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Original Article

Antibody Response of Broiler Chickens against Eight Commercial Infectious Bursal Disease Live Vaccines tested by ELISA

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Abstract

This study was designated to investigate the antibody response of broiler chickens against eight commercial IBD live vaccines. A total of 460 one- day Ross broiler chicks were divided to 9 groups, eight groups were vaccinated with IBD live vaccines and the last one was served as control. Four groups were vaccinated with intermediated vaccines, whereas the other fourth were given intermediated plus vaccines. All vaccinated groups were administrated at 14th day of age via drinking water route. Maternal derived antibody (MDA) and post- vaccination antibody response were tested by ELISA. Blood samples were collected at one day old and at 21st, 28th and 35th day of age post- vaccination. Indirect ELISA test revealed that the mean of maternal derived antibody was 4852±745. Significant differences ($P<0.05$) among means of antibody titers of all vaccinated groups were found at 21st ,28th and 35th day of age compared with that of control group. The results also showed that groups which were vaccinated with intermediated plus vaccines (E and H vaccines) exhibited high level of antibody especially groups 5 and 8 than those which vaccinated with intermediate vaccines. In conclusion, Intermediate plus vaccines induced higher antibody titers than other vaccines, although some intermediate vaccines induced similar titers of antibody .E and H vaccines which were administered to groups 5 and 8 respectively induced better antibody titers.

Keywords: IBD live vaccines; ELISA, antibody; Ross broiler chicks

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Introduction

Infectious bursal disease (IBD) is a major poultry pathogen in the poultry industry (Hein *et al.,* 2002). In practice different vaccination schedules have been recommended and used, but despite these vaccination schedules outbreaks of IBD are still reported (Zaheer and Saeed, 2003). Up to date more than 46 strains of imported IBD vaccines are used to control the disease (Chin, 1993).

Susceptibility to IBDV varies with age, immunological factors, cytokine production of the chickens. Chickens obtained from vaccinated hens had different levels of maternal antibody depending on age, health status, races or genetically factors of the hens. Vaccination represents a very useful method in IBDV controlling (Vegad, 2004). The right strategy for IBD control and its success rate under field conditions depends on hygiene management, IBD field pressure, level and variation in maternally derived IBD antibodies, and the IBD vaccine strains to be used (Block *et al.*, 2007). The efficacy of IBD vaccine in broilers was related to the level of maternal derived antibody (MDA) against IBD at the vaccination date. Vaccination at 1- day-old, 1 and 16-day-old and 16-day-old of chickens that have ELISA titers of MDA of more than 6,000 at 1-day-old may not be effective enough to elicit the antibodies at 30-day-old (Sarachai *et al*., 2010).

Timing of optimal vaccination, doses used and administration routes represent the most important factors in controlling the disease. Active attenuated vaccines give better response, because the inactive ones prove to be less efficient for inducing the active immunity of the chickens with maternal antibodies. Serological methods used for determination of the IBDV titers are seroprecipitation, viral neutralization and ELISA (Muller *et al.,* 2002; Eterradossi and Saif, 2008). The aim of this study was to evaluate the antibody response of 8 commercial IBD vaccines determined by ELISA in broiler chicks.

Materials and Methods

Four hundred and sixty 1-day-old ROSS broiler chickens were allotted into 9 groups namely (1,2,3,4,5,6,7,8 and 9) , 8 groups (from 1-8) were vaccinated with IBD vaccine and the last one (9) was served as control. They were placed into separate sterile cages at the experimental house of the Department of Pathology and Poultry Diseases, College of Veterinary Medicine, Basra University under strict hygienic and standard management conditions.

Eight commercial Freeze-dried live vaccines namely (A,B,C,D,E,F,G and H) were given at 14 days of age via intracrop route, groups 1, 2, 3 and 4 were vaccinated with intermediate strain, while groups 5, 6,7 and 8 were given intermediate plus strain. Group 9 was acted as control unvaccinated group. The vaccines were reconstituted in distilled water to obtain one field dose in 0.5 ml, and given intracrop by a blunted syringe to ensure that all birds has been received the correct dose of the vaccine.

Blood was collected from 10 birds to measure the MDA at the first day of age, as well as at 21st, 28th and 35th days post-vaccination to measure Ab response. It was taken from the main brachial wing vein or by heart puncture using 5ml disposable syringes. Three ml of blood from bird was collect from five randomly selected birds of each group was collected in clean, dry and sterile tubes. The tubes were stoppered and left in slant position for one hour at room temperature and then left for another one hour at 4°C then centrifuged at 3000 rpm for 15 minutes. Serum samples were carefully separated in a small Eppendorf vials, labeled (El-Kady *et al.,* 2007) and heat inactivated and subjected to ELISA test (Rautenschlein *et al.,* 2004).

Enzyme Linked Immuno-sorbent Assay (ELISA) technique was carried out according to the method described by Symbiotic Laboratories Incorporation, USA. Briefly, the antigen coated

plates and ELISA kit reagents were adjusted at room temperature prior to the test. The test sample was diluted five hundred folds (1:500) with sample diluent prior to the assay. A 100 µl of diluted sample was then placed into each well of the plate followed by 100 µl of undiluted negative control into well A1 and A2 and 100 µl of undiluted positive control into well A3 and A4. The plate was incubated for 30 min at room temperature. Each well was then washed with approximately 300 of distilled water for 3 times. 100 µl horseradish peroxidase conjugated anti-chicken IgG was dispensed into each well. The plate was incubated at room temperature for 30 min, followed by washing each well with 300 distilled water for 3 times. A 100 µl substrate solution was dispensed into each well. The plate was then incubated at room temperature for 15 min. Finally 100 µl of stop solution were dispensed into each well to stop the reaction. The absorbance values were measured and recorded at wave length of 405nm using ELISA reader. IBD antibody titers and sample absorbance to positive control absorbance (S/ P) ratio were calculated to interpret the results. (Alam *et al*., 2002).

The data obtained in the study was analyzed using the two-way and one-way analysis of variance (ANOVA) so as to determine the significance of differences between groups of data.

Results and Discussion

The present study revealed that MDA which was measured at the 1st day of age was 4852±745. This result was in agreement with that of Kreider *et al.,* (1991) who divided the ELISA titer of the MDA of 1-day-old chickens into 3 level; the low level (<3,000), intermediate level (3,000-5,000) and high level (>6,000).

The result of this study which could be categorized in the intermediate level of MDA might be attributed to the fact that the parents stock of these birds had high antibody titers which might be due to either vaccination or infection.

The antibody titer of unvaccinated group was gradually declined from 21st (373±0.303) to 28th (209±0.532) and 35th (77±0.048) day of chicken's age. This result was in agreement with those of Amar *et al.,* (2007); Chansiripornchai and Sasipreeyajan, (2009) observed that the MDA was gradually decline in the control non-vaccinated group till the end of the experiment. According to Skeeles *et al.,* (1979) the half-life of MDA was 3-3.5 days .Declination of antibodies may be attributed to several factors such as the proteolytic degradation of antibodies or neutralization due to naturally occurring/persisting IBDV, which possibly would be the primary factor.

Table (1) demonstrated that there were significant differences ($p<0.05$) in the antibody titers among all vaccinated groups at 21st, 28th and 35th day of age post- vaccination. The antibody titers were significantly increased ($p<0.05$) at 21st to 28th and decrease at day 35th post-vaccination for all vaccinated groups. The highest level of antibody titer was shown in group 8(vaccine H) as shown in (Figure1). The antibody titer of chickens, which were immunized at 14th day of age, was significantly increased from day 21st to day 28th and decreased at day 35th of chicken's age for all vaccinated groups.

The result were in agreement with those of Afshin and Mir Hadi, (2011) who indicated that differences between the means of antibody titers of all groups were significant ($P<0.05$), at 7 and 14 days post vaccination in comparison with control group.

Table (1) Mean antibody titers of experimental groups against IBD vaccines at different ages.

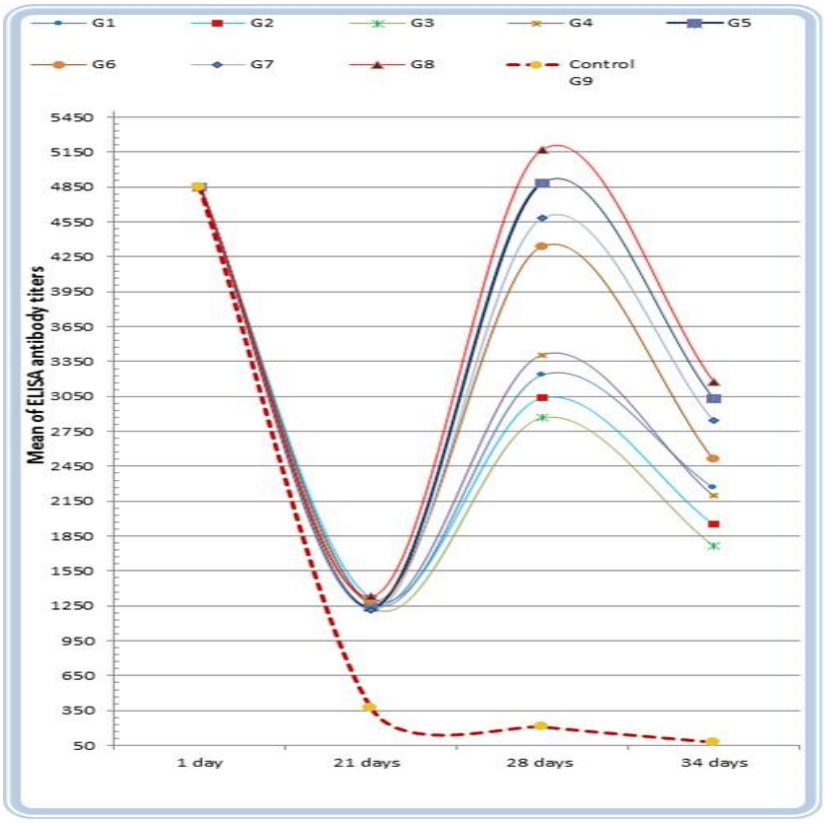
Experimental groups and vaccines	* Mean Ab titer ± SE at different ages		
	21st days	28th days	35th days
G1(A)	1269±0.830 b B	3233±0.167 b C	2265±0.695 b C
G2(B)	1242±0.715 b B	2867±0.897 b C	1766±0.480 b B
G3(C)	1316±0.798 b B	3037±0.318 c C	1948±0.054 b B
G4(D)	1276±0.911 b B	3408±0.259 c C	2197±0.410 b C
G5(E)	1237±0.032 b B	4886±0.585 c BC	3034±0.449 b C
G6(F)	1287±0.902 b B	4340±0.303 c BC	2510±0.267 b C
G7(G)	1212±0.140 b B	4581±0.616 c BC	2841±0.713 b C
G8(H)	1331±0.275 b B	5171±0.028 c BC	3179±0.186 b C
Control G9	373±0.303 a A	209±0.532 a A	77±0.048 a A

Figures with different superscripts in the vertical and horizontal columns were significantly differed at ($p<0.05$) in comparison with the control group. *Five birds in each group.
(A to H refers to vaccines).

Hair-Bejo *et al*., (2004) recommended vaccination of broilers at fourteen days of age because vaccine administration at this age induced high and protective level of IBD antibodies. Al-Mayah, (2009) mentioned that vaccination at the 14th day of age induced high and protective level of IBD antibody titer up to 28th day of age.

This may be due to the ability of vaccine at this time of vaccination to neutralize different levels of MDA.

Figure (1) Antibody titers against IBDV of experimental groups at different times measured by ELISA.



The result of the present study were also in agreement with those of Amer et al., (2007) who stated that the ELISA antibody titers from vaccination with intermediate vaccines were the .lowest at all intervals while the titers of intermediate plus vaccine were the highest
In conclusion, Intermediate plus vaccines induced higher antibody titers than the other vaccines, although some intermediate vaccines induced similar titers of antibody.
The commercial E and H vaccines which were given to group 5 and 8 respectively, were induced better antibody titers. The commercial vaccine names were known .but were not revealed to avoid commercialization. The objective of this experiment was not to determine which vaccine was the best ,but rather to determine whether current vaccines commonly used in the field would correlate with the protection against new IBD outbreaks in 2 weeks vaccinated broiler chicks.

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Original Article

Alteration of some enzymatic activities in whey of ewe's milk Suffered from *Staphylococcal* mastitis

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Abstract

The present experiment was conducted to study variation in milk California mastitis test(CMT) white side test(WST) and chloride test , pH test**,** along with activities of whey enzymes lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and aspartate aminotransferase (AST) in relation to staphylococcal mastitis in lactating ewes. A total of 310 milk samples were collected from the udder halves of 161 dairy ewes at mid period of lactation to determine the percentage of Staphylococcus mastitis. The overall percentage of infection with clinical and subclinical Staphylococcal mastitis was found to be 2.25 % and 12.22% respectively. All samples were subjected to bacteriological examination and the following staphylococcal species were isolated, *coagulase negative Staphylococcus* (1.29% & 27.8%) and *Staphylococcus aureus* (27.8% &12.22%) from clinical and subclinical mastitis respectively. The whey samples were divided into three groups: a non-infected group, subclinical infected group and clinical infected group for estimation of enzymes. Activities of LDH, ALP and AST were significantly higher in milk from the subclinical and clinical mastitis groups for S. aureus and coagulase negative *Staphylococcus*(CNS) (AST:222.09±31.54 ;194±27.15&271.82 ±30.50 ;201.0 ±49.51;ALP:837.08±63.57; 866.01±215.36& 884.22±26.08 ;807.45± 47.05LDH:332.95±5.67& 289.83±32.95;344.2 ±21.17 ;307.62± 72.77) respectively, than in non-infected group(AST: 38.84±2.71; ALP: 187.91±5.54; LDH: 142.59± 5.67).

In conclusions the results of the present study showed that the measurement of AST, LDH and ALP activities in milk samples could be used as reliable method and suitable for detection of ovine subclinical mastitis.

Keywords: Lactate dehydrogenase, Alkaline phosphatase, Subclinical mastitis, clinical mastitis

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Introduction

Mastitis namely, clinical and subclinical, is an economically damaging disease of the dairy industry, which causes physical, chemical and bacteriological alternation in the milk and blood along with morpho-pathological changes in the mammary gland (Guha*et al*.,2012).

Staphylococcus aureus is an opportunistic pathogen in dairy ruminant where it is found in healthy carriage and can be a major cause of mastitis (Seyffert *et al*., 2012). It is classified among the most serious pathogens causing clinical symptoms of various diseases not only in animals, but also in human (VASIĽ, 2007). De Santis *et al*.,(2005) found that the *S. aureus* isolates from sheep with subclinical mastitis are less enterotoxigenic (34.4%) than isolates from acute clinical mastitis (70–80%). Also the coagulase negative *Staphylococci*(CNS) are the most prevalent important pathogen which reported by most scientist (Pradiee *et al*.,2012; Gebrewahid *et al*.,2012).

