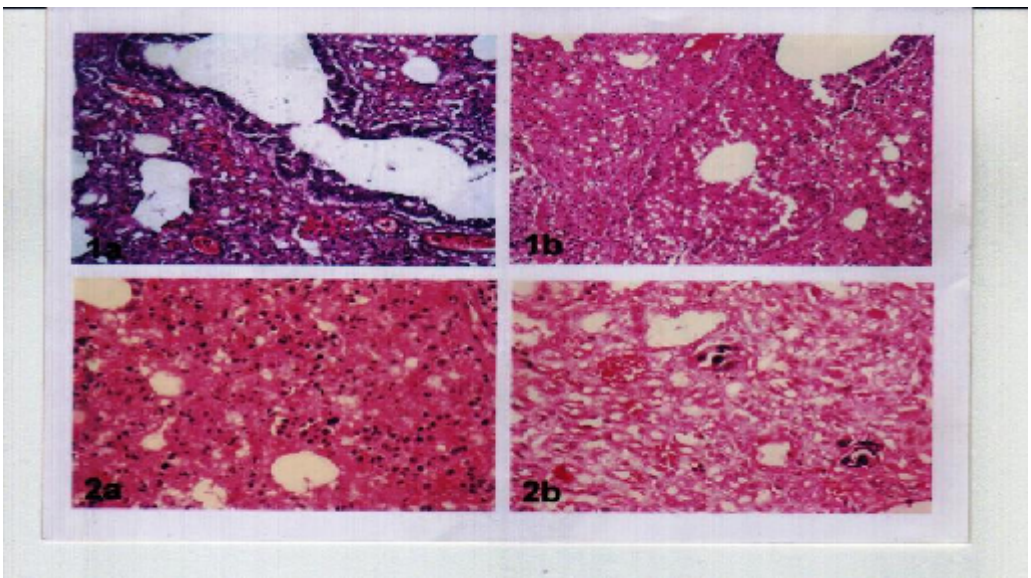

Fig.(1,a & b): Lung of sheep fed on mycotoxin (FB1, T2, ZNE) showing proliferation of the epitheliial cells lining bronchiols was severe, uncontrolled and form nest of epithelial cells giving the feature of preneoplastic stage (H & E X 100).

Fig.(2, a & b): Lung of sheep fed on mycotoxin (FB1, T2, ZNE) showing destruction of the wall of some alveoli with infiltration of inflammatory cells (lymphocytes, macrophages and neutrophils) accompanied with hemorrhage and calcification (H & E X a) 200, b) 400).

Many researchers mentioned that fusarium toxins particularly FB1 produces a wide range of biological effects including nephrotoxicity and liver cancer in rats **(Gelderblom et al., 1996).** The present study revealed glissonian's cirrhosis in liver, vacuolar degeneration and necrobiotic changes of hepatocytes in addition to haemorrhages and oedema in between hepatocytes.).Some liver cells arranged in irregular aceni (preneoplastic stage) (Fig. 3 a & b). Epithelial hyperplasia of bile duct was detected with the formation of newly formed bile ductules. There were aggregation of oval vesicular cells in the portal area with infiltration of mononuclear inflammatory cells and fibrous connective tissue formation. Similar lesions were illustrated caused by FB1 **(Abbes et al., 2006 ; Voss et al., 2001)** and zearalenone **(James and Smith, 1982).** According to data of the **National Toxicology Program (USA) (1982),** ZEN was found to produce hepatocellular adenoma. While**, Abbes et al. (2006**) mentioned that the histological examination of mice kidney that treated with two ZEN doses alone revealed a swelling in the epithelial cells of the proximal tubules, granular degeneration, shrunken glomeruli with the presence of eosinophilic cast in the lumen of tubules and blood vessels dilatation.

