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Molecular diagnosis and genotyping of Bovine viral diarrhea virus

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Abstract:

In the present study isolation, biotyping, antigenic and genomic characterization of BVDV isolates was carried out on buffy coat samples obtained from apparently healthy cattle. The samples were inoculated in MDBK cells for three successive passages and the CPE was recorded indicating that 48%, 62% and 52% were positive CPE in the 1st, 2nd and 3rd passages respectively. The viral antigen was detected in cell culture after propagation also by direct FAT and reveled that (62%) showed positive result. The mean virus titer was recorded after each passage showing values of $<10^2$,10^2 and 10^4 $TCID_{50}$/ml by 1st, 2nd and 3rd passages respectively. The virus was identified by VNT using the reference anti-BVDV-1 serum on cell cultures infected fluids at the third passage. It was found that all samples showed a characteristic CPE of BVDV were neutralized by the used antiserum confirming that they are BVDV-1. Nine buffy coat samples were selected to carry out the nested RT-PCR for detection and genotyping of suspected BVDV. The results indicated the presence of BVDV genotype-I. The positive samples for FAT, VNT and RT- PCR in infected cell culture were stained by H&E for biotyping indicating the presence of the two biotypes (CP& NCP) in the samples. The SNT was carried out on serum samples from the same animals indicating the prevalence of antibodies in 62% by mean titer ranged from < ½ to 1/8. In conclusion, the present study reports the identification of BVDV genotype – I in apparently healthy cattle and presence of P.I. animals.

Keywords: *Bovine viral diarrhea virus*, biotyping, reverse transcriptase polymerase chain reaction (RT-PCR).

Introduction:

Bovine viral diarrhea (BVD) is one of the most imperative worldwide diseases in domestic and wild ruminants, leading to substantial damage in infected herds as well as extensive economic losses for the cattle industry ***(Goyal and Ridpatb, 2005; Ah- med and Zaher, 2008)***. Causing multiple disease and clinical syndromes including embryonic mortalities, abortion, fetal mummification, stillbirths, congenital deformities, respiratory disease (***Flores et al., 2002)*** and hemorrhagic syndrome (***Walz et al., 1999).***

An important condition for maintenance of BVDV in bovine populations is the immunotolerant and persistent infection (PI) that result from transplacental infection of the fetus before onset of immunological maturity. Animals persistently infected with BVDV not only transmit the virus effectively to susceptible in-contact animals, but they harbour clones of BVDV which through serial transplacental passages may be able to replicate for years without immunological selective pressure (***Toplak et al., 2004).*** It is well established that persistently vireamic animals may later succumb to fatal mucosal disease (***Brownlie, 1985).***

BVDV is a small enveloped RNA virus which together with classical Swine fever virus (CSFV) and Border disease virus (BDV) form the genus pestivirus in the family Flaviviridae (***Pringle, 1999).*** BVDV consists of a single stranded positive-sense RNA genome, of approximately 12.5Kb long ***(Collett, 1992).***

BVDV strains have two biotypes, cytopathic (cp) and non-cytopathic (ncp) which can be distinguished on the basis of their effect on cultured bovine cells (***Gillespie et al., 1960).*** The two biotypes are important for the occurrence of MD. The MD is either induced by super infection of persistently infected animals with an antigenically closely related cp BVDV or by generation of a cp mutant from the persisting ncp virus (***McClurkin et al., 1985).***

According to the analysis of the basis of the 5' UTR, BVDV was segregated into two genotypes, BVDV-1and BVDV-2. Sequence homology within each genotype was over 93%, while between genotype 1 and 2 it dropped to 74 % (***Ridpath et al., 1994).***

According to the phylogenetic tree constructed from 420 nt of the E2 glycoprotein gene fragment; the BVDV-1 can be subdivided into BVDV-1 a, c, d, e, f, g and 1 b which is divided into 1b1 and 1b2 and the BVDV-2 is subdivided into BVDV-2a ,2b and 2c. (***Motoshi et al., 2001)***

Due to the complex pathogenesis of BVDV infections and presence of many genovares, laboratory diagnosis of BVDV becomes important in the strategy of control and prevention BVDV infections. Moreover, the knowledge of the type of strains occurring in the field can help to establish effective vaccine and effective control (***Kabango, 2005).***

There are several methods used for diagnosis of BVDV infection which include virus isolation (***Dubovi, 1990),*** virus neutralization (***Brock 1995),*** immunoperoxidase (***Castro et al., 1997),*** ELISA

(***Chu et al., 1985),*** FAT (***Dubovi, 1990)*** and AGPT (***Gutekunst and Malmquist,* 1963)** but all these tests suffer several disadvantages where virus isolation may requires as long as three weeks especially when more than one passage in cell culture is required to recover the virus. It's expensive and unsuccessful when antibodies are present in the examined samples. Moreover, cell cultures in many laboratories are contaminated with low levels of BVDV which interferes with the recovery of field strains of the virus (***Bolin et al., 1985).***

FAT and IP detection are rapid but their specificity and sensitivity are dependent on the quality of the reagents used. Although reagents used in the immunoassays apparently react with group specific antigens, some strains may be missed (***Bolin et al., 1985)***.

The results of virus neutralization test may vary widely depending on the strains of the virus used (***Hassan and Scott, 1986).***

The use of molecular techniques has been increased because of the existing rapidity and accuracy. The nucleic acid based techniques are useful tools for detecting and simultaneously genotyping of BVDV without isolation and propagation in cell cultures. Several researchers reported the use of reverse transcriptase polymerase chain reaction (RT-PCR) (***Hooft et al., 1992)***, multiplex RT-PCR (***Gilbert et al., 1999)*** and nested RT-PCR (***Sullivan and Akkina, 1995)*** for detection and typing of BVDV.

Materials and methods:

Animals: -

A total of 750 apparently healthy Friesian cattle housed under bad conditions in two farms present in Behera Governorate were investigated.

Samples: -

Two sets of blood samples were collected from 50 randomly selected cattle by jagular vein puncture under sterile conditions. One set with EDTA and another set without EDTA for separation of the buffy coat according to ***Rossmanith et al. (2001)*** and sera according to ***Lannette (1964)***, respectively.

BVD virus isolation:-

The obtained buffy coat samples were inoculated on BVDV free cell line of Madine Darby Bovine Kidney (MDBK) supplied by ***Veterinary Serum and Vaccine Research Institute, Abassia, Cairo, Egypt*** according to ***Marcus and Moll (1968).***

These cells were used in virus isolation, virus titration, SNT, and FAT.

Direct fluorescent antibody technique (FAT): -

Fluorescent antibody technique was applied on the buffy coat and on cell culture infected with the 3rd viral passage according to ***Fernelius and Ritche (1964).***

Detection of viral RNA and genotyping of BVDV by nested RT-PCR.

This technique was applied on 9 buffy coat samples from the 50 collected samples. Total RNA was extracted from both reference strain (Iman Strain) and field samples (Buffy coat) using commercial total RNA extraction kit according to the instruction of the manufacturer. Reverse transcription and nested polymerase chain reaction

was carried out according to ***Sullivan and Akkina (1995).***

The nested-PCR oligoprimer sequences are presented in Table 1.

The RT/PCR was performed with ready to Go RT/PCR beads ***(Amersham Pharmacia Biotech)*** according to the manufactures instruction.

The first round of PCR was performed in a 50 µl reaction mix containing 5 µl of P1 primer, 5 µl of P2 primer, 5 µl of ready to go RT-PCR beads and 35 µl RNAse free water. Reactions were also performed in Biometra personal cycler. Conditions for thermal cycler were as follows: one cycle at 94°C for 1 min, thirty cycles in 3 continous phases which included: 94ºC for 30 sec, 55ºC for 30 sec, and 72ºC for 30 sec, a final extention at 72ºC for 10 min. The PCR products were then stored at 4ºC

The second round of PCR was performed also in a 50 µl reaction mix containing 5 µl of product of the first round (diluted 1 in 100 µl distilled water), 5 µl of P2 primer, 5 µl of TS1 primer, 5 µl of TS2 primer, 5 µl of TS3 primer, and 22 µl RNAse free water. Reactions were also performed in the same automated thermal cycler. Conditions for thermal cycler were as follows: one cycle at 94°C for 30 sec, twenty five cycles in 3 continous phases which included: 94ºC for 30 sec, 55ºC for 30 sec, and 72ºC for 30 sec, a final extention at 72ºC for 10 min. The PCR products were then stored at 4ºC until gel electrophoresis were performed. The nested PCR products were separated on 3% agarose gel. The agarose gels contained 10 µg/ml of ethidum bromide to allow visualization of the products under an ultraviolet transiluminator.

Results:

BVDV isolation:-

The buffy coat samples were inoculated into MDBK cell culture. Three successive passages were done in the same cells. It was noticed that 24 samples induced CPE by the first passage, 31 samples induced CPE by the second passage, and 26 samples induced CPE by the third passage. As shown in table (2).

The observed CPE was characterized by onset on second day post infection. The signs of CPE were early rounding and granulation of the infected cells in scattered areas of monolayer then vaculation and foamy appearance were observed in 90% of the cells followed by complete detachment of the monolayer.

Detection of BVDV in infected cell using the direct FAT:-

Direct FAT was applied on 50 infected cell cultures with the 3rd viral passage using anti-BVDV antibodies conjugated with FITC. The results of this technique revealed that 31 samples showed specific fluorescent green reaction

Table (1): Oligonucleotide primers used to amplify and differentiate pestiviruses. According **to *Sullivan and Akkina (1995).***

Primer (A)	*Sequences*	*Genome position (b)*	*Size of amplified DNA product (bp)*
P1	**5'- AAC AAA CAT GGT TGG TGC AAC TGG T -3'**	**1424-1449**	
P2	**5'- CTT ACA CAG ACA TAT TTG CCT AGG TTC CA -3`**	**2221-2250**	
TS1	**5´-TAT ATT ATT TGG AGA CAG TGA ATG TAG TAG CT- 3'**	**1648-1716**	**566 (TS1 & P2) BDV**
TS2	**5' -TGG TTA GGG AAG CAA TTA GG-3'**	**1802-1821**	**448 (TS2 & P2) BVDV- II**
TS3	**5'- GGG GGT CAC TTG TCG GAG G -3'**	**2027-2045**	**223 (TS3 & P2) BVDV-I**

Indicating positive results with a total percentage of (62%). These results are tabulated in table (2) as shown in photo (1).

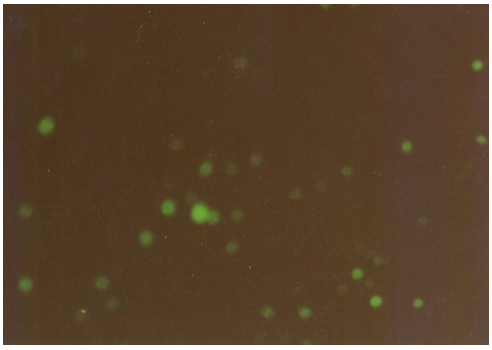

Photo (1): Direct immunofluorescence. The photo show specific fluorescent green reaction indicating positive results.

<u>Detection and genotyping of suspected BVDV by nested RT- PCR.</u>

Nine buffy coat samples were randomly selected to carry out the nested RT-PCR for the detection and genotyping of suspected BVDV. In addition, the technique was including a positive control sample (IMAN strain). This technique indicated the presence of BVDV-RNA type-1 in the examined samples as shown in photo (2&3).

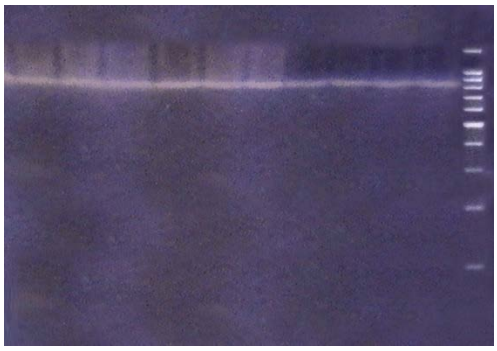

Photo (2): Polyacrylamid gel electrophoresis stained with ethidium bromide. Showing the specific amplification products with specific size (826 bp) of the first round of the nested RT-PCR with primers (P1and P2) indicating that all examined samples are pestiviruses. Lane (1): 100bp DNA ladder, lane (2) positive control (IMAN strain), lanes 3 till 11are examined buffy coat samples.

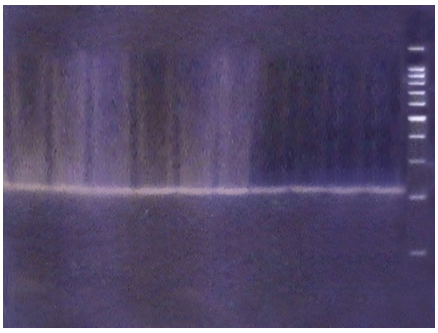

Photo (3): Polyacrylamid gel electrophoresis stained with ethidium bromide. Showing the specific amplification products with specific size (223 bp) of the second round of the nested RT-PCR with primers (P2, TS1, TS2 and TS3) indicating that all examined samples are BVDV-1 Lane (1): 100bp DNA ladder, lane (2) positive control (IMAN strain), lane 3 till 11 are examined buffy coat samples

Serum neutralization test (SNT):-

Both screening and quantitative SNT were carried out on 50 serum samples obtained from the same animals from which the buffy coats were obtained using the microtiter technique. Screening SNT showed that 31 samples were found to be positive for BVDV antibodies. From these samples the quantitative SNT showed that 12 samples had a titer of <2; 7 samples had a titer 2; 6 samples had a titer 4 and 6 samples had a titer 8. These results are tabulated in table (3).

The results presented in tables (3&4) showed the relationship between the presence of BVDV in the samples and BVDV antibodies in the same animals sera. The results revealed that 20 samples were positive for both BVDV and BVDV antibodies, 11 samples were positive for BVDV and negative BVDV antibodies, 12 samples were negative for BVDV and positive for BVDV antibodies and 8 samples were negative for both BVDV and BVDV antibodies.

Discussion and conclusions

In the present study the direct FAT was carried out on 50 buffy coat samples. 26 samples out of them (52%) were found to be positive to BVDV. The results of FAT were obtained within 3 hours.

FAT was Also carried out on all samples propagated in cell culture where 31 samples (62%) showed positive reaction (apple green florescent color).

Trials of virus isolation through the infection of MDBK cell culture with the same buffy coat samples revealed that 26 samples (52%) showed CPE for 3 successive passages.

The difference between the FAT results and detected CPE in cell cultures could be attributed to the presence of 5 samples which did not show CPE. These results agree with those obtained by (***Gerda et al., 1970***) who concluded that the FAT is efficient test in the diagnosis of non-cytopathic BVDV. Moreover, ***Ruckerbauer et al. (1971)*** concluded that the FAT was of a value to detect both cytopathic and non-cytopathic BVDV antigen in primary fetal kidney tissue cultures

Inoculated with field specimens. They proved that FAT was faster than virus neutralization test. In addition, similar results were obtained by (***Snyder et al., 1979)*** who stated that BVDV could be detected 3 days post inoculation into tissue cultures in case of virus isolation, while the virus could be detected within 24 hours post infection in using FAT. They added that FAT sensitivity reached 80% agreement with virus

Table (1): BVDV isolation on MDBK cell cultures.

No. of examined samples	Passage number					
	First passage		Second passage		Third passage	
	No. of positive samples	Percent of positive samples	No. of positive samples	Percent of positive samples	No. of positive samples	Percent of positive samples
50	24*	48%	31*	62%	26*	52%

***** Number of samples which gave CPE.

Table (2): Detection of BVDV antigen in the infected cell using direct FAT.

No. of tested samples	No. of positive samples	% of positive samples
50	31	62%

Table (3): Detection and titration of BVDV antibodies in bovine serum samples:-

No. of Examined samples	No. of the positive sample for BVDV antibodies	Mean of BVDV antibody titer* for positive samples							
		Samples had titer <2		Samples had titer 2		Samples had titer 4		Samples had titer 8	
		No.	Percent	No.	Percent	No.	Percent	No.	Percent
50	31	12	24%	7	14%	6	12%	6	12%

***** Antibody titer = the reciprocal of the final serum dilution which neutralized 100 $TCID_{50}$ of the BVDV.

isolation. In the present work the FAT sensitivity

Table (4): - Results of detection of BVDV and/ or BVDV antibodies in examined animals: -

No. of examined samples	Group A		Group B		Group C		Group D	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent
50	20	40%	11	22%	12	24%	8	16%

Group (A):- Samples show positive virus and positive antibody.
Group (B):-Samples show positive virus and negative antibody.
Group (C):- Samples show negative virus and positive antibody.
Group (D):- Samples show negative virus and negative antibody.

reached 100% and such difference in the 2 results could be attributed to the number of the examined samples from which these samples were obtained. In addition to the inferior health conditions of the animals due to bad conditions of housing, and the method of sample preparation Furthermore (***Tarable et al., 1980***) recommended the use of FAT for detection and diagnosis of BVDV in field tissues specimens collected during the epidemics in Argentina

Among trials of virus isolation from the collected samples in cell culture it was found that the MDBK cell line was considered as one of the most suitable cells for propagation of BVDV ***(Marcus and Moll, 1968; Philip, 1973 and Allam, 2000).***
In the present study, the buffy coat samples were inoculated on BVDV free MDBK cells and

BVDV was detected by CPE and direct immunofluorescence. The recorded CPE was characterized by onset after 48 hours post infection, scattered early cell rounding and cytoplasmic granulation followed by lysis and cell detachment as shown in Photo (3&4).These finding agree with the results obtained by (***Marcuss and Moll, 1968)*** who tried to adapt NADL- BVDV cytopathic strain to MDBK and recorded the CPE. The first observed CPE was recorded by the third day post infection while the severe CPE occurred by the 4^{th} or 5^{th} day post-infection. We reordered the same CPE and accordingly MDBK cell culture was proved for BVDV detection by isolation and serodiagnosis using serum neutralization test. In addition, ***Brock (1995)*** reported that the cytopathic strains of BVDV cause characteristic changes in vitro cell changes such as cytopathic vaculations that are evident in inoculated cell cultures within 24-48 hours.

The results of BVDV isolate propagation in the present study revealed that 24 samples (48%), 31 samples (62%), and 26 samples (52%) showed CPE after the first, second and third successive cell passages, respectively.

The delay of CPE appearance in the third passage of 26 samples less than in the second passage (31) samples may be due to some factors as suggested by ***(Fernelius et al., 1969)*** who stated that such delaying may be due to an abortive infection or incomplete multiplication of the isolates on MDBK cells or incomplete adaptation of the isolates to MDBK cells, ***Johnson et al. (1990)*** stated that such delaying may be due to an absence of the intracellular secretion which is responsible of CPE evolution. Moreover, **Farmer *and Frazier (1986)*** stated that such delaying may be due to sudden reduction in virus titer and/or replication schedule and subsequently BVDV quantity. ***Petkova et al.(1982)*** stated that such delaying may be due to repeated freezing and thawing of the viral harvest could leads to denaturation of the viral proteins and reducing the viral titers and viral RNA yields, while ***Kweon et al.(1997)*** stated that such delaying may be due to long preservation time of harvest at -70 ºC between successive passages could lower the infectivity titer and ice crystals formed could have damage effect on the cell membrane and harmfully affect viral RNA copies, and finally ***Xue and Minocha (1994) and Xue et al.(1997)*** stated that such delaying may be due to inhibition of the viral attachment to the MDBK cell membrane due to the presence of receptor blocking substrates.

For detection of BVDV antibodies, the serum neutralization test was carried out on a total 50 serum samples from the same animals (apparently healthy). The results revealed that 31 samples (62%) were positive. These results corresponded to those reported by ***Baule and Banze (1994)*** who reported BVDV antibodies prevalence from 7-92% and those reported by ***David et al. (1994)*** where they reported that the highest BVDV morbidity was 40% and those reported by ***Radostits and Littlejohnes (1988)*** where they reported that the prevalence of BVD antibodies might be as high as 80%-90%.

Regarding the serum neutralization test applied on the collected serum samples, it was found that 12 samples (24%) have a mean titer of <2, 7 samples (14%) have a mean titer 2, 6 samples (12%) have a mean titer 4 and 6 samples (12%) have a mean titer 8. So, it seems that the recorded mean titer of BVDV antibodies was ranged from (<2 to 8) and this result is agree with that of ***Hopkinson et al.(1979)*** who detected BVDV neutralizing antibodies in the bovine sera by serum neutralization test showing a titer of 1:4. Also similar results were obtained by ***McClurkin et al. (1979)*** who applied serum neutralization test to estimate the seroconversion of cows after mating with persistent infected bulls. The neutralizing antibodies recorded titer was 1:128.

The presence BVDV antibodies in relation to the virus isolation are illustrated in table (10). The animals were classified into four groups. The first group (A): includes animals that were positive toBVD virus isolation and positive antibody. In this case the presence of virus and antibodies is an indication of acute pestivirus infection, as suggested by ***Brownlie, (1991).***

The second group (B): includes animals that were positive to BVD virus and negative antibodies. It

is the group of persistently infected animals, which in most conditions are immune, tolerant and generally lack antiviral antibodies as described by ***Wolf and Buttner (1994).***

The third group (C) includes animals that were negative to BVD virus and positive to its antibodies. These animals were defined as the immune competent ones, which have cleared the virus after infection. The presence of BVD antibodies without virus could be attributed to previous infection or vaccination. Assessment of rise and persistence of neutralizing antibodies to bovine diarrhea virus (BVDV) and border disease virus (BDV) after a two step vaccination using inactivated BVDV/BDV(Mucobovin) and a modified live BVDV vaccine (Vacoviron) was recorded by ***Oguzoglu, et al .(2001).***

The fourth group (D): includes animals that were negative to BVD virus and negative to its antibodies. It is clear that these animals has not exposed to neither pestivirus infection nor vaccination. This group can be considered as basis for a clean herd free from pestivirus infection, which is the basis for control of pestivirus infection ***Radostitis et al. (2000).***

The present study can confirm the presence of the persistent BVDV infection in the tested animals. The persistently infected animals act as reservoir of virus and shed the virus for life exposing other animals in the herd to infection. The same finding was reported by ***Sozan (2002)*** who confirmed the presence of persistent infected animals in Egypt. Moreover, RT-PCR provides a new method for rapid detection and genotyping BVDV.

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A study on antiviral efficacy of *Brucella abortus* ether extract against experimental rabies infection in mice

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Abstract:

The present work deals with Bru-Pel extract in a trial to investigate its effect against rabies virus. The obtained results revealed that Bru-pel has the affinity to induce the highest level of interferon when inoculated 8 hours pre-exposure to rabies infection. However it has no antiviral effect against rabies virus in mice when administrated pre and post-exposure to experimental infection even on zero time. So, it could not be used as post-exposure treatment in case of rabies infection. It was preferable to use specific antiserum with rabies vaccine in emergency cases (post-exposure) for providing the highest rate of protection when administrated on the suitable time (0 to 3 days post-exposure).

Keywords: *Bru-Pel extract; Brucella; Rabies vaccine and Mice.*

Introduction

Rabies is a viral disease that affects the central nervous system (CNS) of mammals and has an extremely high case fatality rate. Once clinical signs develop, there are very few survivors. Vaccines can protect pets, as well as people exposed to rabid animals, but the maintenance of rabies virus in wildlife complicates its control.

Rabies is a life threatening disease caused by RNA virus in the genus lyssavirus that is usually transmitted to human and animals through bites from rabid animals, scratch wounds or licking of the mucous membranes **(Nigg and Walker, 2009)**

In Egypt, rabies is enzootic in jackals and common in dogs as reported by **(Thomas and Rivers, 1952)** and **(El-Kanawati et al.,2000)** who concluded that dogs and wolves are the primary vector animals for transmission of rabies to cattle in the Middle East. It was recommended that all unvaccinated individuals with animal bites receive

immediate treatment with human rabies immunoglobulins (HRIG) injected into and around the wound followed by rabies vaccine. Usually a great attention directed toward protection of animals against infectious diseases especially the fatal of them as rabies infection. Many trials were carried out in this aspect including prophylactic vaccination using specific potent vaccine and post exposure treatment using specific antiserum and immunoglobulin. **(Khodeir and Daoud, 2008)** Some workers studied the antiviral effect of bacterial extracts such as *Brucella abortus* (strain 19) ether extract which produced a non-viable, insoluble residue (Bru-Pel) aiming to limit the severity or treat viral infections **(Youngner et al. 1974)**

It was reported that intravenous injection of living cells of *Brucella abortus* into mice induced high titers of interferon in the circulation **(Stinebring and Youngner, 1964).** The peak of serum interferon appeared 6-8 hours after injection of bacteria, a characteristic indicative of the so called virus type of interferon response **(Youngner, 1970).** Non-viable Brucella cells are also capable of inducing interferon production in mice **(Billiau et al. 1970)**

It was found that extraction of living *Brucella abortus* (strain 456) with aqueous ether yielded a non-viable, insoluble residue (Bru-Pel). When injected into mice, Bru-Pel was found to be effective non-toxic interferon stimulus. The antiviral activity may be associated not only with the production of interferon, but also with a general increase in the level of non-specific resistance in animals **(Youngner et al. 1974).**

The present work is a trial to find an alternative way of intervention in post exposure to rabies infection using *Brucella abortus* ether extract and detecting its efficiency in animal protection.

Material and Methods

1- Brucella ether extract Preparation:

Brucella abortus strain 19, was grown on potato agar infusion medium according to **Alton et al. (1988).** One liter of the medium in a 2- liters Baffled Erlenmeyer flask was inoculated with 5×10^9 viable brucella cells and incubated at 37°C for 48 hours with continuous shaking.

The bacteria were harvested by centrifugation at 5000 rpm and washed once by suspension in cold sterile distilled water with re-centrifugation. They were then suspended in cold water to give a viable count of 1×10^{10} to 2×10^{11} bacteria / ml. The aqueous suspension was treated with diethyl ether by the methods of **Badakhsh and Foster (1970)** and **Ribi et al. (1961).** Two volumes of cold diethyl ether were added to one volume of the cold aqueous

suspension of Brucella, and the mixture was shaken for 60 s in a separatory funnel and then allowed to stand at 25°C overnight. The ether layer was discarded and the aqueous phase containing the extracted bacteria was retained. Nitrogen was bubbled through the aqueous cell suspension until the odor of ether was undetectable. The extracted cells were removed by centrifugation, washed with distilled water, and heated at 100°C for 5 min. in a water bath to destroy the viability of any bacteria that may have survived the extraction process. This heating did not change the biological activity of the material. The aqueous material remaining after removal of the bacteria contained a significant amount of crude Brucella lipopolysaccharide (LPS)

2- Cell culture adapted rabies virus:

Evelyn Rokitincki Abelesth (ERA) strain of rabies virus adapted to BHK-21 cells of a titer 7 $\log_{10}TCID_{50}$/ml was supplied by DPAVR and used to determine the antiviral effect of Bru-pel in infecting BHK cell culture.

3- Cell line:

Baby hamster kidney cell line (BHK-21) established by **Mackpherson and Stocker (1962)** was used for investigating the safety and the invitro antiviral effect of the Bru-pel extract in cell culture.

4-Challenge virus strain (CVS):

CVS mice brain adapted rabies virus with a titer of 10^6 MLD_{50}/ml was supplied by the Department of Pet Animal Vaccine Research (DPAVR); Veterinary Serum and Vaccine Research Institute, Abassia, Cairo. It was used for experimental rabies infection in mice.

5-Inactivated rabies vaccine:

Inactivated cell culture rabies vaccine was supplied by DPAVR and used for post-exposure treatment of experimentally infected mice. Each mouse was injected intraperitoneally with 0.5ml.

6-Rabies antiserum:

Rabies hyper immune serum **(Khodeir and Daoud, 2008)** was used for post-exposure treatment of experimentally infected mice. It had a titer of 130 IU/ml and used at a dose of 30 IU/mouse.

7-Mice:

A total of two hundreds and eighty weaned Swiss albino mice were supplied by DPAVR and used in the different experiments included in the present work.

8-Safety test:

The safety of Bru-pel extract was investigated in-vitro through its inoculation in BHK cell culture to determine any abnormal cellular changes and in-vivo through its inoculation in a group of 10 mice where each mouse received 0.5 ml intraperitoneal and all mice were kept

under observation for any clinical signs or mortalities for two weeks.

9-The effect of pre-exposure administration of Bru-pel against rabies virus infection:

A total number of 30 mice were divided into 6 groups each of 5 mice. Five 10 fold dilutions of Bru-Pel in normal saline started from the undiluted up to 1/10000. Each mouse from the first 5 groups was injected through the intraperitoneal route with a dose of 0.5 ml of each Bru-Pel dilution. The sixth group was kept as control and all animals were subjected for daily observation. After 7 days each mouse of the first 5 groups was injected by CVS intramuscular in a dose of 0.3ml/ mouse, while the 6th group was kept as control with daily observation for all the mice for any clinical signs or mortalities for two weeks.

10- Investigation of the in-vitro antiviral effect of Bru-pel on rabies:

Such investigation was carried out by inoculation of Bru-pel extract (25µl/well) at the same time with the cell culture adapted rabies virus (100$TCID_{50}$/well) on BHK cells; the test included normal cells and virus infected cells as controls and subjected to daily microscopic examination.

11-Testing the post-exposure antiviral efficacy of Bru-Pel extract versus rabies vaccine and antiserum against rabies virus infection

One hundred and eighty mice were divided into 18 groups (10mice/group). The first 17 groups were experimentally infected with 0.3ml of CVS/ mouse through the intramuscular route. The first 8 groups received 0.5ml (25mg) of Bru-Pel/mouse through intraperitoneal route on 0 time; 1; 2; 3; 4; 5; 6 and 7 days post infection. While the second 8 groups received 0.5 ml of rabies vaccine and 0.25 ml of rabies antiserum/mouse through intraperitoneal route on 0 time; 1; 2; 3; 4; 5; 6 and 7 days post infection. The 17th group did not receive any treatment (Positive control, received CVS only). The 18th subgroup was kept without infection and without treatment as test control (Negative control).

12-Detection of induced interferon by Bru-Pel extract:

A forty mice was injected with Bru-pel in the same manner as above, these mice were divided into 4 subgroups (10 mice in each) the first 3 subgroups; were infected with CVS in the same dose and route as above 2; 4 and 8 hours after treatment with Bru-Pel extract respectively while the 4th subgroup was infected without pre-infection treatment.

RESULTS AND DISCUSSION:

Table (1) demonstrates in-vitro safety test which indicated that Bru-Pel ether extract did not induce any cellular changes in the

inoculated BHK cell culture in comparison with the normal cell culture. Also mice inoculated with Bru-Pel did not show any abnormal clinical signs or mortalities all over the experimental period (two weeks) confirming its safety, this results come in agreement with the findings of **Youngner et al. (1974)** who concluded that Bru-Pel was found to be effective non-toxic for mice. It was found that pre-exposure treatment of mice with Bru-Pel extract, 7 days pre rabies virus infection (table-2) did not protect mice against the disease induction. This finding disagrees with **Youngner et al. (1974)** who suggested that antiviral activity may be associated not only with the production of interferon, but with a general increase in the level of non-specific resistance in animals. This disagreement could be attributed to the virus class; where they used Semliki Forest virus of genus Alpha virus, family Togaviridae which is a positive-sense RNA virus while rabies is a negative-sense RNA virus belonging to genus Lyssa virus in the family Rhabdoviridae in addition to the virus tropism where rabies virus has its special pathogenesis. Cell culture assay showed that Bru-Pel extract was unable to inhibit the virus replication. There are no available data discuss the invitro antiviral effect of Bru-Pel in cell culture. The interferon activity of Bru-Pel as shown in (table-3) revealed that it induced 40, 70 and 100% protection against rabies infection 2, 4 and 8 hours respectively after Bru-Pel inoculation. Such investigation showed that antiviral effect of Bru-Pel could be attributed to the induction of interferon as stated by **Stinebring and Youngner (1964)** who found that intravenous injection of living cells of *Brucella abortus* into mice induced high titers of interferon in the circulation. These obtained results agree with **Youngner (1970)** who stated that the peak of serum interferon appeared 6-8 hours after injection of bacteria, which is characteristic indicative of the so called virus type of interferon response In this aspect it could be suggested that induction of interferon as pre-exposure treatment in case of rabies infection is unpractical. Table (4) showed that post-exposure treatment with Bru-Pel extract of infected mice was non-protective where all infected mice were unable to overcome the virus infection even with the treatment on the time of infection. On the other hand, mice treated with the antirabies serum and vaccine were able to withstand the virus infection showing protection rates 100; 100; 100; 90; 40; 10 and 0% on the 0 day; 1^{st}; 2^{nd} ;3^{rd} ; 4^{th}; 5^{th} and 6-7 days post infection respectively.

These findings revealed that the best time for intervention in case of rabies infection, is within the first 3 days post-exposure this result come in agreement with **Khodeir and Daoud**

Table (1): In-vitro and in-vivo safety testing of Bru-Pel extract

Safety test	Tested Bru-Pel extract dilutions				
	Undiluted	1:10	1:100	1:1000	1:10000
In-vitro (in BHK)	No abnormal cellular changes (safe)				
In-vivo (in mice)	No clinical signs or deaths (safe)				

Table (2): Effect of Bru-Pel extract as Pre-exposure treatment in experimentally infected mice 7 days post treatment

Mice groups	Tested Bru-Pel extract dilutions				
	Undiluted	1:10	1:100	1:1000	1:10000
Inf.PT*	All mice in these groups were unable to withstand the virus infection showing typical rabies symptoms within 4-7 days post infection				
Inf.NT**					
Healthy untreated and uninfected mice control remained healthy all over the experiment period					

*****Inf.PT= infected post treatment
******Inf.NT= infected non-treated

Table (3): Effect of interferon induced by Bru-Pel extract in experimentally infected mice

Mice	Time after treatment		
	2hours	4hours	8hours
Number of infected treated mice	10	10	10
Number of survived mice	4	7	10
Protection %	40	70	100
Ten non-treated infected mice showed typical rabies signs			

Table (4): Efficacy of Bru-Pel against rabies Post-exposure to virus infection

Mice treated with	Protection%							
	0DPI*	1DPI	2DPI	3DPI	4DPI	5DPI	6DPI	7DpI
Bru-Pel	0%							
Rabies antiserum&Vacc.	100	100	100	90	40	10	0	0
Infected control	Began to show typical rabies symptoms by the 4th day post-experimental infection and deaths at the 6th and 7th days							
Non-infected control	Remain healthy all over the experimental period							

*****DPI= days post- experimental infection

(2008) and **Albehwar (2009 and 2011).** Who stated that earlier proper treatment of rabies infection result in the highest protection rate.