Determination of enzymes activity might serve as a possible method for detection of subclinical mastitis and other udder diseases (Kitchen *et al*., 1970). It has been reported that the mean activity of lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) were higher in milk from subclinical mastitis (SCM) udders than in milk from health udders (Batavani, 2007).

The aim of this study was to investigate alteration of some enzymatic activities in whey of ewes milk suffered from clinical and subclinical Staphylococcal mastitis.

Materials and Methods

Ewes

One hundred sixty one lactating ewes at 2-6 years of age, from Al-Anbar province were used in this study, ewes were examined clinically to confirm infection with mastitis or apparently normal. The study was carried out over a 6 months period starting from October 2012 to March 2013.

Examination of ewes

Systemic reactions (temperature, pulse and respiratory rate) and local signs on the udder (hotness, redness and swelling) were recorded.

Collection of Samples

Three hundred ten milk samples from 161 ewes were collected aseptically, udder and teats were washed with water and then the teat end were disinfected with cotton soaked in 70% alcohol solution. The first three stripped milk were discarded and 20 ml of milk was collected. These samples transported immediately to the laboratory by cooling box then under aseptic condition (Radostits *et al*., 2007).

Examination of milk samples

Milk samples were examined for:

1-Phyically, chemically and bacteriologically

A- Physical Examination: Which include: Color, odor and consistency of the milk.

B-Chemical tests: Include White Side Test (Coles, 1986), California Mastitis Test (Schalmet al., 1971) and Chloride test (Coles, 1986) and pH test (Coles, 1986) performed on the normal apparent milk samples.

C-Bacteriological examination: Isolation and identification of bacteria from milk samples were performed according to (Quinn *et al*.,2004), All milk samples were cultured on blood agar and nutrient agar, incubated at 37 Cº for 24 hrs. Diagnosis depends on morphological character (shape, color and size of colony) and type of hemolysis on sheep blood agar. Hemolytic colonies were subjected to Gram stain, then suspected isolates subculture on Staph-110 agar ,mannitol salt agar , and chrome agar(specific for *Staphylococcus aureus*) and biochemical tests(catalase, oxidase, Gelatin liquefaction, Urease, O/F test, Sugar fermentation and tube coagulase test) were used for identification of *Staphylococcus aureus* isolates.

2-Biochemical analysis (Enzymes) in whey:

Ten milliliter of milk were centrifuged in cooled centrifuge high speed to separate whey of milk, after that the AST, ALP, LDH were measured by spectrophotometer by using commercial kits (Bio-Merieux, Laboratory reagents and Products, Marcy-I' Etoile, France).

Statistical analysis

All data are represented as means ± SE. One way analysis of variance (One-way ANOVA) by using SPSS program, followed by Least Significant Difference (LSD) test were used to determine differences among means of investigated groups. The level of statistical significant was set at ($P < 0.05$) (Snedecor and Cochran,1989).

Results

Clinical mastitis

Out of 161 ewes examined physically and bactiologically for mastitis, 5 ewes (10 halves) showed clinical mastitis (acute and chronic mastitis) after physical examination. Seven samples showed clinical Staphylococcal mastitis in a percentage of (2.25%) (Table, 1).

Table (1) Percentage of *Staphylococcus aureus* and CNS in clinical cases of mastitis

No.	No. of examined ewes	clinical mastitis	No with *Staphylococcal* spp.	+ve results for *Staph aureus*	+ve results for CNS
Ewes	161	5	4	**2**	**2**
Milk samples	310	10	7	3	4
%		3.22%	2.25%	0.96%	1.29%

Chemical tests

Relation between CMT and bacteriology

The percentage of *S. aureus* was 12.22% in a +ve samples for CMT, While the percentage of Coagulase negative staphylococci (CNS) was 27.8% (Table 2).

Table (3) showed the distribution of *S.aureus* and CNS isolates at different scores of CMT {±, +1, +2, +3}. The CMT +1 and +2 had the highest percent.

The percentage of *S. aureus* in relation to White side test (WST) was 2.66%. While coagulase negative (CNS) isolates give higher percentage than *S. aureus* which reach a percentage of 12.66 %(table 4).

Table (2).Relation between CMT and bacteriology

No of Milk samples examined	+ve *S. aureus* from all +ve CMT	+ve S.aureus from –ve CMT	+ve CNS from all +ve for CMT	+ve for CNS from all –ve CMT
300	11	2	40	13
%	12.22%	0.95%	27.8%	6.2%

Table (3) Relation between CMT scores and Staphylococcal spp. isolation

CMT scores	No of samples +ve to CMT	*+ve for S.aureus*	+ve for CNS
±	21	2	9
+	30	3	10
++	38	6	21
+++	1	0	0
Total	51	11	40
		21.57%	78.43%

Relation between White side test (WST) and Bacteriology

Table (4) Relation between White side test (WST) and Bacteriology.

No of Milk samples examined	Samples +ve for *Staph aureus* from all +veWST	Samples +ve *Staph aureus* and -ve for WST	Samples +ve CNS and +ve for WST	Samples +ve CNS and -ve for WST
300	8	5	38	15
%	2.66%	1.66%	12.66%	5%

Relation of mastitis with enzymes activities

The whey samples were divided into three groups: a non-infected group, subclinical infected group and clinical infected group for estimation of biochemical analysis. Milk serum(whey) activities of LDH, ALP and AST were significantly higher in the subclinical and clinical infected group thanfrom non-infected group in both *S.aureus*and CNS at ($P<0.05$) (Table 5).
Table (6) showed the efficacy of chemical tests and enzymatic activities used for detection of subclinical *Staphylococcal* mastitis, enzymatic activities revealed a higher percentage 100% than other chemical tests for detection subclinical mastitis in relation with isolation of bacteria.

Figure (1) Chemical tests and enzymes activities in relation with isolated bacteria

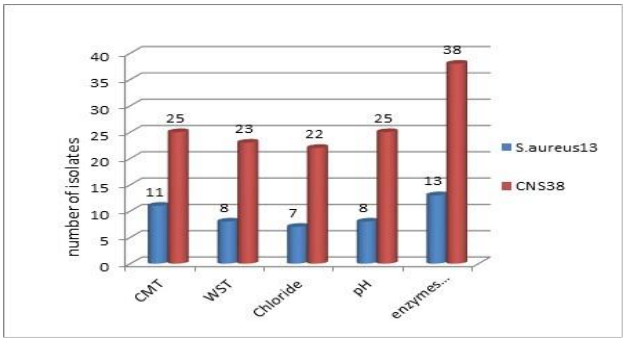

Table (5) Relation of Staphylococcus spp. mastitis with enzymes

Staphylococcus spp.	**AST**	**ALP**	**LDH**
	Means +SE*	Means +SE*	**Means +SE***
Non_ infected	38.84 B 2.71±	187.91 B 5.54±	142.59 B 5.67±
Subclinical mastitis S. aureus(13)	222.09 A 31.54±	837.08 A 63.57±	332.95 A 31.82±
Subclinical mastitis CNS(53)	271.82 A 30.50±	807.45 A 47.05±	344.2 A 21.17±
Clinical mastitis S. aureus(3)	194.0 A 27.15±	866.01 A 215.36±	289.83 A 32.95±
clinical mastitis CNS(4)	201.0 A 49.51±	884.22 A 26.08±	307.62 A 72.77±

The different capital letters refer significant variations at ($P<0.05$)

Table (6) Efficacy of chemical tests and enzymes activities in relation with isolated bacteria

No of bacteria isolated	Chemical tests				+ve for enzymes activities
	+ve samples for CMT	+ve samples for WST	+ve samples for Chloride test	+ve for pH test	
S. aureus 13 %	11 **84.61%**	8 **61.53%**	7 **53.84%**	8 **61.53%**	13 **100%**
CNS 38 %	25 **65.78%**	23 **60.52%**	22 **57.89%**	**25** **65.78%**	38 **100%**

Discussion

In this study, we found that the percentage of subclinical mastitis was higher than clinical mastitis. McDougall et al (2002) reported a prevalence of SCM 19.0% and a similar result obtained by Contreras et al (2007) who noticed a prevalence of SCM 5-30% in goats.

In this study CMT test showed higher prevalence rate of subclinical mastitis than other tests (WST, chloride & pH tests), California mastitis test indirectly detect increased number of leukocytes in mammary secretion there for can be considered as a good test and more accurate diagnostic technique for detection of subclinical mastitis (Schalm *et al*., 1971). CMT scores values in our result was compatible with those obtained by other authors (De la Cruz*et al*.,1994; Fthenakis,1994) and according to these studies the predictive value of positive result is mainly influenced by the prevalence of mammary infections in the flocks. Also our result revealed that scores +1 &+2 of CMT had the highest diagnostic accuracy. This result is in agreement with (Fthenakis, 1994) which recorded that score +2 of CMT was appropriate threshold value for detection of subclinical mastitis.

Intramammary infections caused by *S. aureus* warrant special attention because this bacterium is responsible for both acute clinical mastitis and subclinical mastitis as recorded by (Contreras *et al*.,2007). Our results for CNS isolation agree with a result of Rahim *et al*., (2010) which found that Coagulase-negative staphylococci (CNS) were the most prevalent species. Also similar to results of (Dadkhah,2012) who found that the most prevalent species were Coagulase-negative staphylococci (CNS) (71%), followed by *Staphylococcus aureus* (12%).

Our results of bacterial isolation seem to be lower that results of other researchers,(Watson *et al*.,1990),(Tormod *et al*.,2007), (Yousif,1982), who recorded a percentage (65.3) ,(90%)(57.60%) respectively.

The enzymes (AST,ALP, LDH) are secreted by the epithelial cells of mammary gland. In mastitis, muscle, tissues of mammary gland are damaged which may lead to increase in the level of these enzymes. (Khodke *et al*.,2009). The results of the present study showed that the means of AST ,ALP & LDH activities in milks from ewes with clinical & subclinical mastitis were significantly ($P< 0.05$) higher than those from healthy normal ewes. this indicate that using of determination of enzymes activities in serum milk is a sensitive and reliable method for detection of ovine subclinical mastitis. The results is in agreement with (Batavani *et al*., 2007) who found that the increased in milk enzymes including lactate dehydrogenase, aspartate aminotransferase and alkaline phosphatase in mastitic animals might be linked with tissue damage occurring in mammary tissue. It is also in agreement with result of (Hussain *et al*., 2012) who concludes that the enzymes including lactate dehydrogenase, aspartate aminotransferase and alkaline phosphatase were significantly higher in mastitis than healthy buffaloes. (Katsoulos *et al*., 2009) conclude that the determination of LDH activity in milk serum is a sensitive and reliable method for the detection of subclinical IMI in dairy sheep and goats.

Moreover, (Fruganti *et al*.,1986) found that the increase in LDH and ALP activities were associated with clinical mastitis and to lesser extent with subclinical mastitis. In contrast, we don't agree with a study of (Yang *et al*., 2011) who found that milk AST activity was not significantly different between normal and sub clinical infected udders.

Conclusion

Alteration in enzymatic activity can be used as reliable method for detection of subclinical mastitis in dairy ewes. Early diagnosis of subclinical mastitis in dairy animals may be important in reducing production losses and enhancing prospects of recover herds in order to avoid the development of clinical mastitis.

Acknowledgment

The author would like to thank staff at the College of Veterinary Medicine / Department of Internal and Preventative Medicine in Al-Fallujah for their help and support.

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Original Article

Comparison of Pathogenicity of Four Commercial IBD Intermediate Live Vaccines in Broilers

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Abstract

This study was designed to investigate four commercial intermediate live vaccines against infectious bursal disease (IBD). One hundred and fifty, 1-day-old ROSS broiler chicks divided randomly into 5 groups. Each group was consisted of 30 birds.The birds were vaccinated with an intermediate IBD vaccines namely A,B,C,and D vaccines at 14 days of age *via* intracrop route except group 5 which was acted as control unvaccinated group. Following vaccinations, different parameters were used in this investigation including; clinical signs and gross lesions, lymphoid organs indices and histopathological changes.The result indicated that no clinical signs and gross lesions were observed on vaccinated birds. Significant increase ($P<0.05$) in bursal index at 17^{th} day of age in group 4, whereas a significant reduction ($P<0.05$) at 28^{th} days of age has been noticed which indicated bursal atrophy as compared with control and other vaccinated groups. Spleen index revealed significant reduction ($P<0.05$) at 28^{th} days of age in the same group as compared with control and other vaccinated groups throughout the experiment.Thymus index revealed significant reduction ($P<0.05$) in group1 at 28^{th} days of age as compared with control and other vaccinated groups. Histological examination of bursa of Fabrics (BF), spleen and thymus revealed that all type of vaccines induced different degree of alterations in these organs. The organs in group1, 2 and 4 showed similar degree of changes which characterized by an edema and degeneration in the medullary area of bursal follicles.Spleen of groups1 and 2 showed follicular necrosis and sinusoidal congestion, whereas that of group 4 showed hydropic degeneration in the epithelial layer.Thymus in group1, 2, 4 exhibited congestion and hemorrhage in the medulla with lymphocytic depletion. Bursa of group 3 showed thickened capsule whereas spleen showed hydropic degeneration in the epithelial layer of the blood vessels, whereas the thymus changes represented by focal area of hemorrhage. Study of the pathogenicity of four commercial IBD vaccines showed considerable variation in their pathogenicity. In conclusion, vaccine D proved to be more pathogenic than A, B, and C vaccines.This was supported by bursal, spleen and thymus reduction and bursal score indices.

Keywords: BF; Thymus index; IBD Intermediate; lymphocytic depletion.

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Introduction

Poultry industry has expanded rapidly over the last fourth decades and is playing a vital role in the economy of the country.However the industry is confronted with a variety of problems, particularly the diseases of viral origin. The major problem of this business is the outbreak of infectious diseases, including Infectious Bursal Disease (IBD) (Phatak, 2002).

The IBD is particularly important due to high mortalities,lowered productivity among infected chicks and immunodepression to others (Sahar *et al*., 2004). The IBD virus replicates extensively in IgM (+) cells of the bursa and chickens may die during the acute phase of the disease, although IBD virus-induced mortality is highly variable and depends, among other factors, upon the virulence of the virus strain (Balamurugan and Kataria, 2006). The virus is resistant to a large variety of disinfectants and is environmentally very stable but may be controlled using a proper vaccination schedule. Vaccination represents a very useful method in IBDV controlling (Dacic, *et al*., 2008).

Although live vaccines have been shown to be very efficacious against a variety of poultry diseases, two consequences of vaccinating concurrently with several vaccines are immunosuppression and vaccine interference. Immunosuppression has been associated with the use of live IBDvirus vaccine as this virus replicate in cells associated with immune responses. In addition, vaccines that have similar tropisms are known to interfere with the immune response to each other (Cook *et al*., 2001; Ganapathy *et al*., 2005). Rautenschlein *et al.,* (2003) compared immunopathogenesis of mild, intermediate and virulent strains of classic IBD viruses and showed that the most virulent strain induced the most prominent bursal damage and significant suppression of the mitogenic response and the mild vaccines induced no detectable lesions in the bursa.