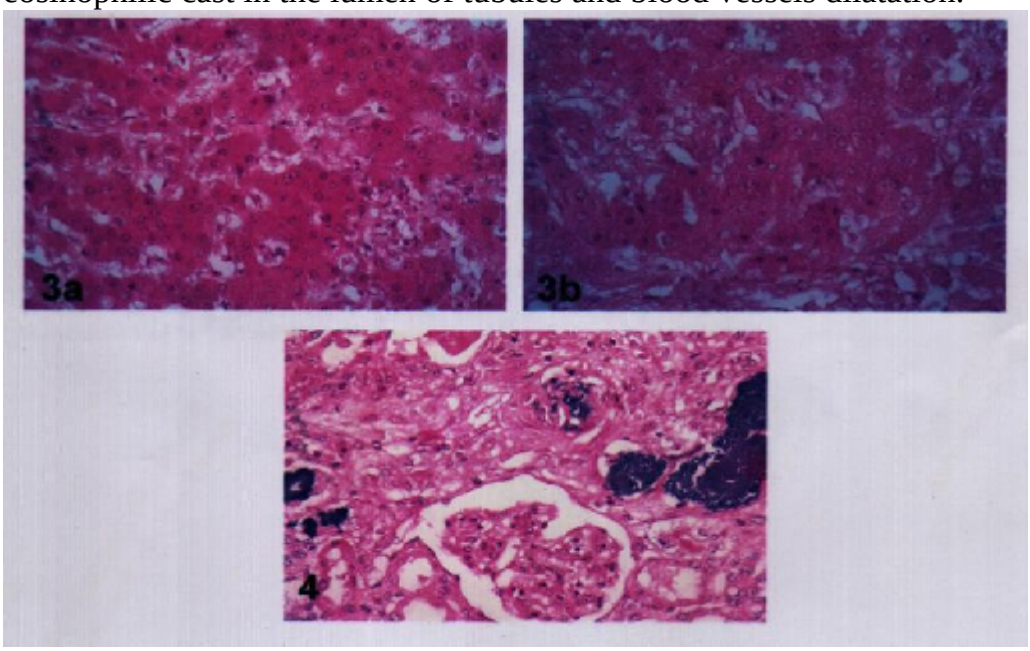

Fig.(3, a & b): Liver of sheep fed on mycotoxin (FB1, T2, ZNE) showing disorganization of hepatic cord (a) with tendency to formation of irregular aceni (preneoplastic stage) (b) (H & E X 400).

Fig. (4): Kideny of sheep feeding on mycotoxin (FB1, T2, ZNE) showing necrosis of renal tubular epithelium, gromerular oedema and calcium deposition. (H & E X 400).

The histopathological examination of kidney revealed dilatation in blood vessels dilatation.Vacuolar degeneration of epithelial cells lining the renal tubules with sloughing in the lumen forming renal casts. Meanwhile, some tubular epithelium revealed necrosis, associated with glomerular oedema and calcium deposition (Fig.4).

Voss et al. (2001), mentioned that FB1 induces apoptosis of hepatocytes and proximal tubular epithelial cells. More advanced lesion in both organs is characterized by simultaneous cell loss (apoptosis and necrosis) and proliferation (mitosis). Microscopic and other findings suggest that an imbalance between cell loss and replacement develops a condition favorable for carcinogenesis. On the molecular level, fumonisins inhibit cermide synthase and disrupt sphingolipid metabolism and theoretically, sphingolipid-mediated regulatory processes that influence apoptosis and mitosis.

The previous literatures recorded that the pollution affect upon the growth rate and health of human being and animals including aneamia , stunted growth , carcinogenic, tremorgenic, haemorrhagic, dermatitic, pulmonary edema, immunosuppressive and hormonal effects (**Hassan, 1998 and 2003 ; Hassan et al., 2003 ;2004;2008 and 2009)**.These findings were confirmed in our study, where , the above results clearly observed the effects of fungal particularly fusarium species and their toxins in sheep at desert districts.

It can induce both toxicologic and immunotoxic effects in a variety of cell systems and animal species as cytotoxic effect to reticulocytes, fibroblasts and lymphocytes and the cellular toxicity appears to be mediated by the inhibition of protein synthesis as reported by **(Ueno, 1983; Rotter et al., 1993; Mogda et al., 2002 ; Hassan et al., 2003 and 2009).** Also, fusarium mycotoxin inhibits cell division, RNA/ DNA synthesis and apoptosis **(Rotter et al., 1996).** Growth retardation and immune suppression are the major toxic effects induced by Fusarium ingestion in farm animals and suppression of the normal immune function

and super induction of pro-inflammatory cytokines have been also suggested as supplementary tools for making a diagnosis as mentioned by **(Widestrand et al., 2003 ; Kinser et al., 2004; Hassan et al., 2004).** This study, focused the highlight of the dangerous effects of fusarium and their mycotoxins pollution of animal feeds and water which allows a certain generalization as to the solution of problems regarding sheep breeding, which is an important contributor to the country's economy (especially at desert districts) in the form of meat, milk, wool and leather, with respect to the effects of environmental factors.

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NEBULIZATION AND INHALATION THERAPY VERSUS CONVENTIONAL MEDICATION OF FELINE ASTHMA WAEL, M. KELANY

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ABSTRACT

Feline asthma syndrome is a life threatening clinical condition characterized by chronic inflammation of the small passageways of the lungs. Although allergens are the prime suspect in the cause of feline asthma, the actual cause is unproven and the condition is believed to be a result of type I immediate hypersensitivity reaction to inhaled allergens. Twenty two diseased and five apparently healthy cats were thoroughly investigated in the present study. The most common clinical presentation was recurrent bouts of coughing (n=13), Cyanosed mucous membranes (n= 7), open-mouth breath (n=7), Squatting with shoulder hunched, neck extended and rapid breathing or gasping for breath (n=11), gagging up foamy mucous (n=3) and exercise intolerance (n=17). Seventeen cats underwent chest radiography. Six cases showed no patterns neither bronchial nor interstitial, nine cases showed bronchial pattern, one case suffer from severe interstitial pattern and one case showed pneumothorax in addition to bronchial pattern. A predominant esinophilic sample was collected from only 4 cats by transtracheal lavage. There were minimal changes in differential white cell counts, except significant esinophilia. Therapeutic plan was directed initially to control asthmatic attack either by conventional medication by injection or nebulization by bronchodilators. Then the pet maintained on oral form of conventional medication or spacer, respectively. The building stone in the present study was avoidance of putative aeroallergens. On the basis of the data of the present cases, it would appear that the diagnosis of feline asthma depends largely on the clinical presentation and radiographic findings. The present study concluded that nebulization and inhalation therapy were more effective and rapid therapy than conventional medication. (n=number).

