

Molecular Studies on E.coli Isolate from Milk of Mastitic Cattle with Special Reference to Associated Biochemical Changes in Kaliobea Governorate

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Abstract: *This investigation was performed in Teaching Hospital and arm of Benha University in Moshtohor. The number of cows in this farm 80 dairy cows. 40 of them had clinical signs of mastitis (inflammation in teats, pain in milking and milk decrease in amount and quality). We examine these cows to identify the cause of these signs. California Mastitis Test (CMT) was performed to determine positive milk samples in the mastitic targeted cows. 20 samples of early lactation stage cows were recovered from 40 CMT- positive milk samples. Biochemical and PCR tests were performed to isolates E. Coli from positive milk samples (CMT) and determined three virulence genes, eae gene, SXT1 and SXT2. The significance of Escherichia coli-induced mastitis and biochemical changes associated to it in cows, due to the presence of virulence genes and resistance to a wide range of 20 antimicrobials, is concluded. E.coli cause biochemical changes in mastitic cow as liver enzymes AST, GPT, TP., Oxidative enzymes as CAT, SOD, GST, LD and kidney function as urea and creatinine. E.coli has effect on inflammatory response in mastitic cow immunity system of by L6, TNF and CRP.*

Keywords: *Mastitis, Serotyping characterization, PCR, biochemical alteration*

1. INTRODUCTION

Mastitis is an inflammation of the mammary glands associated with physical, and chemical and microbiological changes. It is considered the most important disease in dairy herds (Acik et al., 2004)[1]. The most important causative mastitis - causative environmental pathogen is E.coli (Mokovee and Ruegge, 2003) [2]. Escherichia coli is a major etiological agent of intra-mammary infections (IMI) in cows, leading to acute mastitis and causing great economic losses in dairy production worldwide (Blum, 2015 [3]). Particular strains cause persistent IMI, leading to recurrent mastitis. Virulence factors of mammary pathogenic E. coli (MPEC) involved pathogenesis of mastitis as well as those differentiating strains causing acute or persistent mastitis (Burvenich et al., 2003 [4]). The infection occurs after bacteria entrance mammary gland via teat canal, overcoming anatomical barrier so they must evade the cellular and humoral defence mechanism of mammary gland to establish disease (Radostits et al., 2007 [5] and Mbuk, 2016) 6.. Limited number of E.coli strains has ability to adhere and invade bovine mammary epithelial cells and cause persistent infection, have several fimbria and fimbrial adhesion that mediate adhesion to host epithelial cell through cell surface (Milanov, Dubravka 2015 [7] and Dopfer et al., 2000)[8]. This study was performed to detect the causative agent of clinical mastitis in cow by isolation of E.coli from milk of mastitic cow with special reference to biochemical changes associated to it in infected cow. Characterization of E.coli pathogen isolated from mastitic cow chemically and serologically. Investigation of some virulence factor associated to isolated E.coli. Detection of E.coli attaching and enfacing (Intimin) eaeA, STX1 and STX2 virulence factors of E. coli comprise adhesins, which help the bacteria to adhere to and colonize mucosal surfaces, and toxins, which are proteins with the ability to disturb or modify the normal function of the host cell and to help the bacteria to cross the epithelial barrier and to invade the tissue (Kaper et al., 2004)[9]. Clinical E. coli mastitis can range from mild with only local signs to severe disease with systemic clinical signs. In severe cases the outcome can be acute tissue damage and complete loss of milk production or even the death of the diseased cow. The severity of E. coli mastitis depends on the age of the cow and on the lactation stage, i.e. older cows and cows in early lactation are more susceptible to infection (Mehrhad et al., 2002)[10]. The general aim of this study was: 1-To investigate host response to Escherichia coli infection represented in by biochemical

changes and immunity system reponse, 2-To identify possible specific virulence genes and phylogeny types of E. coli associated with severity of clinical mastitis and the intramammary infection.