At the present time, there is more than a company which produces of the IBDV vaccines but there are more than strains differ in the virulence so the strains varying in the effect on the lymphatic system. The veterinary vaccines must be controlled and assessed by neutral and independent researcher in different countries, before admit for commercial use (Martinez *et al*., 2002). This study was designed to determine the pathogenicity of four commercially available IBD live intermediate vaccines administrated at 14-day-old chicks.

Materials and Methods

One hundred and fifty, 1-day-old ROSS broiler chicks were allotted into 5 groups, 4 groups were vaccinated with commercial freeze –dried live vaccines, namely (A,B,C and D vaccines respectively) at 14 days of age *via* intercrop route and the other one was served as control. The commercial vaccines names were known, but were not revealed to avoid comercializam.

The birds were placed into separate sterile cages at the experimental house of the Department of Pathology and Poultry Diseases, College of Veterinary Medicine, Basra University under strict hygienic and standard management conditions. The vaccines were reconstituted in distilled water to obtain one field dose in 0.5 ml, and given intercrop by a blunted syringe to ensure that all birds has been received the dose of the vaccine.

At 17^{th} and 28^{th} day of age, five birds were randomly selected from each group and individually weighted, sacrificed and necropsied. The BF, spleen and thymus were removed and weighed before fixation for histopathological observations. Bursa, spleen and thymus/body

weight ratios were calculated for each bird and expressed as arithmetic means in each group of birds by the following formula:Organ index = organ weight in grams / body weight in grams x1000 (Hedayati *et al*., 2005).

Samples of bursa, spleen and thymus were fixed in 10% buffered formalin for histopathological examination and stained with Haematoxylin and Eosin (HE).The bursa, thymus and spleen were examined using optic microscope for histological study (Luna, 1968).

Effects of used IBD vaccines were assessed in terms of bursal, thymus and spleen indices and microscopic examination of lymphoid organs.

Bursas were scored according to Rosales *et al*. (1989) and assigned as; 1= no lesion (normal); 2 = focal mild lymphocytic depletion; 3 = multifocal, 1/3 to ½ of the follicles show lymphocytes depletion; and 4 = diffuse lymphocytes depletion of all follicles

The data of organ indices were subjected to analysis and the significant differences at ($p<0.05$) which were determined by two ways ANOVA Statistical software sigma stat statistical (Version 19.0, SPSS Inc, Chicago, Illinois, USA, 2010).

Results

Chicks of all vaccinated groups did not exhibit any clinical signs and there was no mortality throughout the experiment.

Bursal index (BI) was increased at 17^{th} day and reduced at 28^{th} day post-vaccination with live Gumboro disease vaccines in all vaccinated groups.Group 4 revealed a significant increase ($P<0.05$) among other groups which was (2.222 ± 0.131) at 17^{th} day .On the other hand this group showed significant reduction ($P<0.05$) which was (1.400 ± 0.089) at 28^{th} days .Significant reduction of spleen index ($P<0.05$) was also noted in G 4 (0.898 ± 0.057) at 28th day of age as compared with control group and other vaccinated groups (Table 1).

The bursal lesions scoring for the G1, G2 and G4 at 17^{th} days post vaccination was 3 lesions, which included an edematous area in fibrous capsule and increasing of interfollicular space due to decrease number of lymphocytes and follicular atrophy (Figure.1). At 28^{th} days the scores of G1, G2 and G4 were 4 lesions, which appeared more severe as increasing of interfollicle space, degeneration in the medullary area and depletion of lymphocytes in bursal follicles, disappearance of demarcation between cortical and medullary area and bursal follicles atrophy (Figure.2,4)

The bursal lesion score of G 3 was 2 lesions at 17^{th} day which appeared as hyperplasia of interfollicular septa, mild depletion of lymphocytes in some bursal follicles (Figure.3). At 28^{th} day was become 3 when characteristic feature of fibroplasia which was responsible for interfollicular connective tissue formation and thickening capsule has been occurred.

Table (1): Results of bursal, spleen and thymus indices following vaccination with commercial live intermediate IBD vaccines at different ages.

Experimental Groups and vaccines	Mean ± SE of bursal index		Mean ± SE of Spleen index		Mean ± SE of Thymus index	
	17th days	28th days	17th days	28th days	17th days	28th days
G1(A)	*1.962 ±0.013a A	1.692 ±0.088a A	1.106 ±0.027a A	0.996 ±0.011a A	3.300 ±0.200a A	2.740 ±0.097b A
G2(B)	2.064 ±0.097a A	1.674 ±0.091a A	1.100 ±0.026a A	0.988 ±0.072a A	3.208 ±0.210a A	2.964 ±0.285a A
G3(C)	1.944 ±0.034a A	1.702 ±0.091a A	1.012 ±0.096a A	1.016 ±0.077a A	3.020 ±0.148a A	2.928 ±0.218a A
G4(D)	2.222 ±0.131b A	1.400 ±0.089b B	1.046 ±0.056a A	0.898 ±0.057b A	3.080 ±0.131a A	2.862 ±0.301a A
Control G5 Unvaccinated	1.870 ±0.021a A	1.974 ±0.017a A	1.144 ±0.085a A	1.140 ±0.037a A	3.364 ±0.133a A	3.444 ±0.218a A

Values followed by different letters (Capital letters= horizontally; small letters= vertically) were significantly different ($p<0.05$) in comparison with the control group.
* Five birds in each group.

Table (2): Bursal lesion scoring in experimental groups at different ages

Age (days)	Experimental groups				
	G1	G2	G3	G4	G5 Control
17th days	**3**	**3**	**2**	**3**	**0**
28th days	**4**	**4**	**3**	**4**	**0**

Control unvaccinated group (G5) in the experiment was showed no alterations in the BF at 17th and 28th day of chicken age (Figure.5)

The histopathological changes of spleen of group1 at 17th day were showed follicular necrosis and lymphocytic depletion in the center of the organ (Figure.6) and mild lymphocytic depletion at 28th day.

Group 2 was exhibited follicular necrosis, depletion of lymphocytes, and sinusoidal congestion along the observation period (Figure.7).

Group 3, was showed severe thickening of the wall of the blood vessels and hydropic degeneration in the epithelial layer of the blood vessels of spleen at 17th day of vaccination with intermediate vaccine. At 28th day, the splenic lesions were seen as sinusoidal congestion with follicular atrophy (Figure.8).

The histopathological changes of G4 at 17th day were showed thickening of the wall of the blood vessels and hydropic degeneration in the epithelial layer (Figure.9) and slight follicular atrophy due to depletion of lymphocytes was present at 28th day.

The control group (G5) did not reveal any alterations during the experimental period in the spleen at 17th and 28th days (Figure.10).

The effect of vaccine on thymic tissue of G 1 was showed congestion in the medulla, lymphocytic depletion and multifocal areas of hemorrhages in the medullary area at 17th day (Figure.11), whereas mild congestion in the thymic tissue has been observed at the 28th day.

The histopathological examination of thymus of group2 at 17th day was exhibited congestion of blood vessels and severe depletion of lymphocytes in the medulla with diffuse hemorrhage. On the other hand, at the 28th day of age, vaculation and depletion of lymphocytes in medulla with diffuse hemorrhage were noticed (Figure.12). Focal areas of hemorrhages in medulla with severe depletion of lymphocytes (Figure.13) were seen in the thymic tissue of group 3 at 17th day .At 28th day of age mild depletion of lymphocytes in medulla was recorded. The tissue of thymus gland of G 4 at 17th day was displayed slight depletion of lymphocytes in medulla and a few number of multifocal areas of hemorrhages. Thymic atrophy and depletion of thymic lymphocytes (Figure.14) was showed at 28th day. The control group was appeared as normal thymic section at 17th and 28th day of age (Figure.15).

The results of the histopathological examination of the bursa, spleen and thymus gland in the vaccinated birds of present study indicated that live IBDV vaccines were enough to cause some pathological lesions in the lymphoid organ.

Discussion

Some vaccines are capable of inducing similar or more severe bursal lesions than those caused by field virus strains (Luengo *et al*.,2001) , and most of the IBDV vaccines showed variable degree of pathogenicity and immunosuppressive effect (Hussain *et al.* ,2001),as well as histopathological changes induced by vaccine virus in the various lymphoid organs are very different, depending on the virulence of vaccine strain (Rus *et al*.,2010).

In the present study, disappearance of clinical signs and mortality might be an indication of safety of the used live IBD virus vaccines and early age resistance (Hassan, 1986; AL-Mayah,2009).

The increasing BI after 3 days of vaccination with intermediated vaccine may be due to the acute inflammation and presence of edema with hyperemia in the bursal tissue, whereas the significant reduction of BI which was recorded in vaccine D in the present study might be explained depending on Nishizawa *et al.,(*2007*)* who explained the reduction of BI by the lower degree of attenuation of vaccines, in which the virus was capable to destroy B-lymphocytes present in BF, leading to reduction in their size. This result was in agreement with that of Mazariegos *et al.,* (1990); Van den Berg (1991) ; Eterradossi *et al.* (1992); Amer *et al.*,(2007) who reported that intermediate strains of IBDV vaccines were sufficient to induce a significant reduction in BI.

The splenic atrophy may occurred due to the severe necrosis in the splenic tissue. This result was in agreement with that of McFerran, (1993) who reported that the reduction of spleen index was due to germinal centers and perivascular sheaths necrosis.

As shown in Table (1) also, there was a significant reduction ($P<0.05$) in the thymic index in group 1 (2.740±0.097) at 28th day of age.

The reduction of the thymic index clearly correlated with decreasing of thymus weight. However, thymus atrophy may occur due to severe depletion and necrosis of lymphocytes in the thymus tissue. The result of thymic index of the present study was in agreement with that of Hedayati *et al.*,(2005) who stated that thymic index of vaccinated groups which administrated at

16th day of age with intermediate vaccine was less than that of control group which indicated thymic atrophy induced by intermediate vaccine.

The result of histopathological changes in this study was in agreement with those of Ezeokoli *et al.,* (1990) who evaluated the histopathological modifications of BF associated with poultry vaccination against IBDV, and described severe lesions in the bursa between three and seven days after vaccination. The results of this study were also in agreement with those of Rautenschlein *et al.,* (2003) who reported that commercial broilers vaccinated with IBDV vaccines of different virulence intermediate and intermediate plus showed variable severity of lesions and the severity of the lesions of the intermediate plus vaccine was of score 4.

Al-Sereah, (2007) observed that chickens vaccinated with intermediate vaccine(Cevac) at 14th day showed more severe lesions such as hyperemia, dilatation of blood vessels and secondary follicular proliferation in BF 3 days post vaccination.

This difference of histopathological changes might be attributed to the number of passages which were used for attenuation.The breed of broiler chickens may have a role in this process. Khan *et al.,* (2007) noticed that local wild strain of IBDV at fifteenth passage causes a loss of complete ability to produce histopathological lesions in BF.

Depending on the virulence of the live attenuated viruses, some vaccine strains can cause bursal damage (Mazariegos *et al.,* 1990).

Splenic congestion and hyperplasia of reticulo-endothelial cells around the adenoid sheath artery in the vaccinated birds of this study were also observed previously by Ley *et al.,* (1983); Nunoya *et al.,* (1992); Hassan *et al.,* (1996), while necrosis was found by Okoye and Uzoukuw (1981); El-Manakhly and Bekheit (1992).

It is worth mentioning that , the above mentioned histologic alterations which observed in the thymus were previously demonstrated by Sharma *et al.,* (1989) ; Goodwin *et al.,* (1996); Goodwin and Hafner (1997); Shaban(2004) ; Amer *et al.,* (2007).

The results of the histopathological examination of the bursa, spleen and thymus gland in the vaccinated birds of present study indicated that live IBDV vaccines were enough to cause some pathological lesions in the lymphoid organs. These results were in agreement with those of Thornton and Pattison, (1975) Ide, (1979); Thangavelu *et al.,* (1998); Mona, (2002); Amer *et al.,* (2007) who mentioned that IBD vaccines induced pathological lesions in the lymphoid organs of chickens vaccinated with intermediate vaccines.

Study of the pathogenicity of four commercial IBD vaccines showed considerable variation in their pathogenicity. Based on the recorded findings, the intermediate vaccines can be divided into 3 pathogenic categories "highly, moderate and low pathogenic". According to the results of group 4, this vaccine considered to be more pathogenic than other intermediate vaccines. In conclusion, vaccine D proved to be more pathogenic than A, B, and C.

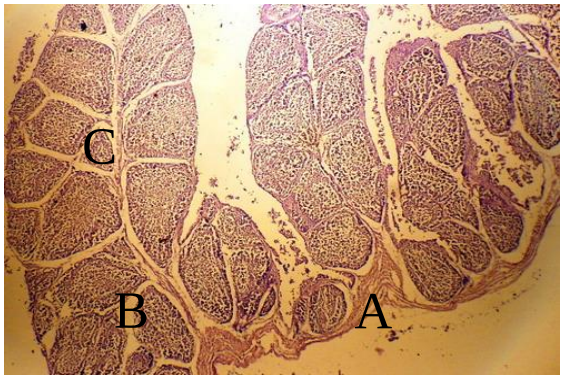

Fig (1): Cross histological section in BF (group1) at 17th day showed: A- Edematous area , B- Increased interfollicular space C-Follicular atrophy- (Score 3). H&E stain, 80x

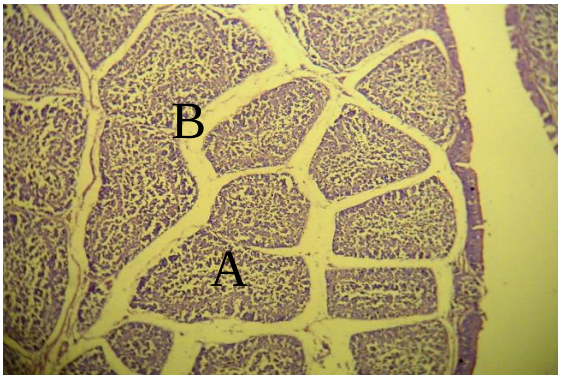

Fig (2): Cross histological section in BF (group2) at 28th day showed: A- Depletion of lymphocytes in medullary area B-Severe increasing of interfollicular spaces -(Score 4). H&E stain, 200x

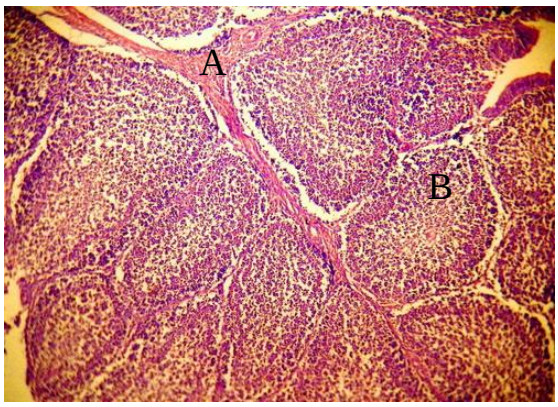

Fig (3)lCross section of BF (group3) at 17th day showed: A- Hyperplasia of interfollicular septa B- Depletion of lymphocytes - (Score 2). H&E stain, 250x