Keywords: Nebulization, inhalation, conventional, radiography, feline, asthma.

INTRODUCTION

Feline asthma is a chronic inflammation of small lung passageways **(Dye et al, 1996; Johnson, 2000; Drowling, 2001; Gardner, 2005; Cohn et al, 2010**). Asthma has been referred to in the literature by a variety of terms including eosinophilic bronchitis, allergic bronchitis, feline bronchitis, feline bronchial asthma, Allergic airway disease, feline chronic obstructive

pulmonary disease **(Dye and Moise, 1992; Padrid, 2000; Gardner, 2005)**. Feline asthma is very similar to humane asthma **(Padrid et al, 1995; Gardner, 2005)**.When an asthma attack occurs, these passageways thicken and constrict, making it very difficult for a cat to breath **(Padrid, 2000a)**. This often leads to respiratory distress, which can become grave in matter of minutes. The lungs may also begin to discharge mucus into the airways, leading to fits of coughing and wheezing **(Dye, 1992)**.

The prevalence of lower airway diseases in the adult cat populations has been estimated to be approximately 1%. Although any breed may be affected, Siamese cats appear to be over-represented **(Moise, 1989; Padrid, 1996, Adamama-Moraitou et al 2004; Hibbert, 2010)**. Feline asthma has been recognized as a clinical entity for over 100 years and is a common cause of coughing and dyspnoea **(Hill, 1906)**. Asthma has been defined as a disorder of the lower airways that causes airflow limitation, which may resolve spontaneously or in response to medical treatment **(Padrid, 2009)**.Asthma is thought to be due to a type I hypersensitivity reaction to inhaled allergens. It is characterized by eosinophilic airway inflammation, spontaneous bronchconstriction and airway remodeling. Young to middle aged cats are most commonly affected **(Adamama-Moraitou et al, 2004; Corcoran et al, 1995).**

An asthmatic attack can range from mild to severe. In mild cases the cat may suffer bouts of coughing, wheezing and labored breathing that come and go, (can sometimes be mistaken for trying to fetch up a hairball). With a severe attack, the cat will have extreme respiratory problems that can, in some, be life threatening **(Corcoran, et al, 1995; Padrid, 2000b)**. So, any coughing cat needs veterinary evaluation **(Gardner, 2005)**. Clinical signs of asthma in cats included bouts of coughing, wheezing, persistent cough, Squatting with shoulder hunched, neck extended, rapid breathing or gasping for breath, gagging up foamy mucus, open mouth breath, blue lips and gums, labored breath after exertion, overall weakness and lethargy **(Dye et al, 1996; Gardner, 2005; Reinero et al; 2009).** Heartworm test should be done if the cat lives in an area endemic for heart worm diseases **(Leib and Monoroe, 1997).**

The most common radiographic feature identified is a bronchial pattern, characterized by bronchial wall thickening and mineralization (doughnuts and tramlines). Hyper-inflation of the lung fields and flattening of the diaphragm may be identified, due to air trapping. Interstitial and focal alveolar patterns may also be seen, hypothesized to be due to airway obstruction by mucus plugs, causing local atelectasis; the right middle lung lobe is most frequently affected. Gas within the esophagus and gastro-intestinal tract may be seen, as a consequence of aerophagia. Pneumothorax may occasionally be identified; however is a rare complication of feline asthma **(Cooper et al, 2003)**. It is very important to remember that up to 16% of affected cats may have no or only very subtle radiographic changes **(d'Anjou et al, 2007)**.

Unfortunately, feline asthma is a chronic progressive disease that cannot be fully cured. Medications can reduce the symptoms of asthma a great deal, but may not be able to eliminate coughing fully. In recent years, veterinarians have found that the most effective therapy for feline asthma may be to use inhalers such as human asthmatics use. A mask and spacer system, called AeroKat®, has been invented to enable cats to use inhalers or puffers. This system is similar to the mask and spacer system used to treat babies and small children **(Kirschvink et al, 2006; Cohn et al,2010; Hibbert, 2010)**.