So, it could be concluded that Bru-Pel extract has no reliable antiviral effect against rabies virus infection and could not be used as post exposure treatment and it is preferable to use specific antiserum or immunoglobulin with emergency vaccination for providing the highest rate of protection that overcome the virus infection especially when administrated on the suitable time (0 to 3 days post exposure).

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Efficacy of Combined Local Prepared Vaccine Against Mannheimia haemolytica and Clostridial Diseases

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Abstract:

The present work aimed to study the protective effect of a locally prepared combined freeze-dried vaccine of M. haemolytica, P. trehalosi and polyvalent Clostridial vaccine. Evaluation of such preparation following the quality control tests revealed that, it was stable, free from foreign contaminants, safe and immunogenic .The prepared vaccine was evaluated in rabbits and the immune response of vaccinated animals to combined vaccine was estimated by ELISA test for M. haemolytica, P. trehalosi antibodies. High antibody levels induced for M. haemolytica and P. trehalosi in combined freeze-dried vaccine compared with combined liquid one. There was a good antibody response to all Clostridial components determined by Serum neutralization test (SNT) and agglutination test in sera of rabbits revealed no substantial differences with respect to the antibody values between combined freeze-dried vaccine and combined liquid vaccine. The obtained results confirmed that the locally prepared combined freeze-dried vaccine of M. haemolytica, P. trehalosi and polyvalent Clostridial vaccine was of good quality and producing high antibody titers.

Keywords: *Pasteurellosis, Clostridial diseases, pneumonia and Combined vaccine.*

INTRODUCTION

Among the bacterial diseases of ruminants, infection with Mannheimia haemolytica, Pasteurella trehalosi and Clostridial strains represent a major bacterial causative agents of highly pathogenic importance causing sever morbidity and mortality rate affecting the meat industries all over the world **(Confer, 2009;Griffin, 2010 and Manteca et.al, 2001).**

Mannheimia haemolytica and Pasteurella trehalosi are well-known pathogens of ruminants found worldwide **(Kodjo et al., 1999).** These two species were formerly included in the single species Pasteurella haemolytica, with the two biochemical types

designated biotypes A and T (the letters standing for arabinose or trehalose fermentation, respectively) **(Adlam, 1989)**. Strains belonging to the T biotype, initially designated as Pasteurella trehalosi **(Sneath & Stevens, 1990),** have been recently reclassified as P. trehalosi on the basis of phylogenetic studies **(Blackall et al., 2007a, b).** In parallel, the taxonomy of M. haemolytica has been further modified **(Sneath & Stevens, 1990; Angen et al., 1999a, b).** Both species can be serotyped by indirect haemagglutination according to their capsular antigens **(Foder et.al., 1999).** M. haemolytica and P. trehalosi are the most common bacterial isolates that cause pulmonary diseases in ruminants worldwide that resulted in economic losses for the cattle backgrounding and feedlot industry due to elevated mortality rates, intensive costs for treatment and prevention as well as reduction of the carcass value **(Confer, 2009).**

Vaccines containing different serotypes are used in the control of pasteurellosis. For the vaccination of cattle and sheep, bacterins, live attenuated, leukotoxin (Lkt), capsule, lipopolysaccaride, subunit vaccines comprised of different surface antigens, sodium salicylate extract and potassium thiocyanate extract vaccines were used. A cell-free vaccine containing leukotoxin and serotype specific surface antigens was shown to be efficacious in preventing pneumonia in calves vaccinated twice followed by intratracheal challenge with live attenuated M. haemolytica **(Diker et al., 2000; Sabri et al., 2000; Donachie et al., 1986).** Researchers found that vaccines provide a good protection against homologous strains while they were insufficiently protective against heterologous ones **(Diker et al., 2000).**

On other hand, Clostridia cause many different diseases that affect beef cattle and sheep of all ages, characterized by sudden death, most occurring worldwide **(Troxel et.al, 2001).** Diseases caused by clostridia can be divided into 4 groups: those affecting the alimentary system (the enterotoxemias), those affecting the parenchymatous organs, those causing myonecrosis and toxemia, and those causing neurologic disorders. Their mode of action is to produce one or more potent toxins when multiplying under favorable conditions **(Parsonson, 2007)**. Excellent control of these diseases by active immunization is of a considerable importance **(Webster and Frank, 1985 and Rahman et al., 1998)**. Vaccination against clostridial diseases has been practiced for many years in sheep **(Blackwell et al., 1983)** and cattle **(Stokka et al., 1994)**. Polyvalent clostridial vaccine containing six toxoids mixture can protect

cattle and sheep against C. perfringens types A, B and D, C. novyi type B, C.chauvoei , C.septicum and C. tetani . The effectiveness of immunization depends on several factors as type of vaccine, route or site of vaccination and adjuvant used **(Chirase et al., 2001).**

The present work aimed to prepare combined freeze-dried bacterin-toxoid vaccine of Mannheimia haemolytica , Pasteurella trehalosi with Clostridial polyvalent vaccine and compare it with the combined inactivated liquid vaccine in terms of inducing a protection for cattle and sheep against the most dangerous diseases, M.haemolytica , P. trehalosi and Clostridial diseases ,affecting them in a dramatic form saving time and efforts.

MATERIAL AND METHODS

1-Mannheimia(M) haemolytica and Pasteurella(P) trehalosi: Local isolates of M.haemolytica and P.trehalosi isolated from infected cattle and sheep respectively were obtained from Vet. Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt. These isolates were used for preparation of experimental vaccine.

2- Clostridial strains: Local isolates of C. perfringens types A, B and D, C. novyi type B, C.chauvoei , C.septicum and C. tetani were obtained from Vet. Serum and Vaccine Research Institute, Abbasia ,Cairo,Egypt. These isolates were used for preparation of experimental vaccine.

3-Rabbits : Fifty two, three-week old Bosket rabbits were supplied by Vet. Serum and Vaccine Research institute, Abbasia , Cairo, Egypt, These animals were divided as follow:

*****12 Rabbits were used in the safety test of the prepared vaccines.

***** 40 Rabbits were used in potency test.

4-Mice: A total of 100 Swiss Albino mice of about 15-20 g body weight supplied by Veterinary Serum and Vaccine Research Institute, were used for serum neutralization test (SNT) for determination of the antitoxin assay.

5- Vaccine preparation:

5.1. Preparation of M.haemolytica and P.trehalosi vaccine in the liquid form:

Inactivated M.haemolytica and P.trehalosi vaccine was prepared according to **(Mosier et.al, 1998)**. The final bacterial suspension was adjusted to contain 10^8 colony forming units/ml then inactivated by adding 0.3% formalin.

5.2. Preparation of M.haemolytica and P.trehalosi vaccine in Freeze-dried form:

A freeze-dried bacterin-toxoid vaccine (containing leukotoxoid , capsular , antigen , soluble , antigens and inactivated cells) of M. haemolytica and P. trehalosi strains prepared

according to **Brown et.al,1995**. The bacterial suspension was adjusted to contain 10^8 cells /ml then inactivated by adding 0.3% formalin.

5.3. Preparation of polyvalent Clostridial vaccine: Inactivated polyvalent clostridial vaccine containing five toxoid components of C.septicum,C.perfringens types A,B and D and C novyi type B and whole culture of C.chauvoei were prepared as described by **(Gaddallah et al.,1974)** while tetanus toxoid was prepared according to **instruction of Rijks Institute (1980)**. Aluminum hydroxide gel was added at 20% concentration as adjuvant according to **(El-Sehemy et al., 2004)**

5.4. Preparation of combined vaccine of M. haemolytica ,P. trehalosi and Clostridial vaccine in liquid form : It was prepared according to **Stone et al. (1978)** by mixing previously prepared inactivated M. haemolytica ,P. trehalosi and Clostridial vaccine vaccines to contain 3ml for clostridia and 2 ml for both M. haemolytica and P. trehalosi per each dose. The vaccine was adjuvanted with aluminum hydroxide gel in a concentration of 20%

5.5 Preparation of combined freeze-dried vaccine of M. haemolytica ,P. trehalosi and polyvalent Clostridial vaccine:

Freeze-dried vaccine of M.haemolytica and P.trehalosi was reconstituted with Clostridial vaccine just prior to use

6- Quality control testing of the prepared experimental vaccine:

6.1-Sterility test: Testing the freedom of the prepared vaccine from foreign contaminants (aerobic and anaerobic bacteria and fungi) was carried out according to **OIE (2013)**

6.2-Safety test: Safety of the prepared vaccines was tested according to **OIE (2013)** through inoculation of double dose subcutaneously in each of rabbits which kept under daily observation for 14 days.

6.3-Potency test:

-Group (1): 10 rabbits inoculated with M.heamolytica and P. trehalosi freeze-dried bacterin-toxoid vaccine which was reconstituted just prior to use with phosphate buffer saline containing 20% alum hydroxide gel.

-Group (2): 10 rabbits inoculated with M.heamolytica and P. trehalosi freeze-dried bacterin-toxoid vaccine which was reconstituted just prior to use with inactivated polyvalent Clostridial vaccine.

- **Group (3):** 10 rabbits inoculated with combined liquid inactivated M.heamolytica, P. trehalosi and polyvalent Clostridial vaccine.

- **Group (4):** 10 rabbits were kept without vaccination as control.

The vaccines were inoculated through the subcutaneous route with a dose of 2 ml / rabbit for group (1), 3 ml/ rabbit for group (2) and 5 ml/ rabbit for group (3) administrated twice with 3 weeks intervals. All rabbits were housed in separate isolates under hygienic measures receiving adequate ration and water. Serum samples were obtained regularly on week intervals to follow up the induced antibody levels up to 9 weeks post the first vaccination.

7- Enzyme linked immunosorbent assay(ELISA):

It was carried out according to **Akan et al (2006)** for the determination of M.heamolytica and P.trehalosi antibody OD in rabbits sera. OD results were evaluated, considering

the highest dilution of sample as positive if the OD rise more than three standard deviations above the mean value of negative serum samples obtained from unvaccinated controls

8-Antitoxin Assay:

The sera of each group of rabbits received Clostridial polyvalent vaccine were collected at 2nd week after the second dose. Pooled sera of each vaccinated groups were tested for the detection of antibodies against all antigens components of the vaccine by using Serum neutralization test (SNT) in Swiss white mice. The antitoxin values for these components were expressed in international units (IU) as described by **British Veterinary Pharmacopeia (2013).** Clostridium tetani antitoxin was titrated according to the **Rijks Institute protocol (1989),** while antibody against C.chauvoei was determined by plate agglutination test according to **Claus and Macheak(1972)**.

9-Statistical analyses

Results of ELISA test in table (1 and 2) were analyzed and compared with parametrical correlation using Student's t test (**Sendecor, 1971**).

RESULTS AND DISCUSSION

Together M. haemolytica and P. trehalosi represent a major bacterial causative agent of bovine and ovine respiratory disease, which is one of the most important causes for economic losses for the cattle backgrounding and feedlot industry. Commercially available vaccines only partially prevent infections caused by P. trehalosi and M. haemolytica **(Confer, 2009).** Clostridial diseases are characterized by sudden onset, short disease fade and high fatality rate which make the probability of treatment success at minimal level **(Troxel et.al, 2001).**

Polyvalent clostridial vaccine is widely recommended as prophylaxis against such

diseases, the prepared polyvalent clostridial vaccine containing six antigens achieved the most suitable requirements to produce high protection rate **(Chirase et al., 2001).**

During the present work a M.heamolytica and P. trehalosi freeze-dried bacterin-toxoid vaccine, combined freeze-dried vaccine of M. haemolytica, P. trehalosi with polyvalent Clostridial vaccine and combined vaccine of M. haemolytica ,P. trehalosi and Clostridial vaccine in liquid form were found to be stable ,free from foreign contaminants (aerobic and anaerobic bacteria and fungi) and safe in vaccinated rabbits where such animals remained healthy all over the experimental period. These observations agree with the recommendation of **OIE (2013).**

ELISA test was performed to follow up the induced immune response of vaccinated rabbits with the prepared M.heamolytica and P. trehalosi freeze-dried bacterin-toxoid vaccine, combined freeze-dried vaccine of M. haemolytica ,P. trehalosi and polyvalent Clostridial vaccine and combined vaccine of M. haemolytica ,P. trehalosi and Clostridial vaccine in liquid form . The results demonstrated in table (1 and 2) showed that high antibody levels were induced by vaccination of rabbits with M.heamolytica and P. trehalosi freeze-dried bacterin-toxoid vaccine either alone or in combination with polyvalent Clostridial vaccine more than that obtained from corresponding combined liquid vaccine starting from the 1st week post vaccination and reached to the maximum value on the 9th week post vaccination. These findings came in agreement with **Diker et al., 2000 and Sabri et al., 2000** who found that vaccine containing leukotoxin and serotype specific surface antigens was shown to be efficacious in preventing pneumonia in animals vaccinated twice followed by intratracheal challenge. The immunogenic response of the prepared polyvalent Clostridial vaccine as shown in table (3) revealed that there was good antibody response to all Clostridial components determined by Serum neutralization test (SNT) for C. perfringens types A, B and D ,C. novyi type B, C.septicum and C. tetani and by using agglutination test for C.chauvoei in sera of rabbits vaccinated with either combined freeze-dried vaccine of M. haemolytica ,P. trehalosi and polyvalent Clostridial vaccine or combined vaccine of M. haemolytica ,P. trehalosi and Clostridial vaccine in liquid form which gave a parallel results. These results came in agreement with that reported by **Chirase et al., 2001** who recommended the use of polyvalent Clostridial vaccine containing six toxoids mixture can protect cattle and sheep against C. perfringens types

A, B and D ,C. novyi type B, C.chauvoei , C.septicum and C. tetani infection.

So, it could be concluded that the combined freeze-dried vaccine of M. haemolytica, P.

Table (1): M.heamolytica antibody OD in rabbits sera as measured by ELISA test

Rabbit groups	Weeks post vaccination								
	1	2	3	4	5	6	7	8	9
Group(1)	0.923	1.233	1.435	2.292	2.345	2.467	2.498	2.501	2.522
Group(2)	0.944	1.345	1.467	2.276	2.367	2.478	2.501	2.511	2.521
Group(3)	0.543	0.893	0.943	1.650	1.785	1.865	1.956	2.115	2.115
Group(4)	0.051	0.061	0.053	0.057	0.060	0.057	0.054	0.062	0.056

Group (1): vaccinated with M.heamolytica and P. trehalosi freeze-dried bacterin-toxoid vaccine
Group (2): vaccinated with combined freeze-dried vaccine of M. haemolytica ,P. trehalosi and polyvalent Clostridial vaccine
Group (3): vaccinated with combined vaccine of M. haemolytica ,P. trehalosi and Clostridial vaccine in liquid form
Group (4): unvaccinated control

Table (2): P. trehalosi antibody OD in rabbits sera as measured by ELISA test

Rabbit groups	Weeks post vaccination								
	1	2	3	4	5	6	7	8	9
Group(1)	0.986	1.356	1.476	2.298	2.352	2.458	2.487	2.505	2.678
Group(2)	0.987	1.367	1.492	2.289	2.345	2.498	2.534	2.578	2.689
Group(3)	0.543	0.893	0.943	1.480	1.534	1.651	1.894	1.978	2.110
Group(4)	0.052	0.543	0.061	0.062	0.060	0.059	0.058	0.060	0.063

Group (1): vaccinated with M.heamolytica and P. trehalosi freeze-dried bacterin-toxoid vaccine
Group (2): vaccinated with combined freeze-dried vaccine of M. haemolytica ,P. trehalosi and polyvalent Clostridial vaccine
Group (3): vaccinated with combined vaccine of M. haemolytica ,P. trehalosi and Clostridial vaccine in liquid form
Group (4): unvaccinated control

Table (3): Antibody titer in sera of rabbits vaccinated with different combined Clostridial vaccines

Rabbit groups	Antibody titer of polyvalent Clostridial vaccines in rabbits IU/ml						
	Alpha Antitoxin Of C.P.A	Beta Antitoxin Of C.P.B	Epsilon Antitoxin Of C.P.D	alpha Antitoxin Of C.N.B	Antitoxin Of C.tetani	Alpha Antitoxin Of C.S	Agglut. Titer of C.Ch (UI)
Group(2)	4	12	6	4	4	3	0.04
Group(3)	3.5	11	6	3.5	4	3	0.05

Group (2): vaccinated with combined freeze-dried vaccine of M. haemolytica ,P. trehalosi and polyvalent Clostridial vaccine
Group (3): vaccinated with combined vaccine of M. haemolytica ,P. trehalosi and Clostridial vaccine in liquid form
C.P.A.: C.perfringens type A
C.P.B.: C.perfringens type B
C.P.D.: C.perfringens type D
C.N.B.: C.novyi type B
C.S.: C. septicum
C.T.: C.tetani
C.Ch.: C. Chauvoie
Agglut.: agglutination titer

trehalosi and polyvalent Clostridial vaccine was of good quality and producing a significant high antibody titers compared to the combined liquid one without any antagonizing effect among them.

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Preparation and Conjugation of Staphylococcal protein A and streptococcal protein G with fluorescein isothiocyanate as non-species specific diagnostic kits

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Abstract:

Staphylococcal protein A "SPA" and streptococcal protein G "SG" are binds to the most mammalian class G immunoglobulin. Therefore "SPA" and "SG" has a unique potential for making "universal" tool, which can be used in various immunoassays which achieve early diagnosis and quick perform till date including coagulation test; Immune fluorescence antibody technique (IFT) and enzyme linked immune sorbent assay (ELISA). In this study "SPA" and "SG" were prepared using two reference strains of Cowan-1 and group C streptococci. Three classical forms of SPA and SG were used including crud; purified and fluorescein isothiocyanate "FITC" conjugates. Such preparations were used in application of coagulation test and fluorescent antibody technique on reference antigens and antibodies (antisera) in order to evaluate their efficacy. The used antisera were obtained from different animal species including cattle; buffaloes; sheep; goat; dogs; horse; rabbit; mice as well as camel sera. SPA and SG also used for differentiation between IgG_1 andIgG_2 subclass in serum of different animal species. In conclusion it could be stated that SPA and SG are safe; easy to be used, high stable, highly sensitive, can be used as rapid field test, of low cost having friendly environmental relationship.

Keywords: *SPA, SG, Fluorescein isothiocyanate, FAT and Coagulation test.*

Introduction

Staphylococcal protein–A "SPA" is a proteinbound to the cell wall of the pathogenic bacterium *Staphylococcus aureus* binds to the Fc portion of most mammalian class G immunoglobulins. Therefore, this protein has a unique potential for making "universal" immunoassays **(Aslam and Dent, 1998).**

Some strains of S-aureus synthesize protein – A, a group specific ligand which binds the Fc region of IgG from many species. It binds to human, mouse, rabbit, cat, cow, dog, goat, Guinea pig, horse and sheep IgG but not bind to chicken (-) and hamster (±) IgG **(Alan and Rabinthorpe, 1996).** Staphylococcal protein–A "SPA" has increasing importance as a tool in both quantitative and qualitative immunological techniques. SPA remains markedly stable on exposure to high temperatures, low pH and denaturizing agents **(Montassier et al, 1994).** Staphyelococcal protein–A was used to develop a coagulation "COA" test for the detection and typing of foot and mouth disease virus (FMDV). Its simplicity and rapidity of performance and its low cost, has a great potential for direct detection and identification of FMDV as screening strategy at FMDV outbreaks **(Montassier et al, 1994).**

Protein G is an immunoglobulin-binding protein expressed in group C and G Streptcoccal bacteria much like protein A but with differing specificity. The native molecule also binds albumin, however, because serum albumin is a major contaminant of antibody sources. The albumin binding site has been removed from recombinant forms of protein G **(Sjobring et al, 1991).**

Fluorescent dyes are commonly employed as labels in primary binding tests, the most important being fluorescein isothiocyanate (FITC). FITC is a yellow component that can be bound to antibodies without affecting their reactivity. When irradiated with invisible ultraviolet light, FITC emits violet green light, so FITC- labeled eddies are used in the direct and indirect fluorescent antibody tests **(Tizard, 1996).**

The present work was designed to prepare “SPA” and “SG” using two reference strains of Cowan-1 and group C streptococci including crud; purified and fluorescein isothiocyanate “FI” conjugates. The efficacy of such preparations will be evaluated through the application of coagulation test and fluorescent antibody technique on reference antigens and antisera from different animal species including cattle; camel; horse; sheep; dogs; horse; rabbit; mice and rats.

MATERIAL AND METHODS

1- Reference bacterial strains:

1.1-Cowan-1 strain of *S.aureus* was supplied by Prof.Dr. Saad, M. A.M (Aimal reproduction Research Institute; Alharam Giza) for preparation of staphylococcal protein A according to **Kassler (1975) and Sting et al (1990).**

1.2-Streptococcal group C strain *Streptococcalequi* (c) was supplied by ATTCC33398 Microbiologic Com 200 Cooper Avenue North. St. Co. Lud for preparation of streptococcal protein –G according to **Sting et al (1990).**

2- Specific antigens and Antibodies (Ags and Abs):

2.1-Viral Ags:

Antigens of foot and mouth disease (FMD) type O, A and SAT2, bovine ephemeral fever (BEF); Rabies; Rift Valley fever (RVF) and rabbit hemorrhagic viral disease vaccine (RHDV) were kindly provided by Veterinary Serum and Vaccine Research Institute, Abasia Cairo.

2.2-Bacterial soluble antigens and antibodies:

2.2.1-Brucella periplasmic protein and positive brucella serum of naturally infected cows were kindly provided by Dr. Nour" Brucella department Animal health Research Institute- Dokki, Giza.

2.2.2-Pasteurellamultocida group type B was kindly provided by Prof. Dr. Emad Mokhtare Bacteriolgy department Animal health institute- Dokki

2.2.3-Tetanus toxoid was kindly supplied by Veterinary Serum and Vaccine Research Institute.

3-Serum:

3.1-Normal rabbit serum was used as control negative.

3.1.2-Serum samples were obtained from different vaccinated animal species cattle (vaccinated with trivalent FMD vaccine and BEF vaccine); camel (vaccinated with rabies vaccine); sheep (vaccinated with RVF vaccine); horse (vaccinated with tetanus toxoid); dog; cat and mice (vaccinated with rabies vaccine); rabbit (vaccinated with rabbit hemorrhagic viral disease vaccine) and camel (vaccinated with rabies and RVF vaccines)

4-Fluorescein isothiocyanate (C_4 H_{11} NO_5 S) E:

(C_4 H_{11} NO_5 S) E was supplied by Merck, Darmstadt for Microscopy (M.Gew.389.39). FI was used for conjugation of SPA and SG proteins according to **Narin (1969).** Acetone dried bovine liver powder at a final concentration of 50mg/ml was used for removal residual untreated fluoresceinisothiocyanate (FITC) and labeled protein according to **Cabel (1974).**

5-Preparation of SPA suspension according to Kessler (1975):

Cowan 1 strain of *S. aureus* was cultivated in one liter of 199 medium supplemented with 0.5% lactoalbuminhydrolysate and 0.5% yeast extract. The inoculated medium was dispended in bottle was incubated at 37C^0 for 24hours with gentle shaken every 3 hrs. Growing bacteria were collected by centrifugation at 800xG for 10 minutes and washed twice with PBS at ph 7.2 containing0.05 (w/v) sodium azide (PBSazide). Bacteria were resuspended to approximately 10% (w/v) concentration in PBS- azide. Fixed bacteria were then washed once with PBSazide without formalin and resuspended again into the same buffer to 10% concentration. The suspended bacteria were killed by heating at 80 ˚C with rapid swirling in water bath for 5 min followed by rapid cooling in PBS azide and finally made to 10% suspension.The SPA suspension could be stored at 4^{0}C where it was stable for at least 4 months. Before use the SPA suspensionshould be treated with NP-40 (0.5%) in Net Buffer ph 8.0; for 20 min at room temperature. The treated SPA suspension was then washed once with 0.05% NP-40 in Net buffer and finally resuspend to the original concentration (10% suspension) in the later buffer.

6-Preparation of SG:

According to **Sting et al (1990)** ATTCC33398 strain of*Streptococcal equi* (c) belonging to serological group G were grown TSB for 20h at 37 ˚C. The bacteria were subsequently centrifuged for 20 min. at 13000 xg and washed repeatedly with distilled water. Hot acid extraction: after suspending the bacteria in 40 ml PBS at PH 2 they were heated for 10 min. at 95 ^{0}c in a water bath. The suspension was subsequently neutralized with 0.5 mol/1N NaOH and the bacteria removed by centrifugation (20 min., 13000 xg) and filtration.

RESULTS AND DISCUSSION

Table (1): Reactivity of protein SPA and SPG With IgG from different animal species

Species	Affinity for protein A		Affinity for protein G	
	IgG_1	IgG_2	IgG_1	IgG_2
Cow	-	++*	+**	+
Sheep	-	++	+	+
dogs	++	++	++	++
Cat	++	++	++	++
Horse	+	++	++	+
Mouse	+	++	++	+
Rabbits	++	++	++	++
Camel	++	++	+++	+++

*++ =Strong reaction ** += Week reaction

It is well known that the reactivity of immunoglobulin-binding proteins for (Igs) of mammalian species is well known. These proteins are staphylococcal protein-A (SPA), streptococcal protein G (SPG), protein AG-peroxidase (SPAG-HRP). These conjugates have a potential use in epidemiological

surveys of zoonotic infections (Angel Alberto Justiz – Vaillant, 2013).

Recombinant protein A/G are reagents more universal than protein A or G individually labeled with horseradish peroxidase as a detection reagent for the presumptive diagnosis of brucellosis caused by smooth brucellae in swine, sheep, goats and cattle as well as for sheep, with antibody to rough brucellae (Nielsen et al, 2004).

Elagamy et al. (1996) stated that camel blood serum or milk IgG had high affinity to proteine – A in addition to IGA did bind to protein A.

It was concluded that the SPA-FA is highly specific more than SPA-ELIZA that is highly sensitive but the drawback of FA in diagnostic reactions was showed in nonspecific reactions this problem can be lightly dissolved by adjusting the amount of conjugate with that of SPA because heavily conjugation of protein and FITC, can be reduced by reducing the ratio of dye to protein, good dialysis leading to the removal of excess amount of free dye, further purification by sing chromatogeaphy as well as using of acetone liver powder also remove nonspecifically stained molecule (**Saad et al., 2000).**

Hanaa et al. (2011) demonstrated that due to the nature of SPA to bind with FC portion of the most mammalian immunoglobulines class G. Therefore, this protein has a good ability to react with wide range of animal species mainly when anti-immunoglouline not available as in case of camel IgG.

It was concluded that protein G is bacterial cell wall protein isolated from group G streptococci, which bind to the Fc region of IgG froma variety of mammalian species. Native protein G has IgG binding domains. For the tertiary structures of protein Aand protein G are very similar, the potential applications of protein G include nearly all of current application of protein -A- compared to protein A and G has greater affinity , ability and binds more strongly for most mammalian IgGs (**Siano Biological, 2014)**

Typical macroscopic patterns of positive and negative coagulation reactions obtained by interaction of the homogenate supernatants of the skin biopsies (100%) with positive and negative sera of rabbits against lumpy skin disease virus. Staphylococcal protein – A Coagulation was rapid (Carried out in 5-10 min) accurate, sensitive, specific and economic and not require special equipment (**Iman and Saad, 2007).**

Iman et al. (2011) aggregated Rhabdoviral particles coated with the specific antibody were observed in addition to their attachment to the surface staphylococcus aurous protein A.

In addition Sigma showed that protein – A is capable of binding to the Fc portion of immunoglobulines, especially IgGs, from large number of species. Protein - A is a highly stable cell surface reseptor produced by several stations of *Staphylococcus aureus*. Protein A may be conjugated with various receptor molecules, including fluorescent (FITC), enzyme marker, (peroxidase without affecting the antibody binding site on the molecule.

SPA / SPG tests when compared with other techniques would be cheaper taking into consideration the price of the reagents involved, which is low and the simplicity of the technique. SPA/ SPG test do not require the production of anti-serum. The SPG test was the most sensitive test, followed by the SPA test. SPG is superior as reagent (**Vallant et al., 2013)**

It was suggested that the discovery of protein A, and subsequently protein G , immune-affinity Chromotography has ground in popularity and is now the standard methodology for the purification of antibodies which many be implemented for selection of different application such as immunodiagnostics (**Darcy et al., 2011)**

Depending on the obtained results and in agreement with **Nielsen et al. (2004);** it could be concluded that Staphylocoocus aureus Cowan strain and streptococcu spp. group G are among a group of bacteria that produce protein with capacity attach to immunoglobuline molecules of various species aiding to rapid detection of bacterial and viral antigens and antibodies in order to reach rapid control of infectious diseases facing different live stocks.

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Clinicopathological studies on chicken infectious anemia disease live virus vaccines

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Abstract:

The present study aimed to shed light on the pathomechanism of chicken infectious anemia disease in chickens experimentally vaccinated with live chicken anemia virus (CAV) -26P4 and -cux-1 strains through investigation of pathogenicity, hematological, blood coagulation and blood biochemical serum parameters. At the75th day of age, 168 chickens were divided randomly into 7 groups, each group contain 24 chickens. Group 1 was subjected to CAV-26P4 vaccinal strain at 75 day of age. Group 2 was subjected to CAV-cux-1 vaccinal strain at 75 day of age. Group 3 was subjected to CAV-26P4 vaccinal strain at 75 & 95 day of age. Group 4 was subjected to CAV-cux-1 vaccinal strain at 75 & 95 day of age. Group 5 was subjected to CAV-26P4 vaccinal strain at 75 day of age and CAV-cux-1 vaccinal strain at 95 day of age. Group 6 was subjected to CAV-cux-1 vaccinal strain at 75 day of age and CAV-26P4 vaccinal strain at 95 day of age. Group 7 was not vaccinated (control). The present study revealed that live CAV-26P4 vaccinal strain inoculated by intramuscular (i.m.) route developed clinical signs and PM lesions, while live CAV-cux-1 vaccinal strain inoculated by oral route did not develop clinical signs but could develop mild PM lesions. Chicken anemia virus-26P4 and cux-1 vaccinal strains cause a significant increase of enzyme linked immunosorbent assay (ELISA) titers mostly at the 5th & 6th weeks post vaccination (PV). A significant anemia, leucopenia, heterophilia, lymphopenia, monocytopenia and thrombocytopenia were observed. All these changes were observed early in birds inoculated with CAV-26P4 vaccinal strain, but were observed later in birds inoculated with CAV-cux-1 vaccinal strain. Both CAV vaccinal strains caused a significant increase of coagulation parameters, liver enzymes and A/G ratio, in addition to a significant hypoproteinemia, hypoglobuliemia. The observed biochemical changes may indicate liver affection.

Keywords: *Chicken anemia virus, Pathomechanism, Hematological parameter and ELISA.*

Introduction

Chicken infectious anemia (CIA), a disease of young chickens, is caused by a unique small circular DNA virus **(Yuasa *et al.*, 1979, and Goryo *et al.*, 1987)**. Chicken anemia virus (CAV) is considered a member of the genus gyrovirus and the family circoviridae and an immunosuppressive pathogen of chickens **(Pringle, 1999; Büchen-Osmond, 2006;**

Schat, 2009, and Hegazy *et al.*, 2010). Chicken is the only recognized natural host, but serological survey has revealed the prevalence of CAV in domestic and wild birds **(Farkas *et al.*, 1998)**. The CAV spreads both vertically and horizontally, vertical transmission occurs following primary infection of in-lay breeding stock and results in clinical disease in their progeny around 2 weeks of age. Horizontal spread usually results in subclinical disease. Both clinical and subclinical disease cause economic loss **(McNulty, 1991)**. The major economic loss caused by this virus is because of severe immunosuppression, which leads to increased mortality due to secondary bacterial or virus infections **(Todd, 2000)**.

Commercial available CAV vaccines stimulate immunity that would be transferred to progeny and protect them from overt CIA disease **(Rasales, 1999)**. Clinical disease of CAV is rare today because of widespread practice of vaccination breeders with the inactivated and live attenuated CAV vaccines **(Franz and Coral, 2003)**. However, live attenuated CAV vaccines have the possibility of reversion to virulence **(Pages *et al.*, 1997)**, and reports have clarified that the pathogenicity of attenuated CAV could be restored after 10 passages in young chickens, so irreversible attenuation of CAV is proving difficult **(Todd *et al.*, 1995 and 1998)**. In addition, recent reports have demonstrated that an attenuated CAV vaccine, 26P4 strain induced anemia and lesion in the lymphoid organs of young chicks **(Hussein *et al.*, 2003)**. These reports activated us to investigate the effect of commercially available live attenuated CAV vaccines in broiler breeders through studding pthogenicity, blood hematology, blood serum chemistry, and blood coagulation mechanisms.

Material and Methods

1- Chicks:

Table (1): Experimental design:

Group No.	Vaccination regime			
	Frequency	Age/days	Type	Rout
1	1X	75	CAV-26P4 strain	i.m.
2	1X	75	CAV-cux-1 strain	D.W.
3	2X	75 95	CAV-26P4 strain	i.m.
4	2X	75 95	CAV-cux-1 strain	D.W.
5	2X	75 95	CAV-26P4 strain CAV-cux-1 strain	i.m. D.W.
6	2X	75 95	CAV-cux-1 strain CAV-26P4 strain	D.W. i.m.
7	----------	---------	----------	----------

CAV: Chicken anemia virus. No: Number. 1X: One time
2X: Two time. i.m.: Intramuscular. D.W.: Drinking water.

Two hundred one-day-old broiler breeder male chicks were obtained from a commercial company (El-Wadi for breeder's production, Egypt). These chicks possessed maternal antibodies against CAV, acquired from their parents that were vaccinated with live and inactivated vaccines according to a specific vaccination program. The chicks were floor reared

under natural day light in strictly isolated experimental rooms, previously cleaned and disinfected and were provided with commercial starter ration. Water and feed were provided adlibitum. Before experimental study, chicks were vaccinated with different vaccines obtained from the local agencies. At 75 day of age, 168 chickens were divided randomly into 7 groups (table 1), each group contain 24 chickens. Chickens were subjected to CAV-26P4 vaccinal strain at 75 or 95 day of age by i.m. injection, 0.5 ml per bird and to CAV-cux-1 vaccinal strain by drinking water. The groups were observed weekly for six weeks post vaccination (PV).