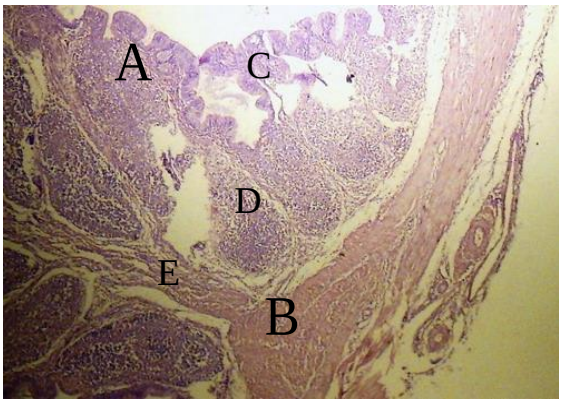

Fig (4): Histological cross section in BF (group4) at28thday showed: A- Hydropic degeneration in parenchymal and epithelial cells B- Hyperplasia C- Cystic degeneracy D- Follicular atrophy E-Edematous area -(Score 4). H&E stain, 100x

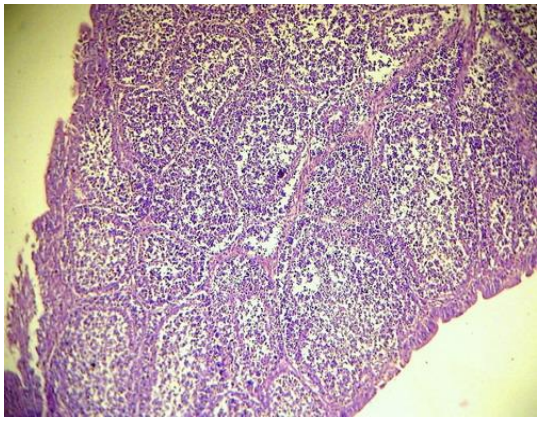

Fig (5): Histological cross section in BF (control) at 17th day showed normal structure of bursal tissue. H&E stain, 200x

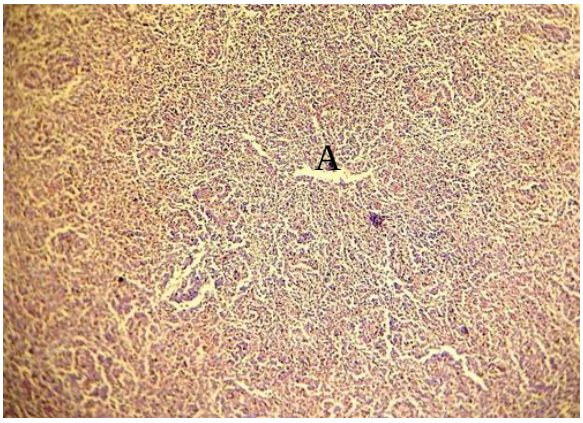

Fig (6) Cross section of spleen (group1) at 17th day showed A. Necrosis and lymphocytic depletion in the center of organ. H&E stain, 100x

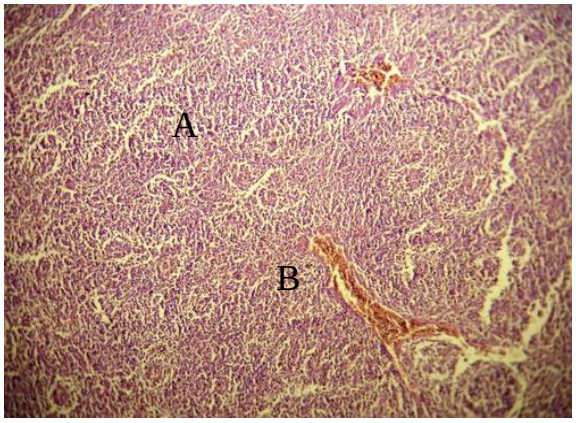

Fig (7):Cross section of spleen (group2) 28th day showed A- Depletion of lymphocytes, B- Sinusoidal congestion. H&E stain, 100x

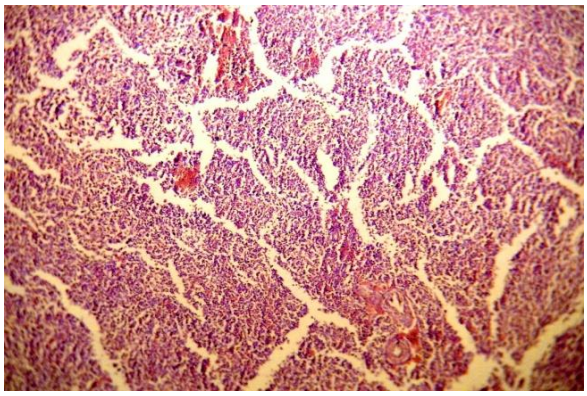

Fig (8): Spleen. Cross section (group3) at 28th day showed A- Follicular atrophy, B-sinusoidal congestion. H&E stain, 100x

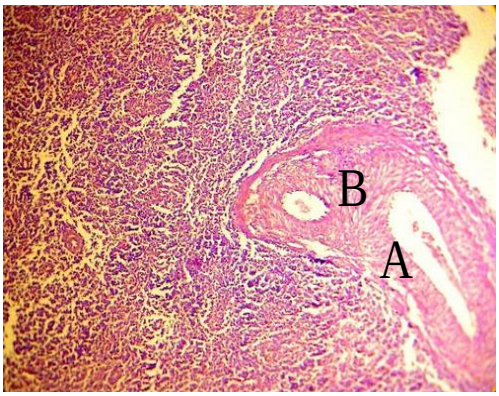

Fig (9): Spleen (group4) at 17th day showed A- Thickening of the wall of the blood vessels B- Hydropic degeneration in the epithelial layer. H&E stain, 100x

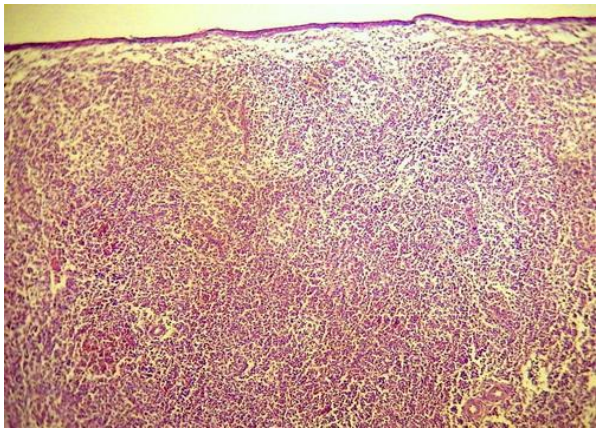

Fig (10):Spleen. Histological cross section (control) at 17th day showed normal splenic structures. H&E stain, 80x

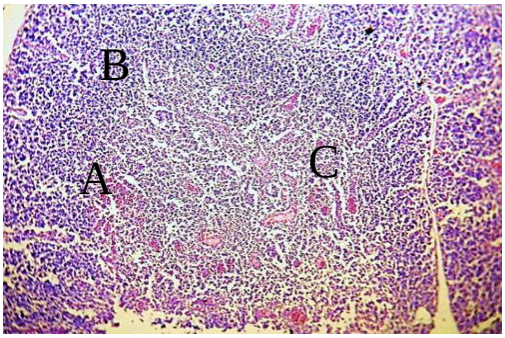

Fig (11):Thymus .Cross section , (group1) 17th day showed: A-Congestion in the medulla B-Severe lymphocytic depletion C- Multifocal areas of hemorrhage in medulla. H&E, 100x

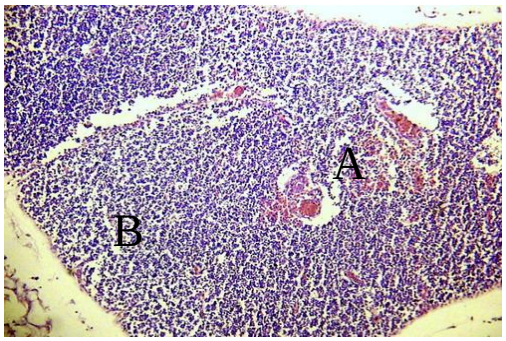

Fig (12):Thymus. Cross section (group2) 28th day showed A- Depletion of lymphocytes in medulla B- Diffuse hemorrhage. H&E stain, 100x

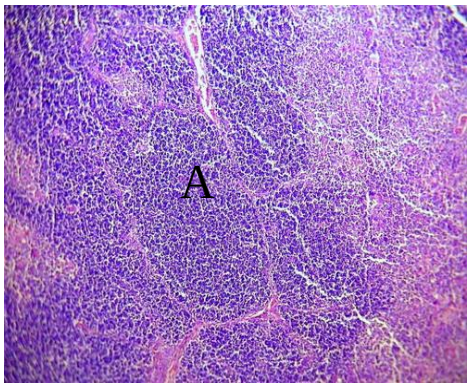

Fig (13):Thymus. Cross section (group3)17th day showed A- Mild depletion of lymphocytes. H&E stain, 80x

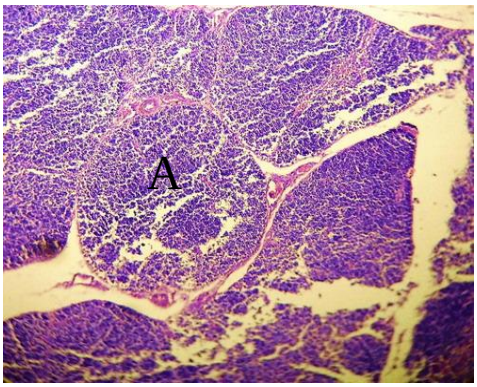

Fig (14):Thymus. Cross section , (group4) 28th day showed A- Thymic follicular atrophy due to depletion of thymic lymphocytes. H&E stain, 80x

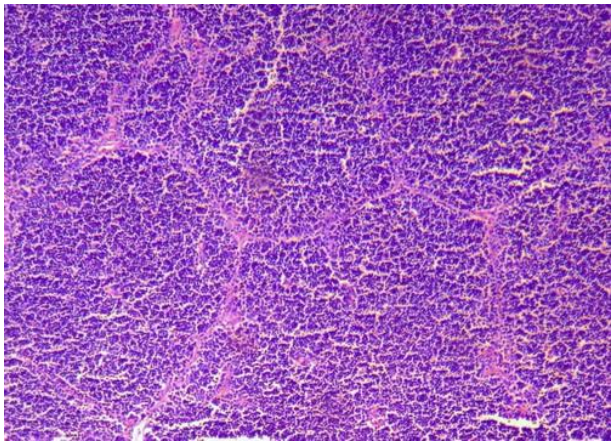

Fig (15):Thymus. Cross section , unvaccinated (control) 28th day showed normal thymic structures. H&E stain, 100x

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Mirror of Research in Veterinary Sciences and Animals (MRVSA)

Original Article

Evaluation of *Echinococcus granulosus* DNA extracts from protoscoles and germinal layer in sheep

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Abstract

Cystic echinococcosis (CE) caused by the metacestode of the dog tapeworm *Echinococcus spp*., is a global zoonotic infection. It is economically important and constitutes a major threat to public health in many countries. Strains characterization is essential for the establishment of a preventive and control strategy in every endemic area.This study was aimed to compare between DNA extracts from Protoscoleces and germinal layer of *E. granulosus* strain in infected sheep. Thirty, fresh fertile hydatid cysts from sheep's infected organs were collected from different abattoirs of Baghdad, Iraq. All cysts were examined by light microscope to investigate the protoscoleces viability. Protoscoleces and germinal layer were seperated and DNA was extracted. Efficiency of the DNA extract was determined by degree of its success in PCR amplification. Genomic DNA mini kit and primers forward JB3 / reverse JB4 were used to extract DNA. The results showed that DNA extract from Protoscoleces were more visible and more concentrated than the germinal layers DNA and appeared at 448bp on electrophoresis. In conclusion, the result of this study revealed that Protoscoles DNA was differed and better than germinal layer DNA.

Keyword : *Echinococcus granulosus*, DNA, , Iraq, Protoscoles.

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Introduction

Hydatid disease is a zoonotic disease known to spread worldwide. It is caused by larval stages (metacestodes) of tape worm parasite of genus *Echinococcus granulosus* (*E. granulosus*). It is one of the most important serious parasitic diseases in the medical, veterinary sciences and with economic consequences in different regions of the world that infect different animal species (Rausch, 1995). These domestic animals include sheep, goats, cattle, swine, buffalos, horses, and

camels (Bryan and Schantz, 1989). Human beings may also serve as dead-end hosts (Binhazim *et al*., 1992). *E. granulosus sensu lato* shows intraspecific variation in relation to host, specificity, epidemiology, morphology, developmental biology, biochemistry and genetics (Thompson and McManus, 2002). Ten different genotypes, among which G1 (sheep strain) have been formerly characterized by several researchers based on genetic characterization and they were mostly depend on the homology of the sequence of the two mitochondrial genes; cytochrome C oxidase subunit 1 (CO1) and reduced nicotinamide adenine dinucleotide subunit 1 (ND1), (McManus, 2002; Lavikainen et al., 2003; Snábel et al., 2009). *E. granulosus* complex has been divided into *E. granulosus* sensu stricto (G1–G3), *E. equinus*(G4), *E. ortleppi* (G5), *and E. canadensis*(G6–G10), according to the new molecular phylogeny of the genus *Echinococcus* (Thompson and McManus, 2002; Nakao et al., 2010; Hüttner et al., 2008). Simseka and Eroksuzb, 2009 were study the molecular characterization of cyst material, mitochondrial cytochrome oxidase subunit 1 (mt-CO1) , gene region amplifying and sequence analyses and they found that sequence corresponding to mt-CO1 gene was identical to a sequence reported for common sheep strain (G1) . This study was designed to compare between DNA extracts from protoscoles and germinal layer of E. granulosus strain that infect sheep.

Materials & Methods

Parasite specimens collection

Thirty fresh fertile hydatid cysts were collected from sheep's infected organs from different abattoirs in Baghdad, Iraq. The viability of protoscoleces were determined by light microscopic test. All Hydatid cysts were washed with distal water. The hydatid cysts fluid was aspirated. Protoscoloces and the germinal layers were collected and washed with sterile phosphate buffered saline (PBS), and stored in 70% (v/v) ethanol at -20 ^{0}C until DNA extraction.