MATERIALS AND METHODS

A total number of 22 clinically affected and 5 apparently healthy cats were admitted to the Vet. Clinic of surgery, anesthesiology and radiology department, faculty of veterinary medicine, Cairo University; and private Vet. clinics in Giza governorate. History, clinical presentation, physical examination, complete blood count and radiographic examination were used to confirm clinically affected cats with feline asthma. All cats were thoroughly investigated including age, gender, breed, respiratory rate/ min, pulse rate/ min, rectal temperature (˚C), lymph nodes, mucous membranes and physical examination by percussion and auscultation of chest according to **Kelly (1984).**

Blood samples were collected from anterior median artery. Complete blood count (CBC) was performed for all blood samples with standard techniques

described by **Feldman et al (2000)**. The CBC included red blood cells (RBCs) count, hemoglobin (Hb) concentration, packed cell volume (PCV), red cell indices (mean corpuscular hemoglobin concentration (MCHC), Mean Corpuscular Volume (MCV)) as well as total (TLC) and differential leukocyte count (DLC).

Chest radiographs

Seventeen cats underwent chest radiography, they were not sedated and a ventro-dorsal and a right lateral view were performed. A scoring system **(according to Foster et al, 2004 and Kirschvink et al, 2006)** ranging for the total combined score from 0 to 6 aimed to evaluate separately bronchial pattern (0: absence of signs, 1: mild [first generation of bronchi visible], 2: moderate [second generation visible], 3: severe [third generation visible]) and interstitial pattern (0: absence of signs, 1: mild [mild interstitial framework visible], 2: moderate [interstitial framework distinguishable from a bronchial pattern], 3: severe [clearly apparent interstitial pattern]). The five other cats with normal radiographic findings were used as a control group.

The clinically affected cats were divided into 2 groups according to therapeutic approach. Firstly, Cats presented in severe asthma required emergency treatment. All stressful procedures such as restrain for injections or radiographs should be avoided until the cat is stable. The first group consisted of 10 clinically affected cats treated traditionally (conventional medication) using initially a single dose of injectable corticosteroids and bronchodilators then maintained on tablet form of corticosteroids and bronchodilators. The second group consisted of 12 clinically affected cats managed by nebulization initially and maintained on inhalers using spacer.

The 2 groups managed as follow:

I) The first group consisted of 10 clinically affected cats treated traditionally (conventional medication). Initial emergency treatment consisted of 0.5 ml/ kg Bwt of Minophylline® ampoules (Aminophylline as injectable bronchodilators, 125mg/ 5ml/ ampoule, by Alex pharmaceutical company) and 0.25 ml/ Kg Bwt of dexamethasone® ampoule (dexamethasone sodium phosphate as injectable corticosteroid, 8 mg / ampoule/ 2ml, by Memphis pharmaceutical company). Then cases

maintained on a third of tablet/ 5 kg Bwt of Quibron® once at night (unhydrous theophylline, as tablet form of bronchodilators, 300 mg/ Tablet Bristol- Mayer Squibb) and tablet/ 5 kg Bwt / 12 hrs of Prednisolone® tablet (prednisolone, 5mg/ tablet by Adco pharmaceutical company). The dose decreased gradually after clinical improvement. This application was described by **Leib and Monoroe (1997)**.

II) The second group consisted of 12 clinically affected cats managed by nebulization (using FLO or aerosol delivery system, Piston Compressor system for aerosol therapy made in Italy by CA- MI) initially and maintained on inhalers using spacer (Averro-spacer or valved holding chamber with pediatric mask for use with metered dose inhalers; Made in Egypt by AVERROES Pharma) in addition of oral one third of a tablet/ 5 kg Bwt of Quibron® once at night (unhydrous theophylline, as tablet form of bronchodilators, 300 mg/ Tablet Bristol- Mayer Squibb). 0.5 ml of Farcolin® solution (Salbutamol soln. 0.5 mg/ ml by Pharco) mixed with 1.5 ml of Saline® soln. (0.9 % Nacl soln., by Otsuka) then used in nebulizer. Cats maintained on 7 puffs of Clenil compositum® as inhaler (Salbutamol 100 µg and Beclomethasone dipropionate 50 µg / dose; 200 doses by Cheisi). The doses decreased gradually according to clinical improvement. This method was described by **Gardner (2005) and Cohn (2010)**. Clinical improvement detected by monitoring of clinical status, physical examination and radiographic examination.

History of parasitic control and vaccination was collected. All cats were dosed one tablet for each 4 kg Bwt of Drontal (20mg Praziquantel and 230 mg Pyrantel Embonate made in Germany by Bayer Healthcare) as broad spectrum anthelmintic. Also cats were vaccinated against Chlamydia psitacci, Feline Parvo virus, Feline calici virus and Feline Rhinotracheitis virus (vaccine of Schering pharmaceutical company).

