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EVALUATION OF SOME MILK ENZYMES AS BIOMARKERS IN DETECTION OF BOVINE SUBCLINICAL MASTITIS

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ABSTRACT

The present study was carried out on One hundred and fifity , one day old chicks to evaluate the immunogenicity of multivalent pilus vaccine containing pili from the three common pathogenic serotypes of E. coli (O1, O2 and O78). Nienty vaccinated birds at 14 days & 28 days of age by 0.5ml s/c of pilus vaccine at base of neck and 60 unvaccinated were divided to 4 subgroups (30 vaccinated & 20 unvaccinated) were used for challenge two weeks after second dose . Each group was challenged each with one type of E.coli strains O1 , O2 and O78. Blood samples were collected during the experiment from the flocks one week before vaccination and weekly for 7th week after the first dose of vaccination for evaluation of immune response by using ELISA test. The prepared multivalent E.coil pilivaccine from combination of $O_{1,}$ O_2 and O_{78}E.coli strains were seemed to cover good range of protection and has been elicited a protective immune response against virulent E.coli challenge with homologous and heterologous strains. strong correlation was found between antibody response vaccinated groups and low lesions score that indicated a good protection

Keywords, *enzyme linked immune sorbent assay (ELISA).Esherichia coli (E.coli).*

INTRODUCTION

Mastitis is a worldwide problem and of major economic threat to dairy farmers. Its incidence depends on the microorganisms, the surrounding environment and the defense mechanisms in the udder tissues and blood. In the dairy industry, both clinical and subclinical mastitis produce great economic losses (**Abd Ellah, 2013).** Subclinical mastitis is difficult to detect due to the absence of any visible indications and requires the availability of a rapid screening test for early onset disease detection **(Viguier et al., 2009).** About 70 to 80% of the estimated $140 to $300 loss per cow per year from mastitis relates to decreased milk production caused by asymptomatic subclinical mastitis **(Leitner *et al.*, 2011).** The bacterial contamination of milk from the affected cows makes it unhealthy for human consumption and has zoonotic importance **(Sharif *et al.*, 2009).** Various method of detection of SCM have been found including California Mastitis Test (CMT) which is considered the best cut off to correctly identify SCM mastitis **(Zaki *et al.*, 2008).** Also, estimation of somatic cell counts (SCC); is an indication of inflammation, measurement of biomarkers or enzymes associated with the onset of the disease and identification of the causative microorganisms **(Viguier *et al.*, 2009).** For many years there has been an interest in using different enzymes in milk as biomarkers for mastitis. Many studies have revealed that enzyme activities in the udder epithelium change markedly due to mastitic inflammation **(Andrei *et al.*, 2011).** More

practical attention has been given to detection of enzyme activity in milk, and many enzymes have been proposed and listed as reliable markers for early diagnosis of subclinical mastitis **(Babaei *et al.*, 2007; Guha *et al.*, 2012).** Among these enzymes, lactate dehydrogenase (LDH) has been suggested as a biomarker for udder health disturbances as well as astatistical model for mastitis detection **(Chagunda *et al.*, 2006).** the quantity and enzymatic activity of the alkaline phosphatase are increasing in the mastitic milk, therefore its measurement can constitute an indicator for identify an infectious process in mammary gland. **(Argherie, 2008).** The correlations between bacterial infections, SCC and enzymatic activities in SCM milk revealed that the mixed bacterial infections; especially with *S. aureus* + *Str. dysgalactiae*, showed higher elevation in SCC as well as enzymes activities than single bacterial infections. They concluded that enzyme parameters can be used as biomarkers for early detection of subclinical mastitis **(Zeinhom et al., 2013).**

MATERIALS AND METHODS

1- Animals: A total of 70 clinically healthy dairy cows of Holstein Friesian breed in special dairy farm and 54 buffaloes from private owners were examined in Dakahlia governorate, Egypt.

2- Milk sampling: Milk samples were collected from 280 quarters of 70 clinically healthy dairy cows of Holstein Friesian breed and from 216 quarters of 54 buffaloes before morning Milking . All animals had no evidence of clinical mastitis at the time of sampling. Teat orifices were cleaned firstly then swabbed with cotton soaked in 70% ethyl alcohol, discarding the first streams of foremilk and then 50 ml of milk was collected aseptically from each teat into sterile tubes . Milk samples were kept cold during transportation, at 4°C and reached to the laboratory to be examined within 2 hours after collection.

3-Detection of subclinical mastitis :

California mastitis test (CMT): CMT was applied directly in the farm to milk samples from each quarter using the method of **Schalm *et al.* (1971).** It is based on the principle that the addition of a detergent to a milk sample with a high cell count will lyse the cells, release nucleic acids and other constituents and

lead to the formation of a 'gel-like' matrix consistency. According to the visible reactions the results were classified in four scores; 0 "negative or trace", 1 "weak positive", 2 "distinct positive" and 3 "strong positive".