DNA extraction

Samples from each individual cyst were processed as an isolate for subsequent characterization. The protoscoleces and germinal layers were rinsed several times with PBS to remove the ethanol prior to DNA extraction. DNA was extracted from protoscoleces and from the germinal layers using the DNA extraction kit according to the manufacturer's protocol. The extraction method is briefly as the fellow: Animals tissue (cyst + protoscoles) (30 mg) were cut and kept in 1.5 ml micro-centrifuge tube provided by micro-pestle. The tissues were grinded and homogenized by adding 200µl of GT buffer and 20µl of proteniase K. The samples were shaken vigorously and incubated at 60 ^{0}C for 30 minute to lyse the tissues. Later on 200µl of GBT Buffer were added with vigorously shaking for 5 minutes. The samples were incubated at 60 ^{0}C for at least 20 minutes until the lysate was clear. Absolute ethanol (200 µl) was added to the sample with continuous vigorously shaking. Samples were placed in GD Column in a 2ml collection tube and centrifuged at 14-16,000 Xg for 2 minutes. Later on, the GD Column was replaced by a new 2ml collection tube, and 400 µl of W1 buffer were added to the GD Column and centrifuged at 14-16,000 Xg for 30 seconds, this steps were repeated for 3 times and 600µl of buffer were added to the GD Column and centrifuged at 14-16,000 Xg for 30 seconds. The flow was discarded and GD Column was placed to a new 2ml collection tube. Then 100µl of pre-heated Elution buffer were added to the center of the column matrix and kept to stand for at least 5minutes to ensure that the Elution buffer was completely absorbed. The samples were centrifuged at 14-16,000 Xg for 30 seconds to elute the purified DNA. The extracted DNAs and PCR products were loaded

on separate 1 and 1.5% TBE (Tris 0.09M-Borate 0.09M-EDTA 0.02M) and 1gm agarose gel were added and kept for 5 minutes in oven and 3µl ethidium bromide was added for staining (all Biobassic / canada). Electrophoresis carried out for 45 minute at 80 V and the bands were visualized in UV Transilluminator.

DNA amplification

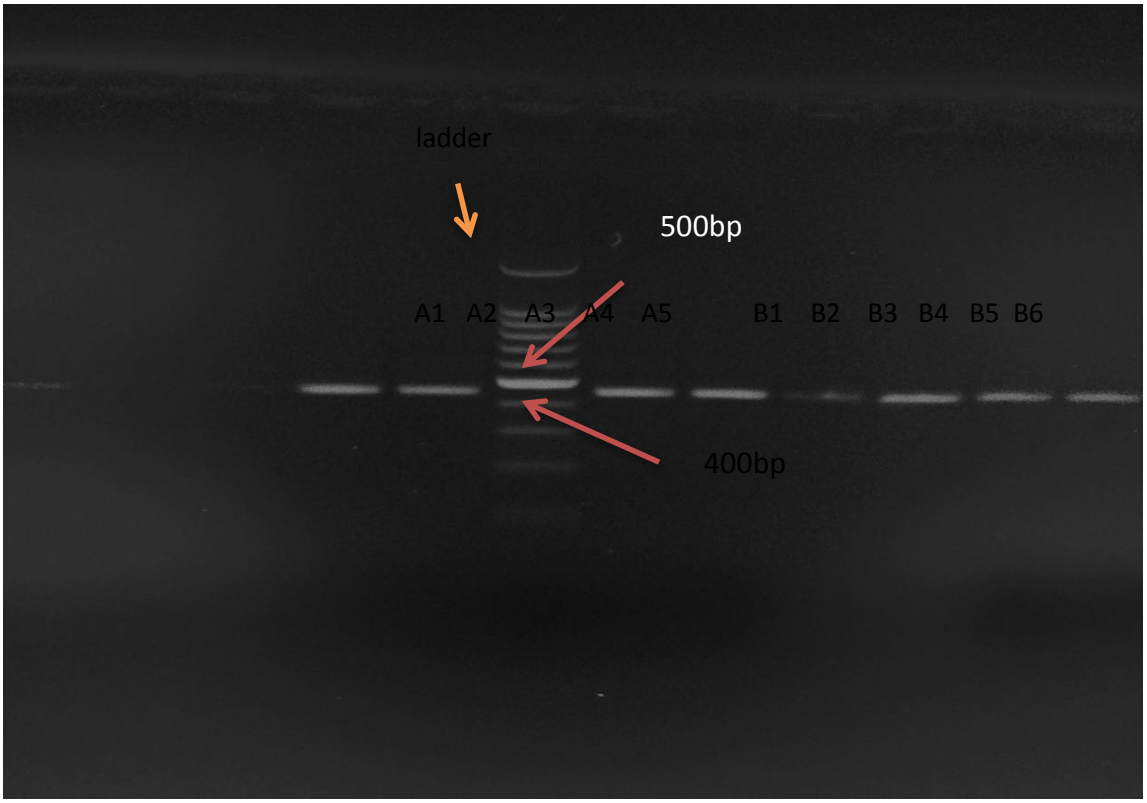
One target sequences of the mitochondrial DNA coding for CO1 PCR-amplified by using Genomic DNA mini kit (origin Bioneer Korea) .Two conserved primers, JB3 (forward)5-TTTTTTGGGCATCCTGAGGTTTAT-3 and JB4 (reverse): 5-TAA AGAAAGAACATAATGAAAATG-3 (Busi et al., 2007), were used to amplify the mtDNA region corresponding to the part of the CO1 gene Bowles et al. (1992). The amplification reactions were carried out in a PCR thermal cycler Dice (bioneer , korea); and it was stained with ethidium bromide and photographed. The PCR programs were: 5 min at 95 ◦C (initial denaturation), 35 cycles of 50 s at 94 ^{0}C, 50 s at 45 ^{0}C and 50 s at 72 ^{0}C and finally 10 minute at 72 ^{0}C (final extension).

Results and Discussion

There is little published information about the genetic characterization of protoscoleses and germinal layer of the E. granulosus in sheep in Iraq. The mitochondrial CO1-PCR with JB3/JB4.5 primers were yielded a 448 bp sized product in the sample. The PCR results of these sample were give positive for *E. granulosus*. The DNA extracted from the protoscoles and some sample of germinal layer give negative result (Figure.1). DNA extracts in this study are in agreement with the results of Simsek and Eroksuz.,(2009), (Genbank accession number: KC660075.1).
The genetic strains of *Echinococcus granulosus* parasites occurring in sheep and cattle in Turkey were determined previously by Vural et al. (2008) using DNA sequencing of part of the mitochondrial Cytochrome C oxidase 1 (cox1) gene. They examined a total of 112 hydatid cysts from sheep (100 isolates) derived from widely distributed sites within Turkey as well as from cattle (12 isolates) from the Turkish province of Kars. Haplotypes were identified which corresponded clearly to the previously described strain G1 in a total of 107 isolates, including 98 isolates from sheep and 9 isolates from cattle. They found that five isolates, including 2 sheep and 3 cattle, were determined to belong to the G3 genotype. The infected animals in this study originating from Baghdad regions of Iraq, had either history of previous rural life or were still living in rural areas. The sheep's strain (G1 genotype) of *E. granulosus* is the Commonly wide distributed strain around the world. It has been found to be dominant strain both in human and animals (Thampson and Mc Manus, 2001; Ahmadi and Dalimi, 2006; Varcasia et al., 2006; Bart *et al*., 2006b; Li et al., 2008). The majority of the samples were 100% identity with sheep strain G1 from protoscoles (GenBank KC660075.1). The present study has given interesting result with CO1 gene amplification (Nejad et al., 2011) and sequencing when applied to the small samples but it is difficult for large samples, to resolve these problem further studies recommended to be done to find specific primer for each strain. Nowadays specific primer for sheep strain were designed by (Dinkel et al.,2004), which is reliable method for molecular epidemiology to be applied on the large sample size. Utuk et al., (2008), examined 179 sheep, 19 cattle, 7 goat, 1 camel, 1 dog and 1 human isolates by using RCR-RFLP of ribosomal ITS1 gene region and mitochondrial CO1 sequence analysis and determined only sheep strain (G1) in all samples. *E. granulosus* sheep's strain were also reported previously as predominant in Kurdistan/Iraq and it was mostly responsible of human hydatid disease in Kurdistan/Iraq(Hama et al .,2012) , which

is in agreement with results reported from neighboring Iran and Turkey(Ergin et al.,2010). In conclusion, the result of this study approved *E. granulosus* sheep's strain in Baghdad province and supports the fact that sheep's strain is the most worldwide distribution.

Fig. 1: Agarose gel electrophoresis of PCR on extracted DNA from 11 isolates germinal layer (A) & protoscoles (B): with DNA size marker (100-bp DNA ladder) (Promega), in the center image. A1, A2 show no band. Visible bands appear in the other samples .



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Mirror of Research in Veterinary Sciences and Animals (MRVSA)

Original Article

Comparative Study of Palm Leaves Extract and Glibenclamide in diabetic female rats induced by alloxan

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Abstract

This study was designed to compare the effect of palm leaves extract and glibenclamide in diabetic female rats induced by alloxan. Twenty four adult female rats were randomly divided into four equal groups (6 rats in each group), three groups were injected intraperitonealy (i.p.)with single dose alloxan(100 mg kg^{-1}B.W) and acted as G1, G2, and G3 and treated orally with (200mg/kg B.W.) palm leaves extract in G1 and (5mg/kg B.W) glibenclamide in G2 , while G3 and fourth group (C) consider as positive(+ve) and negative(-ve) control respectively. Fasting blood sample were collected at 15 and 30 day of experiment (after diabetes induction) for measuring of plasma glucose concentration, aspartate aminotransferase(AST) and alanine aminotransferase(ALT) activity .These parameters were used as a guide for comparison between palm leaves extract and glibenclamide in ameliorating effects of diabetes. The results revealed that i.p. injection of alloxan caused hyperglycemia and significant increase in activity AST, ALT and serum glucose concentration in G3 treated group. The palm leaves extract exhibited significant anti-hyperglycaemic activity in alloxan induced diabetic rats. A significant correction of the plasma glucose concentration and ALT and AST activity was observed in G1 in compare with the G1 treated group at 30 days and G2 glibenclamide treated group. In conclusion, these studies reveals that the palm leave extract was worked as anti-diabetic in the alloxan induce diabetic rats model for minimize the complication associated with the diabetic and related disorder.

Keywords: palm leaves, glibenclamide, alloxan, antidiabetic, rats

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Introduction

Plants represent a good source of potentially useful dietary supplements for improving blood glucose control and preventing long- term complications in diabetics (Gallagher *et al*., 2003). The main characteristics of diabetes are hyperglycemia, polyuria, polydipsia and polyphagia, weight loss, muscle weakness and dyslipidemia (Robert *et al.,* 2005). Chronic hyperglycemia is normally accompanied by increased risk to hypertension, oxidative stress, decreased fibrinolytic activity, increased platelet aggregation, and severe atherosclerosis (Reusch, 2003).The beneficial effect of synthetic drugs like glibenclamide provide good glycemic control but long term use have side effects and thus searching for a new class of compounds is essential to overcome diabetic problems (Prasad *et al*., 2009).

Date palm (*Phoenix dactylifera L.*) is one of the oldest cultivated plants (Riad,2006) plants, *Phoenix dactylifera* was among the most frequently used plants to treat diabetes and hypertension (Tahraoui *et at*., 2007).Antioxidant activities were reported in many plants, among them is date palm fruit (*Phoenix dactylifera*) which possesses a potential antioxidant compounds that capable of scavenging free radicals (Biglari *et at.,* 2008).

This study was designed to evaluate the potential beneficial effects of date palm (*Phoenix dactylifera*) leaves extracts as antidiabetic in the alloxan induce diabetic rats model

Materials and Methods

Plant collection and preparation

Fresh leaves of palm were collected from Kufa Area in Najaf province in Iraq. The leaves were air dried on laboratory bench top, then pulverized into a coarse powder and stored at 4^{o}C until used.

Extraction

Extraction of palm leaves was preformed according to (Markham, 1982) in two steps as following:

Step one

200 g of palm leaves were crushed with 400ml of mixture methanol 95% and distilled water (9:1), mixed for 18h in magnetic stirrer at room temperature, then filtered under vacuum using Whitman No. (1).

Step two

The filtrate residues from step one was mixed again with 200ml of mixture methanol 95% and distill water (1:1) for 18h in magnetic stirrer at room temperature and the filtered was collected as described in step one . Then, the filtrate collected in step 1 and 2 was evaporated in the incubator (42°C) to reach one –third of original volumes. The concentrated extract was separated from low organic materials by addition of chloroform 20:100 (extract: chloroform) in separator funnel, then the mixture was left for one hour to separate in two layers: lower layer contain chloroform and upper layer contain (total polyphenol). The upper layer was separated with chloroform 10:100 (extract: chloroform), from the upper layer, total polyphenol was collected and dried in incubator at (40^{o}C), and then collected as powder.

Experimental animals

Adult female Sprague-Dawley rats weighing 150-200g were housed in clean cages and kept in well ventilated room with a 12 h light/ dark cycle at 22-25°C. The rats were maintained on a standard rat pellets. This study was approved by animal ethical committee / Kufa University.

Induction of diabetic rats

Hyperglycaemia was induced in rats after fasting of the animals for 24 h by a single intraperitoneal (i.p.) injection of alloxan 100mg/kg (B.W) (Stanley et al., 2001). Since alloxan is capable of producing fatal hypoglycemia as a result of massive pancreatic release of insulin, to prevent hypoglycemia the rats were then kept on 5% glucose solution for the next 24 h. Three days after injection, threats with fasting blood glucose higher than 150 mg/dL, were considered as hyperglycemic/diabetic (Stanley et al., 2001). Twenty four adult female rats (18 diabetic rats and 6 control) were divided into four groups for 30 days as follow :(i) G1: Diabetic rats were gavages orally with 200 mg/kg B.W. palm leaves extract. (ii) G2: Diabetic rats were gavages orally with glibenclamide 5mg/kg B.W/day (Sigma Chemical Co., St. Louis, USA) (5 mg/kg B.W). (iii) G3: Diabetic control rats. (iv) C: normal control rats. Fasting blood samples were drawn from heart puncture of rat at days 15 and 30 of experiment for measurement plasma glucose concentration, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity (using semi-automatic chemistry analyzer Belgium using kit Cyan com./Belgium).

Statistical analysis

The results are expressed as the mean values with their standard error. Two-way ANOVA followed by Duncan's variance was performed to compare between treatment groups. Significance was set at $p<0.05$.by used Statistical Package for Social Science (SPSS 20) Ready statistic program 20.

Result

Plasma glucose concentration (mg/dL)

Table (1) shows the mean value of plasma glucose concentration (mg/dL) of control and treated groups.

Intraperitoneal injection of alloxan 100mg/kg B.W caused significant elevation ($P< 0.05$) in plasma glucose concentration in alloxan induced groups compared with the control groups. While oral gavages of palm leaves extract G1 or glibenclamide G2 caused significant decrease in the elevated plasma glucose concentration with mean value (148.46±4.82) compared with +ve control G3 with mean value (185.44±6.01) in day 30 of experiment indicating the hypoglycemic effect of palme leaves extract in alloxan-induced hyperglycemic rats. However this value is higher than the G2 group (130.06±3.25) value which was treated Glibenclamide .