Fecal examination is simple, quick, inexpensive and one of the most important diagnostic procedures to exclude parasitic infestation of lungs and intestines. Examination of a fresh fecal saline smear was yield a diagnosis in some cases. Several drops of saline can be applied to fresh thin fecal smear, a coverslip added, the slide examined microscopically according to **Leib and Monoroe (1997).**

Transtracheal lavage in cats was performed by Transtracheal approach. Transtracheal techniques were performed by clipping and shaving of hair over triangular area of cricothyroid ligament. The skin prepared aseptically using Betadine® antiseptic solution (Povidone iodine by Mundi pharmaceutical company). Stabilization of trachea was done by one hand then needle of jugular catheter (18 guge needle) was inserted by other hand. The needle was advanced slightly into tracheal lumen. Twelve ml syring attached to the catheter containing 2-4ml of sterile saline was injected and aspirated quickly back into the syringe at time of cough. Adequate amounts of fluid (1-2 ml) should be aspirated for cytological evaluation. Direct smears examined on microscope slides for cytological evaluation according to **Padrid et al (1991); Leib and Monoroe (1997)**.

Statistical analysis

It was performed by statistical Package for Social Sciences (SPSS). Mean and standard deviation are descriptive values for quantitative data. ANOVA (Analysis Of Variance) was used for testing means of more than two groups by computer program according to the method described by **Irwan (1996)**.

RESULTS

Apparently healthy cats

Apparently healthy cats were 5 cats of age ranged from 6 months to 12.7 years old (2 females and 3 males) without any apparent clinical signs of feline asthma and of normal laboratory data and normal X-ray. The breeds of apparently healthy cats were 3 Persian cats and 2 Siamese cats.

Clinically affected cats

This group was consisted of 22 clinically affected cats of age ranged from 5 months to 7.8 years old (14 females and 6 males). The breeds of the affected cats in the present study were 13 Persian cats, 7 Siamese cats and one Himalayan cat. The investigated cats revealed significant changes of respiratory, pulse rates and cyanosed mucous membranes in 7 cats. There was a panorama of clinical presentation including open-mouth breath with lateral recumbency in some cats (n= 7), bouts of coughing or persistent cough (n= 13), Squatting with shoulder hunched, neck extended and rapid breathing or gasping for breath (n= 11), gagging up foamy mucus (n= 3),

overall weakness or exercise intolerance (n=17), retching and vomiting occur in 3 cases at the end of a coughing episode (figures 1A, 2A, 3A).

Physical examination of cats with bronchial disease was yield normal results between episodes (n= 5), although most cats have expiratory wheezes (n=14) on thoracic auscultation. In severe cases no breath sounds may be heard (n=7). There were significant changes in respiratory and pulse rates (table 1)

Table (1): General clinical examinations of apparently healthy and clinically asthmatic cats
(Mean ± SE)

Parameters	Clinically healthy cats	Clinically asthmatic cats
1-Respiratory rat (No./min)	32.6 ± 1.21	57.90 ± 2.69**
2- Pulse rate (No. / min)	93.00 ± 1.76	162.73± 6.21**
3- Rectal temperature (° C)	38.52 ± 0.10	38.35± 0.09
4- Mucous membranes	Very faint rosy red	Cyanosis in 7 cats and pallor mm in 15 cats
5- Superficial lymph node	Free	Free

** There were significant increase of respiratory and pulse rates in clinically asthmatic cats.

On the basis of our findings, age, gender and breed of cats with feline asthma were not significantly different from those of cats without feline asthma or control cases.

CHEST RADIOGRAPHS

According to Foster et al, 2004 and Kirschvink et al, 2006 scoring system, the seventeen radiographic examined cats were classified as in table (3).

Table (2): Hematological studies Complete blood count was normal except significant Esinophilia (Mean ± SE)

Parameters	Unit	Control cats	Asthmatic cats
RBCs count	X 10^6/µL	6.43 ± .0.10	5.11 ± 0.23**
PCV	%	40.80 ± 0.32	39.38 ± 0.39
Hb concentration	g/dl	12.45 ± 0.12	11.06 ± 0.32*
MCV	Fl	63.47 ± 0.83	75.29 ± 3.05
MCHC	g/dl	30.52 ± 0.21	28.04 ± 0.55*
TLC	X 10^3/µL	8.72 ± 0.06	9.45 ± 0.43
Neutophils	X 10^3/µL	5.46 ± 0.07	5.60 ± 0.24
Lymphocytes	X 10^3/µL	2.30 ± 0.03	2.36 ± 0.13
Monocytes	X 10^3/µL	0.52 ± 0.02	0.45 ± 0.05
Esinophils	X 10^3/µL	0.44 ± 0.03	1.05 ± 0.06**

- = $P < 0.05$ ** = $P < 0.01$

Table (3): Classification of the seventeen radiographic examined cats according to Foster et al, 2004 and Kirschvink et al, 2006 scoring system:

The score		Description of the pattern	number
Bronchial pattern	0	absence of signs	6
	1	mild [first generation of bronchi visible]	-
	2	moderate [second generation visible]	2
	3	severe [third generation visible]	7
Interstitial pattern	0	absence of signs	6
	1	mild [mild interstitial framework visible]	-
	2	moderate [interstitial framework distinguishable from a bronchial pattern	-
	3	severe [clearly apparent interstitial pattern]).	1

From this table we can see that six cases showed no signs neither bronchial nor interstitial. While nine cases showed bronchial pattern (two moderate and seven severe, Fig. 1B). In the other hand there was one case suffer from severe interstitial pattern (Fig. 2B). One case showed pneumothorax in addition to bronchial pattern (Fig. 3B).