2- Blood samples:

Blood samples were obtained from shank vein. Each Blood sample was divided into two portions. The first one was placed into tubes containing sodium citrate for measuring coagulation parameters and evaluation of hemogram. The second portion was obtained in plain centrifuge tubes, allowed to clot, centrifuged at 3000 rpm for 15 minutes for separation of serum for determination of serum biochemical parameters and serological studies.

3- Vaccines:

Two types of commercial live chicken anemia virus (CAV) vaccines were used in the study:

A- CAV-26P4 strain.

B- CAV-cux-1 strain.

Chicken anemia virus-26P4 and cux-1 strains are attenuated live freeze-dried vaccines against chicken infectious anemia disease, each dose contains ≥ 3.0 log10 TCID50 and min. 10^{45} TCID50, respectively. They were administrated via intramuscular and drinking water, respectively, and were obtained from local agency of Intervet and Lohman Animal Health, respectively.

4- Hematological parameters:

The evaluated hematological parameters in the study included estimation of the packed cell volume (PCV) according to **Campbell and Coles (1986)**, Hemoglobin concentration (Hb) according to **Campbell (1988)**, Erythrocyte and Leukocyte counts (RBCs & WBCs) according to **Natt and Herrick (1952)**, Thrombocyte count according to **Campbell (1995)** and Differential Leukocytic count (DLC) according to **Mulley (1979)**.

5- Coagulative parameters:

The evaluated coagulative parameters in the study included estimation of Bleeding Time (BT) according to **Bigland (1964)**, Clotting Time (CT) according to **Benjamin (1978),**

Whole Blood Recalcification Time (WBRT) according to **Skeels *et al.* (1980),** Plasma Recalcification Time (PRT) according to **Archer (1965)** and Prothrombin Time (PT) according to **Coles (1986)**.

6-Blood serum biochemical parameters:

The evaluated biochemical parameters in the study included estimation of alanine aminotransferase activity (ALT) and aspartate aminotransferase activity (AST) according to **Reitman and Frankel (1957)**, uric acid according to **Caraway (1963)**, total proteins (TP) according to **Henry *et al.* (1978)**, albumin according to **Doumas (1971)**, globulins and albumin : globulin ratio according to **Benjamin (1978).**

7-Indirect Enzyme Linked Immunosorbent Assay (ELISA):

The test was employed for detection of maternal derived antibody (MDA) and CAV antibodies in chicken sera using ELISA kits (Synbiotics Corporation, ProFlock KPL. USA).

8- Haemagglutination Inhibition test (HI):

The test was used for determination of immune response of avian influenza (AI) and Newcastle disease (ND) viral vaccines according to **OIE Terrestrial manual (2008)**.

9- Statistical analysis:

All data were presented as mean ± standard error (SE) and were subjected to analysis of variance in one and two way (ANOVA) test according to **Snedecor and Cochran (1969)**. Treatments means were compared by the least significant difference test (LSD) at 0.05 level of probability.

Table (2) Results of waning of chicken anemia virus maternal derived antibodies:

Age/weeks	Sample No.	ELISA titer (Means)
1	5	3178
2	5	1165
3	5	559
4	5	521
5	5	508
6	5	472
7	5	452
8	5	424
9	5	423
10	5	313

Results and discussion

The present study was designated to shed light on the pathomechanism of the disease in chickens experimentally vaccinated with live CAV-26P4 and -cux-1 vaccinal strains through investigation of pathogenicity, hematological, blood coagulation and blood biochemical serum parameters.

Maternal derived antibodies (MDA) gradually dropped from the 1st week till the 10th week of age. According to Table 2, ELISA mean titers were 3178, 1165, 559, 521, 508, 472, 452, 424, 423 and 313 at weekly intervals, respectively. Maternal derived antibodies played an important role in protection of chickens at early 3 weeks of age **(Yuasa *et al.*,**

1980; Goodwin *et al.*, 1993, and Franz and Coral, 2003). Maternal antibodies prevent clinical signs but do not prevent infection, transmission of the virus, or immunosuppression **(Franz and Coral, 2003, and Sommer and Cardona, 2003)**. Mean values of MDA decrease in linear regression line with approximately 10 days half-life **(Pages *et al.*, 1997)**, whereas **Otaki *et al.* (1992)** and **El-Zahed (2007)** revealed that MDA mean titers waned along the first 30 days of the chicks' life with a half life time approximately 7 days.

ELISA: Enzyme Linked Immunosorbent Assay.

It was shown that all birds vaccinated with CAV-26P4 strain by i.m. inoculation (group 1 vaccinated with CAV-26P4 strain at 75 day-old age, group 3 vaccinated with CAV-26P4 strain at 75 & 95 day-old age and group 5 vaccinated with CAV-26P4 strain at 75 day & cux-1 strain at 95 day-old age) developed clinical signs of anemia two weeks PV. Affected birds appeared depressed, with ruffled feathers and pallor of comb and wattles. These results agreed with **Hussein *et al.* (2003)** who reported that chicks vaccinated with live attenuated CAV-26P4 vaccinal strain showed signs of anemia and lower hematocrit values. Birds vaccinated with CAV-cux-1 strain by the oral route (group 2 vaccinated with CAV-cux-1 strain at 75 day-old age, group 4 vaccinated with CAV-cux-1 strain at 75 & 95 day-old age and group 6 vaccinated with CAV-cux-1 strain at 75 day & 26P4 strain at 95 day-old age) did not develop clinical signs during the experimental period. The present results confirm that i.m. route is the most efficient for inducing clinical disease **(Rosenberger and Cloud, 1989, and Yuasa, 1989)**. This may be attributed to presence of a direct relationship between viral load in particular organs (thymus, clotted blood and pancreas) and the extent of clinical signs **(Tan and Tannock, 2005**). Morbidity and mortality in chickens inoculated with CAV are strongly related with the dose of CAV used for inoculation; that is, the larger the dose, the higher the severity of the disease **(Yuasa *et al.*, 1979)**.

The present investigation indicated that all birds vaccinated with CAV-26P4 strain by i.m. inoculation showed atrophy of the thymus, mild atrophy of bursa, pale bone marrow of the femur, enlargement and paleness of the liver and sometimes subcutaneous and intramuscular hemorrhage which were observed at the 2nd week PV. Birds vaccinated with CAV-cux-1 strain by oral route showed mild atrophy of thymus and mild swelling of liver at the 4th week PV. The

lesions were similar to typical lesions of CAV infection mentioned by **(Tan and Tannock, 2005; Kuscu and Gurel, 2008, and Hegazy *et al.*, 2010),** but differ according to pathogenesis, severity of viral strains and route of inoculation, as the lesions were delayed and less severe in the orally inoculated chickens which was concurrent with a delay in accumulation of CAV genomes in the thymus of these chickens **(van Santen *et al.*, 2004)**.

Chicken anemia virus-26P4 & cux-1 strains were immunogenic to vaccinated birds. According to table 3, there was a significant increase of ELISA titers in birds vaccinated with live CAV-26P4 strain approximately at all weeks PV with the most significant increase observed at the 5th and the 6th weeks PV in group 1, at the 4th, 5th & 6th weeks PV, in group 3 and at the 5th & 6th weeks PV in group 5 when compared with non vaccinated group. The results supported by **El-Zahed (2007)** who indicated that the live attenuated CAV-26P4 vaccine administrated intramuscularly in 11 week-old breeding birds was immunogenic to vaccinated birds, and there was a substantial increase in CAV ELISA reactivity following application of the vaccine. Moreover, the results supported by **Tannock *et al.* (2003)** who stated that group inoculated by the i.m. route, antibody could be detected in 75% and 89% of birds at days 15 and 20, respectively, and 100% by day 25. Results also agreed with **Youssef (1998)** who stated that 100% of chicks vaccinated with CAV-26P4 vaccine found to be seroconverted after 3-4 weeks PV.

The present study revealed that birds vaccinated with CAV-cux-1 strain orally produced a significant increase of ELISA titers approximately at all weeks PV with the most significant increase observed at the 5th week PV in group 2, at the 4th, 5th & 6th weeks PV in group 4 and at the 4th, 5th & 6th weeks PV in group 6. The results were supported by **McConnell *et al.* (1993)** who stated that all chickens given orally CAV developed antibodies. Also **Vielitz *et al.* (1987)** stated that chickens vaccinated with a live CAV (Cux-1) vaccine via drinking water produced antibodies but only from 4 weeks after administration of the vaccine.

The present findings in table 3 indicated that, there was no significant differences of HI titers of avian influenza (AI) and Newcastle disease (ND) between vaccinated groups when compared with each other or with non-vaccinated group. However, within group vaccinated with CAV-cux-1 strain at 75 day-old age there was significant increase of avian influenza HI titers at the 2nd week PV (5.0 ± 0.58) when compared with the 6th week PV

(3.6 ± 0.51). Also within group vaccinated with CAV-cux-1 strain at 75 & 95 day-old age, there was significant increase of Newcastle disease HI titers at the 2nd week PV (5.3 ± 0.67) when compared with the 6th week PV (3.6 ± 0.51). These results indicated that the used CAV vaccinal strains do not induce immunosuppression, yet this work does not dismiss the possibility that future mutation of the vaccinal strains may lead to clinical and immunological problems in the future. There is a need to continue this line of investigation and follow up of vaccines usage consequences in the future. This result does not necessarily contradict reports that the administration of the live attenuated CAV vaccine at 1 day of age caused immunosupression **(Hussein *et al.*, 2003)**. In this work the application of the vaccine was done according to the manufacturer's instruction at 10 weeks of age. Studies on hematological parameters (table 4) showed that birds vaccinated with CAV-26P4 strain developed significant anemia mostly at the 2nd week PV. These results agree with **Youssef (1998) and Hussein *et al.* (2003)** who found lower hematocrit values throughout 10 weeks in chicks experimentally vaccinated with CAV-26P4 vaccine, but disagree with **El-Zahed (2007)** who indicated that the live attenuated CAV-26P4 strain administrated in 11 week-old breeding birds do not cause anemia. Birds vaccinated with CAV-cux-1 strain developed significant reduction of Hb and RBCs mostly at the 4th week PV. These results agree with **Vielitz *et al.* (1987)** who observed anemia due to a live CAV (Cux-1) vaccine when administered to 9-15 weeks old chickens, but disagree with **Yuasa *et al.* (1980); McConnell *et al.* (1993); McKenna *et al.* (2003), and Kapetanov *et al.* (2004)** who stated that all chickens orally inoculated with CAV developed no anemia.

The present study also revealed that birds vaccinated with CAV-26P4 strain (groups 1, 3 & 5) developed anemia early, while birds vaccinated with CAV-cux-1 strain (groups 2, 4 & 6) showed delay of anemia. This may be due to severity difference, or concurrent with delay in accumulation of CAV genomes in the thymus **(van Santen *et al.*, 2004, and Tan and Tannock, 2005)**. Packed cell volumes of less than 25% have been detected in chickens infected with chicken anemia virus **(Yuasa *et al.*, 1979; Danial, 1996; Saini and Dandapat, 2009, and Hegazy *et al.*, 2010)**. The reduction of PCV percentage, Hb concentration and RBCs count may be due to CAV targeting erythroid and lymphoid precursor and progenitor cells in the bone marrow causing severe bone marrow hypoplasia and/or complete aplasia with fully depletion of the erythrocytic and granulocytic

series **(Kuscu and Gurel, 2008; Toro *et al.*, 2008, and Hegazy *et al.*, 2010)**. Also anemia may be attributed to hemorrhage that was seen subcutaneously and sometimes in skeletal muscles especially in groups (1, 3 & 5) vaccinated with CAV-26P4 strain, presumably the hemorrhages in affected chicks resulted from a defect in blood clotting **(McNulty, 1991)**. Most of groups vaccinated with CAV-26P4 & cux-1 strains recovered at the 6th week PV, this may be attributed to recovery of haematopoietic activity in the bone marrow suggesting that anemia induced by CAV may be a transient impairment of haematopoietic function rather than aplastic anemia **(Goryo *et al.*, 1985 and 1987; Otaki *et al.*, 1987, and Goryo *et al.*, 1989).** Studies on coagulation parameters (table 6) recorded significant increase of bleeding time (BT) in all groups vaccinated with live CAV- 26P4 strain, but there was no significant changes of BT in groups 2, 4 & 6 vaccinated with CAV-cux-1. Bleeding time is a screening in vivo test which measures the duration of bleeding from a fresh cut of determined size on the skin. A prolonged bleeding time indicates a platelet disorder or an intrinsic blood vessel problem. It was reported that bleeding time is prolonged in; (a) primary, usually congenital defects of blood vessel wall (pseudohemophilia or von Willebrands' disease), (b) platelet defects either because of lack of circulating platelets (thrombocytopenia), or because of abnormal platelets function such as in thrombocytopathies, (c) after administration of large doses of heparin, and (d) in sever liver diseases **(Borchgrevink and Waller, 1958, and Benjamin, 1978)**. The present study revealed thrombocytopenia that may be considered as the main cause of prolonged bleeding time **(Sankaranarayanan and Nambiar, 1971)**. As shown in table 6, birds vaccinated with CAV-26P4 strain (groups 1, 3 & 5) developed significant increase of clotting time (CT) mostly at the 2nd week PV. In group 2, significant increase of CT was observed at the 4th week PV. There was no significant change of CT in groups 4 & 6 when compared with control group. Clotting time is an invitro test that measures the time required for fibrin clot formation in non anticoagulated blood. The test screens for abnormalities in the intrinsic and/or common pathways of blood coagulation. Prolonged CT may be attributed to deficiency of coagulation factors **(Benjamin, 1978)** or dysfunction in the liver and bone marrow. Chicken anemia virus-26P4 & cux-1 strains caused significant increase of prothrombin time (PT) in all vaccinated groups at the 2nd, 4th & 6th weeks PV (table 6).

Prothrombin time is used to test the concentration of prothrombin in blood as the

Table (3): Effect of chicken infectious anemia disease live virus vaccines on immunity:

Group	CAV ELISA mean titers at weeks PV						AI HI mean titers at weeks PV			ND HI mean titers at weeks PV		
	1st	2nd	3rd	4th	5th	6th	2nd	4th	6th	2nd	4th	6th
1	469 ± 0.66 a BC	181 ± 0.44 ab C	366 ± 1.29 d C	1125 ± 0.40 c AB	2019 ± 0.28 b A	2486 ± 0.39 c A	4.3 ± 0.33 a A	4.2 ± 0.37 a A	3.4 ± 0.51 a A	5.0 ± 0.58 a A	4.4 ± 0.51 a A	3.6 ± 0.51 a A
2	191 ± 1.19 bc C	229 ± 0.72 a C	2372 ± 0.28 a B	2741 ± 0.15 ab B	8236 ± 0.12 a A	6545 ± 0.16 ab AB	5.0 ± 0.58 a A	4.0 ± 0.32 a AB	3.6 ± 0.51 a B	5.0 ± 0.00 a A	5.0 ± 0.45 a A	3.6 ± 0.40 a A
3	198 ± 0.27 ab B	68 ± 0.58 a_c C	177 ± 1.11 d BC	1686 ± 0.28 bc A	3568 ± 0.27 ab A	3628 ± 0.36 a_c A	4.7 ± 0.33 a A	4.0 ± 0.32 a A	3.7 ± 0.24 a A	4.7 ± 0.33 a A	4.0 ± 0.45 a A	3.8 ± 0.40 a A
4	44 ± 0.46 cd D	173 ± 0.75 ab C	1504 ± 0.83 b B	6238 ± 0.22 a A	5174 ± 0.25 a A	8170 ± 0.22 a A	4.3 ± 0.33 a A	4.4 ± 0.51 a A	3.6 ± 0.40 a A	5.3 ± 0.67 a A	4.6 ± 0.40 a AB	3.6 ± 0.51 a B
5	158 ± 0.23 ab CD	71 ± 0.56 b D	511 ± 0.86 c BC	1298 ± 0.87 c B	4395 ± 0.13 ab A	3198 ± 0.42 bc A	4.8 ± 0.48 a A	4.4 ± 0.51 a A	4.0 ± 0.41 a A	4.8 ± 0.25 a A	4.6 ± 0.60 a A	4.2 ± 0.48 a A
6	124 ± 0.36 bc C	143 ± 1.89 b C	1517 ± 0.15 b B	4838 ± 0.19 a A	5546 ± 0.14 a A	7604 ± 0.12 a A	5.0 ± 0.41 a A	4.2 ± 0.20 a A	3.8 ± 0.37 a A	4.5 ± 0.29 a A	4.6 ± 0.51 a A	4.2 ± 0.58 a A
7	40 ± 0.57 d C	34 ± 0.45 c C	150 ± 0.36 d B	140 ± 0.15 d A	130 ± 0.15 c A	100 ± 0.15 d A	4.7 ± 0.25 a A	4.4 ± 0.24 a AB	3.4 ± 0.40 a B	5.0 ± 0.41 a A	4.4 ± 0.51 a A	4. ± 0.45 a A

ELISA: Enzyme Linked Immunosorbent Assay. **CAV: chicken anemia virus.**
ND. : New castle disease. **PV: post vaccination.**
AI: Avian influenza. **HI: Haemagglutination inhibition.**
Values are means ± standard errors.
Means in a column without a common small letter differ significantly (P<0.05).
Means in a row without a common capital letter differ significantly (P<0.05).

Table (4): Effect of chicken infectious anemia disease live virus vaccines on erythrogram and thrombocytes:

Group	PCV (%)			Hb (gm/dl)			RBCs (x10^6/µl)			Thrombocytes		
	2nd	4th	6th	2nd	4th	6th	2nd	4th	6th	2nd	4th	6th
1	20.80 ± 1.16 b C	24.00 ± 1.21 c BC	29.20 ± 1.07 a A	9.78 ± 0.64 b C	11.18 ± 0.53 ab BC	13.16 ± 0.34 ab A	2.42 ± 0.21 b B	2.78 ± 0.19 ab AB	3.12 ± 0.14 a A	29.80 ± 0.58 c A	29.20 ± 0.37 b A	30.00 ± 0.71 b A
2	21.20 ± 2.58 ab C	31.80 ± 1.59 a A	28.20 ± 1.83 a AB	11.98 ± 0.84 a AB	12.57 ± 0.94 a A	11.47 ± 0.77 bc AB	2.70 ± 0.22 ab AB	2.09 ± 0.31 cd C	3.07 ± 0.26 ab A	31.00 ± 0.71 bc BC	29.80 ± 0.58 b BC	29.40 ± 0.40 b C
3	20.80 ± 0.68 b B	28.60 ± 1.94 ab A	28.20 ± 1.11 a A	10.22 ± 1.00 b A	10.37 ± 0.37 b A	12.50 ± 0.84 a_c A	2.38 ± 0.19 b C	3.32 ± 0.22 a A	3.14 ± 0.22 a A	30.00 ± 0.45 bc A	29.20 ± 0.97 b A	29.40 ± 0.51 b A
4	21.80 ± 1.83 ab B	29.40 ± 0.98 ab A	29.80 ± 1.39 a A	12.13 ± 0.82 a AB	10.07 ± 0.30 b C	11.18 ± 0.38 c A_C	2.75 ± 0.13 ab AB	1.86 ± 0.18 d C	2.50 ± 0.17 b B	31.80 ± 1.16 b B	28.80 ± 0.49 b C	29.60 ± 0.40 b C
5	20.60 ± 0.51 b C	30.00 ± 1.76 ab A	29.80 ± 1.36 a A	10.00 ± 0.62 b C	11.10 ± 0.54 ab BC	13.46 ± 0.63 a A	2.40 ± 0.12 b B	3.14 ± 0.16 a A	2.96 ± 0.18 ab A	30.00 ± 0.32 bc A	30.40 ± 0.51 b A	30.40 ± 0.51 b A
6	22.40 ± 1.50 ab B	26.60 ± 1.47 bc AB	30.00 ± 1.45 a A	11.84 ± 1.00 a AB	9.85 ± 0.41 b C	11.17 ± 0.55 c A-C	2.79 ± 0.12 ab AB	2.51 ± 0.24 bc B	3.18 ± 0.08 a A	31.40 ± 0.51 bc A	29.20 ± 0.37 b B	29.20 ± 0.37 b B
7	25.00 ± 0.63 a C	28.20 ± 0.73 ab A-C	29.60 ± 0.51 a AB	12.98 ± 0.39 a AB	12.35 ± 0.41 a A-C	13.31 ± 0.60 a A	3.14 ± 0.18 a A	3.31 ± 0.18 a A	3.31 ± 0.16 a A	36.20 ± 0.49 a A	34.20 ± 0.37 a BC	34.20 ± 0.37 a BC

PCV: Packed cell volume. **RBCs: Red blood cells.**
Hb: hemoglobin.
Values are means ± standard errors.
Means in a column without a common small letter differ significantly (P<0.05).
Means in a row without a common capital letter differ significantly (P<0.05).

time taken by blood to clot is inversely proportional to prothrombin concentration in blood. Prolongation of prothrombin time could be attributed to the affection of liver accompanied CAV infection or vaccination as reported by **Youssef (1998) and Hussein *et al.* (2003)**. Chicks vaccinated with CAV-26P4 strain showed histopathological lesions in liver in the form of hepatocytes swelling to centro-lobular necrosis and apoptosis. Liver is the site of formation of prothrombin **(Hallen and Nilsson, 1964)** and it is the major site of synthesis of other coagulation factors (fibrinogen & factors V, VII, IX, X, XI and XIII). The extrinsic clotting mechanism is particularly compromised in liver disorders **(Benjamin, 1978)**. Prolonged prothrombin time may be due to the lack of coagulation factors **(Bigland and Triantophyllopoulos, 1961)**.

The present investigation revealed that CAV-26P4 & cux-1 strains caused significant increase of whole blood recalcification time (WBRT) at the 2^{nd}, 4^{th} & 6^{th} weeks PV. Group 2 vaccinated with CAV-cux-1 strain at 75 day-old age developed significant increase of WBRT only at the 2^{nd} week PV when compared with non-vaccinated group (table 6). Also table 6 revealed that CAV-26P4 & cux-1 strains caused significant increase of plasma recalcification time (PRT) in all vaccinated groups. WBRT and PRT tests are used for evaluation of intrinsic and extrinsic coagulation mechanisms, as calcium (factor IV) is an important factor for conversion of inactivated coagulation factors to active factors responsible for clot formation. Prolonged WBRT and PRT indicate coagulation factors deficiency that may be attributed to liver and bone marrow affection **(Benjamin, 1978 and Nunoya *et al.*, 1992)**.

The present investigation revealed significant increase of albumin to globulin ratio (A/G ratio) at the 2nd week PV in all groups vaccinated with CAV-26P4 & cux-1 strains. In group 4, there was no significant change of A/G ratio at the 2nd, 4th & 6th weeks PV when compared with non-vaccinated group (table 7). Increased A/G ratio may be attributed to hypoglobulinemia due to liver damage.

Chicken anemia virus-26P4 & cux-1 strains caused no significant change of serum uric acid in all vaccinated groups at the 2nd, 4th & 6th weeks PV when compared with non-vaccinated group (table 7). Uric acid is the end product of nitrogen metabolism and is the major nitrogenous component excreted by fowl and is the primary catabolic product of protein, non-protein, and purines in birds **(Campbell and Coles, 1986).**

It was concluded that live CAV-26P4 strain inoculated by i.m. route developed clinical signs and PM lesions, while live CAV-cux-1 strain given by oral route did not develop clinical signs but developed mild PM lesions. Chicken anemia virus-26P4 and cux-1 strains caused significant increase of ELISA titers mostly at the 6th week PV; significant hematological anemia, leucopenia, heterophilia, lymphopenia, monocytopenia and thrombocytopenia. Such changes were observed early in birds inoculated with CAV-26P4 strain, but were observed later in birds vaccinated with CAV-cux-1 strain. Significant increase of coagulation parameters, increase of liver enzymes (ALT & AST), hypoproteinemia, hypoglobuliemia and increase of A/G ratio were also observed. In the current study, table 7 revealed that CAV-26P4 and cux-1 strains caused significant increase of liver enzymes, serum alanin aminotransferase (SALT) and serum aspartate aminotransferase (SAST) in all vaccinated groups mostly at the 4th week PV. Group 6 showed no significant change of SALT and SAST. Also table 7 revealed that CAV-26P4 and cux-1 strains caused significant hypoproteinemia and hypoglobuliemia mostly at the 2nd week PV in all vaccinated groups. The elevation of liver enzymes besides hypoproteinemia and hypoglobuliemia indicate liver affection by CAV vaccinal strains as supported by **Youssef (1998) and Hussein et al. (2003)** who stated that chicks vaccinated with CAV-26P4 strain, showed histopathological lesions in liver in the form of hepatocytes swelling to centro-lobular necrosis and apoptosis. **Ivins et al. (1978)** stated that a moderate increase of serum AST activity (two to four fold increase) is seen with soft tissue injury, whereas liver necrosis causes a more marked elevation. Hypoproteinemia and hypoglobuliemia may be attributed to liver damage because liver is the major organ in production of proteins such as albumin and globulins **(Sastry, 1983).**

Table (3): Effect of chicken infectious anemia disease live virus vaccines on

Group	CAV ELISA mean titers at weeks PV						AI HI mean titers at weeks PV			ND HI mean titers at weeks PV		
	1st	2nd	3rd	4th	5th	6th	2nd	4th	6th	2nd	4th	6th
1	469 ± 0.66 a BC	181 ± 0.44 ab C	366 ± 1.29 d C	1125 ± 0.40 c AB	2019 ± 0.28 b A	2486 ± 0.39 c A	4.3 ± 0.33 a A	4.2 ± 0.37 a A	3.4 ± 0.51 a A	5.0 ± 0.58 a A	4.4 ± 0.51 a A	3.6 ± 0.51 a A
2	191 ± 1.19 bc C	229 ± 0.72 a C	2372 ± 0.28 a B	2741 ± 0.15 ab B	8236 ± 0.12 a A	6545 ± 0.16 ab AB	5.0 ± 0.58 a A	4.0 ± 0.32 a AB	3.6 ± 0.51 a B	5.0 ± 0.00 a A	5.0 ± 0.45 a A	3.6 ± 0.40 a A
3	198 ± 0.27 ab B	68 ± 0.58 a_c C	177 ± 1.11 d BC	1686 ± 0.28 bc A	3568 ± 0.27 ab A	3628 ± 0.36 a_c A	4.7 ± 0.33 a A	4.0 ± 0.32 a A	3.7 ± 0.24 a A	4.7 ± 0.33 a A	4.0 ± 0.45 a A	3.8 ± 0.40 a A
4	44 ± 0.46 cd D	173 ± 0.75 ab C	1504 ± 0.83 b B	6238 ± 0.22 a A	5174 ± 0.25 a A	8170 ± 0.22 a A	4.3 ± 0.33 a A	4.4 ± 0.51 a A	3.6 ± 0.40 a A	5.3 ± 0.67 a A	4.6 ± 0.40 a AB	3.6 ± 0.51 a B
5	158 ± 0.23 ab CD	71 ± 0.56 b D	511 ± 0.86 c BC	1298 ± 0.87 c B	4395 ± 0.13 ab A	3198 ± 0.42 bc A	4.8 ± 0.48 a A	4.4 ± 0.51 a A	4.0 ± 0.41 a A	4.8 ± 0.25 a A	4.6 ± 0.60 a A	4.2 ± 0.48 a A
6	124 ± 0.36 bc C	143 ± 1.89 b C	1517 ± 0.15 b B	4838 ± 0.19 a A	5546 ± 0.14 a A	7604 ± 0.12 a A	5.0 ± 0.41 a A	4.2 ± 0.20 a A	3.8 ± 0.37 a A	4.5 ± 0.29 a A	4.6 ± 0.51 a A	4.2 ± 0.58 a A
7	40 ± 0.57 d C	34 ± 0.45 c C	150 ± 0.36 d B	140 ± 0.15 d A	130 ± 0.15 c A	100 ± 0.15 d A	4.7 ± 0.25 a A	4.4 ± 0.24 a AB	3.4 ± 0.40 a B	5.0 ± 0.41 a A	4.4 ± 0.51 a A	4. ± 0.45 a A

ELISA: Enzyme Linked Immunosorbent Assay. **CAV: chicken anemia virus.**
ND. : New castle disease. **PV: post vaccination.**
AI: Avian influenza. **HI: Haemagglutination inhibition.**
Values are means ± standard errors.
Means in a column without a common small letter differ significantly (P<0.05).
Means in a row without a common capital letter differ significantly (P<0.05)

Table (4): Effect of chicken infectious anemia disease live virus vaccines on erythrogram and thrombocytes:

Group	PCV (%)			Hb (gm/dl)			RBCs ($x10^6$/µl)			Thrombocytes		
	2nd	4th	6th	2nd	4th	6th	2nd	4th	6th	2nd	4th	6th
1	20.80 ± 1.16 b C	24.00 ± 1.21 c BC	29.20 ± 1.07 a A	9.78 ± 0.64 b C	11.18 ± 0.53 ab BC	13.16 ± 0.34 ab A	2.42 ± 0.21 b B	2.78 ± 0.19 ab AB	3.12 ± 0.14 a A	29.80 ± 0.58 c A	29.20 ± 0.37 b A	30.00 ± 0.71 b A
2	21.20 ± 2.58 ab C	31.80 ± 1.59 a A	28.20 ± 1.83 a AB	11.98 ± 0.84 a AB	12.57 ± 0.94 a A	11.47 ± 0.77 bc AB	2.70 ± 0.22 ab AB	2.09 ± 0.31 cd C	3.07 ± 0.26 ab A	31.00 ± 0.71 bc BC	29.80 ± 0.58 b BC	29.40 ± 0.40 b C
3	20.80 ± 0.68 b B	28.60 ± 1.94 ab A	28.20 ± 1.11 a A	10.22 ± 1.00 b A	10.37 ± 0.37 b A	12.50 ± 0.84 a_c A	2.38 ± 0.19 b C	3.32 ± 0.22 a A	3.14 ± 0.22 a A	30.00 ± 0.45 bc A	29.20 ± 0.97 b A	29.40 ± 0.51 b A
4	21.80 ± 1.83 ab B	29.40 ± 0.98 ab A	29.80 ± 1.39 a A	12.13 ± 0.82 a AB	10.07 ± 0.30 b C	11.18 ± 0.38 c A_C	2.75 ± 0.13 ab AB	1.86 ± 0.18 d C	2.50 ± 0.17 b B	31.80 ± 1.16 b B	28.80 ± 0.49 b C	29.60 ± 0.40 b C
5	20.60 ± 0.51 b C	30.00 ± 1.76 ab A	29.80 ± 1.36 a A	10.00 ± 0.62 b C	11.10 ± 0.54 ab BC	13.46 ± 0.63 a A	2.40 ± 0.12 b B	3.14 ± 0.16 a A	2.96 ± 0.18 ab A	30.00 ± 0.32 bc A	30.40 ± 0.51 b A	30.40 ± 0.51 b A
6	22.40 ± 1.50 ab B	26.60 ± 1.47 bc AB	30.00 ± 1.45 a A	11.84 ± 1.00 a AB	9.85 ± 0.41 b C	11.17 ± 0.55 c A-C	2.79 ± 0.12 ab AB	2.51 ± 0.24 bc B	3.18 ± 0.08 a A	31.40 ± 0.51 bc A	29.20 ± 0.37 b B	29.20 ± 0.37 b B
7	25.00 ± 0.63 a C	28.20 ± 0.73 ab A-C	29.60 ± 0.51 a AB	12.98 ± 0.39 a AB	12.35 ± 0.41 a A-C	13.31 ± 0.60 a A	3.14 ± 0.18 a A	3.31 ± 0.18 a A	3.31 ± 0.16 a A	36.20 ± 0.49 a A	34.20 ± 0.37 a BC	34.20 ± 0.37 a BC

PCV: Packed cell volume. **RBCs: Red blood cells.**
Hb: hemoglobin.
Values are means ± standard errors.
Means in a column without a common small letter differ significantly (P<0.05).
Means in a row without a common capital letter differ significantly (P<0.05).

Table (5): Effect of chicken infectious anemia disease live virus vaccines on leukogram ($x10^3$/µl

Group	WBCs				Heterophils			Lymphocytes			Monocytes			Eosinophils		
	2nd	4th	6th		2nd	4th	6th	2nd	4th	6th	2nd	4th	6th	2nd	4th	6th
1	29.20 ± 1.39 b B	38.40 ± 1.94 ab A	27.00 ± 3.00 ab B		41.80 ± 1.36 a A	41.00 ± 0.71 a A	42.40 ± 0.24 a A	54.00 ± 1.30 b AB	54.20 ± 0.80 c AB	52.40 ± 0.40 c B	4.20 ± 0.37 b B	4.80 ± 0.20 b AB	4.80 ± 0.20 b AB	0 ± 0 a B	0 ± 0 a B	0.40 ± 0.24 a A
2	31.60 ± 1.33 ab B	36.80 ± 1.96 b B	19.40 ± 2.18 bd C		33.00 ± 0.71 b A	33.80 ± 0.66 d A	34.00 ± 0.55 c A	62.00 ± 0.84 a A	61.40 ± 0.68 a A	61.00 ± 0.45 a A	4.80 ± 0.37 b A	4.80 ± 0.20 ab A	4.80 ± 0.20 b A	0.20 ± 0.20 a AB	0 ± 0 aB	0.20 ± 0.20 ab AB
3	29.00 ± 0.84 b CD	38.80 ± 2.01 ab AB	25.80 ± 3.65 a_c D		41.20 ± 0.86 a A	39.60 ± 1.33 ab A	39.00 ± 0.32 b A	54.00 ± 0.84 b A	55.60 ± 1.33 bc A	56.20 ± 0.37 b A	4.60 ± 0.24 b A	4.80 ± 0.20 b A	4.80 ± 0.20 b A	0.20 ± 0.20 a A	0 ± 0 a A	0 ± 0 b A
4	33.20 ± 2.76 ab BC	39.40 ± 1.33 ab AB	14.00 ± 1.00 d D		32.80 ± 0.73 b C	37.40 ± 0.75 c B	38.20 ± 0.37 b B	62.40 ± 0.81 a A	57.00 ± 0.84 b B	57.00 ± 0.32 b B	4.60 ± 0.24 b BC	5.40 ± 0.24 ab A	4.80 ± 0.20 b A_C	0 ± 0 a A	0.20 ± 0.20 a A	0 ± 0 b A
5	29.20 ± 2.60 b B	36.80 ± 1.32 b AB	19.00 ± 1.58 cd C		41.60 ± 0.81 a A	34.60 ± 0.51 d B	34.20 ± 0.20 c B	53.80 ± 0.86 b B	60.40 ± 0.51 a A	61.00 ± 0.32 a A	4.40 ± 0.24 b B	5.00 ± 0.00 ab AB	4.80 ± 0.20 b AB	0.20 ± 0.20 a A	0 ± 0 a A	0 ± 0 b A
6	33.40 ± 1.89 ab B	36.80 ± 1.74 b AB	20.20 ± 1.53 b_d C		33.00 ± 0.32 b B	38.00 ± 0.32 bc A	38.40 ± 0.51 b A	62.20 ± 0.49 a A	56.80 ± 0.58 b B	56.60 ± 0.51 b B	4.60 ± 0.24 b B	4.80 ± 0.20 AbAB	4.80 ± 0.20 b AB	0.20 ± 0.20 a A	0.20 ± 0.20 a A	0 ± 0 b A
7	37.80 ± 0.73 a B	46.00 ± 1.64 a A	28.20 ± 1.28 a C		32.20 ± 0.49 b A	32.80 ± 0.58 d A	33.80 ± 0.49 c A	62.00 ± 0.55 a A	61.60 ± 0.51 a A	60.60 ± 0.40 a A	5.60 ± 0.24 a A	5.60 ± 0.24 a A	5.60 ± 0.24 a A	0 ± 0 a A	0 ± 0 a A	0 ± 0 b A

WBCs: White blood cells.
Values are means ± standard errors.
Means in a column without a common small letter differ significantly (P<0.05).
Means in a row without a common capital letter differ significantly (P<0.05).