2. MATERIAL AND METHODS

2.1. Samples

A total 40 of milk samples were collected from clinically mastitic cows from Quliobeia Kaliobeia Governorate. All samples were collected in sterile macartins and perform (CMT). The positive samples will send as soon as possible to lab to be for examination. Bacteriological examination of milk samples (Qurnn et al. 2002)[11]. The collected samples were incubate aerobically at 37°C for 18-24 hrs. then centrifuge date at 3000 rpm /20 min the cream and supernatant layer were discarded and streak the sediment on blood agar, Mac Conkey agar and EMBagar. The plates were incubated aerobically at 37°C for 24-48 hrs and examined for bacteriological growth. Suspected colonies appeared on different media were picked up and purified by subculture on fresh set of protective and preserved into semisolid agar for Identification of isolated m.o. According to colonial morphological and appearance, growth characterization, hemolytic patterns, microscopically by Giemsa stain and biochemically changes according to (Boerlin et al., 2003) [12]

- Morphologically
- Biochemically identification
 - 1-Catalase
 - 2-Oxidase
 - 3-TSI
 - 3-Urease
 - 5-Indole
 - 6-MR
 - 7-VR
 - 8-Citrate
 - 9-Nitrate
 - 10-Sugar fermentation
- Serological identification according to (Edwards and Ewing 1972)[13]
- PCR molecular identification

2.2. DNA extraction

DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

2.3. Oligonucleotide Primer

Primers used were supplied from **Metabion (Germany)** are listed in table(3)**PCR amplification.** Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (**Takara, Japan**), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler. **For stx1,2 duplex PCR,** primers were utilized in a 50- µl reaction containing 25 µl of EmeraldAmp Max PCR Master Mix, 1 µl of each primer of 20 pmol concentration, 13 µl of water, and 8 µl of DNA template.

3. ANALYSIS OF THE PCR PRODUCTS

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of each PCR product were loaded in each gel slot. A Generuler 100 bp ladder (Fermentas, Thermo Scientific, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

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Table1. Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences 5'-3'	Amplified segment (bp)	Primary Denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
eaeA	ATGCTTAGTG CTGGTTTAGG	248	94°C 5 min.	94°C 30 sec.	51°C 30 sec.	72°C 30 sec.	72°C 7 min.	[14]
	GCCTTCATCA TTTCGCTTTC							
Stx1	ACACTGGATG ATCTCAGTGG	614	94°C 5 min.	94°C 30 sec.	58°C 45 sec.	72°C 45 sec.	72°C 10 min.	[15]
	CTGAATCCCC CTCCATTATG							
Stx2	CCATGACAAC GGACAGCAGT T	779						
	CCTGTCAACT GAGCAGCACT TTG							

4. RESULT

Table2. Characterization of E.coli isolated from mastitic milk

Test	Reaction	+ Ve
Gram stain	Gram –Ve medium size bacilli	100%
Biochemical Identification		
1-catalase	Gas bubbles	100%
2-Oxidase	-Ve	0%
Indol	Red ring	100%
3-MR	Red colour	100%
4-VR	-Ve	0%
5-S.Citrate	-Ve	0%
6-Urease	-Ve	0.0%
7-Tsi	A/A/ gas+H ₂ S -H ₂ S	100%

Table3. Serotypes of E.coli isolated from clinical mastitis cow

Number of mastitic cows	Serotypes	Number	Percent
20	O44	4	20%
	O55	3	15%
	O111	2	10%
	O124	2	10%
	O114	2	10%
	O158	2	10%
	O125	3	15%
	O26	2	10%

Table4. Characterization of E.coli serogroup isolates recovered from milk samples of mastitic cow by PCR assays for *Intamin*, *Stx1* and *Stx2*

Sample No.	Sample ID	Results		
		eaeA	Stx1	Stx2
1	O44	+	-	-
2	O44	+	+	-
3	O55	-	-	-
4	O26	+	-	-
5	O114	-	-	-
6	O146	+	-	+
7	O158	-	-	-
8	O125	-	-	-

Table5. Biochemical changes associated to *E.coli* infection in serum of cows * value of $p < 0.01$ and ** value of $p < 0.001$.