THERAPEUTIC APPROACH:

Improved cases in nebulization and inhalation therapy represented 83% but dead cases represented about 17%. While improved cases in conventional medication represented 50% and dead cases were 50%.(fig. 4 A & B)

Cases	Nebulization and inhalation therapy		Conventional medication	
	Improved cases	Dead cases	Improved cases	Dead cases
Number and Days	10 cases out of 12 improved within 10-17 days	2 cases within first 2 days of therapy	5 cases within 12-24 days	5 cases dead within first week of treatment

Fecal examination revealed negative samples except 2 samples contained Eimeria oocysts. Transtracheal lavage revealed large numbers of esinophils.

*FIGURES of clinical signs, radiography, nebulization and inhalation therapy of clinically asthmatic cats

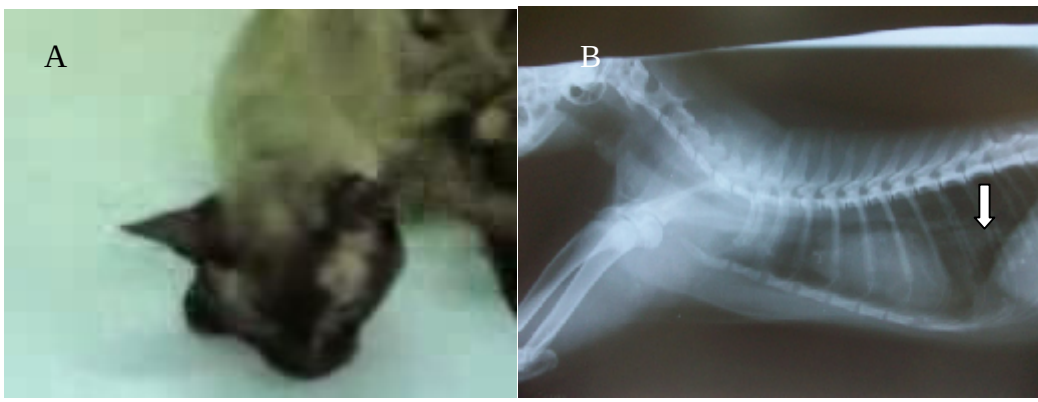

Fig (1): (A) Three years and six months old Siamese queen showed marked respiratory distress and squatting position with shoulder hunched and extended head and neck to catch its breath. (B) Right lateral view plain x-ray film of the same case showed bronchial pattern mainly in the right middle lung lobe (White arrow).

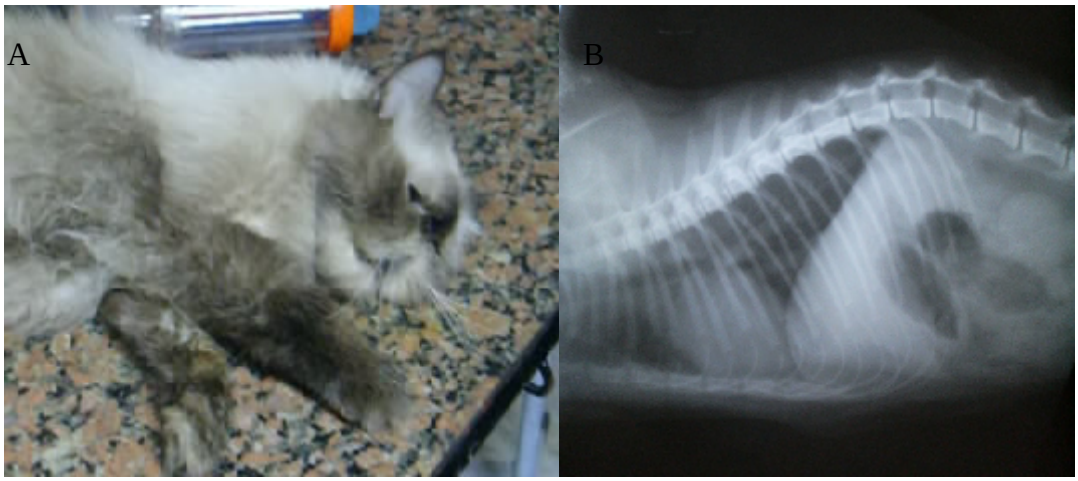

Fig (2): (A) Two years and two months old apathic Himalayan tom cat displayed gasping of air. (B) Right lateral view plain x-ray film of the same case showed bronchial wall thickening and mineralization (doughnuts and tramlines), local atelectasis and clearly apparent interstitial pattern.