Table (6): Effect of chicken anemia disease live virus vaccines on coagulation

Group	BT			CT			PT			WBRT			PRT		
	2nd	4th	6th	2nd	4th	6th	2nd	4th	6th	2nd	4th	6th	2nd	4th	6th
1	181.80 ±14.08 a A	178.20 ±18.49 a AB	129.00 ± 8.75 a C	201.60 ± 18.32 a A	193.20 ±17.76 a A	131.00 ± 14.64 a BC	262.60 ±20.23 a A	231.80 ±7.84 c AB	241.60 ± 9.79 b A	330.60 ± 9.72 a BC	280.20 ±17.67 bCD	257.20 ± 8.70 ac D	360.60 ±12.64 a A	247.80 ± 6.06 c C	245.00 ± 4.11 ab C
2	152.80 ± 1.36 ab A_C	162.40 ±32.56 ab AB	112.00 ± 3.77 a C	140.60 ± 8.41 b-d B	176.20 ±31.25 ab AB	143.00 ± 27.38 a B	182.00 ± 6.63 d AB	158.40 ± 8.29 d B	158.20 ±10.58 e B	258.40 ±11.63 cd A	179.20 ±20.16 c C	223.60 ±19.04 bc A_C	215.80 ±16.46 cd AB	138.20 ± 6.06 e C	191.80 ±11.68 d B
3	184.00 ±22.59 a A	144.60 ±11.14 a_c AB	128.20 ±12.58 a B	209.60 ± 16.44 a A	162.80 ±13.87 a_c BC	145.20 ± 18.39 a C	275.80 ±21.18 a AB	281.00 ± 8.05 b AB	278.40 ± 7.20 a AB	326.00 ±16.00 a BC	350.20 ± 7.45 a B	285.80 ±13.12 a C	372.00 ± 5.83 a A	298.60 ±11.11 b B	261.00 ±10.19 ab B
4	121.60 ± 8.28 b AB	111.40 ±12.41 c B	127.00 ±12.93 a AB	134.40 ± 3.66 d AB	139.00 ±22.21 bc AB	126.60 ± 18.71 a B	198.80 ±14.91 cd B	216.60 ±10.45 c B	204.00 ± 5.26 cd B	270.20 ± 18.66 b-d A_C	218.80 ±22.67 c C	290.20 ± 4.47 a AB	210.60 ±20.83 d BC	191.40 ± 4.31 d C	234.60 ± 8.00 bc B
5	173.00 ±11.68 a A	115.20 ±19.24 c B	121.40 ± 3.74 a B	200.80 ± 23.94 a A	125.20 ±20.96 c C	147.40 ± 11.04 a C	280.00 ±14.28 a BC	334.20 ±10.34 a A	251.40 ±11.85 ab C	372.00 ±17.72 a BC	396.80 ±12.75 a B	280.20 ±17.38 ab E	348.00 ±15.94 a A	356.60 ± 5.13 a A	265.20 ± 4.91 ab B
6	148.80 ±12.62 ab AB	126.20 ± 8.60 bc B	129.60 ±13.25 a AB	171.80 ± 5.61 a_d AB	135.80 ± 8.18 bc AB	145.80 ± 20.99 a AB	200.80 ±14.73 b-d BC	229.20 ± 6.46 c B	233.60 ± 3.84 bc B	238.80 ±21.70 d C	274.80 ±14.64 b BC	275.60 ±26.77 ab BC	238.20 ±15.23 b-d C	220.20 ±11.55 cd C	277.00 ± 7.22 a B
7	116.40 ± 8.79 b A	120.20 ±16.04 bc A	113.80 ± 6.70 a A	136.60 ± 13.54 cd A	123.00 ± 6.75 c A	126.20 ± 6.09 a A	140.80 ±11.09 e C	160.40 ± 7.80 d BC	195.40 ± 6.72 d A	203.20 ± 7.98 e AB	208.60 ±25.18 c AB	208.40 ± 7.26 c AB	189.20 ±14.19 e A	194.40 ± 9.55 d A	200.00 ± 8.51 cd A

Bt: Bleeding time. CT: Clotting time. WBRT: Whole blood recalcification time.
PT: Prothrombin time. PRT: Plasma recalcification time. Values are means ± standard errors.
Means in a column without a common small letter differ significantly (P<0.05).
Means in a row without a common capital letter differ significantly (P<0.05

Table (7): Effect of chicken infectious anemia disease live virus vaccines on blood biochemical parameters

Group	AL			AST			TP			Albumin			Globulin			A/G ratio			Uric acid		
	2nd	4th	6th	2nd	4th	6th	2nd	4th	6th	2nd	4th	6th	2nd	4th	6th	2nd	4th	6th	2nd	4th	6th
1	59.80 ± 0.80 abBC	64.80 ± 0.86 a A	60.80 ± 1.07 abBC	67.80 ± 4.83 abAB	65.60 ± 1.71 a B	71.60 ± 1.17 a A	3.66 ± 0.27 b C	6.30 ± 0.28 b A	5.22 ± 0.20 aAB	2.20 ± 0.02 cd A	2.12 ± 0.04 a A	2.22 ± 0.06 a A	1.46 ± 0.26 b C	4.18 ± 0.30 b A	3.00 ± 0.19 abAB	1.51 ± 0.22 a A	0.51 ± 0.05 a B	0.74 ± 0.05 a B	6.41 ± 0.57 aAB	6.18 ± 0.26 abAB	5.25 ± 0.14 a B
2	57.00 ± 0.45 b C	63.60 ± 0.81 a A	60.40 ± 0.75 ab B	64.20 ± 4.41 ab B	65.40 ± 1.17 a AB	69.80 ± 2.27 a A	4.69 ± 0.54 b B	6.45 ± 0.47 b A	5.58 ± 0.66 aAB	2.47 ± 0.05 ab A	2.17 ± 0.03 a BC	2.15 ± 0.06 aBC	2.22 ± 0.54 b B	4.27 ± 0.49 b A	3.43 ± 0.68 a AB	1.11 ± 0.36 ab A	0.51 ± 0.07 a B	0.63 ± 0.26 aAB	5.84 ± 0.30 a A	5.18 ± 0.19 b A	5.12 ± 0.13 a A
3	61.00 ± 0.71 a BC	65.80 ± 1.28 a A	62.20 ± 0.49 a B	72.40 ± 7.42 a A	65.80 ± 2.44 a A	69.60 ± 0.40 a A	3.99 ± 0.46 b C	5.83 ± 0.27 bAB	4.27 ± 0.33 a C	2.24 ± 0.01 cd A	2.17 ± 0.05 a A	2.29 ± 0.06 a A	1.75 ± 0.46 b C	3.66 ± 0.26 bAB	1.98 ± 0.30 b C	1.28 ± 0.68 ab A	0.59 ± 0.04 a B	1.16 ± 0.18 a A	5.53 ± 0.49 aAB	6.58 ± 0.08 a A	4.55 ± 0.35 a B
4	57.80 ± 0.20 b C	63.80 ± 0.97 a A	60.20 ± 0.73 abBC	60.00 ± 1.52 b A	63.40 ± 2.79 a A	66.80 ± 2.56 a A	4.78 ± 0.50 b B	6.09 ± 0.38 b A	4.50 ± 0.37 a B	2.31 ± 0.05 bcdAB	2.30 ± 0.06 a AB	2.19 ± 0.11 aBC	2.46 ± 0.50 b B	3.79 ± 0.38 b A	2.31 ± 0.47 ab B	0.94 ± 0.14 bc A	0.61 ± 0.03 a A	0.91 ± 0.41 a A	5.32 ± 0.28 a A	5.84 ± 0.31 abA	4.97 ± 0.30 a A
5	59.80 ± 0.80 ab B	63.00 ± 0.32 a A	59.60 ± 1.17 ab B	70.20 ± 7.04 ab A	65.40 ± 1.16 a B	69.20 ± 0.58 a AB	4.15 ± 0.27 b C	5.49 ± 0.48 b B	4.82 ± 0.20 aBC	2.39 ± 0.05 abc A	2.22 ± 0.03 aA_C	2.34 ± 0.19 aAB	1.77 ± 0.24 b C	3.27 ± 0.48 b B	2.48 ± 0.13 abBC	1.35 ± 0.20 ab A	0.68 ± 0.12 a B	0.94 ± 0.10 aAB	5.70 ± 0.53 aBC	6.56 ± 0.64 aAB	4.87 ± 0.30 a C
6	57.80 ± 0.58 b A	60.20 ± 0.73 b A	60.20 ± 1.07 ab A	63.20 ± 1.83 ab B	58.40 ± 3.75 b C	71.40 ± 1.21 a A	4.56 ± 0.16 bBC	5.68 ± 0.53 bAB	4.40 ± 0.40 a C	2.56 ± 0.06 a A	2.30 ± 0.04 a B	2.24 ± 0.09 a B	2.00 ± 0.16 b C	3.38 ± 0.52 b A	2.16 ± 0.46 abBC	1.28 ± 0.15 ab A	0.68 ± 0.12 a B	1.03 ± 0.36 aAB	5.53 ± 0.19 a A	6.07 ± 0.21 ab A	5.23 ± 0.15 a A
7	58.20 ± 0.49 b A	58.20 ± 2.96 b A	59.00 ± 1.00 b A	61.40 ± 0.60 b AB	58.20 ± 0.24 b B	65.60 ± 1.72 a A	6.94 ± 0.78 aBC	8.54 ± 0.43 a A	5.31 ± 0.11 a D	2.15 ± 0.11 d B	2.24 ± 0.05 a AB	2.30 ± 0.05 aAB	4.80 ± 0.72 aBC	6.29 ± 0.47 a A	3.01 ± 0.10 ab D	0.45 ± 0.06 c A	0.36 ± 0.03 a A	0.77 ± 0.03 a A	5.97 ± 0.50 aAB	5.91 ± 0.36 abAB	5.12 ± 0.26 a B

Values are means ± standard errors Means in column without a common small letter differ significantly (P<0.05).

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Microscopical Identification and seasonal dynamics of Gastrointestinal Nematodes in Small Ruminants at Menoufia province.

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Abstract:

Gastrointestinal nematodes are of major concern to the productivity and health for small ruminants, they cause severe symptoms such as watery diarrhea, anorexia, weight loss and even death of affected animals. The present study was carried out for microscopical identification of gastro intestinal nematodes in sheep and goats by floatation technique and faecal culture and determines their prevalence and seasonal incidence. Faecal examination was carried out on 83 sheep and 38 goats of different ages, sex and localities, the infection rate among sheep and goats was (44.57%) and (18.42%) respectively. The seasonal incidence of nematodes was recorded. By floatation technique, the recorded species in sheep were as follow; eggs of *Trichuris ovis* (2.40%), *Haemonchus contortus* (38.55%), *Strongyloides papillosus* eggs (36.14%), *Bunostomum trigoncephalum* eggs (2.40%). *Trichostrongylus sp.* eggs (9.63%), the highest infection rate recorded during summer season followed by spring and winter season was 46.66%, 44.82% and 41.66% respectively. Regarding goats; *Trichuris ovis* eggs (2.63%), *Haemonchus contortus* eggs (7.89%). *Strongyloides papillosus* eggs (13.15%). *Trichostrongylus sp.* Eggs (5.26%), the highest infection rate recorded during winter season followed by spring and summer season with an infection rate of 25%, 22.22% and 16% respectively, the same samples were re-examined by faecal cultures and Baerman's technique, results compared, it concluded that, the combination between two method is better for concise identification than floatation technique alone, also the morphology and morphometry of recovered eggs *and larvae* were recorded.

Keywords: *Diagnosis, Baerman's technique, Fecal culture, Gastrointestinal nematodes and Menoufia province*

Introduction

Gastrointestinal nematode parasitism is arguably the most serious constraint affecting the production of small ruminants worldwide. Economic losses are caused by decreased production, the costs of treatment and the

death of the infected animals, they are the most numerous, complex and variable among the helminth parasites of sheep and goats (**Urquhart et al., 1996)**, inadequately parasite control causing severe symptoms such as watery diarrhea, anorexia, weight loss and even death of affected animals, and should therefore be regarded as major animal welfare concerns. However, more common and of major economical impact are sub clinical infections, which may cause significantly reduced performance of infected animals without obvious symptoms. (**Suarez and Busetti, 1995)**. Traditional methods for detesction of the type and level of infection in a sheep flock require laborious laboratory extraction, culture and microscopic examination of eggs or larvae from faecal samples. This study aimed to identify gastrointestinal nematodal infections in sheep and goats by floatation technique and faecal culture, to identify the recovered eggs and larvae and determine the prevalence and seasonal incidence.

Materials and methods

1- **Samples collection**:-

Faecal pellets samples were collected from 83 sheep and 38 goats either from rectum directly or as soon as possible after defecation in plastic containers that labeled with all data about the animal (Age, sex, species, date, and locality).

2- **Floatation technique: (Urquhart *et al.*, 1996)**

Two grams of fresh faeces is added to 10 ml of saturated sodium chloride solution and following thorough mixing the suspension was poured into a test tube and more floatation solution added to fill the tube to the top. A cover slip is then added on the top of the surface of the liquid and the tube and the cover slip left standing for 15 minutes. The cover slip is then removed vertically and placed on a slide and examined under the compound microscope systematically.

3-Faecal culture technique: (MAFF, 1986).

The faeces were broken finely, using either a tongue depressor, very dry faeces should be moisted with water; if they are very wet, wood shaves should be added until the correct consistency is obtained. The glass culture or honey jars are then filled with the mixture and placed in an incubator at 27°c for 7 days. The larvae were recovered by Baerman's apparatus.

4- Baerman's technique: (**MAFF 1986), and (Urquhart *et al.*, 1996)**

Baerman's apparatus consisted of a ring stand and a ring supporting a large glass or plastic funnel. The funnel's stem is connected by a piece of rubber tubing to a tapered tube (a cut

of Pasteur pipette). The rubber tubing is clamped shut with a pinch clamp. A piece of metal screen is placed in the funnel to serve as a support for the sample. The funnel is then filled with warm water at 30 ˚c to a level 1 to 3 cm above the sample. The content of faecal culture was placed in the gauze on the metal screen over the funnel. The apparatus was kept undisturbed over night. Afterward, the clamp was removed and collect the first few drop into clean glass tube. A small drop of suspension of larvae was placed on a microscope slide and mixed with a drop of gram's iodine solution (1gm iodine, 2 gm potassium iodide and 300 ml distilled water). The slide was covered by cover slip and examined under a microscope. Each larva was examined, recognized and identified according to standard keys **(Soulsby 1986).**

Results

The results in table (1) showed that the infection rate of nematodes in sheep and goats by floatation technique that was carried out on 83 sheep and 38 goats, it was (44.57%) and (18.42%) respectively. Concerning to the nematode infection between sheep, *Trichuris ovis* eggs 2.40%, *Haemonchus contortus* 38.55%, *Strongyloides papillosus* eggs 36.14%, *Bunostomum trigoncephalum* eggs 2.40%, *Trichostrongylus sp.* eggs 9.63%, mixed infections between nematodes were recorded, the seasonal incidence show the highest infection rate during summer season followed by spring and winter season with an infection rate of 46.66%, 44.82% and 41.66% respectively. The nematode infections among as recorded in table (1), 7 out of 38 goats was infected with nematodes with an infection rate of 18.42%. *Trichuris ovis* eggs 2.63%, *Haemonchus contortus* eggs 7.89%, *Strongyloides papillosus* eggs 13.15%, *Trichostrongylus sp*. eggs 5.26%. The seasonal incidence show the highest infection rate during winter season followed by spring and summer with an infection rate of 25%, 22.22% and 16% respectively.

The morphology of some recovered eggs of nematodes *(Trichuris ovis, Haemonchus contortus, Trichostrongylus sp., Strongyloides papillosus* and *Bunostomum trigoncephalum)* was displayed in figure (1). The morphological examination of the recovered larvae (the anterior and posterior extremity of the body, the number of intestinal cells, and the shape of esophagus) as well as the micrometrical data (total body length, esophagus length, and the length from anus to the tail sheath tip) were recorded. Table (2, 3), including *Haemonchus contortus, strongyloides papillosus, Trichostrongylus axei, Bunostomum trigoncephalum, Ostertagia circumcincta, Cooperia curticei*

and Chabertia ovina larvae. Concerning sheep, 67 out of 83 sheep with an infection rate of 80.72%. *Haemonchus contortus* (61.44%), *Strongyloides papillosus* (56.62%). *Trichostrongylus axei* (12.04%), *Chabertia ovina* (3.61%), *Cooperia curticei* (2.40%), *Bunostomum trigoncephalum* (2.40%), *Trichuris ovis* (egg containing larva) (2.40%), and 1 sheep were identified with *Ostertagia circumcincta* with an infection rate of 1.20%, for mixed infection, the highest infection rate was *Haemonchus contortus* (61.44%) followed by *strongyloides papillosus* (56.62%) and the lowest infection rate was recorded by *Ostertagia circumcincta* (1.20%). Concerning goats, 23 out of 38 goats were infected with nematodes with an infection rate of 60.52%. *Haemonchus contortus* (39.47%). *strongyloides papillosus* (36.84%). *Trichostrongylus axei* (5.26%). *Chabertia ovina* (2.63%), *Cooperia curticei* (2.63%). *Ostertagia circumcinca* (2.63%). *Trichuris ovis* (egg containing larva) (2.63%). About the mixed infection, the highest rate was recorded by *Haemonchus contortus* (39.47%) followed by *Strongyloides papillosus* (36.84%) and the lowest infection rate was recorded by *Ostertagia circumcincta*, *Cooperia curticei* and *Chabertia ovina* that had the same percent (2.63%). Tables (2, 3) showed seasonal variations of the recovered larvae; the highest infection rate recorded during summer season (90%) followed by spring (79.31%) and winter season (70.83%). For goats; the highest infection rate of *nematodes* recorded during spring season (66.66%) followed by summer (60%) and winter season (50%).

Discussion

Gastrointestinal nematode parasitism of sheep and goats is a serious constraint affecting their production worldwide; the economic losses are due to decreased production, the costs of treatment and the death of the infected animals, little knowledge on the prevalence and seasonal dynamics of parasitism with these nematodes lead to inadequately parasite control measures that causing severe problems even death of affected animals. Traditional methods for detection of the type and level of infection in a sheep flock require laborious laboratory extraction, culture and microscopic examination of eggs or larvae from faecal samples. Until now little researches were done on the prevalence and dynamics of these nematodes in Menoufiya province; so, this study aimed primarily to identify morphological and morphometrical data on the gastrointestinal nematodes in sheep and goats by floatation technique and faecal culture, to identify the recovered eggs and larvae and determine the prevalence and seasonal incidence in the province.

Table (1) Seasonal infection rate of gastrointestinal nematode by floatation technique

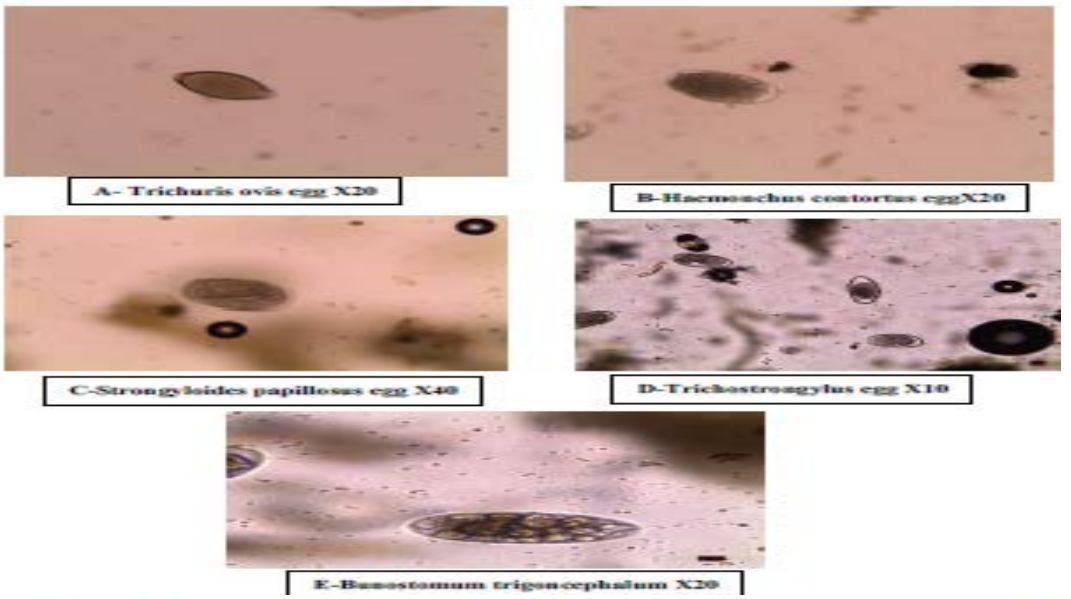
		Faecal examination (floatation technique)		
	Season	Number examined	Number infected	Percentage of infection
Sheep	**Winter**	**24**	**10**	**41.66%**
	Spring	**29**	**13**	**44.82%**
	Summer	**30**	**14**	**46.66%**
	Total	**83**	**37**	**44.57%**
Goats	**Winter**	**4**	**1**	**25%**
	Spring	**9**	**2**	**22.22%**
	Summer	**25**	**4**	**16%**
	Total	**38**	**7**	**18.42%**

Table (2) Seasonal infection rate of gastrointestinal nematode larvae by faecal culture

		Faecal culture		
	Season	Number examined	Number infected	Percentage of infection
Sheep	**Winter**	**24**	**17**	**70.83%**
	Spring	**29**	**23**	**79.31%**
	Summer	**30**	**27**	**90%**
	Total	**83**	**67**	**80.72%**
Goats	**Winter**	**4**	**2**	**50%**
	Spring	**9**	**6**	**66.66%**
	Summer	**25**	**15**	**60%**
	Total	**38**	**23**	**60.52%**

Table (3) Infection rate of gastrointestinal nematode larvae of different examined ruminants

Parasitic nematode species		Animal species			
		Sheep		Goats	
		No. examined (83)		No. examined (38)	
		No. infected	% of infection	No. infected	% of infection
	Trichostrongylus axei	10	12.04%	2	5.26%
	OstertAgia circumcincta	1	1.20%	1	2.63%
	Haemonchus contortus	51	61.44%	15	39.47%
	Cooperia curticei	2	2.40%	1	2.63%
	Chabertia ovina	3	3.61%	1	2.63%
	Strongyloides papillosus	47	56.62%	14	36.84%
	Bunostomum trigoncephalum	2	2.40%	-	-
	Total animal infected	67	80.72%	23	60.52%



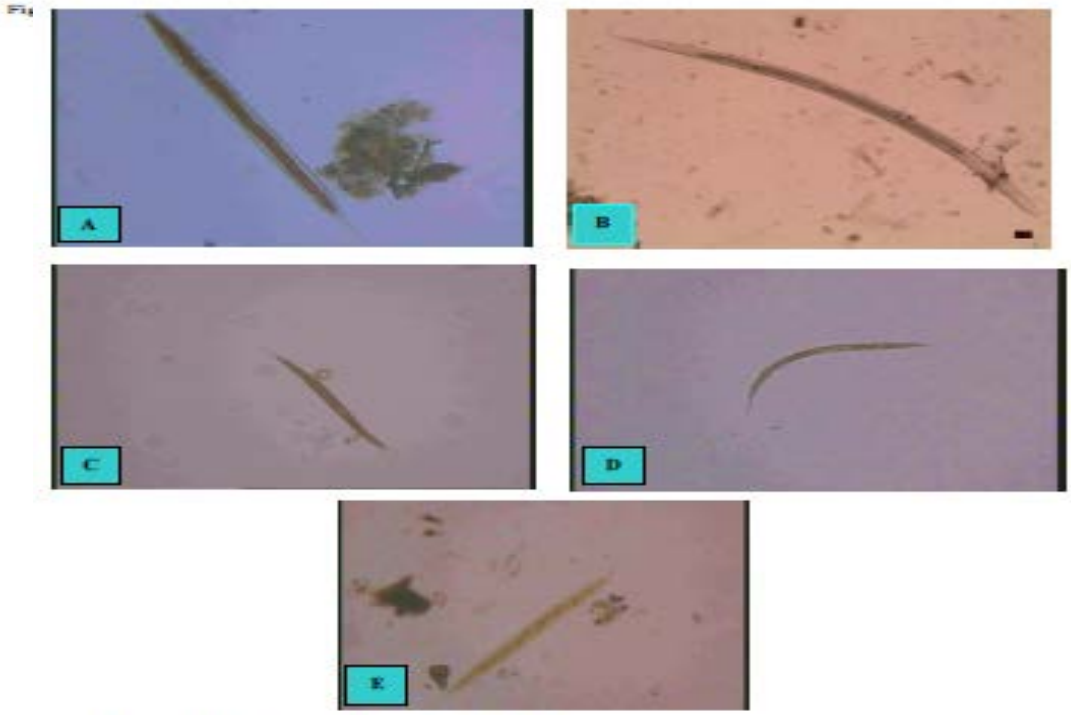


Figure (2): Some recovered larvae from faecal cultures.
A- Haemonchus contortus larva X10
B-Nematodirus filicollis larva X10
C-Cooperia curticei larva X10
D- Trichostrongylus axei larva X10
E- Strongyloides papillosus larva X10

Results showed that, rate of infection in sheep is lower than in goats, this may be due to a high number of farmer rearing sheep with huge number than goats, the recorded species in sheep were as follow; eggs of *Trichuris ovis* , *Haemonchus contortus*, *Strongyloides papillosus*, *Bunostomum trigoncephalum*, *Trichostrongylus sp.*, these also were recovered by several worker and they were in agreement with theh present work such as; **Soulsby (1986); Rossanigo and Gruner (1996**) , **and Urquhart et al., (1996)**. **Indre et al., (2009)** who found that *Trichostrongylus* sp. larvae recorded highest infection rate of 37%. Concerning to Haemonchus contortus, higher infection rate of 78% was recorded by **Vercruysse (1985)** in Senegal. Lower infection rate of 9.7% was recorded by **Groski et al., (2004)** in Poland. The infection rate of *Trichuris ovis* was in agreement with that recorded by **Fritsche et al., (1993)** in Gambia which was 12%.Higher infection rate of 55% was recorded by **Leriche et al., (1973)** in Cyprus.Lower infection rates of 3.9%, 4.2% and 4.59% were recorded by **El-Manyawe (1999)** in Cairo governorate, Egypt, **Epe et al., (2004**) and **Mazyad and El-Nemr (2002)** in north Sinai, Egypt. All fecal samples was examined by floatation technique to reveal eggs and re examined by fecal culture and Baerman's technique, by comparing results obtained, we found that; the combination between two method is better for concise identification than floatation technique alone, in sheep, the highest infection rate recorded during summer season followed by spring and winter season, this can be explained by the nature of larvae of this nematodes that it need a high temperature, and humidity that are most suitable in summer and spring, regarding goats; the highest infection rate recorded during winter season followed by spring and summer season, this may be inadequate samples collection during summer, and spring, it was in agreement with **Le Riche, et al., (1973), who were done** A helminth survey of sheep and goats in Cyprus, and the seasonal distribution of these helminthes.

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Prevalence of Different Flatworms Infecting Ruminants In Menoufia Governorate.

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Abstract:

Flatworms in ruminants cause a great variety of clinical signs including diarrhea, unthriftness, weight loss, anemia, and may cause intestinal obstruction and deaths in heavy infections**.** Little studies was done on flatworm of ruminants in Menoufia governorate, so the present study was aimed to identify flatworms of ruminants, their prevalence, seasonal and monthly incidence in this governorate, it was carried out on 1200 animals (656 cattle, 271 buffaloes, 159 sheep and 114 goats) of different ages, breeds and sexes, the period of survey extended from April 2006 to March 2007 by examination of slaughtered animals. Results revealed that, trematodes recovered were *Fasciola hepatica*, *Fasciola gigantica*, *Paramphistomum cervi*, *Cotylophoron cotylophorum* and *Carmyerius gregarius*. The highest infection rate was among sheep (97.48 %), while the lowest was goats (26.31%). The recovered cestodes were *Moniezia expansa*, *Moniezia benedeni*, *Avitellina centripunctata, and Moniezia denticulata.* The highest infection rate was among sheep (62.89%), while the lowest was among buffaloes (3.69%). Regarding seasonal and monthly incidence of trematodes showed that, the highest infection rate recorded during winter and spring season (30.09%) and the peak of infection observed during December(66.66%), for cestodes infection, the highest infection rate of *Moniezia expansa* recorded during autumn, and winter season and the peak of infection observed during November and December.

Keywords: *Prevalence, Ruminants, Fecal culture, Flatworms and Menoufia province*

Introduction:-

Ruminants such as cattle, buffaloes, sheep, and goat are the most important animals raised and used for meat production in Moslem countries and considered the main source of animal protein in Egypt. The gradual increase in the human population with the increased consumption of animal proteins (from) in front of the limited abilities of animal production required more research interest in

the problem concerning the capacity of the animal to produce **Hassan (1973),** and **Hiekal (1980)**. Flatworms of ruminants cause a great variety of clinical problems and may cause intestinal obstruction and deaths in heavy infections. (**Urquhart et al., 1996**), the most destructive enemies of our animal wealth and the biggest hinderness for successful production are parasitic infection where, its percentage in the Egyptian field animals fluctuated according to many factors including irrigation, season, frequency of exposure of animal to infection, immune condition of the animal, the geographic location and climatic conditions **(Cawthorne, 1984).** The indirect losses by flatworm parasitism are not easily diagnosed, as the infection run usually a silent long standing course including loss in body weight, lowered fertility, reduced work capacity, involuntary culling, treatment costs, retardation of growth, impaired milk and meat production **(El-Molla et al., 1981),** increase their susceptibility to other diseases with increase of mortality rate **(Khan, 1993)** and diarrhea specially in calves **(Al-farwachi, 2000).** So, the aim of this study was to identify flatworms in ruminants and determine their prevalence, seasonal and monthly dynamics in Menoufia governorate.

Material and methods

1-Animals and localities:

This study carried out on: 656 cattle, 271 buffaloes, 159 sheep and 114 goats of different ages and breeds from slaughtered ruminants at abattoirs of Ashmoun, Shebin el-koom, Kafr dawoud and Minoof, from April 2006 to April 2007.

2- Parasitological examinations

A-Samples collection and preservation: (Khan, M.Q. 1993)

Worms from slaughtered ruminants was collected from different organs and during evacuation of gastrointestinal tract, then washed thoroughly several times in normal saline solution, placed in refrigerator overnight for complete relaxation. The length and breadth of the worms were measured before fixation. Each sample collected in a separate glass container that labeled with date of collection, site of collection, age of animal, species of animal and sex of animal.

B- Identification of collected flatworms: (Pritchard and Kruse, 1982)

1-Fixation and Staining:

The washed relaxed worms were compressed between glass sheets and flooded with the fixative (10% formalin) over night, after the removal of the fixative solution, then transferred to 70% ethyl alcohol, then stained with Mayer's acid carmine stain 48 hrs.

2-Dehydration and mounting:

The samples were dehydrated by serial passage for 1/2 hour in ascending grades of ethyl alcohol 50%, 70%, 80%, 90%, 95% and absolute. The specimens were mounted after this in Canada balsam under cover slips, left over night at 37ċ in the incubator to dry, and then stored for microscopic examination. They were measured using a standardized eye piece micrometer.