G Groups Parameters	CAT	SOD	LDH	ALP	TP	CREATINE	GOT	GST	GPT	UREA	MDA
CONTROL negative groups	50.00 ±4.103	41.00 ±.512	43.50 ±.272	4.545±.169	8.463±.224	0.5225 ±0.04715	56.50±.331	241.0 ± 6.24	18.75 ± .287	24.50 ± .661	64.50 ± 4.252
E.coli Infected groupa	19.83± 1.470**	16.67±1.085**	115.6± 8.721**	3.513 ±0.116*	6.428 ±0.1523*	1.370 ±0.1610*	81.40 ±8.68**	156.0 ±5.310**	53.20±3.137**	44.67± 3.252**	139.6 ±6.258**

Table6. In study Van ELISA for the quantitation of bovine TNF- α in plasma was modified for serum as described in (Carstensen et al., 2005)16 limit of the ELISA was 0.5 ng/ml for the serum.

Groups Parameters	Control negative	E.coli infected
IL6	81.50 ± 5.362	136.2 ± 6.320*
TNF	33.25 ± 2.056 4	72.67 ± 6.412 *
CRP	20.25 ± 3.568	96.00 ± 6.261*

* Value of $p < 0.01$

Table1 explain Primers sequences, target genes, amplicon sizes and cycling conditions which used in preparation of DNA, Our results in **Table 2and 3** showed that Characterization of *E.coli* isolated from mastitic milk by chemical tests which diffrentiation it from other cause of mastitis and other enterobactereaeae ,table 2 determine strain of *E.coli* by Serotypes of *E.coli* isolated from clinical mastitis cow . **table 4** showed that virulant genes present in strains O44eae, O44eaeand *Stx1*, O55, O26eae, O114, O146 eae and *Stx2*, O158, O125 .From begaining of table 5 and 6 table biochemical changes associated to infection appear in cows that infected with mastitis **Table 5 : showed** biochemical changes associated to *E.coli* infection in serum of cows while Table 6 :showed inflammatory response associated to *E.Coli* infection and immunity response . . Fig 1 showed abnormal changes in teat infected with *E.coli* showed inflamed and redness teat when compare with normal teat in other figure while Figure 2: agrose gel electrophoresis showed Intamin (*eaeA*, *Stx1* and *Stx2*) genes from extracted DNA of *E.coli* serogroup (O55, O26, O114, O146 O158, and O125).



Figure1. Mastitis in cattle

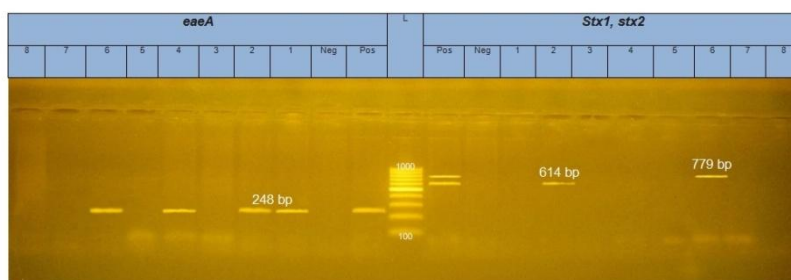


Figure2.

5. STATISTICAL ANALYSIS

5.1. Statistical Analysis Should be at Material and Methods Section

The statistics was applied by means of SPSS software (SPSS ver. 16, Inc., Chicago, IL). T- Test was used for each group at a significant value at $p < 0.05$ Steel (1997) [17]