All milk samples showed negative CMT were considered as control and were subjected into two parts, the first part for SCC and the second part for enzymatic analysis. The Milk samples showed positive CMT were subjected into two parts, the first part for enzymatic analysis and the second part for bacteriological examination.

4-Bacteriological examination of milk samples:

The Milk samples showed positive CMT were used to detect any possible bacteriological causal agent. The samples showed negative CMT were used as control.

Milk samples:

(10 µl) were cultured from each positive CMT milk sample on blood agar, Mannetol salt agar, Edward's media and MacConkey agar plates according to **National Mastitis Council (1999).** Plates were incubated aerobically at

37°C for 24-48 h. The plates were examined for gross colony morphology, pigmentation and haemolytic characteristics at 24-48h. Presumptive identification of bacterial isolates was according to their colonial characteristics, Gram's reaction and morphology. Identification was confirmed by additional laboratory tests according to **Quinn *et al.* (1994); Abera *et al.* (2010) and Persson *et al.* (2011).**

Enzymatic assays: It was applied to all milk samples. Firstly, milk samples were defatted by centrifugation at 3000×g for 10 min at 4°C. Defatted milk samples were used for enzymatic estimation. LDH and ALP enzymes activities were estimated using commercial colorimetric assay kits (An EKP Diagnostic company and ELITech Clinical Systems SAS-Zone Industrielle- 61500 SEES FRANCE

For LDH and ALP, respectively). The standardized protocol provided with the kit was followed for estimation. The final results were reported in units per l of milk (U/l milk).

Table (1): Prevalence of subclinical mastitis (SCM) according to California mastitis test (CMT) at quarter level in dairy Cows and Buffaloes.

Criteria	Cows	Buffaloes
Total number	280	216
-ve CMT	196	160
+ve CMT	84	56
Prevalence	30%	26%

Table (2): Frequency distribution of microbial isolates from subclinical mastitis milk samples of dairy cows and buffaloes at quarter level.

	Microbial isolates	Cows		Buffaloes	
		No.	Frequency (%)	No.	Frequency (%)
Single isolates	Staphylococcus Spp.	32	38%	22	39%
	Streptococcus Spp.	23	27%	16	29%
	E-coli	18	22%	9	16%
	Corynebacterium Spp.	1	1%	2	4%
Mixed isolates	Staph.+ E-coli	3	4%	4	7%
	Strept. + E-coli	7	8%	3	5%
	Overall total	84	100%	56	100%

Table (3):LDH (U/L) and ALP (U/L) values (mean values ± S.E.M) in control group of cow's milk and in those with subclinical mastitic milk samples caused by different Bacteria.

Group	LDH	ALP
Control	157.500 ± 5.1460^{d}	170.250 ± 4.578^{d}
Staphylococcus	478.281 ± 7.6187^{a}	539.219 ± 8.919^{a}
Streptococcus	417.391 ± 6.3950^{b}	489.348 ± 463.58^{b}
E-coli	390.833 ± 4.3770^{bc}	389.333 ± 5.470^{c}
Corynebacterium spp.	300.000 ± 00^{c}	330.000 ± 00^{c}
Staph.+ E-coli	423.333 ± 8.8192^{ab}	370.000 ± 2.887^{c}
Strept. + E-coli	377.143 ± 5.3293^{bc}	345.000 ± 4.082^{c}

$^{a, b, c, d}$: Variables with different superscript in the same column are significantly different at $P < 0.05$.

Table (4): LDH (U/L) and ALP (U/L) values (mean values ± S.E.M) in control group of buffalo's milk and in those with subclinical mastitic milk samples caused by different Bacteria.

Group	LDH	ALP
Control	169.750 ± 5.2875^{d}	169.750 ± 6.762^{c}
Staphylococcus	440.000 ± 3.3900^{a}	561.094 ± 15.933^{a}
Streptococcus	395.000 ± 4.1703^{b}	485.652 ± 10.337^{b}
E-coli	361.667 ± 2.6813^{c}	487.778 ± 10.697^{b}
Corynebacterium	355.000 ± 5.0000^{bc}	470.000 ± 10.000^{ab}
Staph.+ E-coli	387.500 ± 6.6144^{bc}	476.250 ± 23.038^{ab}
Strept. + E-coli	378.333 ± 6.0093^{bc}	510.000 ± 11.547^{ab}

$^{a, b, c, d}$: Variables with different superscript in the same column are significantly different at $P < 0.05$.