3- Identification:

The species of flatworms were identified according **to Soulsby (1986), Urquhart et al., (1996**) **Khalil; Jones and Bary (1994).**

Results

1-Trematodes:-

The recovered trematodes were *Fasciola hepatica, Fasciola gigantica, Paramphistomum cervi, Cotylophoron cotylophorum and Carmyerius gregarious.* The results in tables (1), (2), (3) showed that the prevalence, seasonal and monthly incidence of different trematodes of ruminants. *Cotylophoron cotylophorum and Carmyerius gregarious were recorded only in cattle*. Concerning to the trematodes infection among cattle, it was (71.64%), among which (18.29%) *Fasciola hepatica*, (19.81%) *Fasciola gigantica*, (16.76%) *Paramphistomum cervi*, (13.71%) *Cotylophoron cotylophorum* and (3.04%) *Carmyerius gregarius*. Seasonal and monthly incidence of trematodes in cattle showed that, the highest infection rate of *Fasciola hepatica* recorded during winter season (30.09%) and the peak of infection observed during December (66.66%), the highest infection rate of *Fasciola gigantica* recorded during spring season (25.87%) and the peak of infection observed during May (35.71%), the highest infection rate of *Paramphistomum cervi* recorded during spring season (24.47%) and the peak of infection observed during May (59.52%), the highest infection rate of *Cotylophoron cotylophorum* recorded during spring season (25.17%) and the peak of infection observed during May (85.71%), the highest infection rate of *Carmyerius gregarius* recorded during winter season (5.82%) and the peak of infection observed during December (18.51%). Regarding to the trematodes infection among buffaloes, it was (18.45 %), among which (3.69%) *Fasciola hepatica*, (7.38%) *Fasciola gigantica*, (7.38%) *Paramphistomum cervi.* The seasonal and monthly incidence showed that the highest infection rate of *Fasciola hepatica* recorded during autumn (7.57%) and the peak of infection observed during September (15.78%), the highest infection rate of *Fasciola gigantica* recorded during summer season (12.72 %) and the peak of infection observed during June (26.31%), the highest

infection rate of *Paramphistomum cervi* recorded during autumn season (16.66%) and the peak of infection observed during October (35.71%). While in sheep, (97.48 %) of examined sheep were infected with trematodes, among which (62.89%) *Fasciola hepatica*, (25.15%) *Fasciola gigantica* and (9.43%) *Paramphistomum cervi*. The results in tables (2, 3) showed the seasonal incidence of infection of different helminthes of sheep. Concerning to the trematode infection, the highest infection rate of *Fasciola hepatica* recorded during autumn season (77.41%) and the peak of infection observed during November (100%), the highest infection rate of *Fasciola gigantica* recorded during autumn season (22.58%) and the peak of infection observed during September (100%), the highest infection rate of *Paramphistomum cervi* recorded during spring season (18.18%) and the peak of infection observed during April (41.66%). Concerning the trematodes infection among goats table (1) showed that (26.31%) of examined goats were infected with trematodes, (8.77%) *Fasciola hepatica* and (17.54%) *Fasciola gigantica.* The results in tables (2, 3) showed the seasonal and monthly incidence of *Fasciola hepatica* and *Fasciola gigantica* in goats. the highest infection rate of *Fasciola hepatica* recorded during autumn season (13.04%) and the peak of infection observed during October (16.66%), the highest infection rate of *Fasciola gigantica* recorded during autumn season (30.43%) and the peak of infection observed during September (50%). Measurements of recovered trematodes were recorded in table (6).

2-Cestodes

The recovered cestodes were *Moniezia expansa, Moniezia benedeni* and *Moniezia denticulate and Avitellina centripunctata*. The results in tables (1), (4) and (5) showed the prevalence, seasonal and monthly incidence of different cestodes of ruminants. (32.01%) of examined cattle were infected with cestodes, among which (9.14%) *Moniezia expansa*, (7.62%) *Moniezia benedeni* and (15.24%) *Moniezia denticulata.* Regarding to seasonal and monthly incidence of cestodes among cattle, the highest infection rate of *Moniezia expansa* recorded during autumn season (11.30%) and the peak of infection observed during November (20.68%), the highest infection rate of *Moniezia benedeni* recorded during winter season (9.70%) and the peak of infection observed during December (18.53%), the highest infection rate of *Moniezia denticulata* recorded during autumn season (20.86%) and the peak of infection observed during October (32.25%). (3.69%) of examined buffaloes were infected with

cestodes. Cestodes infection among buffaloes was *Moniezia benedeni* only. The highest infection rate of *Moniezia benedeni* in buffaloes recorded during autumn (13.63%) and the peak of infection observed during October (28.57%). Results showed (62.89%) of examined sheep were infected with cestodes, (12.57%) *Moniezia expansa*, (12.57%) *Moniezia benedeni*, (18.86%) *Moniezia denticulata* and (18.86%) *Avitellina centripunctata*, the highest infection rate of *Moniezia expansa* recorded during autumn (22.58%) and the peak of infection observed during September (55.55%), the highest infection rate of *Moniezia benedeni* recorded during summer season (23.43%) and the peak of infection observed during June (47.61%), the highest infection rate of *Moniezia denticulata* recorded during autumn season (29.03%) and the peak of infection observed during September (55.55%), the highest infection rate of *Avitellina centripunctata* recorded during winter season (54.83%) and the peak of infection observed during January (75%). Results in table (1) showed that, goats were infected with cestodes with (41.22%), (8.77%) *Moniezia expansa*, (17.54%) *Moniezia benedeni*, (8.77%) *Moniezia denticulate* and (6.14 %) *Avitellina centripunctata*. the highest infection rate of *Moniezia expansa* recorded during summer season (11.62%) and the peak of infection observed during June (40%), the highest infection rate of *Moniezia benedeni* recorded during summer season (25.58%) and the peak of infection observed during July (58.82%), the highest infection rate of *Moniezia denticulata* recorded during winter season (15.78%) and the peak of infection observed during February (25%), the highest infection rate of *Avitellina centripunctata* recorded during summer season (9.30%) and the peak of infection observed during June (30%). Measurements of the recovered cestodes in millimeters were recorded in table (7).

Discussion:-

Little is known about the prevalence, seasonal, monthly incidence and identification of different flatworms affecting ruminants in Menofia governorate due to few studies was done in this field in the province that will be benificial to the local veterinary authorities in the province and so will help in the prevention and control of this parasitic worms in the region, from this point of view the aim of the present study was done and this work was constructed. The recovered flatworms in this study were *Fasciola hepatica, Fasciola gigantica, Paramphistomum cervi, and Cotylophoron cotylophorum, Carmyerius gregarious, Moniezia expansa, Moniezia benedeni, Moniezia denticulate, and*

Avitellina centripunctata. Concerning The prevalence of flatworm infection in cattle; many studies were done in different parts of the world on the prevelance of *Fasciola hepatica* and *Fasciola gigantica* as, **Munguia-Xochihua et al., (2007)** in Mexico, **Pfukenyi et al., (2006)** in Zimbabwe and **Mungube et al., (2006)** in Kenya and **Kithuka et al., (2003)**, **Epe et al., (2004)** in Kenya, the infection rate in our study was in agreement with that records. For *Paramphistomum cervi* and *Cotylophoron cotylophorum* prevalence in cattle, it was in agreement with study by **Aly (1993)**, **Mazyad and El-Nemr (2002)** in Egypt, **Garrles, (1975)** in Bangladesh, and **Celep (1984)** in Turkey. Regarding buffaloes, several studies on prevalence of *Fasciola hepatica, and Fasciola gigantica* were done as; **Akhter et al., (2001)** in Pakistan, **Haiba and Selim (1960)** in Egypt, **Al-Barwari (1977**) in Iraq and **Ismail et al., (1978)** in Jordan, *Prevelance of Paramphistomum cervi* was recorded by **Haridy et al., (2006)** in Gharbia governorate, Egypt and **Aly (1993)** in Dakahlia governorate, Egypt, and **Akhter et al., (2001)** in Pakistan, they obtained nearly the same results as we obtained. Regarding to sheep, and goats, **Cabrera et al., (2003)** in Uruguay**, Epe et al., (2004**), **Groski et al., (2004)** and **Munguia-Xochihua et al., (2007)**, **Wilson and Samson (1971)** in Mexico, had recorded the prevalence of *Fasciola hepatica*, but for *Fasciola gigantic*a, **Shehata (1954)** in Cairo, Egypt and **Al-Barwari (1977**) in Iraq, **Haiba and Selim (1960)** in Egypt and **Schillhorn et al., (1980)** in Nigeria, on the other hand, **Mazyad and el-Nemr (2002)** in North Sinai, Egypt, **Haridy et al., (2006)** in Gharbia, Egypt, has recorded *Paramphistomum cervi* prevalence. In our opinion, the high infection rate that obtained in this study is due to the presence of fresh water snails intermediate host for most of these trematodes as *Lymneae caiuidii, L. truncatula, bulinus truncatus* in rivers and water canals in most of the delta region of Egypt where Menoufiya is located, that used as a source of drinking water for these animals, and the discrepancy in the infection rate of recovered flatworms of ruminants between the previous authors and this study may due to the differences in the location of examination, methods of examination, breed of examined animals, number of the examined animals, age of the examined animals and the environmental conditions concerning the prevalence of tape worm (cestodes) infection in cattle; many studies on the prevelance were done in different parts of the world on *Moniezia benedeni*, *Moniezia denticulata*, and

Moniezia expansa, by **Aydenizoz and Yildiz (2003)** in Turkey.

Table (1) Prevalence of different recovered flatworms of ruminants

	Cattle Number examined 656		buffaloes Number examined 271		Sheep Number examined 159		Goats Number examined 114	
	Number infected	Percentage of infection	Number infected	Percentage of infection	Number infected	Percentage of infection	Number infected	Percentage of infection
Fasciola hepatica	120	18.29	10	3.69	100	62.89	١٠	٨,٧٧
Fasciola gigantica	130	19.81	20	٧,٣٨	40	25.15	٢٠	١٧,٥٤
Paramphistomum cervi	110	16.76	20	٧,٣٨	15	9.43	0	0
Cotylophoron cotylophorum	90	13.71	0	0	0	0	0	0
Carmyerius gregarious	20	3.04	0	0	0	0	0	0
Total trematode infection	**470**	**71.61**	**50**	**18.45**	**155**	**97.48**	**30**	**26.31**
Moniezia expansa	60	9.14	0	0	20	12.57	10	٨,٧٧
Moniezia benedeni	50	7.62	10	3.69	20	12.57	٢٠	١٧,٥٤
Moniezia denticulate	100	15.24	0	0	30	18.86	١٠	٨,٧٧
Avitellina centripunctata	0	0	0	0	30	18.86	٧	٦,١٤
Total cestode infection	**210**	**32.01**	**10**	**3.69**	**100**	**62.89**	**47**	**41.22**

Table (2) Seasonal incidence % of infection of different trematodes of ruminants

	Cattle					Buffaloes			Sheep			Goats	
Season	*F. hepatica*	*F. gigantica*	*P. cervi*	*Cotylophoron cotylophorum*	*Carmyerius gregarius*	*F. hepatica*	*F. gigantica*	*P. cervi*	*F. hepatica*	*F. gigantica*	*P. cervi*	*F. hepatica*	*F. gigantica*
winter	30.09	18.44	23.30	0	5.82	0	4.05	4.05	67.74	9.67	9.67	5.26	15.78
spring	12.58	25.87	24.47	25.17	2.09	2.63	3.94	2.63	54.54	21.21	18.18	10.34	24.13
Summer	13.22	15.59	9.83	9.49	3.05	5.45	12.72	5.45	57.81	4.68	4.68	6.97	6.97
autumn	27.82	24.34	19.13	22.60	0.86	7.57	10.60	16.66	77.41	22.58	9.67	13.04	30.43

Table (3) monthly incidence % of infection of different trematodes of ruminants

	Cattle					Buffaloes			Sheep			Goats	
	F. hepatica	*F. gigantica*	*P. cervi*	*Cotylophoron cotylophorum*	*Carmyerius gregarius*	*F. hepatica*	*F. gigantica*	*P. cervi*	*F. hepatica*	*F. gigantica*	*P. cervi*	*F. hepatica*	*F. gigantica*
January	10.25	12.82	38.46	0	2.56	0	3.84	7.69	100	12.50	12.50	0	14.28
February	24.32	18.91	13.51	0	2.70	0	3.44	0	46.66	6.66	6.66	25	25
March	5.26	12.28	8.77	0	0	0	3.22	3. 22	62.5	12.50	0	20	100
April	15.90	34.09	11.36	0	2.22	4.76	4.76	0	50	8.33	41.66	8.33	8.33
May	19.04	35.71	59.52	85.71	4.76	4.16	4.16	4.16	53.84	7.69	7.69	8.33	8.33
June	26.92	24.03	9.61	8.65	4.80	5.26	26.31	10.52	95.23	9.52	9.52	10	10
July	8.88	6.66	10	10	3.33	5.88	5.88	0	52.38	23.8	9.52	5.88	5.88
August	2.97	14.85	9.90	9.90	0.99	5.26	0	10.52	27.27	9.09	4.54	6.25	6.25
September	37.5	62.50	33.33	83.33	0	15.78	10.52	5.26	66.66	100	11.11	10	50
October	30.64	11.29	14.51	9.67	1.61	3. 57	17.85	35.71	66.66	91.66	8.33	16.66	16.66
November	13.79	20.68	17.24	0	0	5. 26	5. 2 6	0	100	50	10	14.28	14.28
December	66.66	25.92	14.81	0	18.51	0	5. 26	5. 26	75	12.50	12.50	0	12.50
total	18.29	19.81	16.76	13.71	3.04	3. 69	7. 38	7. 38	62.89	25.15	9.43	8.77	17.54

Table (4) Seasonal incidence % of infection of different cestodes of ruminants

	Cattle			Buffaloes	Sheep				Goats			
	Moniezia expansa	*Moniezia benedeni*	*Moniezia denticulata*	*Moniezia benedeni*	*M. expansa*	*M. benedeni*	*M. denticulata*	*Avitellina centripuncta*	*M. expansa*	*M. benedeni*	*M. denticulata*	*Avitellina centripuncta*
winter	10.67	9.70	13.59	5.40	9.67	6.45	19.35	54.83	5.26	15.78	15.78	0
spring	9.09	5.59	8.39	2.63	9.09	6.06	18.18	6.06	6.89	10.34	6.89	3.40
Summer	7.45	7.45	16.94	9.09	10.93	23.43	14.06	14.06	11.62	25.58	6.97	9.30
autumn	11.30	8.69	20.86	13.63	22.58	3.22	29.03	6.45	8.69	13.04	8.69	8.69

Table (5) Monthly incidence % of infection of different cestodes of ruminants

	Cattle			**Buffaloes**	**Sheep**				**Goats**			
	Moniezia expansa	***Moniezia benedeni***	***Moniezia denticulata***	***Moniezia benedeni***	***M. expansa***	***M. benedeni***	***M. denticulate***	***Avitellina centripunctata***	***M. expansa***	***M. benedeni***	***M. denticulata***	***Avitellina centripunctata***
January	7.69	7.69	5.12	3.84	12.50	12.50	25	75	14.28	14.28	14.28	0
February	10.81	5.40	5.40	6.89	6.66	0	13.33	60	0	25	25	0
March	10.52	5.26	17.54	3.22	12.50	0	25	0	20	20	0	0
April	9.09	4.54	0	0	8.33	8.33	16.66	16.66	0	8.33	8.33	8.33
May	7.17	7.14	4.76	4.16	7.69	7.69	15.38	0	8.33	8.33	8.33	0
June	7.69	4.80	19.23	10.52	23.8	47.61	9.52	28.57	40	0	10	30
July	8.88	2.22	11.11	5.88	9.52	23.80	9.52	4.76	0	58.82	5.88	5.88
August	5.94	14.85	19.80	0	4.54	0	22.72	9.09	6.25	6.25	6.25	0
September	16.66	12.50	8.33	5.26	55.55	0	55.55	0	0	10	10	10
October	6.45	8.06	32.25	28.57	8.33	8.33	16.66	0	16.66	16.66	16.66	0
November	20.68	6.89	6.89	0	10	0	20	20	14.28	14.28	0	14.28
December	14.81	18.53	37.03	5.26	12.50	12.50	25	25	0	12.50	12.50	0
Total	9.14	7.62	15.24	3. 69	12.57	12.57	18.86	18.86	8.77	17.54	8.77	6.14

Table (6) Measurements of the recovered trematodes in millimeters

	Fasciola hepatica	*Fasciola gigantica*	*Paramphistomum cervi*	*Cotylophoron cotylophorum*	*Carmyerius gregarius*
Length	13-35 (24)	25-55 (40)	7.25-11	7-11	9-12
Breadth	4.5-14.5 (9.5)	3-11 (7)	2.5-3.5	2.5-5	2.5-3.5
Oral sucker	0.35x 0.55-0.45x 0.70 (0.40x0.625)	0.47x 0.70-0.50x 0.85 (0.485x0.775)	0.52x 0.55- 0.70x0.80	0.45x 0.55- 0.75x 0.80	0.35x 0.40- 0.40x 0.55
Length of esophagus	0.50-0.65 (0.575)	0.40-0.70 (0.55)	0.17-0.30	0.20-0.35	0.25-0.40
Length of intestinal caeca	-	-	4.70-5.75	5.00-6.00	3.27-3.50
Ovary	-	-	0.30x0.33- 0.45x0.55	0.40x0.50- 0.50x0.65	0.40x0.45- 0.50x0.55
Testes	-	-	Oral 0.80x1.00 Caudal 0.90x1.30	Oral 0.90x1.20 Caudal 1.25x1.30	0.70x1.20- 0.90x1.75
Ventral sucker	0.90x 0.95-1.00x 1.05 (0.95x1.00)	0.85x 0.90-1.45x 1.50 (1.15x1.20)	-	-	-
Posterior sucker	absent	absent	1.50x 152- 1.55x 1.60	1.00x 1.25- 1.60x 1.65	0.70x 1.40- 0.75x 1.70
Genital sucker	absent	absent	Absent	0.10x0.15- 0.20x0.22	absent

Table (7) Measurements of the recovered cestodes in millimeters.

		Moniezia expansa	*Moniezia benedeni*	*Moniezia denticulata*	Avitellina centripunctata
Scolex	Breadth (mm)	0.75-0.90	0.47-0.60	0.45-1.00	1.5-2.5 (2)
	Length (mm)	0.47-0.55	0.35-0.40	0.40-0.95	1-1.20 (1.1)
	Sucker (mm)	0.24x0.25-0.34x0.35	0.15x0.16-0.19x0.20	0.18x0.19-0.45x0.48	0.51x0.52-0.65x1.20 (0.58x0.86)
Neck	Breadth (mm)	0.35-0.40	0.30	4-12	0.70-1.20 (0.95)
	Length (mm)	4-8	5-15	4-12	5-9 (7)
Mature segment	Breadth (mm)	5-10	6-13	5-19	1.00-2.5 (1.75)
	Length (mm)	1-2.5	1-1.5	1.5-3	0.10-0.20 (0.15)
	Ovary (mm)	0.40x0.80-0.90x1.00	0.50x0.80-1.00x1.50	0.70x 0.80-0.95x1.00	0.05x0.06-0.05x0.08 (0.05x0.07)
Gravid segment	Breadth (mm)	6-12.5	5.5-26	7.5-19	1-2 (1.5)
	Length (mm)	1-2.5	1-3	1.5-3	0.15-0.20 (0.175)
Paruterine organ	Diameter (mm)	-	-	-	0.13x0.19-0.30x0.35 (0.21x0.27)

In sheep and goats, recorded by **Heikal (1980)** in Behera, **Hassan (1973**), and **Mazyad and el-Nemr (2002)** in north Sinai, Egypt**, Umur and Gicik (1995)**, **Tinar et al., (1993), Aydenizoz and Yildiz (2003)** in Turkey, and **Cantoray et al., (1993)** in Kenya, **pal and Qayyum (1993)** in Pakistan. The infection rate of *Avitellina centripunctata* was recorded by **Hassan (1973)**, El-**Manyawe (1999) in Cairo, Egypt** and **Umur and Gicik (1995)** in turkey, **Leriche et al., (1973)** in Cyprus.

Regarding seasonal and monthly incidence of trematodes showed that, the highest infection rate recorded during winter and spring season, and the peak of infection observed during December, this is may be due to this period (spring, and winter) is the rainy season in the year and a high amount of water is available for snail reproduction and maintenance of larval stages of trematodes to producing the encysted metacercariae on vegetable and tree leaves on water to become infective for ruminants and continue the life cycle and more infection, on the other hand, if water canals and rivers is dried or even the air temperature is high as in autumn and summer season, the metacercariae cannot encysted and so infection rate is low. for cestodes infection, the highest infection rate of *Moniezia expansa* recorded during autumn, and winter season and the peak of infection observed during November, and December, and this may be due to the humidity and moderate temperature that may be suitable for oribated mite (intermediate host) in which the cysticercoids is encysted.

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Studies on clinical and subclinical mastitis in Menoufia Governate with application of PCR for diagnosis

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Abstract:

Bovine mastitis is a serious problem in the dairy herds all over the world. In this study 105 mastitic milk samples were collected from small holder's cows for bacteriological and molecular diagnosis. The prevalence of clinical and subclinical mastitis were 20.5% and 32% respectively which were detected by clinical examination and California Mastitis Test respectively. Bacteriological results revealed that *Staphylococcus aureus* was the most common isolated bacteria from both clinical and subclinical mastitis. *Streptococcus* species, *Pseudomonas* species, *E. coli* and *Enterobacter* species were also isolated. Application of multiplex PCR was effective in identification of bacteria causing mastitis directly from milk samples and from extracted DNA of bacteria

Keywords: *Mastitis, Prevalence, Staphylococcus aureus, Multiplex PCR.*

Introduction

Mastitis is a global problem in dairy herds as it adversely affects animal health, and milk production **(Sharma et al., 2012).** High losses attributed to mastitis includes reduction of milk yield, low grade of milk quality, premature culling and treatment costs **(Fetrow, 2000).** Wide variety of bacteria can be involved in udder infection, but the most common bacteria causing mastitis in Egypt are *Staphylococcus Species, Streptococcus Species and Escherichia coli* **(Ahmed and Mohammed, 2008).** Application of PCR for mastitis diagnosis will be helpful for rapid application of the preventive measures of the disease **(Qing-Hil et al., 2008)** PCR tend to

be specific, sensitive and rapid for detection of *bacteria* in raw milk which helps in and control of the infection **(Yu-Ping et al., 2007)**.

Material and methods

1- Milk samples:

A total of 105 mastitic milk samples were collected from small holder's cows in Menoufia province according to **Edmondson and Bramely (2004)**. Clinical signs of acute mastitis were detected in 41 cases by clinical examination and 64 samples from subclinical cases which were detected by California Mastitis Test (CMT).

2- Bacterial identification: was carriedout according to **Cruickshank et al., (1975).** through culturing onto blood agar, Baired parker agar medium, Edward's medium and MacConkey agar medium , Gram's staining and biochemical tests

3- DNA Extraction from isolated bacteria and from milk samples were preformed according to **Riffon et al., (2001).** DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56˚C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

4- Multiplex PCR: the reaction mixture consists of 10 µl DNA template, 25 µl of 2X Taq master mixes, 1 µl of each primers, and 13 µl of DNA free water. The PCR was carried out with preliminary step at 95˚C for 2 minutes, followed by 35 cycles consisting of: 1 minute of denaturation at 95˚C, 1 minute of denaturation at 45˚C, 30 seconds of denaturation at 72˚C, and the final extension at 72˚C for 10 minutes. The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 10 µl of the products was loaded in each gel slot. A 100 bp and 100DNA Ladders (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra), and the data was analyzed through computer software.

Results:

The prevalence rate of clinical mastitis in dairy cows was 20.5% depending on the clinical signs of mastitis which appeared on the animals. While the prevalence rate of subclinical mastitis depending on CMT was 32%. Bacteriological examination of milk samples collected from both clinical and subclinical cases resulted in isolation of *Staphylococcus aureus*, *Streptococcus* species, *Pseudomonas* species, *E. coli* and *Enterobacter*. Table (2) illustrated the percentage of each bacterial species in milk samples of both clinical and subclinical cases.

Table (1): Primers: sequences and target genes for *staphylococcus aureus, streptococcus species* and *E coli* according to **Pradhan et al., (2011).**

Primer name Specify	Sequences	Target gene
SU-F *Staphylococcus aureus*	TTC GTA CCA GCC AGA GGT GGA	16s-23s ISR rRNA 229bp
SU-R *Staphylococcus aureus*	TCT TCA GCG CAT CAC CAA TGC C	
ST-F *Streptococcus Spp*	GAT ACA TAG CCG ACC TGA GA	16s rRNA 561bp
ST-R *Streptococcus Spp*	AGG GCC TAA CAC CTA GCA CT	
ECO-F *E.Coli*	TCT GCG GGA GTC TCA GGG ATG GCT G	(tra T)gene 313bp
ECO-R *E.coli*	GTA TTT ATG CTG GTT ACC TGT TT	

Table (2): Results of bacteriological examination of milk samples from both clinical and subclinical mastitis.

M.O	Clinical mastitis		Sub clinical mastitis	
	Number of isolates	%	Number of isolates	%
Staphylococcus aureus	23	29.11	17	21.51
Streptococcus Species	13	16.5	16	20.2
Pseudomonas Species	0	0	6	7.6
E.coli	1	1.27	1	1.27
Enterobacter species	1	1.27	1	1.27

Application of multiplex PCR resulted in amplification of target genes at expected sites for *Staphylococcus aureus*, *Streptococcus* species and *E.coli* (229 bp, 561 bp and 313 bp respectively) in both DNA extracted from bacterial isolates and raw milk samples fig (1,2).

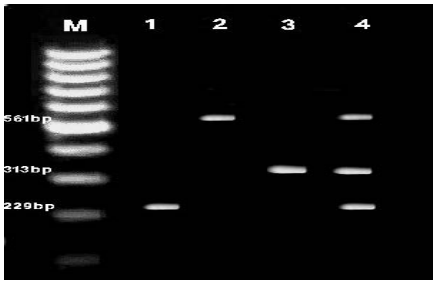

Fig (1): Results of PCR from bacterial culture by different primers combination. Lane M: 100-bp DNA ladder, Lane 1: *S.aureus*, Lane 2: *Streptococcus Species*, Lane 3: *E.coli*, Lane 4: Mixture of the three isolates *S.aureus*, *Streptococcus Species* and *E.coli*.

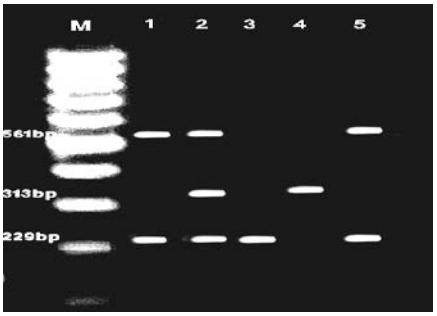

Fig: (2): Results of P.C.R. on milk samples with different primer combination. Lane M: 100-bp DNA ladder, Lane 1: *S.aureus* at 229 bp and *Streptococcus Species* at 561 bp Lane 2: *S.aureus* at 229 bp *Streptococcus Species* at 561 bp and *E coli* at 313 bp, Lane 3: *S.aureus* at 229 bp, Lane 4: *E .coli* at 313 bp, Lane 5: *S.aureus* at 229 bp and L *Streptococcus Species* at 561 bp.

Discussion:

Mastitis is a highly prevalent problem in dairy cattle and one of the most important threats affecting the world's dairy industry **Wallenberg and Vanior (2002**). The present study reported a prevalence rate of clinical mastitis of 20.5%. Nearly similar results of clinical mastitis (20.43%) were obtained by **Ahmed and Mohammed (2009**) in Friesian cattle. While low prevalence rates were detected in Netherlands (12.7) **(Miltenburg et al., 1996**) and Ethiopia (10.3%) (**Delelesse 2010).** Prevalence of subclinical mastitis was (32%) which is nearly similar (30% and 31.75%) to that obtained by **Seddek et al., (2000)** and **Hussein et al., (2009) respectively.** A lower prevalence rate (9.96%) was reported by **Ahmed et al., (2008**). On the other hand a higher prevalence rate of 75.9% was reported by **Karimuribo et al., (2008)**. There was a large variation in the prevalence of clinical and subclinical mastitis rates which may be attributed to some mangemental factors such as using of dry cow therapy, feeding patterns, heifer replacement rates, environmental condition surroundings the animals, prevalent microorganism **McDougall (1999**). Bacteriological examination revealed that Staphylococcus *aureus* is the most prevalent isolate in clinical and sub clinical (29.11%), and (21.51%) respectively. This was in agreement with **El-Seedy et al., (2010) and Zeryehun et al.,) 2013**). *Streptococcus species* were identified (16.5% and 20.2%) in clinical and subclinical mastitis respectively. These results agree with **Zeryehun et al., (2013)** as they recorded a percentage of 39.9% of *Streptococcus species* isolated from clinical and subclinical mastitis. A lower percentage of *Streptococcus species* (14.2%, and 5.5%) were recorded by **Matios et al., (2009); Harini and Sumathi (2011)** respectively. Six isolates of *Pseudomonas Species* (7.6%) were identified from subclinical mastitis case.These results agree with **Kivaraia and Noordhuizen (2007) and Zeryehun et al., (2013**) as they recorded 7.5% *Pseudomonas aeruginosa* of the total bacterial isolates. *E. coli* and *Enterobacter* aerogens were identified from both clinical and subclinical mastitis (1.27%) for each organism. A higher percentage of (5.9% and 18.7%) were recorded by **Anakalo and Gathoni (2004) and Ahmed and Mohammed (2009)**. These findings are supported by **Bradley and Green (1997**) who stated that *Coli form* particularly *E. coli, Enterobacter aerogense* were the chief organisms that caused environmental mastitis. Application of multiplex PCR on bacterial isolates showed *Staphylococcus aureus* at 229 bp, *Streptococcus Species* at 561bp and *E. coli*

at 313 bp. These results are in agreement with (**Alaa et al., 2008 and Phuektes et al., 2001)**. Also application of multiplex PCR on milk samples directly without the need for culture step showed *Staphylococcus aureus*, *Streptococcus species* and *E coli* were detected at 229 bp, 561 bp and 313 bp) respectively. The obtained results are in agreement with **Yamagishi et al., (2007) and Amin et al., (2011**).

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A review on Clostridium perfringens toxins with special reference to Beta 2 toxin

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Abstract:

Clostridium perfringens toxins are the main cause of lesions and symptoms associated with diseases caused by its infection. There are two main groups of toxins; major and minor. The major toxins are alpha (CPA), beta (CPB), epsilon (ETX), and iota (ITX) toxins. Theses toxins are lethal and necrotizing agents. minor toxins such as eta, theta (θ), kappa, lambda, mu, nu toxins, neuraminidase, sialidase, enterotoxin, non-alpha-delta-theta hemolysin, and metabolic active substance. Recently there are newly discovered toxins Such as enterotoxin and a cytotoxic beta -2 toxin. Theses toxins may play an important role in pathogenesis of the diseases and need more investigation. Here we summarized important data about *Clostridium perfringens* toxins.

Keywords: *Clostridium perfringens, Toxins, Beta-2 toxins and Pathogensis.*

Introduction:

Clostridium perfringens is a spore forming; anaerobic bacteria widely distributed in the soil and the digestive tract of many domestic animals and they produce a number of toxins that result in enteric and histotoxic disease. *Clostridium perfringens* isolates are classified into five types designated A, B, C, D and E according to their ability to produce the four major lethal toxins: the alpha (CPA), beta (CPB), epsilon (ETX), and iota (ITX) toxins. The activities of the major lethal toxins are the basis of the pathogenesis of the classical enterotoxaemia attributed to this organism. Recently, it has been recognized that *Clostridium perfringens* produces other toxins of probable importance in animal disease, such as enterotoxin and a cytotoxic beta -2 toxin, the latter encoded by the cbp2 gene (**Radostitis et al., 2007).** On the other hand *Clostridium perfringens* produces minor toxins such as eta, theta (θ), kappa, lambda, mu, nu toxins, neuraminidase, sialidase,

enterotoxin, non-alpha-delta-theta hemolysin, and metabolic active substance **(Louis 1979).**

Clostridium perfringens alpha toxin (CPA):

Alpha-toxin, produced in large amounts by type A strains, is a phospholipase C; and is believed to be the major factor responsible for the tissue pathology in myonecrosis (gas gangrene) caused by this organism **(Stevens et al., 1988)**. All *Clostridium perfringens* strains possess the gene encoding CPA. This gene (cpa) is chromosomally encoded and located close to the origin of replication, which is one of the most stable regions within the bacterial chromosome **(Canard and Cole 1989).**

CPA is a 43 kDa protein comprised of 370 amino acids. It contains two domains, an alpha-helical N-terminal domain harboring the phospholipase C active site, and an alpha-sandwich C-terminal domain which is involved in membrane binding **(Uzal et al., 2010).**

Alpha-toxin has been shown to affect myocardial function, causing hypotension and bradycardia, resulting in shock, a common and often fatal feature of gas gangrene **(Stevens et al., 1988).** The toxin also increases vascular permeability, as can be demonstrated by i.d. injection **(Sugahara et al., 1977).** CPA is a classic example of a toxin that modifies cell membranes by enzymatic activity. This toxin is a zinc-dependent phospholipase C which degrades phosphatidylcholine and sphingomyelin, both components of the eukaryiotic cell membranes causing damage on the membrane of erythrocytes and other cells from many animal species. The net result of this action is cell lysis, by degradation of membrane phospholipids. CPA also activates several other membrane and internal cell mechanisms that lead to hemolysis. In addition, CPA activates the arachidonic cascade resulting in the formation of thromboxanes, leukotrienes and prostaglandins, which activate the inflammation cascade and produce vasoconstriction **(Tiball et al., 1999, Sakurai et al., 1993, Oda 2008 and Sakurai et al., 2004).** CPA associated with yellow lamb disease, gas gangrene in humans and malignant edema of domestic animals including sheep, goat, cattle and horse **(Uzal et al., 2010)**. Its role still frequently blamed for enteritis, abomasitis and/or enterotoxemia in cattle **(Manteca et al., 2002** and **Songer and Miskimins 2005**), horses **(Bacciarini 2003** and **water et al., 2005)**, goats **(Songer 1998)** and pigs **(Saenz et al., 2007)**.

Table (1): Major and minor toxins produced by *Clostridium perfringens* (**modified after Charles 1990):**

Toxins	**Biological activity**	**Toxin type ***				
		A	**B**	**C**	**D**	**E**
Major toxins: Alpha	Lethal, lecithinase (phospholipase C)	+	+	+	+	+
Beta	Lethal, necrotizing, trypsin labile	-	+	+	-	-
Epsilon	Lethal, permease, trypsin activatable	-	+	-	+	-
Iota	Lethal, dermonecrotic, binary, ADP-ribosylating trypsin activated.	-	-	-	-	+
Minor toxins: Gamma	Not defined, existence questionable	-	±	±	-	-
Delta	Hemolysin	-	±	+	-	-
Eta	Not defined, existence questionable	±	-	-	-	-
Theta	Hemolysin (O_2 labile), cytolysin	±	+	+	+	+
Kappa	Collagenase, gelatinase	+	+	+	+	+
Lambda	Protease	-	+	-	+	+
Mu	Hyaluronidase	±	+	±	±	±
Nu	DNAS	±	+	+	±	±
neuraminidase	N-Acetylneuraminic acid glycohydrolas	+	+	+	+	+
Other: Enterotoxin	Enterotoxic, cytotoxic	+	NT	+	-	NT

*****+. Produced by most strains; +, produced by some of the strains; -. no strain of the indicated toxin type has been shown to produce the toxin. NT, Insufficient studies have been done with strains of the indicated toxin type.