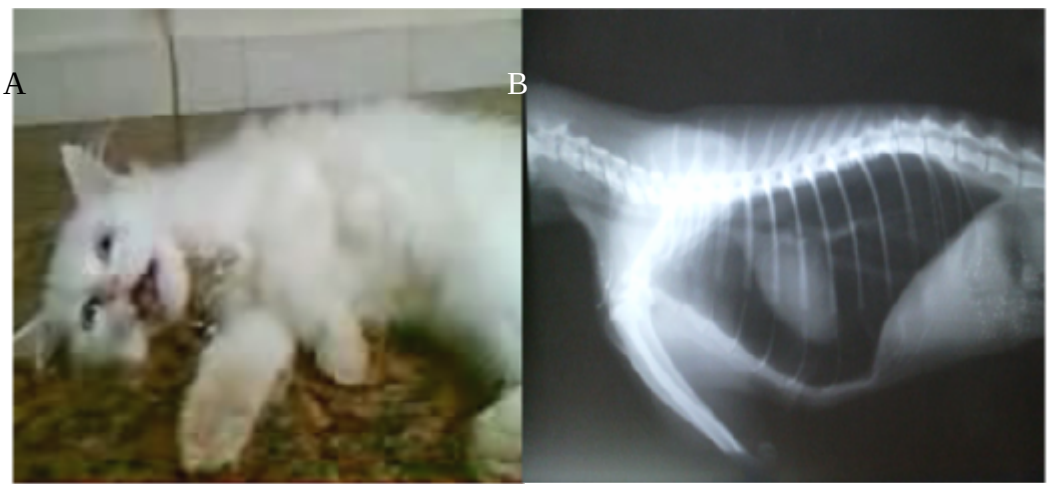

Fig (3): (A) Six months female Persian kitten showed open mouth breath with overall weakness (lateral recumbency). (B) Right lateral view plain x-ray film of the same case showed bronchial wall thickening and hyper-inflation of the lung fields (pneumothorax).

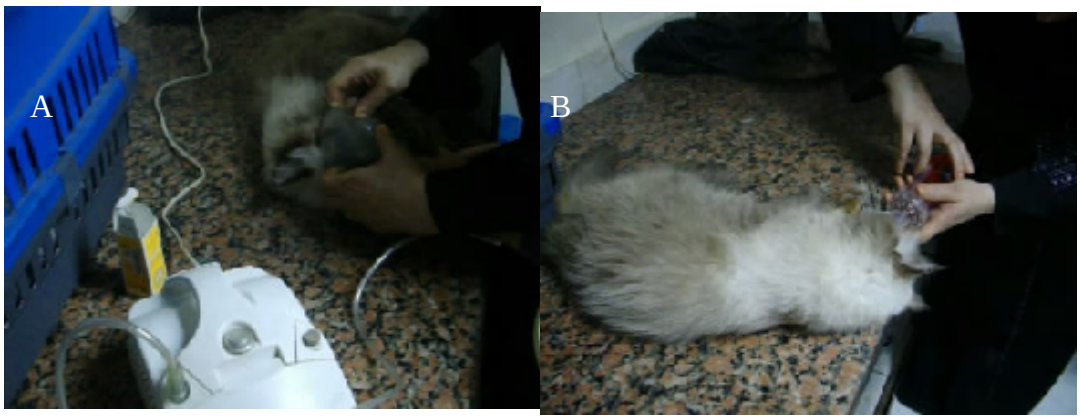

Fig (4): (A) The same Himalayan tom cat in Fig. 2 initially treated (emergencely) by nebulization using saline® and bronchodilator (Farcolin®). (B) Then maintained on spacer using bronchodilator and corticosteroid (Clenil compositum® spray).

DISCUSSION

Feline asthma syndrome is a life threatening condition **(Corcoran et al, 1995; dye et al, 1996)**. When the cat has asthma, the small passageways of the lungs were thickened, and collapsed when the cat inhales, making it difficult for the pet to catch its breath. In severe cases, there were coughing, exercise intolerance, open-mouth breath and cyanosis of mucous membranes. In these cases, large numbers of bronchioles plug mucus and smooth muscle that surrounds these tubes go into spasm restricting breathing **(Corcoran et al, 1995; Johnson, 2000; Padrid, 2000a)**. Cats during acute asthma attack have very hard time breathing. They assume a praying position and concentrate on obtaining the air they need in deliberate breaths. These breaths are deep, labored and abdominal.

Intrathoracic airway obstruction may be due to bronchial smooth muscle hypertrophy, increased mucus production, bronchial inflammation and edema, leading to bronchoconstriction. Bronchial obstruction prevents movement of air out of the lower airways during expiration, which can lead to air trapping and subsequent emphysema and pneumothorax (rare) **(Leib and Mnoroe, 1997; Padrid, 2000a).** Pneumothorax was recorded in one case in the present study which parallel to the results of **Cooper et al (2003)** who reported pneumothorax as a complication of feline asthma in 5

cats out of 421 cases (1.2 %). These changes are associated with severe clinical manifestations that often do not respond to treatment

Other cats have only a mild cough or high pitched wheeze that comes and goes. From the present study, it estimated that very low percent of cases of feline asthma were recorded in Persian cats which is the common breed reared in Egypt. **Padrid (1996), Gardner (2005) and Hibbert (2010)** concluded that feline asthma represents one percent of feline diseases although this percent could be increasing. The incidence of the disease is highest in Siamese cats.