6. DISCUSSION

The significance of *Escherichia coli*-induced mastitis in cows, associated with the presence of virulence genes, this targeted surveillance of rural dairy farms confirmed the significance of *E. coli* infection in mastitis of cows Elie et al., (2015) *Escherichia coli* is a major etiological agent of intramammary infections (IMI) in cows, leading to acute mastitis and causing great economic losses in dairy production worldwide (Bradley, 2001). Our result concluded that *E. coli* is the most cause of mastitis by field diagnosis CMT found 20 out of 40 samples the causative agent of mastitis was pathogenic *E. coli* and confirmed by characterization of *E. coli* and biochemical analysis to determine strain of *E. coli*. This aforesaid results came in agreement with other reports which recorded that *E. coli* is among the most common infectious agents isolated from severe mastitis cases in modern dairy farms (Bradley and Bradley, 2002). The California Mastitis Test (CMT) provided a useful tool for farmers and veterinarians for measuring the level of inflammation in the udder (Elie, 2015) [22]. In current study found increases in inflammatory parameters IL6, TNF and CRP factors. In our opinion, this elevation may be created as a result of proinflammatory response to infection with *E. coli* and stimulation of immunity system. These aforesaid results came in agreement with other reports recorded that LPS triggers formation of proinflammatory and inflammatory cytokines, produced predominantly by monocytes and macrophages (Persson-Waller et al., 2003 [23]; Gonen et al., 2007) [24]. Cytokines, such as tumor necrosis factor alpha (TNF- α), initiate the inflammatory response (Paape et al., 2003) [25], which induces the acute phase response (APR) by activating the production of acute phase proteins (APP) and LPS-binding protein (LBP) (Bannerman et al., 2003 [26], Bannerman et al., 2004; Eckersall, 2001 and Hiss et al., 2004). All the above mentioned alterations mainly have a drawback effect on the biochemical and oxidative serum constituents specially SOD, LDH, ALB, TP, CREATINE, GOT, GST, GPT, Urea and MDA. These factors were cow-dependent, like the speed of the inflammatory response, lactation stage and age of the cow, are thought to determine the severity of *E. coli* mastitis (Burvenich et al., 2003). The study advanced our standing of the mastitic effect of *E. coli* on cows. *E. coli* virulence genes which were detected by PCR were Intimin, SxT1 and SxT2 these toxins were isolated from strains (O44, O55, O111, O124, O114, O158, O125, O26) were considered as very important virulent factors of *E. coli*. Most of the pathogenic *E. coli* possesses several kinds of pathogenic mechanisms and virulence factors. Intimin is a protein encoded by *eae* gene (Ghanbarpour and Oswald, 2010) [30]. It facilitates the adherence of attaching and effacing *E. coli* to the epithelial cells. It is proven that the *eae* gene in *E. coli* plays a definite role in induction of cattle mastitis (Correa and Marin, 2002) also this result came agree with (Kaper et al., 2004) who concluded that virulence factors of *E. coli* comprise adhesins, which help the bacteria to adhere to and colonize mucosal surfaces, and toxins, which are proteins with the ability to disturb or modify the normal function of the host cell and to help the bacteria to cross the epithelial barrier and to invade the tissue. There was a clear significant correlation between the CMT scores and the *E. coli*, The presence of *eae* Intimin gene in *E. coli* involved in mastitis of dairy cows is of paramount importance, *E. coli* with Intimin gene are able to form small microcolonies on the surface of infected epithelial cells, followed by localized degeneration of the microvilli cumulating in an attaching and effacing (A/E) Elie et al. (2015). All the *E. coli* isolates with the virulence genes *stx* and *eae* showed resistance to a higher number of antimicrobials than those which were *stx*-negative (Solomakos et al., 2009).

7. CONCLUSION

It is recommended in disease-control programs of dairy to study the *E. coli* involvement in mastitis, and to include in the surveillance the detection of virulence genes that are decisive in economic losses in veterinarian.