Table (2): Main properties of major toxins of *Clostridium perfringens* (modified after Sakurai, 1995*):*

	CPA	**CPB**	**ETX**	**ITX**	**PFO**	**CPE**	**CPB2**
Molecular weight	42.528kDa	35 kDa	34 kDa	Ia: 47.500 Ib: 71.500	54kDa	35 kDa	28 kDa
LD 50*(mice)	3µg	1.87µg/ip; 30ng-50 µg/iv	100 µg	Ia: 620 µg Ib: 940µg	Not reported	50µg	0.3µg
Biological activity	Lethal, necrotizing, haemolytic &contraction of smooth muscle	Dermonecrotic, edema & enterotoxic cytotoxic	Dermonecrotic, lethal, edema & contraction of smooth muscle	Lethal & necrotizing	Lethal & necrotizing	Erythema, lethal & enterotoxic	Dermonecrotic, edema, enterotoxic
Thermostability	Stable	Inactivated at 60ºC Stable at -80 ºC for 2 months	Up to week at room temperature, several months at -20 ºC and years at -80 ºC	Ia: stable Ib: labile	unknown	Inactivated at 60 ºC. <1 week at room temperature. 1 month at -20 ºC Years lypholized	Unknown
Reaction to trypsin	Susceptible	Sensitive inactivated	Resistant Activated	Resistant activated	Susceptible	Resistant activated	Sensitive Inactivated
*per kg of mouse	Ip: intraperitineal injection			Iv: intravenous injection			

Table (3): Toxin-types and diseases caused by Clostridium perfringens (**modified after Jasmina 2012)**

C. perfringens types	Animal species	Disease	Toxins: confirmed and suspected
A	Poultry	Necrotic enteritis	CPA, Net B
	Piglets	Enterocolitis	CPA, CPB2
	Horses	Neonatal hemorrhagic diarrhea	CPA, CPB2
	Calves	Abomasal ukceration and tempany	CPA, CPB2
	Humans	Gangrene	CPA, Theta
		Food poisoning	CPA, CPE- positive for food poisoning
		Antibiotic associated diarrhea	CPE, CPB2- associated
B	Lambs	Dysentery	CPA, CPB, ETX
	Sheep	Chronic enteritis	CPA, CPB, ETX
C	Poultry	Necrotic enteritis	CPA, CPB
	Neonatal pigs, Lambs, Calves, Goats, Foals	Hemorrhagic or necrotic enterotoxaemia	CPA, CPB
	Adult sheep	Acute enterotoxaemia	CPA, CPB
	Human	Enteritis necroticans	CPA, CPB
D	Calves, Goats, Adult cattle, Young lambs	Enterotoxaemia	CPA, ETX
E	Calves	Enterotoxaemia	CPA, ITX

Clostridium perfringens beta toxin (CPB):

Beta-toxin is a major lethal toxin produced by both type B and C strains of Clostridium perfringens. The cpb gene encodes a prototoxin of 336 amino acids that includes a 27-amino acid signal sequence removed during secretion, resulting in a mature beta toxin of ~35 kDa **(Hunter et al., 1993** and **Sakurai & Duncan 1978)**. CPB is encoded by the cpb gene, which is carried on virulence plasmids **(Sakurai & Duncan 1978** and **Katayama et al., 1996)** of different molecular size.

In spite of the importance of beta toxin in veterinary medicine, the biological activity of this protein is poorly defined **(Steinthorsdottir et al., 1995; Gkiourtzidis et al., 2001).** According to **Hauschild (1971)** the combined effects of beta- and epsilon-toxins are responsible for the diseases caused by C. perfringens type B, such as lamb dysentery and enterotoxemia of foals, goats, sheep and calves. CPB is very trypsin sensitive, so low trypsin levels or the presence of trypsin inhibitors likely contribute to disease by favoring CPB persistency in the gastrointestinal tract. CPB-induced cytotoxicity for HL-60 cells, HUVEC (human umbilical vein cell) and intestinal I407 cells endothelial through forming pores in the cell membranes. Those pores induce K+ efflux and Ca2+, Na+ and Cl- influxes, which then

produce cell swelling and lysis. **(Steinthorsdottir et al., 2000 and Nagahama et al, 2003a).** CPB is nonhemolytic for rabbit or sheep erythrocytes **(Steinthorsdottir et al., 1995)**. CPB is very trypsin sensitive, so low trypsin levels or the presence of trypsin inhibitors likely contribute to disease by favoring CPB persistency in the gastrointestinal tract.

Beta toxin is responsible for the diseases caused by *Clostridium perfringens* type B; that causes an often fatal hemorrhagic dysentery in sheep, and possibly in other species and by *Clostridium perfringens* type C which including Struck of sheep and enterotoxaemia of lambs, calves and piglets and necrotic enteritis of man and fowls. **(Maria et al., 2004).**

Clostridium Perfringens Epsilon toxin (ETX):

Both type B and D strains of *Clostridium perfringens* produce epsilon-toxin. The ETX gene (etx) is localized on conjugative plasmids **(Sayeed et al., 2008** and **Miyamoto et al., 2008)**, with most type B isolates possessing the same kb etx plasmid that also carries the gene encoding beta2 toxin (**Miyamoto et al., 2008**). It is produced as a prototoxin that is activated by proteolytic enzymes produced by the same organism (the kappa- and lambda-toxins) **(Willis 1969).** It can be activated by adding trypsin to the culture. The prototoxin is a protein consisting of 311 amino acids, with a molecular mass of 34.25 kDa (**Habeeb 1975)**. Activation of the prototoxin with trypsin involves breaking of the peptide bond between the 14th and 15th amino acids from the amino terminus (Lys-14-Ala-15) and releasing an 'activation peptide' of 14 residues **(Bhwon and Habeeb 1977).**

Epsilon-toxin is the third most potent clostridial toxin, after botulinum and tetanus toxins. A characteristic feature of epsilon toxin is its potent neurotoxicity, which is not observed for other structurally well-defined pore-forming toxins **(Miyata et al., 2001)**. This toxin exhibits toxicity toward neuronal cells via the glutamatergic system **(Miyamoto et al., 1998; Miyamoto et al., 2000)** or extravasation in the brain **(Finnie et al., 1999).** The pore-forming activity of the toxin based on the following observations: (i) ε-toxin can form a large complex in the membrane of Madin-Darby canine kidney cells, and permeabilizes them **(Petit et al. 1997; Nagahama et al., 1998)**; (ii) the large complex formed by ε-toxin is not dissociated by SDS-treatment, which is a common feature of pore-forming toxins **(Petit et al. 1997)**; and (iii) the CD spectrum of ε-toxin shows that it mainly consists of β-sheets **(Habeeb et al.,**

1973), as observed for pore-forming β-barrel toxins.

This toxin causes blood pressure elevation, increased contractility of smooth muscle, vascular permeability increase, as well as brain and lung edema in multiple animal species, while in goats ETX also causes colitis **(Uzal et al., 2004)**. ETX-producing C. perfringens type D strains are the most common cause of clostridial enterotoxemia in sheep and goats **(Uzal and Kelly 1997)**; In cattle there are few reports about natural cases of type D enterotoxemia, and information about clinical and pathologic findings of the disease in this species is scant and frequently contradictory (**Keast and McBarron 1954 , Griner et al., 1956 and Griesemer and Krill 1962**).

Clostridium Perfringens Iota toxin (ITX):

The iota-toxin is produced only by *Clostridium Perfringens* type E and has been implicated in fatal calf, lamb and guinea pig enterotoxemias **(Bosworth, 1943; Madden, 1970).** ITX is a clostridial binary toxin has a common structure consisting of two independent protein components that are not covalently linked, one being the binding component (Ib,100 kDa), and the other the enzymatic component (Ia, 45 kDa); both components are required for biological activity **(Barth et al., 2000).** These components are proteolytically activated by trypsin or chymotrypsin, removing a 20 kDa Nterminal peptide from the binding component (80 kDa for the active form) and 9 to 11 N-terminal residues from the enzymatic component **(Gibert et al., 2000).** These components encoded by two genes in a plasmid, organized in an operon. Two genes, with the same orientation, coding for Ia (454 amino acids) and Ib (875 amino acids) and separated by 243 noncoding nucleotides, were identified. **(Sakuria et al., 2009).**

Iota-toxin enters host cells and induces toxicity by exploiting the cell's endogenous pathways as follows: (1) Ib binds to a receptor at plasma membranes, then moves to lipid-rafts and

forms a heptamer, (2) the N-terminal domain of Ia binds to the Ca2+-binding motif in the N-terminal region of Ib, (3) Ia bound to the Ib oligomer is internalized in cells by receptor-mediated endocytosis, (4) the complex of Ia and Ib is transported to the early endosomes, where acidification promotescytosolic entry of Ia, and 5) the C-domain and part of the N-domain of Ia bind to G-actin in the cytosol and ADP-ribosylate it, thereby blocking the polymerization of actin, and eventually intoxicating cells. **(Sakuria et al., 2009).**

Infections by Clostridium perfringens type E are usually assumed to be mediated by ITX,

although no definitive evidence in this regard has been provided. Clostridium perfringens type E produces enterotoxemia in rabbits. Although the role of ITX in these infections has not been elucidated; toxinotype E is an occasional cause of hemorrhagic enteritis and sudden death in beef calves. Type E disease has rarely been described in sheep and goats. **(Uzal et al., 2010).**

Clostridium Perfringens Beta 2 Toxin (CPB2):

Toxin discovery:

The history of beta 2 toxin started with Popoff et al., during the 1980s as they obtained a protein of approximately 28kDa, which is significantly smaller than CPB (~35kDa). The newly discovered protein (27.6 kDa) was at first considered a product of CPB (34.8 kDa) proteolysis; but the CPB2 amino acid sequence showed minimal homology with CPB (15% identity). The protein and the encoding sequence were named CPB2 and cpb2, respectively, due to comparable biological effects to CPB **(Gibert et al., 1997).**

Structure and genetic of the toxin:

The CPB2 protein consists of a 265 amino acid polypeptide, which after secretion loses 30 amino acids giving it a molecular mass of 27.6 kDa **(Lebrun et al., 2007a)**. It is unstable, being susceptible to proteolytic cleavage **(Gibert at al., 1997)**.

The cpb2 toxin gene is transcribed during vegetative growth, especially during the exponential phase and regulation by the VirS/VirR two-component regulatory system (**Ohtani et al., 2003)**. The cpb2 gene is present on *Clostridium perfringens type D* isolates on plasmids 48 kb to 110 kb in size **(Sayeed and McClane 2007).**. In these isolates, the gene is either present on the same plasmid carrying the etx gene or on a different plasmid. In type E isolates, cpb2 gene is present on plasmids sizes varying from 70 kb to 90 kb. The gene is always present on different plasmid carrying the iota toxin gene (itx) **(Li et al., 2007)**. In recent studies the cpb2 gene was found to be present on a 65 kb plasmid that also carries the etx gene, in *Clostridium perfringens type B* isolates **(Uzal et al., 2010).**

Mechanism of action of Beta 2 toxin:

There is no significant homology between the novel CPB2 and beta toxin or other known bacterial toxins **(Gibert et al., 1997, Shimizu et al., 2002b and Shimizu et al., 2002a). Schotte et al., 2004 and Gibert et al. 1997** suggested that the toxin behaves similarly to CPB and demonstrated the cytotoxic effects of CPB2 on Chinese hamster ovary and human intestinal embryonic 1407 cell lines. This

cytopathology included cell rounding, membrane blebbing and detachment from the culture matrix. The toxin did modify small G proteins such as actin, either by ADP-ribosylation or by UDP- glucosylation. Other cell lines, such as CaCo2 cells have also been shown to be sensitive to the cytotoxic effects of CPB2 **(Fisher et al., 2005).** Based on these studies, it was suggested that CPB2 toxin could act as a potential pore-forming toxin similar to other enterically-active clostridial toxins. The toxin was shown to be highly susceptible to proteolytic cleavage by trypsin **(Gibert et al. 1997).**

Prevalence and pathological effect induced by Beta 2 toxin among animals:

Clostridium perfringens beta 2 toxin has been responsible for enteric diseases in wide range of animals and man. The clinical symptoms related to the action of the toxin are pasty to watery bloody diarrhea, abdominal pain and loss of condition **(Uzal et al., 2010).** The beta2 toxin gene of *Clostridium perfringens* was detected in all isolates originated 1213 field samples taken from diseased or perished live stock. Animal species examined, comprising pigs, the small ruminants sheep and goats, cattle, horses, rabbits, alpacas and lamas, and fallow deer. Among all the animal species included in this study, pigs attracted attention by a high quota of 74.2% (610 of 822) cpb2-positive C. perfringens isolates in comparison to the other animal species tested, revealing a quota of 20.8% (72 of 346) **(Sting 2009)**

There was evidence for importance of beta 2 toxin in bovine enterotoxaemia; since necrotic and haemorrhagic lesions developed after infection with beta 2 positive *Clostridium perfringens* strain **(Manteca et al., 2002).**

Gkiourtizidis et al., 2001 found that the recently discovered, beta 2-toxigenic type of *Clostridium perfringens* was represented in 6% of all isolates of *Clostridium perfingens* associated with lamb dysentery. But **Das et al., 2009** found that Beta 2 toxigenic *Clostridium perfringens* incriminated in the chronic enteritis of goats. In which in PCR, both cpa and cpb2 toxin genes were detected from all the isolates.

In equine **Cornella et al., 1999 attributed** the pathogenicities of *Clostridium perfringens* isolates from horses to the beta 2 toxin. They found beta 2 toxigenic *Clostridium perfringens* mainly in horses with typical and atypical typhlocolitis, representing 52% of the isolates. In **2003 Bacciarini et al.,** confirmed the role of beta 2 *Clostridium perfringens* in development of typhlocolitis and hemorrhagic fibrinonecrotic duodenitis-proximal jejunitis in horses by detection of beta 2 toxin in horse intestinal tissues by immuhistochemical staining; in which 69 sections from horses

were stained and beta 2 toxin was observed in 40 animals. **Murray et al., 2011** recovered beta 2 toxigenic *Clostridium perfringens* type A in large numbers from the intestine of a neonatal foal with colitis and beta -2 toxin was demonstrated in the lesions by immunohistochemical staining.

Garmory et al., 2000 showed a significant association between *Clostridium perfringens* possessing the gene encoding b2-toxin and diarrhoea in piglets. Results obtained by **Michael et al., 2003** support a significant association between CPB2-positive *Clostridium perfringens* isolates and diarrhea in piglets. **Hendriksen et al., 2006** found that alpha- and beta2-toxin-producing *Clostridium perfringens* was isolated from all tested herds with piglets with neonatal diarrhoea.

Clostridium perfringens enterotoxin (CPE):

Granum *1986* and **Hauschild and Hilsheimer 1971** found that *Clostridium perfringens* enterotoxin composed of a protein with a molecular mass of 35 kDa and determine its amino acid sequence. The toxin consists of one peptide of 309 amino acids with a molecular weight of 34,262. The peptide has one free sulfhydryl group. The activity of the enterotoxin is enhanced threefold by treatment with trypsin (Granum et al., 1981).The trypsinized toxin then consists of 284 amino acids and two short peptides of 10 and 15 amino acids. The gene (cpe) encoding CPE can reside on either the chromosome or on plasmids. In type A isolates, there are two major cpe plasmid families: i) a family of ~75 kb plasmids that also carry the beta2 toxin gene (cpb2); ii) a family of ~70 kb plasmids lacking the cpb2 gene. Some type C and D isolates also carry a plasmid-borne cpe gene although those plasmids do not appear to be closely related to the cpe plasmids of type A isolates. Interestingly, most type E isolates carry silent cpe sequences on their iota toxin-encoding plasmids, which are often related to cpe plasmids of type A isolates **(Uzal et al., 2010).** The mechanism of action of the enterotoxin seems to involve direct binding of the toxin to receptors on the surface of intestinal epithelial cells. Binding is followed by insertion of the entire molecule into the cell membrane, but no internalization into the cell. A sudden change of ion fluxes occurs, affecting cellular metabolism and macromolecular synthesis. As intracellular calcium ion levels increase, morphological damage occurs, resulting in greatly altered membrane permeability and loss of cellular fluid and ions and moderatesized molecules up to 3.5 kDa. Under some conditions a loss of protein molecules may occur. **(McClane et al., 1988a and McClane et al., 1988b).**

Enterotoxin has been studied almost exclusively in type A strains. It has been established that type C and D strains also can produce enterotoxin. Only a few strains of types B and E have been tested for enterotoxin production, and all were negative (**Charles 1990).**

All tested mammalian species are sensitive to CPE. This toxin affects all small intestinal regions but is most active in the ileum. CPE has only weak effects on the colon of rabbits or human colonic tissue. In the ileum, CPE causes villus shortening and epithelial desquamation and such damage appears necessary for accumulation of fluids and electrolytes in the lumen **(Uzal et al., 2010)**.

CPE toxin is important for *Clostridium perfringens* type A food poisoning or nonfoodborne gastrointestinal disease isolates to cause diarrheal symptoms in experimental animals. Coupled with extensive epidemiologic evidence, these findings support CPE as an important virulence factor for C. perfringens type A food poisoning or CPE-associated nonfoodborne human gastrointestinal disease disease. In contrast to the well-established role of this toxin in human GI disease, the data implicating CPE in animal disease remains more ambiguous. there are case reports suggesting CPE may cause GI disease in both domestic animals (including dogs, pigs, /horses, and goats) and possibly wild animals (such as penguins, leopards and tortoises) **(Uzal et al., 2010)**. Type A isolates and CPE in the small intestines of goat kid suffering from necrotic enteritis **(Deguchi et al., 2009)**. Additionally, fecal CPE and CPE-positive fecal isolates have been associated with canine diarrhea and it has been suggested that CPE-positive strains can cause recurrent diarrhea in dogs **(Marks et al., 2002** and **Weese et al., 2001b**). Finally, in horses, fecal CPE was detected in ~20% of adults with diarrhea and ~30% of foals with diarrhea (Weese et al., 2001); thus supporting a role for this fecal toxin contributing to disease in those animals, while no fecal CPE was detected in healthy adult horses or foals.

Minor toxins produced by Clostridium perfringens:

The minor toxins produced by *Clostridium perfringens* are listed in Table 1. At least some strains of all toxin types produce theta, kappa, mu, nu, and neuraminidase. Delta-toxin is a hemolysin produced by type B and C strains, but not by type A, D, and E strains. Lambda has not been detected in type A and C strains. It is a proteinase that digests gelatin, hemoglobin, and casein to some extent, but not collagen (**Smith and Williams 1984**). Kappa-toxin (collagenase), mu-toxin (hyaluronidase), and lambda-toxin (protease)

appear to play roles in pathogenesis because of their ability to break down host tissues. Gamma- and eta toxins have been proposed to account for discrepancies in neutralizing results obtained with specific antisera, but specific active substances related to those supposed entities have not yet been isolated (**McDonel 1980).**

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Bacteriological Quality and Safety of Raw Cow's and Buffalo's Milk Sold in Menoufia Governorate, Egypt

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Abstract:

This study focused on assessment of the microbiological quality, including the incidence of pathogens, of commercial raw cow's and buffalo's milk in Menoufia Governorate, Egypt. A total of 70 (35 each) milk samples were collected from different sites and analyzed for microbiological quality and isolation of pathogenic bacteria. Microbiological analysis revealed that the mean aerobic plate count was $2.01\pm0.836\times10^7$ and $4.03\pm1.37\times10^7$ cfu/ml for cow's and buffalo's milk, respectively. Enterobacteriaceae were detected in 31 (88.57%) and 31 (88.57%), with mean count values of $2.22\pm0.92\times10^6$ and $1.03\pm0.267\times10^7$ cfu/ml in cow's and buffalo's milk samples, respectively. Coliforms were detected in 88.57% and 88.57%, with mean count values of $5.57\pm3.56\times10^5$ and $8.86\pm1.71\times10^5$ cfu/ml in cow's and buffalo's milk samples, respectively. *E. coli* was detected in 8 (22.9%) and 5 (14.3%), *S. aureus* was detected in 22 (62.86%) and 20 (57.14%), with mean count values of $5.69\pm1.97\times10^4$ and $1.58\pm0.50\times10^5$ cfu/ml in cow's and buffalo's milk, respectively. On the other hand, *Salmonella* and *L. monocytogenes* were not detected in the examined samples.

Keywords: *Bacteriological quality, E. coli, S. aureus, Salmonella, Listeria monocytogenes, cow's and buffalo's milk.*

Introduction

Milk is a major component in human diet all over the world, it was considered as complete food for human from birth to senility, as it contains all the nutrients required for growth and maintenance of the body health (Jay, 2000).

Milk of cattle, buffalo, goat, sheep and camel contains almost same but varying concentration of the chemical constituents. Milk differs widely in composition due to different factors including species of animal, breed, individuality, stage of lactation,

frequency of milking, age, seasonal variations, feed, interval of milking, disease and abnormal conditions and administration of drugs and hormones (Ensminger, 1993).

Cow's milk has long been considered a highly nutritious and valuable human food, and is consumed by millions daily in a variety of different products. Its nutrient composition makes it an ideal medium for bacterial growth, and therefore it can be considered one of the most perishable agricultural products because it can so very easily be contaminated (Bramley & McKinnon, 1990 and Heeschen 1994).

Buffalo's milk receives increasing research interest and investment in various countries, owing mainly to its attractive nutrient content (Amarjit & Toshihiko, 2003). Buffalo is the second most important dairy species in the world. Egypt is among the largest producer countries of buffalo milk, with both buffalo herds and buffalo milk production listed forth worldwide in 2008, after those of India, Pakistan and China (FAOSTAT, 2008).

Raw milk could be a source of undesirable or even pathogenic bacteria which implicated in milkborne diseases. A number of bacteria including *S. aureus*, *Escherichia coli*, *Listeria monocytogenes* and *Salmonella* have been recovered from raw milk and some of these have been determined to be pathogenic and toxicogenic, and implicated in milkborne gastroenteritis (De Buyser et al., 2001; Harrington et al., 2002)

Microorganisms may gain entry into raw cow's and buffalo's milk from various sources either directly from dairy animals experiencing sub clinical or clinical mastitis, or from faecal contamination, particularly around the teats, and from the farm environment particularly the water source and utensils used for the storage of milk on farm or during transportation (Oliver et al., 2005).

In view of the growing public awareness about food safety and quality, a better knowledge of the microbiological quality of milk is of great significance for further development of its hygienic processing to safeguard the consumers. Therefore the objectives of this study were to 1) determine the microbiological status of cow's and buffalo's milk sold in supermarkets in Menoufia governorate, Egypt and 2) study the prevalence of foodborne pathogens, especially *E. coli*, *S. aureus*, *Salmonella* spp. and *L. monocytogenes* in cow's and buffalo's milk.

Materials and Methods

Collection of samples:

Seventy raw cow's and buffalo's milk samples (35 each) were collected from dairy shops and supermarkets from different areas in Menoufia Governorate. Collected samples were transferred to the laboratory of Food Hygiene

& Control Department at University of Sadat city in an ice box for bacteriological examination.

Bacteriological examination:

Initially, 25 ml of each raw milk sample was dispensed into a sterile flask containing 225 ml of 0.1% peptone water and mixed thoroughly. Subsequent serial decimal dilutions of each sample were prepared in 0.1% peptone water.

Viable cell counts were performed by the standard pour plate method after serial dilutions in the following conditions: Aerobic plate count (APC) was carried out on plate count agar according to the plate count method APHA 2001 (Morton, 2001).

Enterobacteriaceae count was carried out on Violet Red Bile Glucose (VRBG) Agar according to the plate count method APHA 2001 (Kornacki & Johnson, 2001).

Coliform bacteria were enumerated by the most probable number (MPN) multiple-tube fermentation method according to US standard method (US FDA, 2002). The identification of *E. coli* was confirmed by colony morphology on eosin methylene blue agar (EMB) and performing biochemical tests according to Holt et al. (1994). Serological identification of isolated *E. coli* was done according to Varnam & Evans (1991).

S. aureus count was carried out by direct plate count method on Baird Parker agar supplemented with egg yolk tellurite emulsion according to the plate count method APHA 2001 (Lancette & Bennett, 2001).

Detection of Salmonella:

Detection of Salmonella was done using the presence/absence method (US FDA, 2011). The suspected isolates were identified according to Forbes et al. (2007).

Detection of Listeria monocytogenes:

Detection of *L. monocytogenes* was done according to the most widely used approaches which based upon FDA method (Lovett, 1987) modified by Hitchins (1990). Identification of suspected colonies was done according to Hitchins (1995)

Results and Discussion

The analyzed samples were in general highly contaminated with the tested bacterial groups (Table 1). The aerobic plate count (APC) is an indication of the sanitary conditions under which the food was produced (Andrews, 1992). The results obtained in this study showed that all examined samples of raw cow's and buffalo's milk were contaminated with aerobic mesophilic bacteria, and the APC/ml ranged from 8.8×10^4 to 2.78×10^8 and 4.9×10^4 to 4.3×10^8 with mean count values of $2.01\pm0.83\times10^6$ and $4.03\pm1.37\times10^7$ respectively (Table 1). The highest frequency distribution of APC in examined raw cow's

and buffalo's milk samples were 74.28% and 68.57%, lies within the range of 10^6 to $<10^8$ and 10^6 to $<10^8$ respectively (Figures 1&2).

These findings for raw cow's milk, agree to some extent with those reported by Godefay and Molla (2000), Chye et al. (2004), Mennane et al. (2007) and Abd El-Krim et al. (2008), while relatively lower counts were reported by Kivaria et al. (2006), El-Diasty & El- Kaseh (2007), while comparatively higher counts were recorded by Tarek (2000) and Sobeih et al. (2002). The obtained findings for raw buffalo's milk were approached those reported by Adesiyun (1994), Awadall (2002) and Muhammad et al. (2009), relatively lower counts were reported by Boycheva et al (2002), Chatterjee et al. (2006) and Han et al. (2007). Comparatively higher counts were recorded by Tarek (2000) and Ibrahim (2010).

According to the limits proposed by Egyptian Standards (ES, 2010), recommended by the Egyptian Organization for Standardization and Quality "EOSQ", SPC of raw milk must not exceed 1×10^5 cfu/ml milk. Only 2.86% and 8.57% of the examined raw cow's and buffalo's milk samples complied with the standard, respectively.

The same percentage (88.6%) of the examined raw cow's and buffalo's milk samples were contaminated with Enterobacteriaceae with counts ranged from 9.0×10^2 to 2.5×10^7 and 3.5×10^3 to 5.310^7 with mean count values of $2.22 \pm 0.92\times10^6$ and $1.03\pm0.27\times10^7$ respectively (Table 1). The highest frequency distribution of Enterobacteriaceae count of the examined raw cow's and buffalo's milk samples were 77.14% and 62.85%, lies within the range of 10^4 to $<10^7$ and 10^6 to $<10^8$ (Figures 1&2)

The obtained findings for raw cow's milk are concomitant with those reported by Allam (1999) and El-Diasty & El-Kaseh (2007). Comparatively higher findings were recorded by El-Zubeir & Ahmed (2007). For raw buffalo's milk, comparatively higher findings were recorded by El-Shazly (2007). The obtained higher incidences and counts may be attributed to the unhygienic condition under which milk was produced, handled and stored, and is an indicative for direct or indirect feacal pollution of milk, neglection of hygienic measures and possible presence of enteric pathogens (Jay, 2000). The incidence of coliforms were detected at the same percentage (88.6%) and the counts ranged from 2×10^2 to 1.1×10^7 and 9×10^2 to 5×10^6 with mean count values of $5.57\pm3.56\times10^5$ and $8.86\pm1.71\times10^5$ for examined raw cow's and buffalo's milk samples respectively (Table 1).

Table 1. Bacterial loads of commercial raw cow's and buffalo's milk

Bacterial counts	Cow milk (n=35)					Buffalo milk (n=35)				
	Positive samples		Min.	Max.	Mean ±SEM	Positive samples		Min.	Max.	Mean ±SEM
	No.	%				No.	%			
APC	35	100	8.8×10^4	2.78×10^8	$2.01 \pm 0.84 \times 10^7$	35	100	4.9×10^4	4.3×10^8	$4.03 \pm 1.37 \times 10^7$
Enterobacteriaceae	31	88.6	9×10^2	2.5×10^7	$2.22 \pm 0.92 \times 10^6$	31	88.6	3.5×10^3	5.3×10^7	$1.03 \pm 0.267 \times 10^7$
Coliforms	31	88.6	2×10^2	1.1×10^7	$5.57 \pm 3.56 \times 10^5$	31	88.6	9×10^2	5×10^6	$8.86 \pm 1.71 \times 10^5$
S. aureus	22	62.9	1.7×10^3	3.4×10^5	$5.69 \pm 1.97 \times 10^4$	20	57.1	1×10^3	6×10^5	$1.58 \pm 0.50 \times 10^5$

No. of examined samples = 35

*SEM= Standard error of the mean

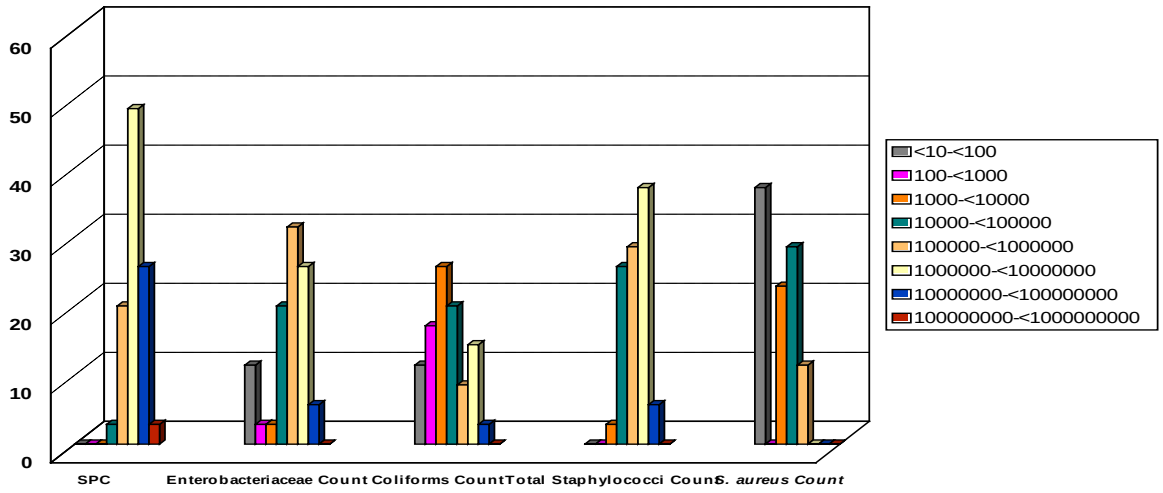

Fig. (1) Frequency distribution of bacterial load in cow's milk samples

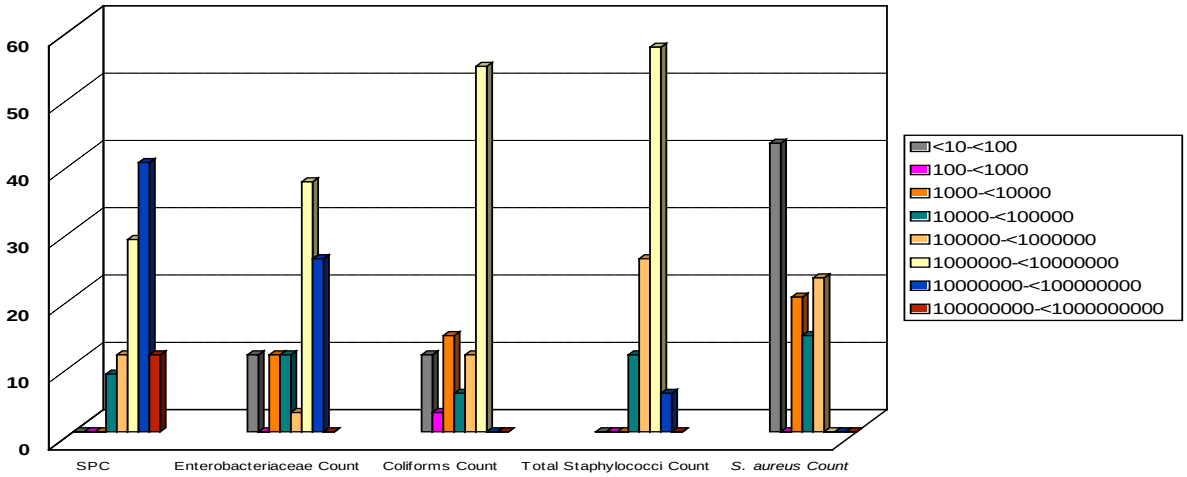

Fig. (2) Frequency distribution of bacterial load in buffalo's milk samples

The highest frequency distribution of coliforms in raw cow's and buffalo's milk samples were 62.85% and 65.72%, lies within the range of 10^2 to $<10^5$ and 10^5 to $<10^7$, respectively (Figures 1&2). Nearly similar findings for raw cow's milk were recorded by Al-Tarazi et al., (2003) and Abd El-Krim et al. (2008). Comparatively higher counts were recorded by Saudi & Mowad (1990) and El-Diasty & El-Kaseh (2007) and relatively lower counts were recorded Tarek (2000) and Mennane et al. (2007). The obtained findings for raw buffalo's milk agree to some extent to that obtained by Tarek (2000) and Awadall (2002). Relatively higher counts were reported by Hafez (1984), Farag (1987), and El-Shazly (2007), while relatively lower counts were obtained by El-Sayed & Ayoub (1993) and Wira & Orasa (2009).

Coliforms are abundant in the environment which including dust, manure, hair coat, exterior of the udder and milkers hand. Moreover, coliforms in milk can reduce its keeping quality due to production of sharp flavored substances (Blood et al., 1983). Furthermore, presence of coliforms and faecal coliforms beyond certain level could be of public health hazard, as they may cause dreadful diarrheal diseases (Robert et al., 1977).