Episodes of asthma are triggered by inhalation of allergens in the air or by stress. Some common allergens are grass and tree pollens, smoke, fumes, Cigarettes, mold, polish, dust mites, dust, potpourri, paint, carpeting, feather pillows, aerosols of various sorts such as perfumes, deodorants and flea spray. Heat, cold and exertion can all trigger an attack **(Dye, 1992; Padrid et al, 1995; Adamama-Moraito et al; 2004 Padrid, 2009)**. Food can cause allergic reactions in cats even if the food has been fed for years. Sensitivity to the food ingredients may last forever and must be omitted from the diet permanently. Common food ingredients that cause asthma symptoms included wheat, milk, gluten, tuna, and the preservatives added to cat food.

In the present study, feline asthma confirmed by the marked clinical presentation especially in severe cases. The asthmatic cat is bought by little restrain to X-ray tray to avoid stress which may be the end of life of severely asthmatic cat. As it may result in the release of stress hormones which in turn led to bronchoconstiction which aggravate the case.

Frequently, radiographs may demonstrate diffuse prominent bronchial markings consistent with inflammatory airways. Radiographic signs of increased lung lucency and flattening and caudal displacement of the diaphragm represent hyperinflation and suggest air trapping. It is worth recalling that the feline heart sits in the mid thorax because of the presence of inflated lungs on either side. When a lung lobe collapses and the lung volume decreases, the heart may shift its position within the thorax to take over this new space. Thus, a mediastinal shift is evidence of atelectasis rather than consolidation. In more extreme cases, you may appreciate fluffy

ill defined heavy interstitial infiltrates in multiple lung lobes. The cause of these changes in cats with lower airway disease is apparently due to multiple small areas of atelectasis in multiple lung lobes resulting from multiple diffuse small mucus plugs.

Feline asthma must be differentiated from other diseases of the same clinical manifestations. Firstly, the present study rule out cardiac cough by physical examination and absence of cardiac murmurs or gallop rhythm. Pleural diseases also differentiated by absence of muffled respiratory or cardiac sounds. Although bronchial asthma is confirmed by the results of a complete blood counts, fecal floatation, thoracic radiography. Esinophilia is identified in approximately 20% of cats with bronchial disease. Stool analysis was used to exclude Paragonimiasis and Aleurostrongylosis **(Corcoran et al, 1995; Dye, 1996; Foster, 2004; d'Anjou et al, 2007)**

Saline can be used to wash cells from deep within the lungs for a microscopic examination (transtracheal wash). The presence of large numbers of esinophil white blood cells is characteristic of the disease. Stool analysis excluded parasitic agents that cause esinophilia. So, the present study denoted hypersensitivity reaction (mostly immediate type I). These findings were in parallel with the results of significant esinophilia in the present hematological studies. We must capture the incriminated aeroallergens as the etiology of asthma.

From the present data, clinical improvement in nebulization and inhalation therapy is more obvious in cases with severe asthmatic attacks. As Salbutamol and Beclomethasone dipropionate directed into the target organ and exerted its action rapidly. It was resulted in the solution of rigid asthma except in 2 cases. Only 2 dead cats attributed to marked bronchial obstruction which prevents air movement **(Leib and Monoroe, 1997; Foster et al, 2004)**. While conventional medication could solve 50% of mild to moderate cases. But it failed to treat 50% of severe cases of feline asthma as bronchial asthma cannot be cured by injectable and tablet form due to severe bronchoconstriction.

Feline asthma in our study was confirmed and differentiated with other diseases of the same manifestations by clinical presentation, findings of thorough clinical examination, x-ray, complete blood count and

transtracheal lavage. From the present study, nebulization and inhalation therapy is more rapid, effective and cheapest than conventional medication. The percent of cure in nebulization and inhalation therapy was 83% while in conventional medication was 50%. Although nebulization and inhalation therapy is more effective, the conventional medication is easier in application. This is because a short period is required to restraint asthmatic cats for injection or oral medication while nebulization and inhalation therapy needs more time **(Gardner,2005; Kirschvink et al, 2006; Padrid, 2006; Reinero et al, 2009; Cohn et al, 2010; Hibbert, 2010)**.

CONCLUSION

Allergens are the main culprits in feline bronchial disease. The present panorama of diagnostic approach depends mainly on clinical presentation and radiographic examination. The present study concluded that nebulization and inhalation therapy were more effective and rapid therapy than conventional medication as it is directed to the target organ.

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