RESULTS & DISCUSSION

Subclinical mastitis is a condition in which there is no detectable inflammatory change in the udder and no observable abnormalities in the milk. However there are many changes encountered in milk from udder with subclinical infection. Cellular, biochemical, immunological and enzymatic change are present in such milk. These changes are detected in most of screening and routine tests applied to diagnosis Subclinical mastitic cases. Often it is more prevalent than the clinical form, it usually precedes the clinical form , it reduces milk production and adversely affect milk quality. Subclinical mastitis is 3 to 4 times more common than clinical mastitis and causes great losses in the dairy herds **(Joshi and Gokhale, 2004).** Total milk loss from quarters affected with Subclinical mastitis has been estimated to vary from 10% to 26%. Approximately 75% of the economic loss from Subclinical mastitis is attributable to loss of milk production. The most significant Subclinical abnormality of the milk is the increase in SCC, the most common measure of milk quality and udder health **(Radostitis et al, 2000).**

CMT which is most common test used for diagnosis of subclinical cases depend on detection of increased leukocytic count in milk of such cases. In this study, 280 quarter milk samples from cows and 216 quarter milk samples from buffaloes were examined by CMT and results showed that 84 samples (30%) were positive for cows and 56 samples (26%) were positive for buffaloes **Table(1).**

Karimuribo et al .(2006): concluded that CMT is still superior screening diagnostic aid for subclinical mastitis, hence it could be most the reliable test to be conducted to investigate subclinical mastitis in dairy farms. In this study , based on CMT the prevalence of subclinical mastitis at quarter level was 30% and 26% for cows and buffaloes , respectively.

This results is in agreement with previous studies by **Mdegela et al.(2009), Mekebib et al. (2009), Bitew et al. (2010), Girma (2010) and Mir AQ et al. (2014)** who reported prevalence of 30, 34.8 , 32.4, 34.4 and 30.73%, respectively. Higher prevalence were reported by **Zeryehun et al. (2013), Abrahmsen (2014), Zenebe et al. (2014) ,** while lower prevalence were reported by **Biffa et al. (2005), Alemu et al. (2013), Elbably et al. (2013) and Zizet (2015) for cows.**

While in buffaloes, this results is in agreement with previous studies by **Khan &Muhammad(2005), Beheshti et al. (2011) and Hamed & Zaitoun (2014)** who reported prevalence of 27, 27.36 and ranged from 11.11 to 37.21% , respectively. Higher prevalence were reported by **Oezenc et al. (2008), Chavoshi & Husaini (2012), Elsayed et al. (2015),** while lower prevalence were reported by **Elhaig and Selim (2015) and EL-Naker et al.(2015).**

Because mastitis is a complex disease involving interactions of several factors, mainly of management, environment, and factors relating to animal and causative organisms, its prevalence is expected to vary from place to place.

In this study, the prevalence of subclinical mastitis was lower on quarter level in buffaloes than in cows. This conclusion is

supported by previous studies **(Moroni et al. 2006),** which reported that the buffaloes showed higher absolute and relative resistance to SCM.

Early diagnosis of mastitis is a must for reduction of production losses and for enhancing the prospects of recovery. Also, the identification of subclinically infected gland is urgently require for successful control of mastitis in dairy animals **(Ahmed et al., 2008**). pathogenic organisms in milk can derived from the cow itself, the human hand or the environment **(Bradely, 2002).** Otherwise, from public health hazard view, the assessment of SCM etiological pathogen aids to classify the healthy sound milk samples from those of public health hazard as the limits recommended by **European countries standards (IDF, 1996)** and **(Egyptian standards, 2001)**.

Dingwell et al. (2003): reported that the bacteriological culture is the gold standard method for identifying IMI. The major mastitis causing organisms are S. aureus , streptococci, E-coli, Corynebacterium species **(Shrestha and Bindari , 2012).** In this study, 84 cow quarters milk samples and 56 buffaloes quarters milk samples were subjected to bacteriological examination to isolate mastitis pathogens, results showed that the most frequently isolated bacterial species in cows were Staphyloccocus spp. (38%) and streptococcus spp. (27%) followed by E-coli (22%) and Corynebacterium species (1%) as single infection. In addition, Staphyloccocus spp.+ E-coli (4%) and streptococcus spp. + E-coli (8%) as mixed infection **Table (2).** These bacterial infections were recorded also by **Elango et al. (2010), Shrestha and Bindari (2012) and Shereen (2014).**

While in buffaloes**,** the most frequently isolated bacterial species were Staphyloccocus spp. (39%) and streptococcus spp. (29%) followed by E-coli (16%) and Corynebacterium species (4%) as single infection. In addition, Staphyloccocus spp.+ E-coli (7%) and streptococcus spp. + E-coli (5%) as mixed infection **Table (2).** These bacterial infections were recorded also by **Ahmed et al. (2008)**, **Ali et al. (2011) and Aliaa et al. (2013).**