The predominant isolated coliform strains in the examined raw cow's and buffalo's milk samples were. *E. coli, Citrobacter amalonaticus, C. freundii, Escherichia adecarboxylata, E. coli inactive, Enterobacter aerogenes, Ent. agglomerans, Ent. cloacae, Ent. gergoviae, Klebsiella oxytoca, K. pnumoniae sub spp. ozaenae, and K. pneumoniae sub.spp. pneumoniae* at percentages of (22.86& 14.29%), (14.29& 8.57%), (0& 5.71%), (2.86& 0%), (8.57%& 8.57%), (11.43& 5.71 %), (28.57& 14.29%), (0& 5.71%), (17.14& 11.43%) (11.43& 14.29%), (11.43& 20%) and (8.57& 20%), respectively (Table 2). Serological typing of isolated *E. coli* showed that they belonged to EPEC serotypes O119, O55 and O127:K63 and EIEC O124:K72 while the remaining were untypable (Table 3). The presence of presumably pathogenic *S. aureus* in 62.86% and 57.14% of examined raw cow's and buffalo's milk with counts ranged from 1.7×10^3 to 3.4×10^5 and 1.0×10^3 to 6.0×10^5 with mean count values of $5.69\pm1.97\times10^4$ and $1.58\pm0.50\times10^5$ respectively (Table 1), indicates the poor hygienic quality under which such milk was produced and also may indicate udder inflammation as staphylococcus spp. are one of the main etiological agents of intramammary infections.

Table 2. Incidence of Coliform organisms in the examined cow's and buffalo's milk samples

Isolates	Cow milk (n=35)		Buffalo milk (n=35)	
	Positive samples		Positive samples	
	No.	%.*	No.	%*
Escherichia coli	8	22.9#	5	14.3
Citrobacter amalonaticus	5	14.3	3	8.57
C.freundii	0	0	2	5.71
Escherichia adecarboxylata	1	2.86	0	0
E.coli inactive	3	8.57	3	8.57
Enterobacter aerogenes	4	11.4	2	5.71
Ent. agglomerans	10	28.6	5	14.3
Ent. cloacae	0	0	2	5.71
Ent. gergoviae	6	17.14	4	11.4
Klebsiella oxytoca	4	11.43	5	14.3
K. pneumoniae sub.spp. ozaenae	4	11.43	7	20
K. pneumoniae sub.spp. pneumoniae	3	8.57	7	20

* **% calculated according to samples number**.

Table 3. Serodiagnosis of some *E. coli* strains isolated from the examined cow's and buffalo's milk samples.

E. coli serotype	Cow milk (n=35)	Buffalo milk (n=35)	Strain pathotype
	No. of strains (%) #	No. of strains (%)	
O_{55}:K_{59}	-	1 (2.9)	EPEC
O_{119}:K_{69}	1 (2.9)	-	EPEC
O_{124}:K_{72}	1 (2.9)	2 (5.7)	EIEC
O_{127}:K_{63}	1 (2.9)	-	EPEC
Untypable	2 (5.7)	1 (2.9)	-
Total	5 (14.3)	4 (11.4)	

EPEC =Enteropathogenic *E.coli*
# % calculated according to samples number.

Table 4. Incidence of *Salmonella* spp. and *L. monocytogenes* in the examined cow's and buffalo's samples

Isolated bacteria	Cow milk (n = 35)		Ewe's milk (n = 35)	
	No.	%	No.	%
Salmonella spp.	0	0	0	0
L. monocytogenes	0	0	0	0

The highest frequency distribution of *S. aureus* in examined raw cow's and buffalo's milk samples were 51.43% and 57.15%, lies within the range of 10^3 to $<10^5$ and 10^3 to $<10^6$ respectively (Figures 1&2).

Nearly similar findings for raw cow's milk were obtained by Desmasures et al. (1997), Ali (2000) and Mohamed et al. (2002),

relatively higher counts and incidence were reported by Halawa (1987), Capurro et al. (2000) and Mennane et al. (2007),. Relatively lower counts and incidence were obtained by El-Bagoury (1992), and Belickova et al. (2000). For raw buffalo's milk, nearly similar findings were obtained by Adesiyum (1994) and Awadall (2002), relatively higher counts and incidence were reported by Halawa (1987), El-Bagoury (1988) and Jorgensen et al. (2005). Relatively lower counts and incidence were obtained by Gupta (1986) and Youssef et al. (2010).

Comparing the obtained results with Egyptian Standard (ES, 2005) recommended by the Egyptian Organization for Standardization and Quality "EOSQ", which stipulated that the number of *S. aureus* must not exceed 100 cfu/ ml, only 37.14% and 42.86% of examined raw cow's and buffalo's milk samples, respectively, complied with the standard.

Salmonella and *L. monocytogenes* were not detected in any of examined samples (Table 4). These findings, agree with results recorded by Nero et al. (2008), D'Amico & Donnelly (2010). Raw milk must be *Salmonella* and *L. monocytogenes* free (ES, 2005). Consequently all examined raw cow's and buffalo's samples complied with the standard in this point.

Conclusion

Results obtained in this study highlight the poor microbiological and sanitary quality of raw cow's and buffalo's milk sold in supermarkets in Menoufia governorate, and showed that the prevalence and counts of Enterobacteriaceae, coliforms and *S. aureus* were higher compared to some other studies. Therefore more efforts should be taken to increase sanitary and hygienic measures during production, transportation and storage of cow's and buffalo's milk to safe guard the consumers.

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Studies on the potential protective effect of curcumin and zinc against nickel- induced nephrotoxicity in rats

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Abstract:

Nickel (Ni) is a widely distributed metallic environmental pollutant and reported to be the highest potential risk to health of populations. This work aimed to investigate the ameliorative effect of curcumin (80 mg/kg. b.w. P.O. every other day) and/or zinc (227mg/L in D.W.) against $NiCl_2$ (1200 ppm (Ni) in D.W.)- induced nephrotoxicity in rats. One hundred and twenty female albino rats were used. They were divided into two main groups (A &B) 60 rats each. Each group was subdivided into sex subgroups, 10 rats each. Group A (administrated the antioxidants concomitantly with nickel for 8 weeks), group B (administered the antioxidants 2 weeks prior to nickel exposure and 8 weeks with nickel). Subgroup A1& B1 (Normal control), A2 & B2 (Control +ve), A3 & B3 (Ni + corn oil), A4 & B4 (Ni + curcumin), A5 & B5 (Ni + zinc) and A6 & B6 (Ni + curcumin + zinc). $NiCl_2$- exposed rats showed significant elevation in serum urea, creatinine levels and serum, renal MDA levels and CAT activities. Moreover, $NiCl_2$ exposure induced significant decrease in serum and renal GSH levels. Furthermore, histopathological examination of renal tissues showed necrobiotic changes in renal tubules with hypercellularity of glomeruli. Treatment (concomitantly with Ni) and pre-treatment (Pre- Ni exposure) with curcumin and/or zinc improved the altered biochemical parameters and renal histopathological changes. In conclusion, curcumin and or/zinc administration reversed $NiCl_2$-induced nephrotoxicity in rats via their ameliorating antioxidant effects. Pre-treatment with curcumin and zinc combination provided the greatest protection.

Keywords: *Nickel, curcumin, zinc, nephrotoxicity, antioxidants.*

Introduction

Metals, especially heavy metals, are serious environmental and public health hazards because their released concentrations into the environment from industrial processes often exceed the permissible limits **(Velma et al.,**

2009). Industry, mining, advanced agriculture, household waste, and motor traffic are all among the activities considered to be major sources of metals pollution **(Sevcikova et al., 2011)**. Generally, metals exert toxic effects by generating reactive oxygen species (ROS) that induce oxidative damage **(Jabeen et al., 2012)**.

Nickel is one of the most common heavy metal contaminants in polluted environments **(Mishra and Dubey 2006)**. It is used in a wide variety of metallurgical processes such as electroplating and alloy production as well as in nickel-cadmium batteries and household products manufactures **(Das et al., 2008)**. Although nickel is considered as an essential heavy metal **(Goyer, 1991)**, its toxicity is frequently approved **(Parthiban and Muniyan, 2011; El Ballal et al., 2012 and Adeyemi and Elebiyo, 2014)**.

Nickel is a known haemato-, immune-, geno-, nephro-, hepato-, reproductive and pulmonary toxic as well as carcinogenic agent **(Kasprzak, et al., 2003; Hostynek, 2006; Gupta et al., 2006, 2007; Das et al., 2007; El Ballal et al., 2012 and Adeyemi and Elebiyo, 2014)**. It exerts its toxic effects by causing cell death and/or damage, as nickel induces oxidative stress via ROS production accompanied by lipid peroxidation (LPO) and modification in the antioxidant capacity **(Prasad et al., 2006, 2007)**.

Data suggest that exogenous antioxidants may play an important role in the prevention of cellular damage by interacting with free radicals and by terminating the chain reaction of LPO **(Das et al., 2001)**. Zinc **(Sidhu et al., 2004)** and curcumin **(Rao et al., 2008**) were previously reported as exogenous antioxidants against Ni- induced oxidative stress.

Curcumin, a major yellow pigment and active component of turmeric powder extracted from rhizome of *Curcuma longa* Linn. Previous *in vitro* and *in vivo* studies suggested that curcumin possesses hepatoprotective **(Marotta et al., 2003)**, nephroprotective **(Farombi and Ekor, 2006)**, antitumor **(Choi et al., 2006)**, anti-inflammatory **(Stix, 2007)** and antioxidant **(Jefremov et al., 2007)** properties.

Zinc is an essential mineral that is naturally present in some foods,

added to others, and available as a dietary supplement. Zinc exerts nephroprotective **(Michael et al., 1985)**, hepatoprotective **(Szuster-Ciesielska et al., 2009)** and antioxidant **(El Ballal et al., 2012)** properties. Also, zinc accelerates wound healing **(Wintergerst et al., 2007)** and regulates apoptosis **(Hambidge and Krebs, 2007).**

A number of natural and chemical compounds **(Rao et al., 2006, 2008, Jia and Chen 2008 and Adeyemi and Elebiyo, 2014)** have been used to ameliorate nickel toxicity. However, there is scanty literature concerning the protective activity of curcumin and zinc gainst Ni- induced nephrotoxicity especially the ideal time of their administration to give the best protection. In the light of this, our work aimed to study ameliorating role of curcumin and/ or Zinc (both on concomitant and pre Ni-exposure treatment) against Ni– induced nephrotoxicity in rats.

Materials and Methods

1. *Animals*:

A total of 120 adult female albino rats weighing between 140-160g were used in this study. They were obtained from Faculty of Veterinary Medicine, Cairo University, Giza, Egypt. All animals were housed in polypropylene cages with mesh wire tops in well ventilated room and provided with balanced ration and clean water *ad libitum*. They were kept under observation for two weeks before the beginning of experiments for atmospheric and handling accommodation.

2. *Chemicals*:

Nickel in the form of Nickel chloride hexahydrate ($NiCl_2$. $6H_2O$) and zinc in the form of Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) were purchased from EL Naser Company, Egypt.

Curcumin in a powder form was purchased from EL Gomhorya Company, Egypt. Curcumin was dissolved in corn oil (vehicle). Diagnostic kits for assaying serum and tissue biochemical parameters were purchased from Biodiagnostic Company, Dokki, Giza, Egypt. All other chemicals and reagents were of analytical grade and commercially available.

3. Experimental design and animal grouping:

One hundred and twenty female albino rats were used. They were divided into two main groups (A & B) 60 rats each. Each group was subdivided into sex subgroups, 10 rats each. Group A (administrated the antioxidants concomitantly with nickel for 8 weeks), group B (administered the antioxidants 2 weeks prior to nickel exposure and 8 weeks with nickel). Subgroup A1& B1 (Normal control), A2 & B2 (control +ve, $NiCl_2$- intoxicated), A3 & B3 ($NiCl_2$+corn oil), A4 & B4 ($NiCl_2$+curcumin), A5 & B5 ($NiCl_2$+zinc) and A6 & B6 ($NiCl_2$+curcumin+zinc).

The dose and rout of exposure of $NiCl_2$ (1200 ppm (Ni) in D.W.), curcumin (80 mg/kg. b.w.P.O. every other day) and zinc (227mg/L in D.W.) were selected according to those previously used by **Cempel and Janicka**

(2002); Kalpana and Menon (2004) and Sidhu et al. (2004), respectively.

4. *Samples collection and preparation:*

After eight weeks in treated subgroups and ten weeks in pre-treated subgroups, animals were fasted overnight, anaesthetized and sacrificed for samples collection. Blood sample were collected without anticoagulant and centrifuged at 3000 rpm for 15 min in a cooling centrifuge for serum separation and estimation of serum biochemical parameters. Kidneys of each rat were divided into two parts, one part stored in ice bags at -20^{0}C for assaying renal tissue biochemical investigations while the other part was kept in 10% formalin for the histopathological examination.

5. *Biochemical analysis*:

5.1. Serum urea and creatinine levels were measured using the colorimetric methods of **Young (2001)**.

5.2. Serum and tissue MDA, GSH contents and CAT activities were determined according to the procedure described by **Ohkawa et al. (1979); Beutler et al. (1963) and Aebi (1984)**, respectively.

6. *Histopathological examination*:

Kidney Specimens intended for the histopathological examination were fixed in 10% neutral buffered formalin solution, processed and stained by hematoxylin and eosin (H&E) stain according to **Bancroft and Marilyn (2002)**.

7. *Statistical analysis*:

Values were presented as mean ± standard error (SE). Statistical differences of Ni-nephrotoxic effect and the ameliorative effect of curcumine and/or zinc were analyzed by one-way ANOVA according to **Snedecor and Cochron (1987)**. All statistical analyses were performed using SPSS (Statistical package for Social Sciences) Version 16 released on 2007. Statistical significance was considered at $P < 0.05$.

Results

Our results revealed that $NiCl_2$- induced renal adverse toxic effects as evidenced by biochemical and histopathological changes. In addition, the obtained results approved the curative and protective effects of curcumin and/or zinc administrations against such toxic effects **(Tables 1, 2 and figures 1-14)** this study declared a significant increase in serum urea and creatinine levels in Ni- intoxicated rats compared to the normal control ones **(figure 1)**. Also, investigation of MDA; GSH levels and CAT activities revealed significant elevation in serum and renal MDA levels with a concomitant decrease in GSH contents and significant increase in CAT activities **(figures 2, 3, 4)**.

Table 1: Effects of nickel administration alone; with curcumin and/or zinc on serum urea and creatinine (mg/dl) levels of different treated and pre- treated groups of albino rats.

Groups	(A) First (treated) group						(B) Second (pre-treated)group					
parameters	A1	A2	A3	A4	A5	A6	B1	B2	B3	B4	B5	B6
Serum Urea (mg/dl)	38.85 ± 3.63	53.15 ± 0.878[a]	49.34 ± 0.71	45.61 ± 1.97 [b]	47.92 ± 1.14	44.58 ± 0.96 [b]	38.85 ± 3.63	53.15 ± 0.878 [a]	48.78 ± 2.09	43.18 ± 0.862 [b]	44.94 ± 1.51 [b]	40.97 ± 0.224 [b]
Serum Creatinine (mg/dl)	1.69 ± 0.069	1.97 ± 0.075 [a]	1.89 ± 0.059	1.75 ± 0.037 [b]	1.79 ± 0.03	1.73 ± 0.077 [b]	1.69 ± 0.069	1.97 ± 0.075 [a]	1.86 ± 0.051	1.71 ± 0.060 [b]	1.72 ± 0.059 [b]	1.68 ± 0.054 [b]

Subgroup A1& B1 (Normal control), A2 & B2 (control +ve, $NiCl_2$- intoxicated), A3 & B3 ($NiCl_2$+corn oil), A4 & B4 ($NiCl_2$+curcumin), A5 & B5 ($NiCl_2$+zinc) and A6 & B6 ($NiCl_2$+curcumin+zinc).

The values are presented as mean ± S. E.

a, significantly different from Sub-group A1 or B1

b, significantly different from Sub-group A2 or B2 at P< 0.05.

Table 2: Effects of nickel administration alone; with curcumin and/or zinc on serum and renal MDA levels, GSHcontents and CAT activities of different treated and pre- treated groups of albino rats.

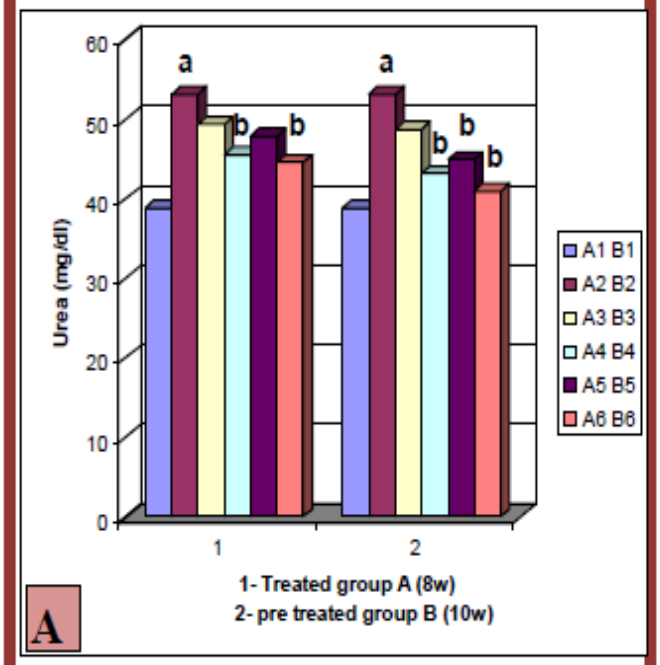
Groups	(A) First (treated) group						(B) Second (pre-treated)group					
Parameters	A1	A2	A3	A4	A5	A6	B1	B2	B3	B4	B5	B6
Serum MDA (nmol/ml)	5.79 ± 0.664	9.40 ± 0.753[a]	7.81 ± 1.42	6.56 ± 0.617[b]	6.91 ± 0.49[b]	6.12 ± 0.110[b]	5.79 ± 0.664	9.40 ± 0.753[a]	7.79 ± 1.14	5.42 ± 0.225 [b]	6.57 ± 0.943[b]	5.39 ± 0.209 [b]
Renal MDA (nmol/g)	10.95 ± 0.47	19.11 ± 3.33 [a]	16.92 ± 1.74	13.98 ± 0.15[b]	15.22 ± 0.46	13.18 ± 2.67 [b]	10.95 ± 0.47	19.11 ± 3.33 [a]	16.49 ± 0.66	12.49 ± 0.52[b]	13.11 ± 1.64 [b]	11.38 ± 0.62 [b]
Serum GSH (mg/dl)	11.89 ± 0.524	6.83 ± 0.096 [a]	7.69 ± 0.495	9.18 ± 1.24 [b]	8.62 ± 0.475	10.23 ± 0.404[b]	11.89 ± 0.52	6.83 ± 0.096 [a]	7.91 ± 0.379	12.49 ± 1.84 [b]	11.92 ± 0.096[b]	13.69 ± 3.12 [b]
Renal GSH (mg/g)	26.19 ± 1.04	18.46 ± 0.423[a]	22.13 ± 1.00	24.62 ± 2.43[b]	22.35 ± 1.12	25.95 ± 1.39 [b]	26.19 ± 1.04	18.46 ± 0.423[a]	22.89 ± 0.989	26.03 ± 2.03 [b]	25.11 ± 0.771[b]	27.64 ± 4.49 [b]
Serum CAT (U/L)	0.222 ± 0.034	0.449 ± 0.023[a]	0.413 ± 0.042	0.322 ± 0.010	0.280 ± 0.069[b]	0.264 ± 0.062[b]	0.222 ± 0.034	0.449 ± 0.023[a]	0.410 ± 0.068	0.286 ± 0.043[b]	0.252 ± 0.039 [b]	0.238 ± 0.019[b]
Renal CAT (U/g)	0.809 ± 0.078	1.04 ± 0.039 [a]	0.954 ± 0.089	0.880 ± 0.049	0.840 ± 0.095	0.828 ± 0.024[b]	0.809 ± 0.078	1.04 ± 0.039 [a]	0.940 ± 0.089	0.832 ± 0.024[b]	0.829 ± 0.092[b]	0.817 ± 0.016[b]

Subgroup A1& B1 (Normal control), A2 & B2 (control +ve, $NiCl_2$- intoxicated), A3 & B3 ($NiCl_2$+corn oil), A4 & B4 ($NiCl_2$+curcumin), A5 & B5 ($NiCl_2$+zinc) and A6 & B6 ($NiCl_2$+curcumin+zinc).

The values are presented as mean ± S. E.

a, significantly different from Sub-group A1 or B1

b,significantly different from Sub-group A2 or B2 at P< 0.05.



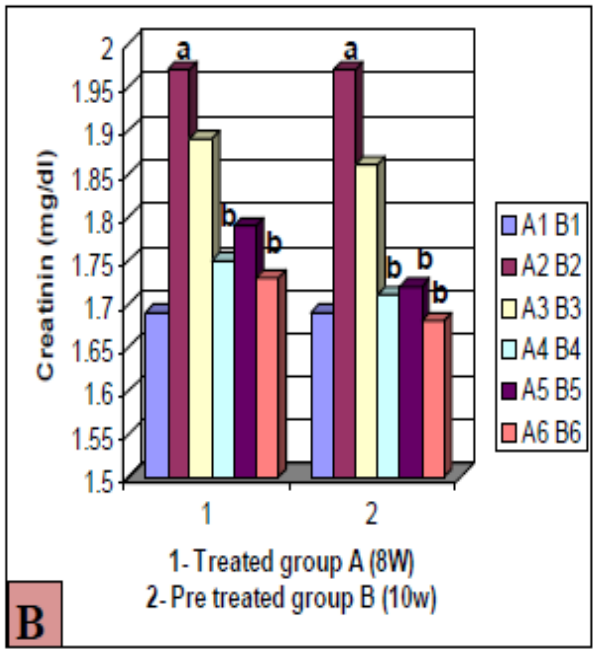
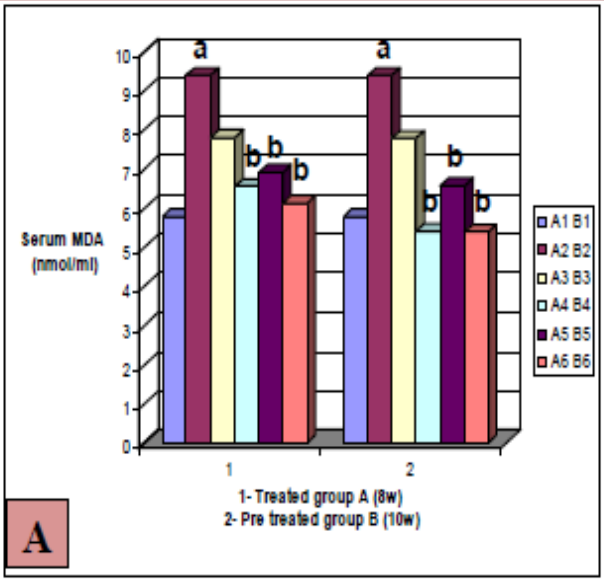


Figure (1): Effect of $NiCl_2$ exposure alone; with curcumin and/or zinc on serum urea (A) and creatinine (B) levels of different treated and pre- treated groups of albino rats.
a, significantly different from Sub-group A1 or B1 at $P< 0.05$.
b, significantly different from Sub-group A2 or B2 at $P< 0.05$.



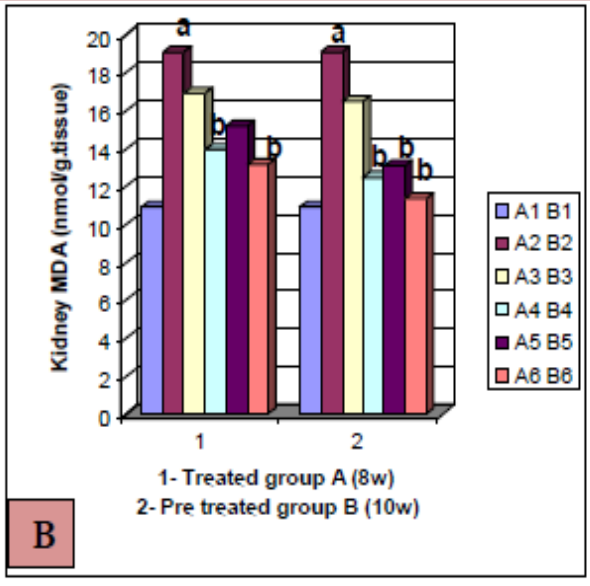


Figure (2): Effect of $NiCl_2$ exposure alone; with curcumin and/or zinc on serum (A) and renal (B) MDA levels of different treated and pre- treated groups of albino rats.
a, significantly different from Sub-group A1 or B1 at $P< 0.05$.
b, significantly different from Sub-group A2 or B2 at $P< 0.05$.

Renal histological examinations showed severe pathological alterations **(figure 5, 6)**.

Minimal ameliorative effect was observed in corn oil- treated (concomitantly with Ni) or pre-treated (Pre- Ni exposure) groups that was recorded as insignificant improvement in all the studied parameters **(figures 1-4)** and minimal improvement in the renal histological structure **(figures 7, 8)** compared to Ni-intoxicated groups.

The curative (Given concomitantly with Ni) and protective (Given pre- Ni exposure) effects of curcumin were indicated by the significant decrease in the elevated serum urea and creatinine levels, serum and renal MDA with concomitant increase in GSH levels of curcumin- treated or pre- treated rats compared to Ni- intoxicated rats **(figures 2,3)**. Also, curcumin administration improved serum and renal CAT activities and restored it within the normal range **(figure 4)**. In addition, curcumin attenuated nickel- induced renal histopathological changes **(figures 9, 10)**.

To a lesser extent, treatment (Given concomitantly with Ni) and pre-treatment (Given pre- Ni exposure) of Zn to Ni-intoxicated rats indicated reduction in the elevated serum urea and creatinine levels which was only significant in the pretreated group compared to Ni- intoxicated one **(figure 1)**. Also, zinc exerted antioxidant effect that was recorded as decrease of MDA levels and increase of GSH levels in serum and kidney tissue that was more prominent in Zn-pretreated group compared to Ni- intoxicated rats **(figures 2,3)** and restored CAT activities to be within the normal level **(figure 4)**. Moreover, Zn improved the renal histoarcheticture **(figures 11, 12)**.

Marked and better ameliorative effects were obtained on pre-treatment with curcumin and/ or zinc than in respective treated animals, Also, Pre-treatment with curcumin and zinc combination provided the greatest protection that was indicated by significant improvement in all the altered biochemical parameters **(figures 1-4)** as well as the renal histopathological alterations **(figures 13,14)**.

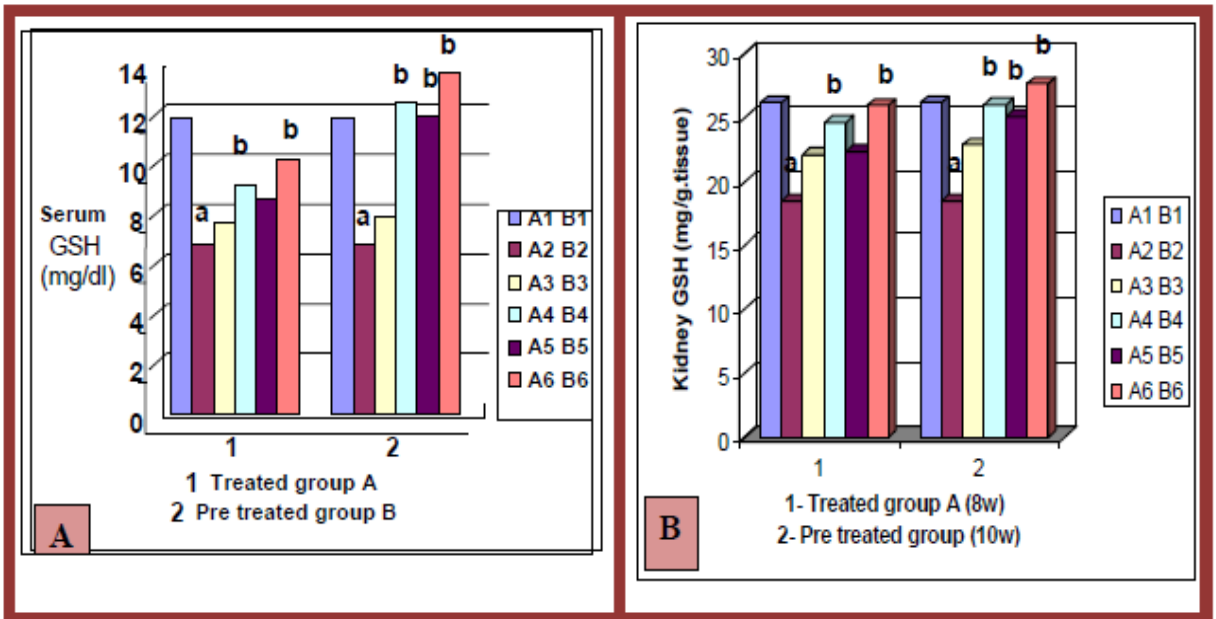


Figure (3): Effect of $NiCl_2$ exposure alone; with curcumin and/or zinc on serum (A) and renal (B) GSH levels of different treated and pre- treated groups of albino rats.
a, significantly different from Subgroup A1 or B1&b, significantly different from Subgroup A2or B2 at P< 0.05.

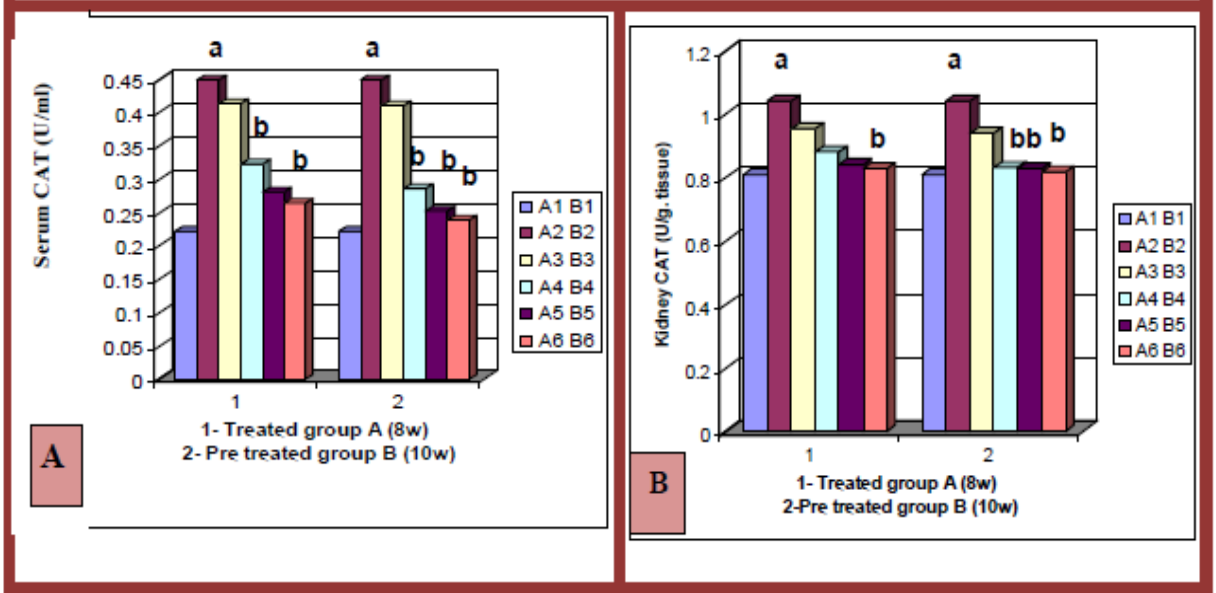


Figure (4): Effect of $NiCl_2$ exposure alone; with curcumin and/or zinc on serum (A) and renal (B) CAT levels of different treated and pre- treated groups of albino rats.
a, significantly different from Subgroup A1 or B1&b, significantly different from Subgroup A2or B2 at P< 0.05.

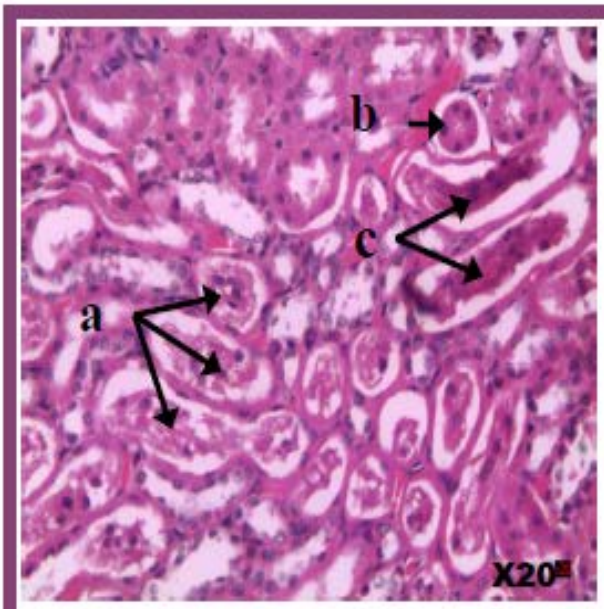


Fig (5): Kidney Photomicrograph of Ni- intoxicated rats showing shedding of epithelium (a), necrosis (b) and hyaline cast (c) in renal tubules.

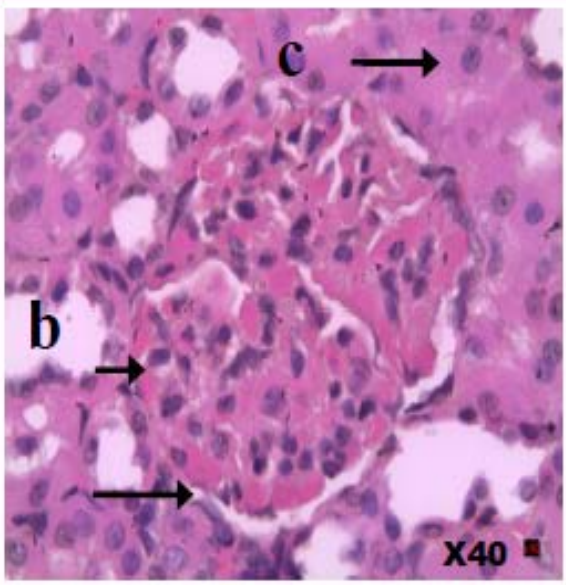


Fig (6): Kidney Photomicrograph of Ni- intoxicated rats showing congestion (a) and hypercellularity (b) of glomeruli and cloudy swelling of the epithelial cell of PCT with obliteration of their lumens (c).