REFERENCES

- [1] **Acik, M.N., Yurdakul N.E., Akici L. C., Onat N., Dogan, O., etinkaya B. C.** 2004 traT and CNF2 genes of *Escherichia coli* isolated from milk of healthy cows and sheep. Department of Microbiology, Faculty of Veterinary Medicine, University of Firat, 23119 Elazig, Turkey
- [2] **Mokovec, J.A., Ruegge, P.L.** (2003) Results of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001. *J. Dairy Sci.* 86:3466-3472.
- [3] **Blum SE1, Heller ED2, Sela S3, Elad D4, Edery N5, Leitner G6.** 2015. Genomic and Phenomic Study of Mammary Pathogenic *Escherichia coli*. *PLoS One*. Sep 1;10(9):e0136387. doi: 10.1371/journal.pone.0136387. eCollection 2015.
- [4] **Burvenich, C., Van Merris, V., Mehrzad, J., Diez-Fraile, A., Duchateau, L.,** 2003. Severity of *E. coli* mastitis is mainly determined by cow factors. *Vet. Res.* 34, 521-564.
- [5] **Radostits OM, Gay CC, Hinchcliff KW, Constable PD** (2007). Diseases caused by fungi. *Veterinary Medicine: A textbook of the diseases of cattle, horses, sheep, pigs and goats*. 10th edition. Saunders Elsevier Ltd. Philadelphia, USA. pp. 842-860.
- [6] **Mbuk, E. U.1, Kwaga, J. K. P.1, Bale, J. O. O.2, Boro, L. A.3 and Umoh, J. U.** 2016. Coliform organisms associated with milk of cows with mastitis and their sensitivity to commonly available antibiotics in Kaduna State, Nigeria. *Journal of Veterinary Medicine and Animal Health* Vol. 8(12), pp. 228-236, December
- [7] **MILANOV Dubravka*, PRUNIĆ Bojana, VELHNER Maja, TODORVIĆ Dalibor, POLAČEK Vladimir** 2015, INVESTIGATION OF BIOFILM FORMATION AND PHYLOGENETIC TYPING OF *ESCHERICHIA COLI* STRAINS ISOLATED FROM MILK OF COWS WITH MASTITIS, *Acta Veterinaria-Beograd* 65 (2), 202-216.
- [8] **Döpfer, D., Almeida, R.A., Lam, T.J.G.M., Nederbragt, H., Oliver, S.P. and Gaastra, W.,** 2000. Adhesion and invasion of *Escherichia coli* from single and recurrent clinical cases of bovine mastitis in vitro. *Vet. Microbiol.* 74, 331-343
- [9] **Kaper, J.B., Nataro, J.P., Mobley, H.L.,** 2004. Pathogenic *Escherichia coli*. *Nat. Rev. Microbiol.* 2, 123-140.
- [10] **Mehrzad, J., Duchateau, L., Pyörälä, S., Burvenich, C.,** 2002. Blood and milk neutrophils chemiluminescence and viability in primiparous and pluriparous dairy cows during late pregnancy, around parturition and early lactation. *J. Dairy Sci.* 85, 3268-3276.
- [11] **Qurnn P.J.; Morkey, B.K. Cater M.E. Donnelly W.J.C. and Leonard F.C.** (2002) *Veterinary Microbiology and Microbial diseases*. 1st Low stat University Press Blackwell Scienc
- [12] **Boerlin P. Kuhnert, P. Hussy, D. and Schaellibaum, M.** 2003) Methods of identification of *S. aureus* isolates in cases of bovine mastitis. *J. Clin. Microbiol.* 41(2) : 767-771.
- [13] **Edwared P.R. and Ewing W.H.** (1972). Identification of Enterobacteriaceae. *Burgesspubi, Co., Minneapolis, Minnesota*, 103-104.
- [14] **Bisi-Johnson, M.A.; Obi, C.L.; Vasaikar, S.D.; Baba, K.A. and Hattori, T.** (2011): Molecular basis of virulence in clinical isolates of *Escherichia coli* and *Salmonella* species from a tertiary hospital in the Eastern Cape, South Africa. *Gut Pathogens* 2011, 3:9.
- [15] **Dipineto, L.; Santaniello, A.; Fontanella, M.; Lagos, K.; Fioretti, A. and Menna, L.F.** (2006). Presence of Shiga toxin-producing *Escherichia coli* O157:H7 in living layer hens. *Letters in Applied Microbiology* 43 (2006) 293–295.
- [16] **Carstensen, L., Rontved, C.M., Nielsen, J.P.,** 2005. Determination of tumor necrosis factor- α responsiveness in piglets around weaning using an ex vivo whole blood stimulation assay. *Vet Immunol. Immunopathol.* 105, 59-66.
- [17] **Steel R, Torrie J, Dickey D** (1997). Principles and procedures of Statistics: A Biometrical Approach, 3rd ed., McGraw-Hill, New York, NY.
- [18] **Elie K. Barbour, Tamar J. Kassabian, Houssam Shaib, Zeina Kassaify, Archana Iyer, Esam Azhar, Steve Harakeh, Taha Kumosan.** (2015) The Significance of *Escherichia coli*-induced Mastitis in Cows Associated with the Presence of Virulence Genes and Wide Range-resistance to Twenty Antimicrobials