Our results showed that the most prevalent isolated pathogens , from milk samples of cows and buffaloes suffering from subclinical mastitis, were Staphyloccocus spp., streptococcus spp. and E-coli **Table (2).** This results is in agreement with previous studies by **Hameed et al. (2006), Abdel-Rady and Sayed (2009), Ayano et al. (2013), Srinivasan et al. (2013) and Shereen (2014).**

The invasion of polymorphnuclear leukocytes and macrophages is one of the essential body defense against subclinical mastitis , during the inflammatory process the damaged cells of the udder tissues secret enzymes such as lactate dehydrogenase **(Oliszewski et al, 2002).** Subclinical mastitis milk shows evidence of direct passage of blood into the milk as indicated by the changes of some blood proteins and enzymes level **(El Zubeir 2005**).

For many years there has been an interest in using different enzymes in milk as biomarkers for mastitis. Many studies have revealed that enzyme activities in the udder epithelium change markedly due to mastitic inflammation **(Andrei et al, 2011).** More practical attention has been given to detection of enzyme activity in milk and many enzymes have been proposed and listed as reliable markers for early diagnosis of subclinical mastitis **(Babaei et al., 2007 and Guha et al., 2012).**

lactate dehydrogenase (LDH) has been suggested as a biomarker for udder health disturbances as well as a statistical model for mastitis detection **(Chagunda et al., 2006).**

Table (3) and (4): showed that the correlations between bacterial infections and enzymatic activities in SCM milk. Concerning the milk enzymes, all bacterial infections showed high milk enzymatic activity . Generally, staphylococcus spp was associated with the highest activity of LDH and ALP in cows with a level of 478.3 ± 7.62 and 539.22 ± 8.92 U/L , respectively .This come in agreement with the findings of **Babaei et al. (2007) and Nabih & EL Rahman (2015).** While in buffaloes, staphylococcus spp was associated with the highest activity of LDH and ALP in cows with a level of 440 ± 3.4 U/L and 561.1 ± 16 U/L, respectively . This come in agreement with the findings of **Guha et al. (2012) and Aliaa et al. (2013).** While corynebactrium spp. showed the lowest activity of LDH and ALP in cows with a level of 300 ± 00 U/L and 330 ± 00 U/L , respectively . In buffaloes, corynebactrium spp. showed the lowest activity of LDH and ALP with a level of 355 ± 5.0 and 470 ± 10 U/L , respectively . This come in agreement with the findings of **Kato et al. (1989) and Pyörälä (2003).**

In conclusion, SCM influences milk enzyme activity. From the current study it is evident that there is not a single indicator to detect SCM. Although, LDH and ALP were found to be indicators of SCM, all are being better and more reliable in the present study as they showed the highest agreement amongst all with IDF criteria of SCM diagnosis. It is recommended that all of mentioned enzymes are considered as markers for screening large herds for SCM.

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الملخص العربي
تقييم بعض إنزيمات الألبان كدلائل حيويه للكشف عن التهاب الضرع الكامن فى الماشيه

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أجريت هذة الدراسه على عدد٢٨٠عينه لبن تم تجميعها من ٧٠ بقره حلاب و٢١٦عينه لبن تم تجميعها من ٥٤ جاموس حلاب وتم فحصها بواسطه اختبار الكاليفورنيا للكشف عن التهاب الضرع الكامن. وقد أظهرت النتائج تواجد التهاب الضرع الكامن فى ٨٤عينه لبن بقرى بنسبه ٣٠% و٥٦ عينه لبن جاموس بنسبه ٢٦% . وقد تم تعيين بعض الإنزيمات منها انزيم اللاكتات دى هيدروجينيز وانزيم الفوسفاتيز القاعدى فى جميع العينات ولكن تم الفحص البكتريولوجى فى العينات الإيجابيه لاختبار الكاليفورنيا فقط. وقد أظهرت جميع العينات الإيجابيه لاختبار الكاليفورنيا ارتفاعا ملحوظا فى الإنزيمات قيد الدراسه منها انزيم اللاكتات دى هيدروجينيز وانزيم الفوسفاتيز القاعدى. وقد درست العلاقه بين الفحص البكتريولوجى ونشاط الإنزيمات وتبين منها ان العدوى بالمكورات السبحيه شهدت معدلات اعلى من العدوى بالميكروبات الاخرى. ويستخلص من هذه الدراسه ان إنزيمات الألبان التى تم دراستها تصلح لأن تكون كاشف للتعيين المبكر لحالات التهاب الضرع الكامن.