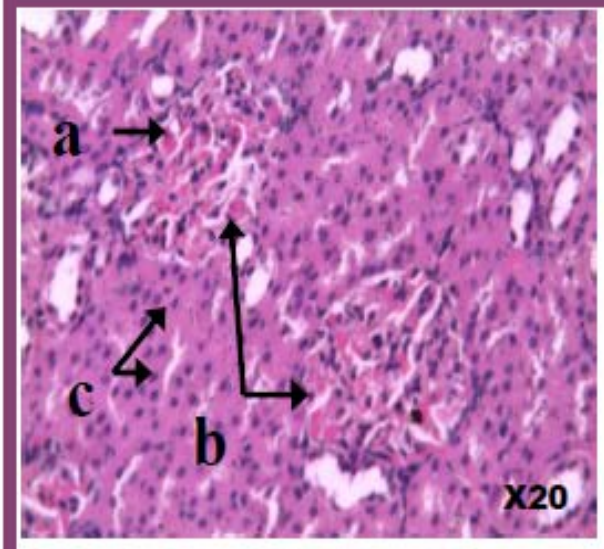


Fig (7): Kidney Photomicrograph of rats treated with corn oil plus Ni showing congestion (a), hypercellularity (b) of glomeruli and cloudy swelling of the epithelial cell of PCT with obliteration of their lumens (c) .

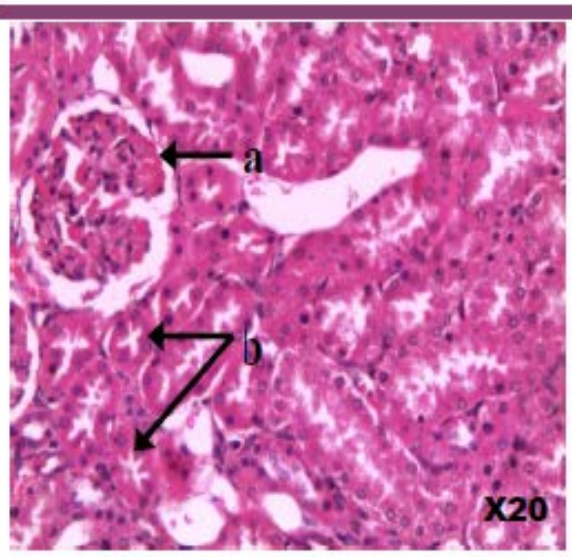


Fig (8): Kidney Photomicrograph of rats pre-treated with corn oil (2w) then with Ni (8w) showing hyper-cellularity of glomeruli and narrowing of the Bowman's space (a), cloudy swelling (b) and necrobiotic changes in PCT.

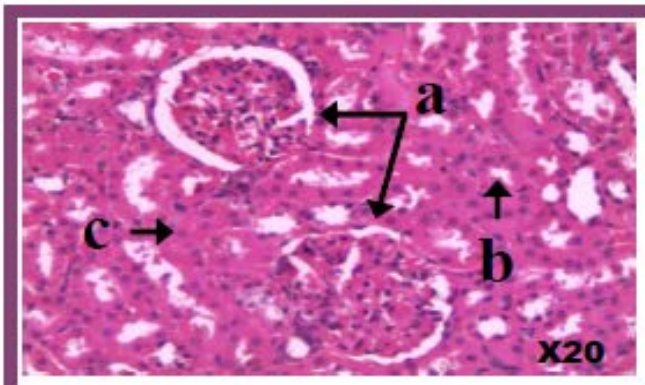


Fig (9): Kidney Photomicrograph of rats treated with curcumin plus Ni showing slight degree of hypercellularity of glomeruli(a) and cloudy swelling(b) and single cell necrosis (c).

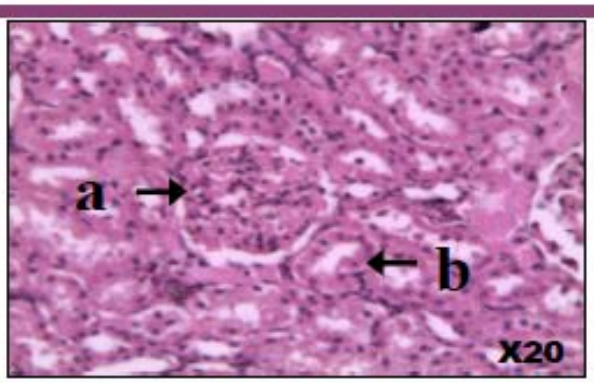


Fig (10): Kidney Photomicrograph of rats pre-treated with curcumin (2w) then with Ni (8w) showing slight degree of hypercellularity of glomeruli(a) and cloudy swelling (b).

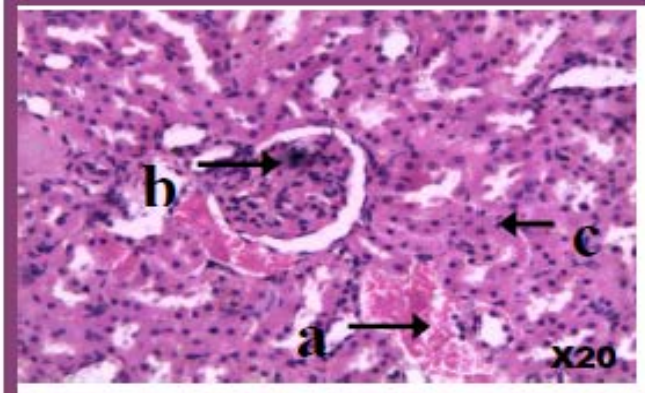


Fig (11): Kidney Photomicrograph of rats treated with zinc plus Ni showing hyperemia in the interstitial blood capillaries(a), moderate degree of glomerular hypercellularity (b) and single cell necrosis (c).

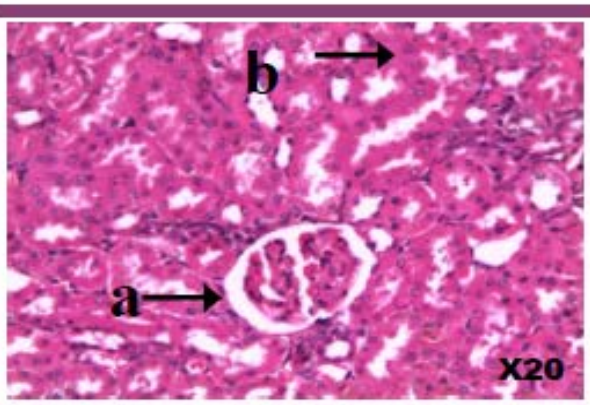


Fig (12): Kidney Photomicrograph of rats pre-treated with zinc (2w) then with Ni (8w) showing slight degree hypercellularity of glomeruli (a) and single cell necrosis (b).

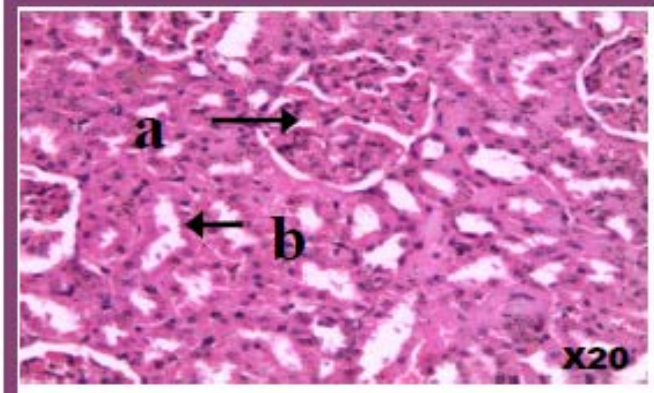


Fig (13): Kidney Photomicrograph of rats treated with curcumin and zinc plus Ni showing hypercellularity of some glomeruli(a) and slight necrobiotic changes (b).

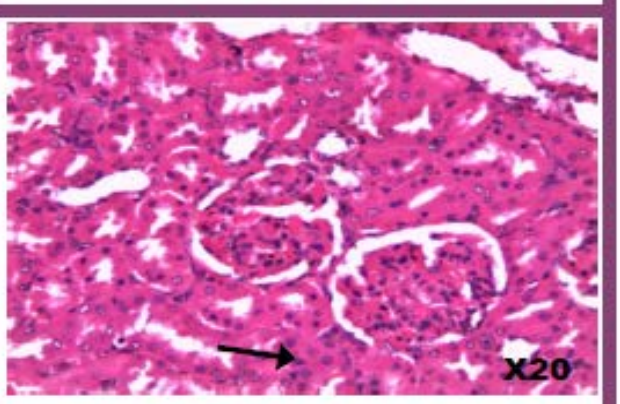


Fig (14): Kidney Photomicrograph of rats pre-treated with curcumin and zinc (2w) then with Ni (8w) showing single cell necrosis.

Discussion:

The role of pollution as a health risk factor is of special interest. Numerous toxic pollutants, such as nickel, cadmium and lead are being released into the environment as a result of industrialization, combustion of fossil fuels, crude oil and coal.

Nickel (Ni) is considered as one of the widely distributed metallic pollutants in the environment. Several approaches involving its nephrotoxicity have been documented **(Prasad et al., 2006, Amudha and Pari, 2011 and Adeyemi and Elebiyo, 2014)**.

As a result of the frequent and continuous exposure to Ni due to its indiscriminate uses and industrial discharges into the environment with the resulting environmental pollution and health hazards, this study aimed to investigate $NiCl_2$- induced nephrotoxicity in rats with trials for protection using curcumin and/or zinc against such toxic effects.

Our results revealed that the nephrotoxic effect of Ni was in the form of biochemical changes in serum urea and creatinine levels, serum and renal MDA, GSH contents and CAT activities, as well as renal histopathological changes.

Serum urea and creatinine levels are among the major biochemical indices commonly used to evaluate renal functions **(Gross et al., 2005 and Asante et al., 2014)**. In agreement with our result, **Adeyemi and Elebiyo (2014)** observed elevated serum urea and creatinine levels following Ni exposure. The negative effect of nickel ions on glycoprotein metabolism may explain the nephrotoxic effects of excessive exposure **(Savolainen, 1996)**.

Previous studies revealed that oxidative damages are implicated in the pathogenesis of Ni- induced nephrotoxicity **(Prasad, 2006, 2007 and Amudha and Pari, 2011)**. In general, Ni induces oxidative damages in various tissues by either increasing ROS production **(Das et al., 2001)** or depleting the endogenous antioxidants **(Parthiban and Muniyan, 2011)**

In the present study, Ni administration disrupted the prooxidant/ antioxidant balance of cells resulting in oxidative damages that was represented by the significantly increased serum and renal MDA levels, CAT activities and reduction in their GSH contents.

The increase in MDA, the breakdown product of lipid peroxidation and the reduction in GSH were previously recorded in kidney of rats exposed to nickel intoxication **(Amudha and Pari, 2011)**.

In our study the decreased level of serum and tissue GSH contents of Ni- intoxicated rats may be due to its excessive utilization in detoxification of peroxide radicals produced

after Ni toxicity. The decreased GSH contents may be the cause of enhanced renal LPO and increase MDA levels **(Dwivedi and Sarkar, 2009)**.

Surprisingly, our result showed increase in serum and renal CAT activities that could be attributed to several interpretations. **Prasad et al. (2006, 2007)** and **M'Bemba-Meka et al. (2005)** mentioned that Ni administration resulted in increase the formation of H_2O_2**.** Also, zinc deficiency following exposure to Ni causes leakage of H_2O_2 from a NADPH-dependent cytochrome P450 enzyme system **(Hammermueller et al., 1987)** which could stimulate CAT activity to cope with the increase of oxidative stress **(Sidhu et al., 2004)**. In addition, CAT is a homotetrameric ferriheme-containing enzyme, requiring iron for its synthesis **(Fridovich, 1998),** Nickel toxicity increases tissue iron levels **(Athar et al., 1987)** and as a consequence, it increases CAT activities**.**

The recorded improvement in the biochemical parameters and renal histopathological changes following corn oil administration to Ni– exposed animals could be attributed to its antioxidant constituents of alpha-tocopherol, beta-tocopherol, gammatocopherol and delta-tocopherol (vitamin E) **(Traber, 2006)**.

In our study, curcumin attenuated renal dysfunction along with reduction of lipid peroxidation and restored MDA, GSH and CAT in Ni-intoxicated rats near to normal. This observation is in line with the previous reports, which approved that curcumin decreased LPO possibly by its antioxidant properties **(Farombi and Ekor, 2006 and Hussein and Abu-Zinadah, 2010)**.

The antioxidative properties of curcumin could be attributed to various mechanisms. Curcumin suppresses iron- induced LPO due to chelation of iron **(Sreejayan and Rao, 1994)**, acts as free radicals scavenger **(Majeed et al., 2000)**, reduces the utilization of vitamins C and thus maintains their levels **(Rukkumani, 2003)** and increases GSH synthesis **(Zheng et al., 2007)**. Thus, curcumin exerted its protective effect against Ni-induced toxicity by modulating the extent of LPO and augmenting the antioxidant defense system.

The ability of curcumin to chelate iron **(Jiao et al., 2006, 2009)** that is essential for CAT synthesis and to scavenge H_2O_2 **(Reddy and Lokesh, 1994)** could explain the recorded reduction in CAT activity following curcumin administration both in treated and pre-treated groups.

In consistent with our results, **Michael et al. (1985)** reported that oral zinc supplementation counteracted the Ni– induced renal oxidative damages in rats, approving its antioxidant

properties **(Milbury and Richer, 2008).** This could illustrate the recorded improvement of all the altered biochemical parameters and renal histopathological changes following Zinc administration either in treated (Given Zn concomitantly with Ni) or pre-treated (Given Zn pre- Ni exposure) groups.

It has been suggested that zinc may protect sulfhydryl groups against oxidation and inhibit the production of ROS by transition metals, in addition to its function as a membrane stabilizer **(Bray and Bettger, 1990 and Oteiza et al., 1995)**. Zinc acts as a cofactor of antioxidant enzymes and essential for SOD synthesis and thus for elimination of free radicals **(Tainer et al., 1983 and Leung, 1998)**.

Following administration of both curcumin and zinc together, the obtained results confirmed significant improvement in all of the adversely affected parameters and the toxic pathological changes especially in the pre-treated rats. The attenuation of N- induced toxic effects may be due to an agonistic effect between curcumin and zinc that strengthened their antioxidant properties.

Conclusion

In conclusion, the results of this study revealed that Ni- induced nephrotoxicity could be ameliorated by curcumin and/or zinc supplementations. Significant and better improvement was obtained on pre-treatment with the two antioxidants alone or together. However, Pre-treatment with curcumin and zinc combination provided the greatest protection.

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Effects of feeding rates on behavior and growth performance of Nile Tilapia (Oreochromis niloticus)

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Abstract:

The present study was carried out to investigate the effect of different feeding rates on behavior and growth performance of Nile tilapia. Fish were classified into three groups (n=10/group, 2 replicates). 1st, 2nd and 3rd groups received 3.4%, 3.6% and 3.8% feeding rates, respectively. Behavior of fish, body weight, body dimensions and water quality were recorded. Weight gain, relative growth rate and feed conversion ratio were calculated. The results revealed that feeding, foraging and resting behaviors were significantly higher in fish fed 3.8% than in 3.4% and 3.6% groups. However, chasing and mouth pushing activities decreased in 3.8% group in comparison with 3.4% and 3.6% groups. Body weight of fish fed 3.8% and 3.6% feeding rates were significantly higher than 3.4%. Weight gain and relative growth rate were significantly increased by increasing feeding rates. It was concluded that feeding Tilapia on 3.8% gave the highest growth performance and enhanced the fish behavior with no effects on water quality.

Keywords: *Nile Tilapia, Feeding rates, Behavior and Growth performance.*

Introduction

Feed is one of the most important factors responsible for both profitability and environmental sustainability of a farm. Therefore, there is a growing interest in developing selection programs aimed at selecting individuals that are more feed efficient, i.e. that need to consume less feed to achieve the same growth rates as less efficient individuals. As a consequence less feed is necessary to produce a unit of output **(Wall *et al.*, 2010)** and less waste is discharged into the environment **(Nkrumah *et al.*, 2006)**.

In aquaculture feeding rate is an important factor affecting the growth of fish, and thus determining the optimal feeding rate is essential to the success of any aquaculture operation. The quality of the diet and feeding management adopted by producers can

significantly affect growth, survival and feed conversion of culture Tilapia fish.

Determining the optimum pattern of feed added to fish ponds is one of the most important tasks in pond management. Therefore, some concern should be paid to reduce this expense by feeding the correct amount at the right time to insure maximum efficiency. Meanwhile, realization of the optimum feeding regime for cultured fish would help to reduce feed wastes, costs and maximizing feed conversion efficiency **(Abdel Hakim *et al.*, 2004)**.

Feeding rate is an important factor affecting the growth of fish, and thus determining the optimal feeding rate is imperative to the success of any aquaculture operation. Consequently, some farmers used mixed feeding timetable of varying high and low dietary protein levels in feed **(De Silva, 1985**; **Gabriel *et al.*, 2000)** and optimizing feeding rate **(Qin and Fast, 1996; Dong-Fang *et al.*, 2003)** to improve production with low cost. However, poor fish farmers find these methods difficult to apply and expensive to achieve.

On the other hand, inappropriate feeding practices in aquaculture may lead to overfed which results in feed wastes in pond water and consequently contamination of aquatic environment as well as high production costs **(Ali *et al.*, 2010)**. On contrary, insufficient feeding lead to poor growth and high fish mortalities which make losses in the aquaculture business **(Eroldogan *et al.*, 2006; Gandra *et al.*, 2007)**. Additionally, the patterns of ingestive behavior are related to nature of its characteristic food **(Hafez, 1975)**. In the same time, every fish species has its particular food preferences and feeding behaviors. In general, feed management or feeding techniques have two main objectives: i) to encourage rapid and positive consumption and thus reducing leaching of nutrients wastage; ii) to provide greater potential for growth by minimizing the metabolic costs.

Fish perform all their bodily functions in water. Because fish are totally dependent upon water to breathe, feed and grow, excrete wastes, maintain a salt balance, and reproduce, understanding the physical and chemical qualities of water is critical to successful aquaculture. To a great extent, water determines the success or failure of an aquaculture operation **(Swann, 1990)**. In the same time, some water quality factors are more likely to be involved with fish losses such as dissolved oxygen, temperature, and ammonia. Others, such as pH, alkalinity and hardness affect fish, but usually are not directly toxic. Each water quality factor

interacts with and influences other parameters, sometimes in complex ways **(Buttner *et al.*, 1993)**. The aim of this study was to investigate the effect of different feeding rates on behavior and growth performance of Nile tilapia (Oreochromis niloticus) in relation to water quality.

Materials and Methods

Sixty fish were randomly sampled and divided into three groups [10 fish per aquarium (100 cm length x 30 cm width x 40cm height)] with two replicates per treatment. Fish were fed three different rates 3.4%, 3.6% and 3.8% of fish body weight. The experiment lasted for 10 weeks to study the effect of these feeding rates on Nile Tilapia behavior and their growth performance. All groups were fed on commercial diet. The amount of food given was adjusted every two weeks after determination of new fish weights in each group.

Data recording: Behavioral observations were recorded by using video camera (Sony, Japan) for 10 minutes for each aquarium two times per day at morning (9:00-11:30 am) and at afternoon (2:00-4:30 pm); the recording was twice per week. Sampled behavioral categories for all fish were scanned by one observer. The behavior of the fish in each aquarium was recorded using instantaneous sampling method. Each behavior was observed at 60 second intervals.

Fish were taken from each aquarium for individual weight every two weeks. Body weights were taken at 0, 2, 4, 6, 8 and 10 weeks of experiment. Fish were handled by using wetted towel to avoid stress then putted in plastic container with wetted sponge on electronic weighing balance (ACCULAB, Sartorious group, Germany). According to **(Lee *et al.*, 2000)** the following items were calculated: weight gain= Wf - Wi ; relative growth rate= (Wf - Wi)/ $^1/_2$ (Wf + Wi) X 100 and feed conversion ratio (FCR) = [dry feed fed (g)/ weight gain (g)] where Wf= final weight; Wi= initial weight.

Body dimensions of fish were measured at the end of study. Fish were put in 20L worm water (25°C) with Tricaine Methanoesulfonate (0.5g/10 L, Finquel, USA) for anesthesia. Fish left for about 5-10 minutes then taken to measure body dimensions by using ruler and electronic digital caliper (GENERAL, Switzerland). According to **(Ricker, 1979)** the body dimensions were described as the followings: Total length: The length from the tip of fish mouth till the end of the tail, with mouth closed and the tail lobes pressed together. Standard length: The length from the tip of fish mouth with mouth closed till caudal peduncle of tail. Length of jaw: The

length from the tip of fish mouth with mouth closed till angle of mouth. Head length: The length from the tip of fish mouth with mouth closed till the operculum (gill cover) border.

Water quality parameters were measured daily for each aquarium. PH was measured by Water Proof PH-Temperature Pocket tester with replaceable probe (AD12, Romania). Dissolved oxygen (D.O) was measured by Professional Portable Dissolved Oxygen Meter (HD3030, Trans-instruments Company, Singapore). Nitrite (NO2) and Nitrate (NO3) concentrations were assayed according to instruction of manufacture (Hersteller, sera GmbhH D 52518 Heinsberg, Germany).

Statistical analyses: Data were analyzed by using SAS system (2001). The proportion of fish that performed a behavioral pattern (feeding, foraging, elimination, resting, chafing, schooling, surfacing, chasing, mouth pushing or fin tugging) per minute was calculated. All data (behavioral patterns, growth performance and water quality parameters) were analyzed with ANOVA using a generalized linear model (GLM-procedure). Results were considered statistically significant when p-values were below 0.05.

Results

Results from Table (1) indicated that different feeding rates had significant effect on fish behavior. Feeding and foraging behaviors were higher in fish fed 3.8% than fish fed 3.4% and 3.6% (P=0.001, P=0.02, respectively). On the other hand, elimination was significantly more in 3.6% than 3.4% (8.92±0.87, 6.50±0.87%, respectively, P=0.05). Comfort behavior generally was not affected by different feeding rates except resting (Table 1). Resting behavior was significantly higher in 3.8% than 3.4% (25.79±1.34, 20.67±1.34%, respectively, P=0.007). However, surfacing, schooling and chafing were not significant affected by feeding rates (P>0.05). Feeding rates had significant effects on aggressive behavior as shown in Table (1). Chasing behavior was more in 3.4% feeding rate than 3.8% (34.47±2.05, 28.22±2.05%, respectively, P=0.03). Additionally, proportion of mouth pushing in 3.6% groups was high in compared with 3.8% groups (13.52±1.46, 7.29±1.46%, respectively, P=0.003). Conversely, fin tugging (biting) was not affected by different feeding rates.

Results from Table (2) showed that different feeding rates had significant effects on Tilapia body weight. At second and fourth weeks, fish fed 3.8% feeding rate were heavier than fish fed 3.6% feeding rate (P=0.05). On the other

hand, 3.8% and 3.6% feeding rates increased fish body weight in comparison with 3.4% feeding rate at 8th and 10th weeks (P=0.006 and P=0.04, respectively).

Feeding rates had significant effect on weight gain and relative growth rate although, it had no significant effect on feed conversion ratio (P>0.05). The weight gain was more in feeding rate 3.8% than 3.6% and 3.4% (55.84±2.52, 48.48±2.39 and 41.29±2.39g, respectively, P=0.038). Fish fed 3.8% had higher relative growth rate than 3.6% and 3.4% (125.48±1.81, 120.02±1.72 and 112.48±1.72% respectively, P=0.033) (Table 3). In the same time, feeding rate 3.6% improved weight gain and relative growth rate in compared with feeding rate 3.4%.

Results summarized in Table (4) indicated that different feeding rates had a significant effect on fish body dimensions. Fish in 3.8% and 3.6% groups were longer than fish in 3.4% group (15.71±0.25, 15.63±0.23 and 14.93±0.23cm, respectively, P=0.03). Also, the standard length was more in 3.8% and 3.6% feeding rates than 3.4% feeding rate (12.91±0.18, 12.73±0.17 and 12.23±0.17cm, respectively, P=0.028). Conversely, head and jaw lengths were not affected by different feeding rates (Table 4).

Data presented in Table (5) revealed that water quality parameters were not significantly affected by feeding fish on different feeding rates (P>0.05).

Discussion

The difference between feeding rates in this study was very little (0.2%) however, a clear effect on Tilapia behavior and performance were recorded. Generally, feeding and foraging behavioral activities were improved by increasing feeding rates. These results reflected on fish performance where body weight, weight gain and relative growth rate were enhanced. These findings were agreed with **Marimuthu *et al.*, (2011) and Andem *et al.*, (2013)** who reported that feeding rate presented a significant effect on the growth performance parameters. In group 3.8%, aggressive behaviors including chasing and mouth pushing were reduced. However, first stage of aggressive behavior (chasing) in this study was observed markedly in fish fed 3.4% compared to fish fed 3.8% and 3.6%. These results may be attributed to long stay of food in front of fish that motivate the fish to spend more time in feeding and foraging activities than showing aggression. Similarly, **Fanta (1997)** who reported that as soon as feeding ends, *B. soporator* fish keep in repose and aggressiveness becomes almost non-existent.

Table (1): Effect of different feeding rates on Tilapia behavior (LSMeans):

Behaviors (%)	Feeding rates			s.e.d	P-value
	3.4 %	3.6 %	3.8 %		
Ingestive: **Feeding**	25.06^{b}	26.69^{b}	39.62^{a}	2.47	0.001
Foraging	24.45^{b}	29.05^{b}	37.66^{a}	2.58	0.02
Elimination	6.50^{b}	8.92^{a}	8.53^{ab}	0.87	0.05
Comfort: **Resting**	20.67^{b}	22.44^{ab}	25.79^{a}	1.34	0.007
Chafing	1.90	2.40	3.88	1.04	NS
Schooling	1.95	1.77	2.19	0.51	NS
Surfacing	6.24	4.68	6.14	1.29	NS
Aggressive: **Chasing**	34.47^{a}	31.30^{ab}	28.22^{b}	2.05	0.03
Mouth pushing	10.69^{ab}	13.52^{a}	7.29^{b}	1.46	0.003
Fin tugging	33.84	33.55	37.77	2.23	NS

NS: non significant. s.e.d: standard error of difference. a-b: LS Mean with different superscripts were significantly different.

Table (2) Effect of different feeding rates on Tilapia fish body weight (g) (LSMeans):

Weeks	Feeding rates			s.e.d	P-value
	3.4%	3.6%	3.8%		
Initial	18.91	17.79	18.51	0.50	NS
2	25.07^{ab}	23.33^{b}	25.61^{a}	0.80	0.048
4	31.74^{ab}	30.42^{b}	33.35^{a}	1.06	0.053
6	41.73	41.21	43.14	1.45	NS
8	48.10^{b}	53.01^{a}	57.20^{a}	1.77	0.006
10	57.16^{b}	64.12^{a}	70.09^{a}	2.63	0.04

NS: non significant. s.e.d: standard error of difference. a-b: LS Mean with different superscripts were significantly different.

Consequently, Tilapia fish fed 3.8% did not utilized intraspecific aggression for defense of a particular position relative to neighboring conspecifics. Therefore, Tilapia in group 3.8% spent more time in feeding and foraging activities that covered a territorial area and reduced territorial aggression **(Rincon and Grossman, 2001).** Tilapia in 3.6% and 3.4% groups exhibited less feeding and foraging behaviors which associated with high level of aggression. These findings indicated that feeding rates had a benefit effect on behavioral adaptation of fish by increasing feeding and foraging activities and reducing level of aggression. This is the opposite as generally observed, as social facilitation or co-operation during prey location is usually followed by more intense aggression shortly after feeding in many species, as *e.g. Gadus morhua* **(Brawn 1961, 1969).** Comfort behaviors were not affected by increasing feeding rates except resting. Where fish fed 3.8% of their biomass showed more resting behavior.

Table (3) Effect of different feeding rates on weight gain, relative growth rate {RGR} and feed conversion ratio {FCR} of Tilapia (LSMeans):

Growth performance	Feeding rates			s.e.d	P-value
	3.4%	3.6%	3.8%		
Weight gain (g)	41.29[c]	48.48[b]	55.84[a]	2.52	0.038
RGR (%)	112.48[c]	120.02[b]	125.48[a]	1.72	0.033
FCR	1.70	1.60	1.51	0.073	NS

NS: non significant. s.e.d: standard error of difference. a-b-c: LS Mean with different superscripts were significantly different

Table (4): Effect different feeding rates of on Tilapia body dimensions (cm) (LSMeans):

Body dimensions	Feeding rates			s.e.d	P-value
	3.4%	3.6%	3.8%		
Total length	14.93[b]	15.63[a]	15.71[a]	0.23	0.03
Standard length	12.23[b]	12.73[a]	12.91[a]	0.17	0.028
Jaw length	1.10	1.15	1.10	0.05	NS
Head length	4.20	4.32	4.31	0.06	NS

NS: non significant. s.e.d: standard error of difference. a-b: LS Mean with different superscripts were significantly different

Table (5): Effect of different feeding rates on water quality parameters (LSMeans):

Water quality parameters	Feeding rates			s.e.d	P-value
	3.4%	3.6%	3.8%		
Nitrite(mg/l)	1.71	1.60	1.75	0.27	NS
Nitrate(mg/l)	36.82	34.87	38.42	5.71	NS
Dissolved oxygen(ppm)	5.29	5.20	5.34	0.15	NS
PH	7.80	7.74	7.81	0.03	NS

NS: non significant. s.e.d: standard error of difference.

By comparison between aquarium and pond, low enriched environment in the aquarium allowed Tilapia to be showed behavioral adaptation by spending more time in resting in addition to feeding and foraging activities than aggression as shown in 3.8% group. Conversely, in 3.4% and 3.6% groups fish spent more time in social interactions because these fish did not found a suitable shelter for hiding and resting as holes or stones as in their normal home pond. Natural behavior is positively motivated and promotes biological functioning as it is the best way of portraying the preferences and evolutionary capabilities of a species to adapt to a given environment **(Bracke and Hopster 2006)**.

In this study, numerically the best feed conversion ratio was recorded in fish fed 3.8%. In the same time, there were no significant differences in feed conversion rate between different feed application rates. These results were differed with **Anderson and Fast (1991)** who reported that feeding ration greater than optimum feed level would increase the waste food and increase the feed conversion ratio in Chinese catfish. These variations may be attributed to species differences and more feeding and foraging activities that may be play role in reducing the food waste.

Moreover, during the current study, the fish body weight, weight gain and relative growth rate were enhanced by increasing feeding rate.

Fish fed 3.8% feeding rate had higher body weight, weight gain and relative growth rate than fish fed 3.4% and 3.6%. These findings were in accordance with **Abdel-hakim *et al.*, (2006)** who found that Tilapia body weight and weight gain were enhanced by increasing feeding rates in poly culture system and **Marimuthu *et al.*, (2011)** who suggested that high feeding rates till 12% in African catfish fingerlings improving growth rate including body weight and specific growth rate.

Total and standard lengths were significantly increased by increasing feeding rates. Generally, the body dimensions (total and standard lengths) were depended on body weight of fish. These results were disagreed with **Fafioye and Oluajo (2005)** who found that the rate of increase in body length of *Clarias gariepinus* was not proportional to the rate of increase in body weight. These differences may be attributed to species difference.

Water quality is considered as an important factor in rearing of fish under culture conditions **(FAO, 1999)**. Overfeeding and waste food disrupts the water quality **(Ng *et al.*, 2000)**. Nitrite enters a fish culture system after feed is digested by fish and the excess nitrogen is converted into ammonia, which is then excreted as waste into the water. Total ammonia nitrogen is then converted to nitrite (NO2) which, under normal conditions, is quickly converted to non-toxic nitrate (NO3) by naturally occurring bacteria **(Robert *et al.*, 1997)**. Fish digest the protein in their feed and excrete ammonia through their gills and in their feces. In this study, the water quality parameters including nitrite, nitrate, dissolved oxygen and pH were within the appropriate ranges for Tilapia culture and no apparent influence of these parameters on Tilapia growth was recorded especially in fish fed higher rate (3.8%). These results may be attributed to increase feeding and foraging activities of fish in 3.8% group and behavioral adaptation of fish to high level of food which led to decrease food wastage that may deteriorate water quality.

The proportion of total ammonia nitrogen in the toxic form increases as the temperature and pH of the water increased **(Robert *et al.*, 1997)**. Therefore, in the present study, the amount of ammonia which excreted through fish gills may be still in non toxic form because water temperature was unchanged and PH of water was within normal range for Tilapia throughout the experiment. By contrast these results, **Marimuthu *et al.*, (2011)** found that the increased rate of feed associated with increased amount of feed offered to fishes reflected on water quality with respect to dissolved oxygen, but the

parameters as temperature and pH were within the normal ranges for catfish culture. These differences may be attributed to fish species difference and high level of feeding and foraging activities of fish that recorded in the current study.

In general, Tilapia fish had ability of behavioral adaptation to high feeding rates by increasing feeding and foraging activities that reflected on enhancing of growth performance by improving fish body weight, weight gain and relative growth rate of fish in 3.8% group. In the same time, high level of feeding and foraging activities decreased the possibility of water deterioration occurrence as water quality parameters did not changed by increasing feeding rates in this study. Moreover, long stay of food in front of fish fed 3.8% feeding rate had a benefit effect on fish behavior especially decreasing interspecies aggression between fish in 3.8% group. Consequently, the stress that may be occurred due to fish aggression was reduced.

Conclusion

It was concluded that increasing feeding rates in Tilapia improved feeding and foraging activities. Furthermore, first stage of aggressive behavior (chasing) was reduced by increasing feeding rates. The adaptation of fish to high feeding rates by increasing of ingestive behavior reflected on Tilapia growth performance where body weight, weight gain, relative growth rate and feed conversion rate were enhanced. Consequently, these results suggested that feeding Tilapia on 3.8% of their biomass gave the highest growth performance and enhanced the fish behavior in the present study.

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