Table (1) Effect of palm leaves extract and glibenclamide on plasma glucose concentration (mg/dL) in alloxan induced diabetic rats.

Days / Groups	15	30
G1	158.90±4.15aAB	148.46±4.82aB
G2	144.13±3.87aB	130.06±3.25bC
G3	164.66±5.16aA	185.44±6.01bA
C	107.01±6.36aC	113.67±4.54aD

-C= control.
- G1=Animal intraperitonealy injected with alloxan (100mg/kg B.W.) + Palm extract (200mg/kg orally).
- G2=Animal intraperitonealy injected with alloxan (100mg/kg B.W.) + glibenclamide (5mg/kg orally).
- G3=Animal intraperitonealy injected with alloxan (100mg/kg B.W.).
-Capital letter denote difference between groups, P< 0.05.
-small letter denote difference within groups, P< 0.05.
± SE

Plasma aspartate aminotransferase (AST) and plasma alanine aminotransferase(ALT) activity(U/L)

Tables (2) and (3) illustrate the mean value of plasma aspartate aminotransferase (AST) and plasma alanine aminotransferase (ALT) activity (U/L) of control and treated groups.
The results show that intraperitoneal injection of alloxan caused significant (P< 0.05) increase in AST and ALT activity in all treated groups compared with control group at day 15 of experiment. Glibenclamide orally gavage in G2 group caused significant (P< 0.05) decrease in AST activity with mean value (16.26±0.93)compared with control +ve G3groups (21.58±1.41) at day 30 of experiment as well as, non-significant(P> 0.05) difference between G1 and G2 which received palm leaves extract and glibenclamide respectively when compared each other at the same period.

Table (2) Effect of palm leaves extract and glibenclamide on plasma aspartate aminotransferase (AST) activity (U/L) in alloxan induced diabetic rats.

Days / Groups	15	30
G1	30.95±2.42aA	18.87±0.83bAB
G2	26.13±1.08aA	16.26±0.93bBC
G3	27.17±2.26aA	21.58±1.41bA
C	14.37±1.28aB	13.54±0.56aC

-C= control.
- G1=Animal intraperitonealy injected with alloxan (100mg/kg B.W.) + Palm extract (200mg/kg orally).
- G2=Animal intraperitonealy injected with alloxan (100mg/kg B.W.) + glibenclamide (5mg/kg orally).
- G3=Animal intraperitonealy injected with alloxan (100mg/kg B.W.).
-Capital letter denote difference between groups, P< 0.05.
-small letter denote difference within groups, P< 0.05.
± SE

Palm leaves extract orally gavage group G1 and glibenclamide G2 revealed significant (P> 0.05) decrease in the elevated ALT activity, (10.12±0.52) and (8.60±0.61)respectively in compare with

group G3 (14.85±0.45) . In addition, plasma alanine aminotransferase ALT in G1 and G2 appears near to the value of the control group C (9.54±1.02) at the end of experiment.

Table (3) Effect of palm leaves extract and glibenclamide on plasma alanine aminotransferase (ALT) activity (U/L) in alloxan induced diabetic rats.

Days / Groups	15	30
G1	19.59±2.04aA	10.12±0.52bB
G2	18.15±1.05aA	8.60±0.61bB
G3	20.57±0.86aA	14.85±0.45bA
C	9.09±0.57aB	9.54±1.02aB

-C= control.
- G1=Animal intraperitonealy injected with alloxan (100mg/kg B.W.) + Palm extract (200mg/kg orally).
- G2=Animal intraperitonealy injected with alloxan (100mg/kg B.W.) + glibenclamide (5mg/kg orally).
- G3=Animal intraperitonealy injected with alloxan (100mg/kg B.W.).
-Capital letter denote difference between groups, $P< 0.05$.
-small letter denote difference within groups, $P< 0.05$.
± SE

Discussion

Alloxan monohydrate is one of the chemical agents used to induce diabetes mellitus. It induces diabetes by partial destruction of the β-cells of Islets of Langerhan's (Szkudelski, 2001). It causes to elevation of blood glucose level, decreased protein content, increased levels of cholesterol and triglycerides (Dhanabal, 2007).

The results of this study revealed that palm leaves extracts have good anti-diabetic activity and exhibited significant anti-hyperglycaemic activity in alloxan-induced hyperglycemic rats; they can also improve the condition of Diabetic mellitus as indicated by parameters like aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity. Similar study had been performed by Rosalina, *et al*., (2011). They showed that Oil palm leaves (OPL) ethanolic extract treatment dose-dependently reduced blood glucose and oxidation in the STZ rats, and restored antioxidants enzymes levels. The optimum dose was 100mg/kg, which effectively reduced liver and kidney damage to the level of normal rats.

The renewal of β cells in diabetes have been studied in several animal models. The total β cell mass reflects the balance between the renewal and loss of these cells. It was also suggested that regeneration of islet ß cells following destruction by alloxan may be the primary cause of the recovery of alloxan-injected guinea pigs from the effects of the drug (Gorray, 1986).

This study demonstrated that palm leaves extract at orally dose (200 mg/kg B.W.) is effective and shows similar curative effects as standard drug glibenclamide orally dose (5mg/kg B.W.), which was used as a comparative drug. The anti-diabetic curative effective of palm leaves extract could be due to the possibility that some β-cells are still surviving to act upon by palm leaves extract to exert its insulin releasing effect. It is well known that certain flavonoids exhibit hypoglycemic activity and are able to help regenerate the beta cells of the pancreas.The significant antihyperglycaemic effect of palm leaves is probably due to it flavonoids contents.

In conclusion, the results of this study strongly suggest that palm leaves extract is potentially useful for the alleviation of diabetic and its secondary complications. Further work is needed to investigate the actual active components in the palm leaves. The present investigation reports the first anti-hyperglycemic activity of palm leaves which may be a new potential alternative in the treatment and management of diabetes.

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Mirror of Research in Veterinary Sciences and Animals (MRVSA)

Review Article

First Cohort students graduated from the new Faculty of Veterinary medicine / University of Kufa

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Abstract

Thirty four students made history on July 04 2013 when they graduated from the new faculty of veterinary medicine in university of Kufa (FVMUK). The University of Kufa/ faculty of veterinary medicine students, who enrolled in October 2008, are the first cohort to have successfully completed the five year veterinary degree. The five-year course is unique in that students get hands on experience with animals from day one and graduation project in year five. Students awarded degree as Bachelor of Veterinary Medicine and Surgery. Assistant Professor Ahmed Al-Azam, Acting Dean and assistant Professor of Veterinary Pathology, said: "This is a momentous day for the University of Kufa, students, their families and the veterinary profession. We were given an exceptional opportunity to establish a new veterinary faculty. Recognition by the Iraqi veterinary medical syndicate was the highest possible embracing they could award, their approval and today's graduation ceremony is the capstone of five years successful hard work for staff and students.

Keywords: Cohort, Capstone, Graduate, Kufa, Veterinary, FVMUK.

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Introduction

The development and progress of human civilization has been associated with the ability of man to exist together with animals. The human being realized his need for animals, whether hunting, nomadic or as a farmer. Iraq has a large population of animals: sheep, goats, cattle, water buffaloes, horses, donkeys, mules, and camels (Khamas W. A. & Nour A, (2004) 31(4). www.utpjournals.com/jvme/tocs/314/301.pdf.). Iraq's rich and distinctive livestock population is largely a result of a sound nucleus from which the most common farm animal species were

produced (Al-salihi, 2012).Iraq established its education system in 1921, and the first college of veterinary medicine was established in the 1950s within Baghdad university. This college has provided veterinary education and prepared graduates and veterinarians for more than 55 years (Al-salihi, 2012). The veterinary medical education in Iraq faces challenges from different factual vicinities and due to the society needs, there are currently 15 veterinary colleges in the country, which differ with respect to the number of students, staff members and equipment they have. The FVMUK was established in 2008 to serve the society and preserve animal welfare in the surrounded geographical area.
The intent of this article is to provide an overview of faculty of veterinary medicine / university of Kufa after first Cohort student's graduation, and suggestions on how the faculty may be improved.

Faculty of veterinary medicine in Kufa city

The FVMUK was established in 2008 and students, who enrolled in October 2008, are the first cohort to have successfully completed the five year veterinary degree in July 04 2013. The Faculty established in Kufa city in Al-Najaf province (Figure1.). The faculty is located in the heart of Kufa city near the Euphrates River and close to the Faculty of dentistry. Kufa (al-Kūfah, former Mesopotamian city) is one of most important Islamic cities in Iraq, about 170 kilometres (110 miles) south of Baghdad, and 10 kilometres (6.2 miles) northeast of Najaf. It is located on the banks of the Euphrates River. The estimated population in 2003 was 110,000. The city was the final capital of ʿImam Alī ibn Abī Ṭālib, and was founded within the first hundred years of the 622 Hijra. In this city, there is also the school where Jaber Iben Hayan studied. Westerners call him "the father of chemistry". Kufa was the city of the most beautiful Arab calligraphy, in which the Koran was written, as well as the broad outline of Arab grammar Abu El-Assouad Addaouali, whose grammatical school competed with the Basra school. The Najaf province and all its villages including kufa city is considered as one of the most important agriculture areas and livestock production, in addition to the Najaf desert, which contents a variety of natural resources and large number of camels dominant there.

Figure1. Shows, the location of FVMUK in the heart of Kufa city near the Euphrates River.

Veterinary Educational system

The FVMUK in Kufa, as other Iraqi colleges of veterinary medicine follow classical standard curriculum. Courses in veterinary education are divided into academic and clinical components, which are studied over five years and are accompanied by summer practical training courses and final year research, project (Figure 2.). Currently, the faculty runs only the undergraduate studies and services institute in the Al-Najaf province. The faculty has well established six departments which are: Anatomy and histology, Physiology and pharmacology, Microbiology, Public health, Pathology and poultry diseases and clinical sciences.

Figure 2. Shows the courses, that are taught in the FVMUK and the Hierarchy education system from first year until year fifth year.

First year

Course	Lecture (h)	Lab (h)	Total credit
Biology	2	2	6
General chemistry	2	2	6
Anatomy	2	2	6
Animal management	2	2	6
Poultry management (2nd semester)	1	2	2
Computer	1	2	4
Democracy and human rights	2	0	4
Total	12	12	34

Second year

Course	Lecture (h)	Lab (h)	Total credit
Biochemistry	3	2	8
Physiology	4	3	10
Anatomy	2	3	7
Histology	2	3	7
Animal nutrition	2	2	6
Genetics (1st semester)-	2	0	2
Embryology (2nd semester)-	1	0	1
Statistics (2nd semester)-	2	2	3
Total	18	14	44

Third year

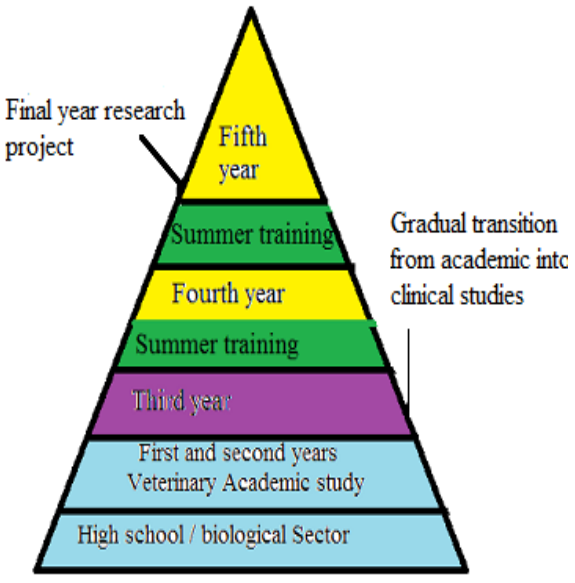
Course-	Lecture (h)	Lab (h)-	Total credit
Pathology	3	3	9
Pharmacology	3	2	8
Parasitology	3	2	8
Microbiology	3	3	9
Immunology (1st semester)	2	2	3
Toxicology (2nd semester)	2	0	2
Clinic (2nd semester)	2	0	1
Total	16	14	40

Fourth year

Course-	Lecture (h)	Lab (h)-	Total credit
Clinic	0	4	4
Infectious diseases & epidemiology	3	2	6
Theriogenology	2	2	6
Poultry diseases	2	2	6
Medicine	3	0	6
Surgery	3	2	8
Clinical pathology	1	2	4
Zoonotic diseases (2nd semester)	2	0	2
Total	16	12	42

Fifth year

Course	Lecture (h)	Lab (h)	Total credit
Clinic	0	13	13
Veterinary public health	2	2	6
Obstetrics	1	2	4
Fish diseases (1st semester)	1	2	2
Medicine	3	0	6
Surgery	2	2	6
Research project	1	0	2
Morbid anatomy & forensic medicine (2nd semester)	1	2	2
Veterinary ethics (2nd semester)	1		1
Summer Clinic	0	3	2
Total	12	26	44



Hierarchy of educational system in faculty of veterinary medicine / University of Kufa

The total number of subjects are 40 and made up of 204 units in total during the five years

Staff and facilities

The FVMUK has a number of academic staff who teaches in different departments (Figure 3.) as well as a support staff and technician who assist in the teaching processes. The faculty is well equipped with modern equipment that is necessary for teaching processes as well as for research activities. The FVMUK has classrooms, laboratories, research facilities, offices and library. The faculty has established the research committee and animal ethical committee to ensure keeping the animal welfare issue and quality assurance.

Figure3. Show FVMUK's academic staff and some other colleagues from college of veterinary medicine/ University of Baghdad during the one day seminar about the application of drugs and its side-effects on public health

What is the next?

From day one of establishment, the FVMUK attempts to improve the quality of veterinary education in different aspects in order to provide next generation of veterinarians with appropriate knowledge and skills to perform their duties with a high degree of professional quality.

The ‘‘One Medicine’’ concept was reintroduced during the second half of the 20th century by Dr. Schwabe in his book Veterinary Medicine and Human Health and is credited with renewing recognition that the ‘‘cumulative effect of all practitioners of medicine is aimed at quality of human life and survival’’ (Schwabe, 1984). Schwabe always mentioned that ‘‘the critical needs of man include the combating of diseases, ensuring enough food, adequate environmental quality, and a society in which humane values prevail’’ (Schwabe, 1984). Schwabe's vision that human and veterinary medical practitioners are obliged to work together in sharing information to ensure

the physical, mental, social, economic and inner health for all life have never been more important than it is now in our changing global environment. Most school of medicine and veterinary medicine responded to this concept and has seen a gradual development of their curriculum in the context of global education (Jorge A. Hernandez, Traci M. Krueger , Sheilah A. Robertson , Natalie Isaza, 2009).

