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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
Investigation was performed in Teaching Hospital and arm of Benha University in Moshtohor. The number of cows in this farm consists of 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 virulance 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

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 causitave 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 occur 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 persistant infection, have several fimbria and fimbrial adhesion that mediat 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 causitive agent of clinical mastitis in cow by isolation of *E. coli* from milk of mastatic cow with special refrence to biochemical changes associated to it in infected cow. Charactarization of *E. coli* pathogen isolated from mastitic cow chemical and serlogicaly. Investigation of some virulance 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 (Mehrzad *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.

Material and methods

Samples

A total 40 of milk samples were collected from clinically mastitic cows from Quliobea Kaliobea 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 centrifugedate at 3000 rpm /20 min the cream and supernatant layer were discarded and streak the sediment on blood agar, MacConkey agar and EMBagar. The plates were incubated aerobically at 37^0C 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]

1-Morphologically

2-Biochemically identification

1-Catalase	2-Oxidase
3-TSI	3-Urease
5-Indole	6-MR
7-VR	8-Citrate
9-Nitrate	10-Sugar fermentation

3-Serological identification according to (Edwards and Ewing 1972) [13]

4-PCR molecular identification

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^OC 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.

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.

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.

Table 1: 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	ATGCTTAGTGCTGGTTTAGG	248	94˚C 5 min.	94˚C 30 sec.	51˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**[14]**
	GCCTTCATCATTTCGCTTTC							
Stx1	ACACTGGATGATCTCAGTGG	614	94˚C 5 min.	94˚C 30 sec.	58˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**[15]**
	CTGAATCCCCCTCCATTATG							
Stx2	CCATGACAACGGACAGCAGTT	779						
	CCTGTCAACTGAGCAGCACTTTG							

Result

Table 2: Characterization of *E. coli* isolated from mastatic 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.%
7-Tsi	A/A/ gas+H -H2S	100%

Table 3: Serotypes of *E. coli* isolated from clinical mastitis cow

Noumber of mastatic cows	Serotypes	Noumber	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%

Table 4: Characterization of *E. coli* serogroup isolates recovered from milk samples of mastatic cow byPCR 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	-	-	-

Tabie 5 : 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 ± 0.047 15	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 **

Table 6: 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

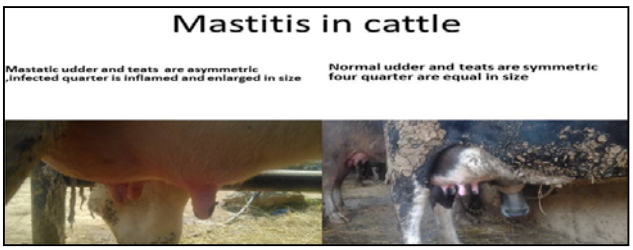

Fig 1 *

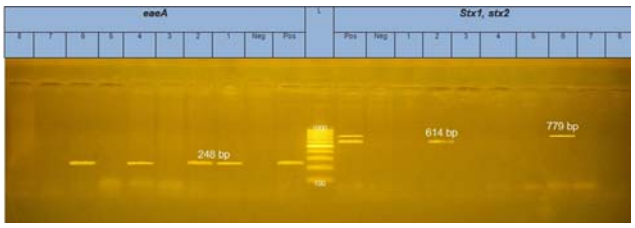

Fig 2

Table 1 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 diffrantiation it from other cause of mastitis and other enterobactereacae, 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 Tabie 5: showed biochemical changes associated to *E. coli* infection in serum of cows while Tabie 6 :showed inflamatory 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).

Statistical analysis.

(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].

Disscusion

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) [18] *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(Bradley, 2001) [19]. Our result concluded that *E. coli* is the most cause of mastities by feild diagnosis CMT

found 20 out of 40 samples the causitive agent of mastities was pathogenic *E. coli* and confirmed by charachtarization of *E. coli* and biochemical analysis to determined 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 etand 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 founded increases in inflamatory 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 recoreded that LPS triggers formation of proinflammatory and inflammatory cytokines, produced predominantly by monocytes and macrophages (PerssonWaller *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) [28]. 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 cows-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* virulance genes which were detected by PCR were Itamin,SxT1 and SxT2 these toxins were isolated from strains(O44, O55, O111, O124, O114, O158,, O125, O26) were considered as very important virulant 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 Oswalt, 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) [30] also this result came agree with (Kaper *et al*., 2004) [9] 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) [18]. 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) [31].

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 vetrinarian.

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