- [19] **Bradley AJ, Green MJ: 2001**, Adaptation of Escherichia coli to the bovine mammary gland. Journal of Clinical Microbiology 39: 1845-1849.
- [20] **Bradley, A.J., Leach, K.A., Breen, J.E., Green, L.E., Green, M.J., 2007**. Survey of the incidence and aetiology of mastitis in dairy farms in England and Wales. Vet. Rec. 160, 253-258
- [21] **Bradley, A. 2002** Bovine mastitis an evolving disease ,The veterinary Journal 164,116-128
- [22] **Persson Waller, K., Colditz, I.G., Lun, S., Östensson, K., 2003**. Cytokines in mammary lymph and milk during endotoxin-induced bovine mastitis. Res Vet Sci. 74, 31-6.
- [23] **Gonen, E., Vallon-Eberhard, A., Elazar, S., Harmelin, A., Brenner, O., Rosenshine, I., Jung, S., Shpigel, N., 2007**. Toll-like receptor 4 is needed to restrict the invasion of Escherichia coli P4 into mammary gland epithelial cells in a murine model of acute mastitis. Cellular Microbiology, doi: 10.1111/j.1462-5822.2007.00999.x.
- [24] **Paape, M., Bannerman, D., Zhao, X., Lee, J.W., 2003**. The bovine neutrophil: +structure and function in blood and milk. Vet. Res. 34, 597-627.
- [25] **Bannerman, D.D., Paape, M.J., Hare, W.R., Sohn, E.J., 2003**. Increased levels of LPS-binding protein in bovine blood and milk following bacterial lipopolysaccharide challenge. J. Dairy Sci. 86, 3128-3137.
- [26] **Bannerman, D.D., Paape, M.J., Lee, J.W., Zhao, X., Hope, J.C., Rainard, P., 2004**. Escherichia coli and Staphylococcus aureus elicit differential innate immune responses following intramammary infection. Clin. Diagn. Lab. Immunol. 11, 463-472.
- [27] **Eckersall, P.D., Young, F.J., McComb, C., Hogarth, C.J., Safi, S., Weber, A., McDonald, T., Nolan, A.M., Fitzpatrick, J.L., 2001**. Acute phase proteins in serum and milk from dairy cows with clinical mastitis. Vet. Rec. 148, 35-41.
- [28] **Hiss, S., Mielenz, M., Bruckmaier, R.M., Sauerwein, H., 2004**. Haptoglobin concentrations in blood and milk after endotoxin challenge and quantification of mammary Hp mRNA expression. J. Dairy Sci. 87, 3778-3784.
- [29] **Ghanbarpour R, Oswald E**. Phylogenetic distribution of virulence genes in Escherichia coli isolated from bovine mastitis in Iran. Res Vet Sci. 2010; 88:6-10.
- [30] **Correa MGP, Marin JM**. O-serogroups, eae gene and EAF plasmid in Escherichia coli isolates from cases of bovine mastitis in Brazil. Vet Microbiol. 2002; 85:125-132.
- [31] **Solomakos N, Govaris A, Angelidis AS, Pournaras S, Burriel,AR, Kritas SK, Papageorgiou DK**. Occurrence, virulence genes and antibiotic resistance of Escherichia coli O157 isolated from raw bovine, caprine and ovine milk in Greece. Food Microbiol. 2009; 26:865 - 871.

Citation: M. Radwan, "Molecular Studies on E.coli Isolate from Milk of Mastitic Cattle with Special Reference to Associated Biochemical Changes in Kaliobea Governorate", *International Journal of Research Studies in Biosciences (IJRSB)*, vol. 5, no. 8, p. 33, 2017. <http://dx.doi.org/10.20431/2349-0365.0508005>.

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