In Iraq the veterinary medical educational follow a classical uniform standard curriculum, the change is urgent to empower the curriculum with a global perspective of the veterinary profession, as well as with a humanist education that can help students recognize the importance of respect for cultural differences and the reasons for different degrees of development and growth in the world. This can only be achieved by adoption the concept of curriculum "**modernization and integration toward veterinary professionalism and societal responsibility"**. In addition, the veterinary education most redirected from teaching objectives to major learning objectives and adopted a modern learning techniques. The FVMUK is a young faculty and can be established it strategies in the context of global education, which is one of the university of Kufa objectives. It requires formulating, implementing, redirecting and integrating its educational system from traditional teaching to a modern learning technique and considering it as the first unique faculty of veterinary medicine in Iraq that turn off the traditional or classical curriculum and moving into the initiative veterinary international education.

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Mirror of Research in Veterinary Sciences and Animals (MRVSA)

Original Article

Histopathological changes in the Intestine and lung of mice infected experimentally with *Salmonella mbandaka*

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Abstract

Salmonella mbandaka has been isolated and identified from human in Iraq. The purpose of the present study was to investigate the histpathological changes in the internal organs of mice experimentally infected with *Salmonella mbandaka*. Thirty mice of both sexes with age range (6 – 8) weeks old were divided randomly into two groups: "group A" (15 mice) inoculated orally with infective dose (ID) (1.3×10^7 cells) and "group B" (15 mice administrated orally with 0.5 ml PBS) and considered as a control group. Both infected and non infected mice were Sacrificed after 1 week ,2 ,4 ,6 and 8 weeks post inoculation. After 1 &2 weeks post infection , results revealed a slight desquamation of intestinal mucosal epithelia together with tissue debris accumulated in lumen accompanied by hyperplasia and hyper atrophy of goblet cell, sub mucosal edema accompanied with blood vessels congestion surrounded with intense cellular infiltration. PMNs infiltration mainly in mucosa and sub mucosa of intestine and around bronchi associated with congested blood vessels in lung. While the characteristics manifestations during 4, 6 & 8were lymphoid hyperplasia of intestine tissue together with MNC pervious aggregation in lung. In conclusion, this study revealed a different changes in organs of mice infected with *S. mbandaka* , this indicate the virulence of this bacteria to cause a disease in mice and its ability to invade and replicate in intestine and lung.

Keyword : *Salmonella mbandaka*, *histopathology*, *Salmonella* infection.
Abbreviations: PMN= polymorph nuclear cell; PI= post infection, CFU= colony forming unit, MN= mononuclear cell, PBS= phosphate buffer saline

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Introduction

Salmonella mbandaka was distributed worldwide in human and animals (Hayward *et al* 2013; Le Doare *et al* ,2013). The microbiota of the mammalian intestinal tract represents a formidable barrier to colonization by pathogens. To overcome this resistance to colonization, bacterial pathogens use virulence factors to induce intestinal inflammation, which liberates nutrients for selective use by the infecting microbe (Bliska and Velden, 2012). Systemic infections represent severe manifestations of salmonellosis. Intracellular *Salmonella* present in immune cells, e.g. macrophages and dendritic cells, may facilitate systemic infection by carrying the microorganism from the intestinal tract throughout the whole body. Dendritic cells are important migratory phagocytes that are widely distributed throughout the body in lymphoid and non-lymphoid tissues (Sundquist *et al*., 2004). , in Iraq, S*almonella mbandaka* was isolated at first time from stool samples of diarrheal children by (Al- Talib,2011). In Iraq, data regarding the use of this species as a model of *Salmonellosis* in animal is very scarce. Therefore, this work aimed to study and investigate the histpathological effect of S*almonella mbandaka* in intestine and lung organ of mice.

Material and methods

Bacterial isolates

Salmonella mbandaka was obtained from zoonosis laboratory –College of Veterinary Medicine-University of Baghdad. Diagnosis was confirmed according to Quinn *et al.* (2004) and serotyping was done in the Central Public Health Laboratories (National Center of *Salmonellae* in Baghdad). The infective dose of *S. mbandaka* is (1.3×10^7 cells) was estimated according to (Shallal, 2011; Yousif & Al-Naqeeb, 2010).

Laboratory animals

Thirty mice (BALB/c) of both sexes, 6–8 weeks old, obtained from the National Center of Researches and Drugs Monitor in Baghdad. Before starting the experiment, the mice were adapted for two weeks by rearing in separated clean and disinfected cages, fed on commercial assorted pellets and clean water was supplied by *ad libitum*. Then, the mice were divided randomly into 2 groups. Group A was given orally 0.5ml (containing of 1.3×10^7 CFU/ml) of *S. mbandaka* while group B administrated 0.5 ml of PBS, and acted as controls.

Histopathological studies

From each group, three mice were sacrificed by neck dislocation at 1, 2, 4, 6 and 8 PI. Organs were removed under aseptic conditions and kept in 10% buffered formalin for 24 h. Then, routine histopathological process was performed to obtain slides stained with haematoxylin and eosin (H&E) for histological evaluation (Bancroft *et al.*, 1994).

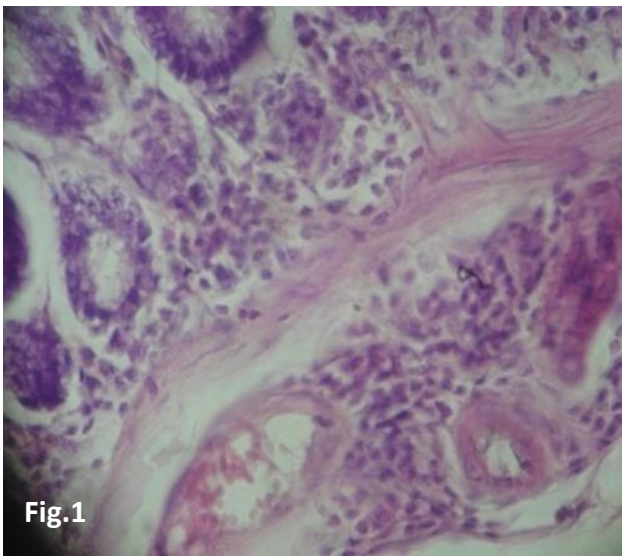
Ethical approval

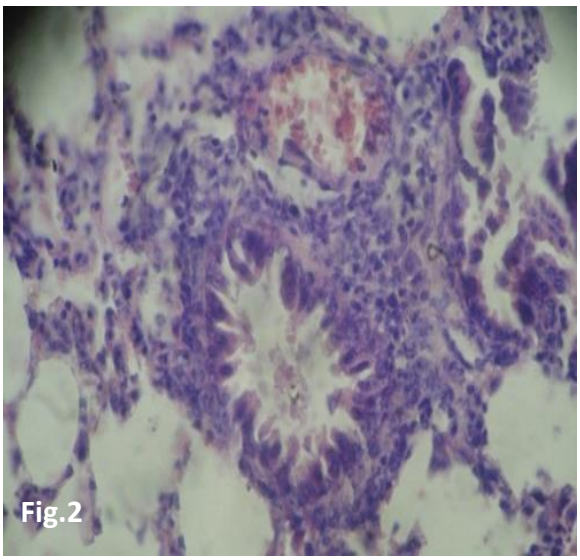
This study was approved by the ethical and research committee of Veterinary Medicine College/University of Baghdad.

Results and discussion

The histopathological examination of organs infected with *S.mabandaka*, 1 week PI was characterized by slight desquamation of intestinal mucosal epithelia together with tissue debris accumulated in lumen accompanied by PMNs infiltration mainly in mucosa and sub mucosa of intestine, hyperplasia and hyper atrophy of goblet cell, sub mucosal edema accompanied with blood vessels congestion surrounded with intense cellular infiltration (Figure.1), and sub epithelial PMNs and MNCs with degeneration and necrosis of mucosal gland were frequently observed. The lung histolesions showed severe PMNs infiltration around bronchi associated with congested blood vessels, active alveolar hyperemia with sloughing of bronchial epithelia (Figure.2).

Figure 1: Photomicrograph of intestine of mouse infected with *S.mbandaka* at 1 week PI shows sub mucosal edema accompanied with MNCs aggregation and congestion blood vesicles(H&E 40X) .
Figure 2: Photomicrograph of lung of mouse infected with *S.mbandaka* at 1 week PI shows cellular aggregation a round bronchi &b.v with sloughing of bronchial epithelial (H&E 40X).

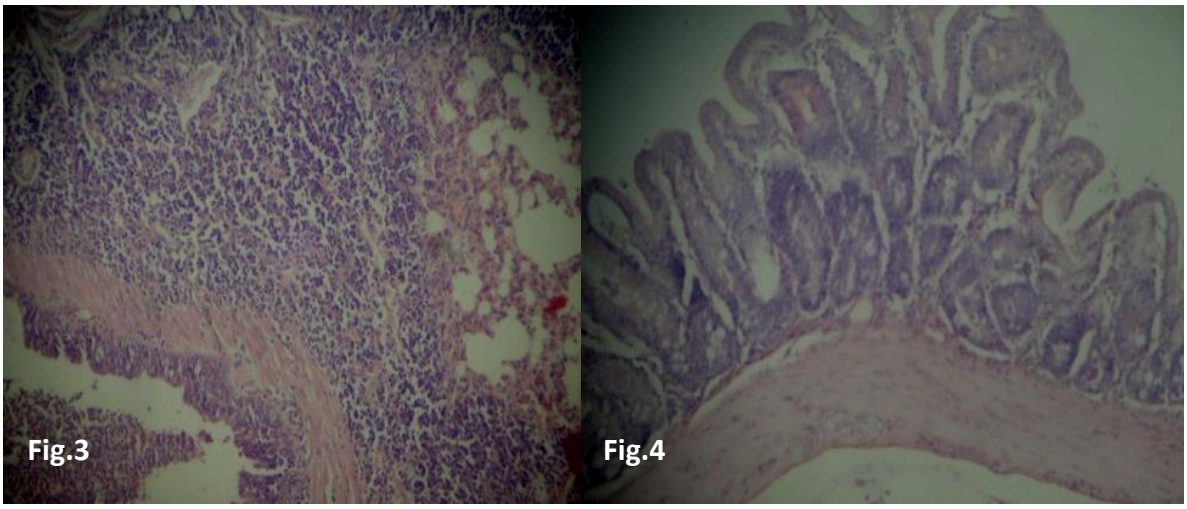




The characteristic manifestation of infective animals sacrificed after 2weeks post infection with S. mbandaka characterized by slight cellular infiltration with sever hyperplasia mucosal gland of intestine (Fig. 3). The lung showed presence of suppurative bronchopneumonia in which intense neutrophil infiltration in bronchiol & acinar tissue with bronchiectasis accompanied with mucopurulent exudate in their lumen to gather with fibro muscular hyperplasia of bronchiolar wall (Fig. 4).

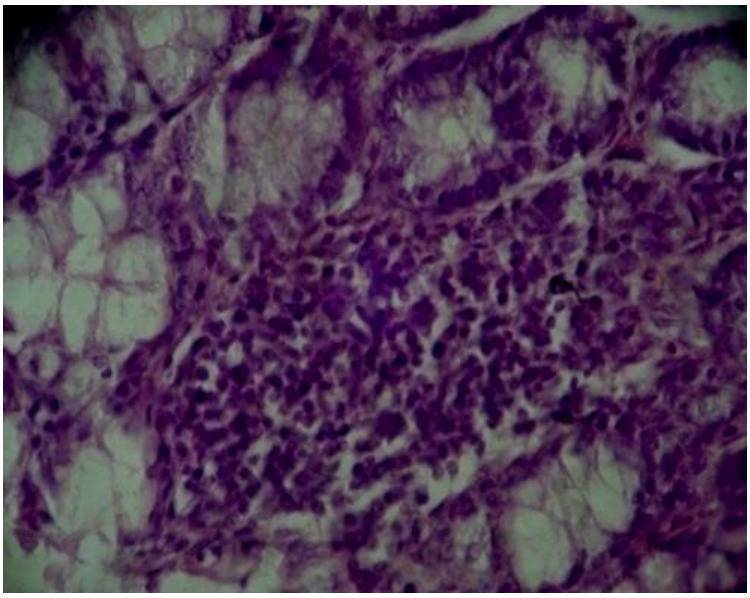
Figure 3: Photomicrograph of intestine of mouse infected with *S.mbandaka* at 2 week PI shows slight cellular infiltration with shortening of mucosal villi. (H&E 40X).

Figure 4: Photomicrograph of lung of mouse infected with *S.mbandaka* at 2 week PI shows suppurative bronochopn accompanied with bronchiectasia (H&E 40X).



After 4 weeks post infection with S.mbandaka, the scarified mice showed intense lymphocytic aggregate in the sub mucosa that appear as nodular forming with sever hyper atrophy of mucosal goblet cells with various degree of sloughing of intestine (Fig. 5).

Figure 5: Photomicrograph of intestine of mouse infected with *S.mbandaka* at 4 week PI shows intense lymphatic aggregate with nodular appearance together with sever hyperatrophy of mucosal goblet cells. (H&E 40X).



Histopathological examination of infective animals sacrificed after 6 weeks PI characterized by intense lymphoid hyperplasia in payer's patches of intestine(Fig. 6). The lung showed intense MNCs perivascular aggregate with congestion blood vesicles (Fig. 7).

Figure 6: Photomicrograph of intestine of mouse infected with *S.mbandaka* at 6 week PI shows intense lymphoid hyperplasia in pyers patches (arrow) (H&E 40X).

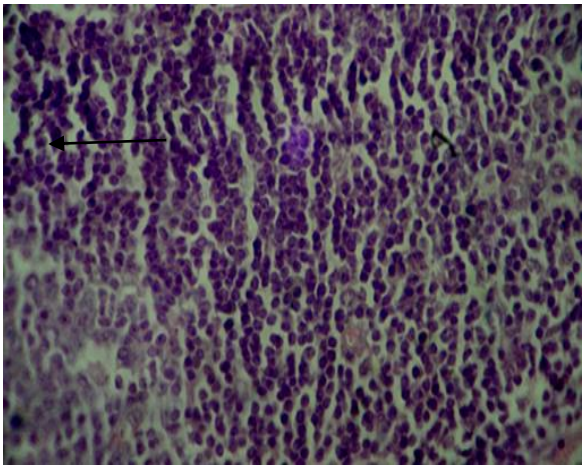
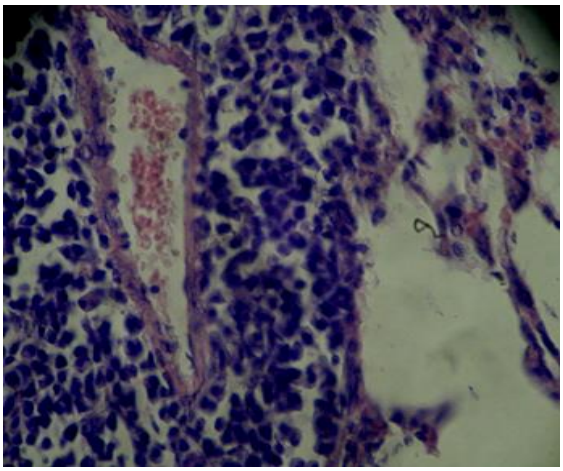
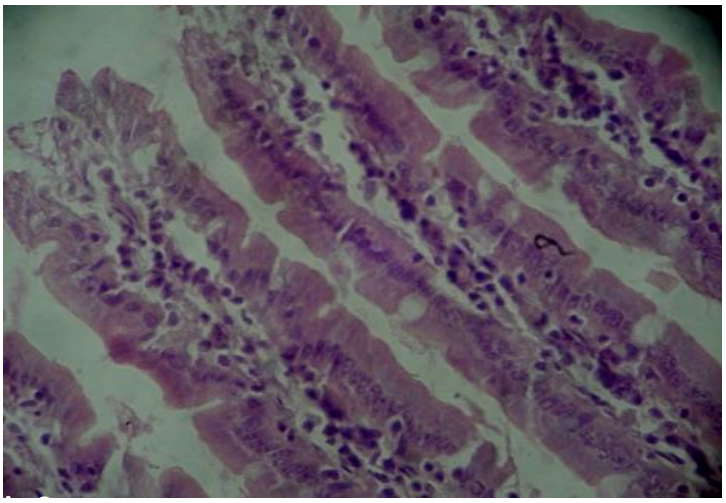


Figure 7: Photomicrograph of lung of mouse infected with *S.mbandaka* at 4 week PI shows intense MNCs perivascular aggregate with blood vessles congestion (H&E 40X).



The histopathological examination of infective animals sacrificed after 8 weeks PI showed no clear pathological changes in most organs except minimal desquamated mucosal layer with sub epithelia cellular infiltrate of intestine (Fig.8).

Figure 8: Photomicrograph of intestine of mouse infected with *S.mbandaka* at 8week PI shows minimal desquamated mucosal layerwith sub epithelia cellular infiltrate . (H&E 20X).



According to histopathological examination the lesions showed that *Salmonella mabandaka* which used in the current study can produce significant changes in the internal target organs of experimental infected mice mainly in intestine and this may be attributed to its primary multiplication in the lumen of intestine that causes changes in the composition of the lumen and enhance inflammation in the mucosa and L.p efficiently disseminate to another host, ensuring success for pathogen invasion (Bliska &Velden 2012).

Salmonella colonizes the Peyer's patches of the intestine and penetrates the gut barrier via M-cells from which it can disseminate to the local mesenteric lymph nodes and then to the spleen and liver, transported by phagocytic cells and when *Salmonella* invades the blood stream, it reaches different and distal target organs or tissues where it is able to multiply and cause more or less severe systemic focal infections (Rodriguez *et al*., 2006). The ability of DC to migrate throughout the body potentially facilitates the spread of *Salmonella* to various parts of the body ; While in the DC the *Salmonella* does not appear to replicate but remains viable, possibly in a small colony variant state with reduced metabolic activity and increased persistence (Tierrez and Garcia-del Portillo, 2005).

The macrophages or denderitic cells enter certain organ systems, the *Salmonella* can spread to adjacent cells and trigger apoptosis, which leads to increase pathology among the infected cells (Sheppard *et al*., 2003). Moreover the results also recorded various degree slough of intestinal mucosa as well as well as shortening of villi, and this was in consistence with observation by (Cousaemeni *et al*., 1982) who suggest the shortening and loss of microvilli is in accordance with decreased alkaline phosphatase activity, and this enzyme is located in the plasma membrane of the microvilli and is considered as a measurement of the digestive-absorptive surface. In addition Yousif and Al- Nageeb ,(2010) mentioned the ultrastructural changes in the ileum of mouse inoculated with infected dose of *S. hader* that killed after 72 hours post infection were similar to those described in the previous intervals. More evident damage of the ileum was observed after passage of 96 hours there were loss of some microvilli, marked dilatation and vacuolization of the endoplasmic reticulum with dispersion of microvilli and loss of the other mainly structures of the injured enterocytes due to presence many intracellular bacteria and after 120 hours post infection revealed hypertrophy of goblet cell, dilatation of endoplasmic reticulum, severe

cytoplasmic vacuolization, thickening of the nuclear membrane and there was several *Salmonella* containing vacuoles.

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Mirror of Research in Veterinary Sciences and Animals (MRVSA)

Original Article

Effects of metformin treatment on Iron, Zinc and Copper status concentration in the serum of female rats with induced polycystic ovary syndrome

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Abstract

This study conducted to investigate the effects of metformin drug on serum Iron, Zinc and Copper concentration in Estradiol Valerate(EV) induced polycystic ovary syndrome(PCOS) in virgin rats. Thirty virgin rats were randomly allotted to constitute Normal control (NC-I) group and induced polycystic ovary (PCO-I and PCO-II) groups having 10 rats in each group. Rats from NC-I group were administered intramuscularly with 0.2 ml of corn oil whereas polycystic ovary was induced in rats from PCO-I and PCO-II groups by administering single intra-muscular injection of estradiol Valerate 4mg/rat. The rats from PCO-I and PCO-II groups were left for 60 days for development of polycystic ovary syndrome. Animals from PCO-I group were then administered with 0.2 ml normal saline as oral gavage for 15 days, these animals were kept as PCO control group animals whereas those from PCO-II groups received metformin (50mg/kg B.wt) as oral gavage for 15 days, these animals served as metformin treated PCO group animals. All the rats were thereafter sacrificed for collecting blood from inferior vena-cava. Serum samples from each rat were assessed for iron, zinc and copper status in each experimental group. The results revealed a significant ($p \leq 0.05$) increase in serum Fe and Zn and a significant ($p \leq 0.05$) decrease in serum Cu concentration in PCO group 1 compared with control non-treated group. The PCO group2 treated with metformin showed a significant ($p \leq 0.05$) decrease in serum Fe concentration as compared with those in animals from group NC-I and PCO-I. While, no significant differences were found in serum Zn concentration between all treated groups. On the other hand, a significant ($p \leq 0.05$) increase in serum Cu concentration appeared in metformin treated group compared with PCO group 1 which appears significant decrease compared with control group.

Key words*:* polycystic ovary syndrome, metformin, Iron, Zinc & Copper concentration.

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Introduction

Polycystic ovary syndrome (PCOS), a complex, multifactorial endocrinopathy of reproductive aged women, is a multifactorial reproductive endocrinopathy of aged women. It is the most common cause of infertility (Jacqueline, et al, 2012). PCOS is a metabolic disorder associated with insulin resistance attributable to ovarian hyperinsulinemia, however, in women with PCOS metformin treatment restores the cyclic nature of menstruation and increases ovulation (Vandemolen, et al, 2001). Metformin is a biguanide currently used as oral antihyperglycemic agent, in additionally, Metformin is an anti-diabetic drug commonly used to treat cycle disorders and anovulation in women with PCOS (Stefano, et al, 2009). Multiple concomitant therapies have been applied in PCOS, for such a syndrome is used of Insulin Sensitizer Drugs (ISDs) and drugs lowering androgen secretion, they were later associated with beneficial effects in the treatment of PCOS(Mandakini, 2005).

Metformin has been shown to significantly reduce basal hepatic glucose production, increased peripheral glucose disposal and reduced intestinal absorption (Ripudaman, and Silvio, 2003). The essential or beneficial effect of many trace elements has been established mainly in laboratory or farm animals rather than in humans, the most important of them are Iron, Zinc and Copper. These trace elements are necessary for the growth and function of the brain where deficiency or excess of these elements resulted in nervous disorders (Takeda, 2004). Many researches have indicated that serum body iron are elevated in patients with PCOS (Angeles, et al, 2009; Manuel, et al, 2011). Other authors opined that metabolic disorder including hyperinsulinemia is probable cause of iron accumulation in PCOS patients (Faranak, et al, 2011; Saeed, et al, 2011)).

A recent report described reduced levels of Zn in obese and insulin resistance subject (Chausmer, 1998). Whereas, other authors have recorded increase in the serum Zn concentration in diabetics (Vikkorinova, et al, 2009). Interestingly it was reported that diabetics have elevated levels of copper (Obeid, et al, 2008) and it could be that copper is in fact linked to metabolic syndrome and diabetes (Wijesekara, et al, 2009). Copper is an essential trace element, capable of fluctuating between the oxidized Cu2+ and the reduced Cu+ state, being co-factor for many enzymes. More, the deficiencies and the excess of Cu are associated with specific clinical manifestations (Guojun, et al, 2004). This study was designed to evaluate effects of metformin treatment on iron, Zinc and Copper status concentration in the serum of female rats with induced polycystic ovary syndrome.

Materials and methods

Thirty virgin adult cycling female rats (200±15 g B.wt.) were housed (4 rats/cage) under optimum identical conditions (12/12 light, dark cycle, 22 ± 2 C°) wherein these are allowed free access to pelleted rat chow and tap water. Animals showing at least four regular 4-day cycles were randomly allotted to constitute Normal control (NC-I) group and induced polycystic ovary (PCO-

I and PCO-II) groups having 10 rats in each group. Rats from NC-I group were administered intramuscularly with 0.2 ml of corn oil whereas polycystic ovary was induced in rats from PCO-I and PCO-II groups by administering single intra-muscular injection of estradiol Valerate 4mg/rat. The rats from PCO-I and PCO-II groups were left for 60 days for development of polycystic ovary syndrome. Animals from PCO-I group were then administered with 0.2 ml normal saline as oral gavage for 15 days, these animals were kept as PCO control group animals whereas, those from PCO-II groups received metformin (50mg/kg B.wt.) as oral gavage for 15 days, these animals served as metformin treated PCO group animals. All the rats were thereafter sacrificed for collecting blood from inferior vena-cava. Serum was separated by centrifugation 5000 rpm and stored at -20 C for assessing Fe, Zn and Cu concentration in serum.
This study was approved by research and ethical committee in College of Veterinary Medicine/ University of Basrah, no: 25/11/2011.

Determination of serum Fe, Zn and CU Levels

The serum samples from each group were subjected to wet digestion for releasing iron, zinc and copper from the protein matrix (Akinloye, et al, 2011), these trace elements were then evaluated in serum by atomic absorption spectrophotometer (AAS) using a Buck Model 211-VGP spectrophotometer according to operator's manual (February, 2005 VER 3.94 C by Analyst: Gerald J. De Menna), with a detection limit of 0.05 ppm for Fe (Akinloye, et al, 2009).

Biostatistical analysis

The data were expressed as mean ±Standard Deviation (SD) and analyzed using two way analysis of variance (ANOVA). Least significant difference (LSD) was used to test the differences among means for ANOVA indicated a significant ($P<0.05$), using computerized SPSS version 11.

The Results

From different groups (Table-1) reveals significantly ($P\leq0.05$) increased serum iron levels in the rats from induced Polycystic ovary (PCO-I and II) groups as compared to that in normal control (NC-I) group; among PCO groups it was significantly higher in untreated animals (PCO-I group) as compared to that in metformin treated animals (PCO-II group). Serum Zn levels in animals from normal control (NC-I) group was significantly higher than that in animals from untreated polycystic ovary (PCO-I) group, however, serum Zn levels in animals from metformin treated (PCO-II) group did not differ significantly from that in animals from normal control (NC-I) and untreated polycystic (PCO-II) groups. Serum Cu level in animals from normal control group (NC-I) was significantly ($P\leq0.05$) higher than that in animals from metformin compared with control group and PCO group.

Table (1): The effect of treatments for 15 days with metformin on some trace elements concentration in female rats with induced polycystic ovary syndrome.
(Mean ±SD, n=10).

Parameter / Groups	Fe/mcg/ml	Zn/ppm	Cu/ppm
Control	b 656.25±11.79	b 6.92±0.25	a 26.61±1.36
PCO 1	a 697.00±7.43	a 8.18±0.23	c 15.24±0.77
PCO2	c 256.25±3.75	ab 7.50±0.29	b 20.25±0.64
LSD Least significant difference	38.12	1.02	5.01

Values expressed in the, b, c; mean significant differences at the (P≤0.05) level.

Discussion

In the present study, substantial evidence was provide support the increase in Fe, Zn serum concentration associated with decreased Cu concentration in female rats suffering from induced PCO compared with control (table 1). Pervious study was mentioned that insulin resistance plays a major role on the increased body Fe stores of rats with PCOS. On the other hand, the reduced menstrual losses and /or oligomenorrhea may contribute to increased Fe concentration (Mandakini, 2005). Guojun, et al (2004) explained that patients with PCOS could have led to increase iron concentration. The result of this study is compatible with the other researcher who reported that patients with PCOS showed decreased circulating hepcidin levels and increase ferritin to hepcidin molar ratios compared with control (Manuel, et al, 2011). The results of this study is in agreement with (Gőzdemir et al, 2013) they explained that the hepcidin plays a role in the regulation of metabolism and acts as an inflammatory marker in polycystic ovary syndrome.

Moreover, Zn is one of the most important trace elements required as catalytic, structure and regulatory ion for the activities of more than 300 enzyme proteins transcriptional factors (Pouteymour, et al, 2011). Current study, revealed increase in the serum Zn concentration in PCO group, this may be associated with disturbance of glucose metabolism represented by insulin resistance, dyslipidemia and endocrine disturbance leading to oxidative stress and finally increase in Fe, and Zn. Mounting evidence indicates that higher body Fe, Zn stores are associated with increased risk of other insulin resistant disorder such as high blood glucose and lipid profile (Yves, et al, 2011). Table(1) shows that after 15 days intake of metformin, female rats with induced PCOS using EV a significant reduction in serum iron, and significant increase in Zinc but serum Copper demonstrated a significant decrease compared with induced PCO non treated and control group. This finding may indicate that metformin prevent the absorption of iron from intestinal lumen leading to decrease in iron body concentration, and this finding is in agreement with other authors (Lugu, et al, 2007). Luca, and Francesca, (2008) have recently found that metformin increasing insulin sensitivity may decrease intestinal iron absorption in patients PCOS. Tahira, et al (2011) reported that three months intake of metformin in PCOS patient caused a reduction ferritin may improve glycemic and insulin sensitivity.

Oral administration of metformin act to improvement the serum level of both Zn and Cu. It may act as antioxidant as well as improving metabolic activity in the body of the rat. Many reported found

that Zn and Cu have important role in cellular metabolic regulation. Fatemah, et al (2010) found positive beneficial effects on feature of metabolic syndrome in PCOS after Zn supplementation. Also, reported in large clinical study, both low consumption of dietary Zn and low serum Zn concentration were associated with increase diabetic and hypercholesterolemia. Tahira, et al (2011) suggested that giving additional Zn has more benefits and effectiveness in patients with PCOS. Pouteymour, et al (2011) mentioned that Zn supplementation improve inflammatory reaction in PCOS patients. Finally, metformin could act as a regulator in cellular body led to improve metabolism and Zn absorption. PCOS is a metabolic disorder may result in dysregulation of systemic copper homeostasis. Metformin treatment improves the serum Cu concentration in PCO patients. In conclusion the present data suggested that metformin has negative effects on iron but it was improved other minerals (Zn and Cu).

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