Molecular studies on E.coli isolate from milk of mastatic cattle with special reference to associated biochemical changes in kaliobea governorate

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Abstract

This investigation was performed in Teaching hospital and farm of Benha university in Moshtohor the number of cows in this farm 80 dairy cows that 40 of them had clinical signs of mastitis (inflammation in teats ,pain in milking and milk decrease in amount and quality) .When examine these cows to identify the disease which cause 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 out of 40 samples recovered from 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 wide range resistance to 20 antimicrobials, is concluded. E.coli cause biochemical changes in mastatic cow as (liver enzymes AST,GPT,TP, ant. oxidative enzymes as CAT, SOD,GST,LDand kidney function as urea and creatinine .E.coli has effect on inflammatory response in immunity system of mastitic cow by increase IL6,TNF and CRP.

Keywords: Mastitis. Serotyping characterization,PCR and 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 environmental mastitis 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 battier so they must evade the cellular and humeral 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 cell[s and

cause persistant infection ,have severa l fimberia and fimberial 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 :Detection the causitive agent of clinical matitis 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 mastatic cow chemical and serlogicaly .Investigation of some virulance factor associated to E.coli isolated . Detection of E.coli attaching and enffacing (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]. [10].

The general aim of this study was 1-To investigate host response to Escherichia coli infection represented in 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 mastatic cows from Quliobea governorate all samples collected in sterail macartins and perform (CMT) the positive samples will send as soon as possible to lab to be examination .Bacteriological examination of milk samples (Qurnn et.al 2002)[11]the collected samples were incubate aerobically at 370 C for 18-24 hrs then centrifugate at 3000 rpm /20 min the cream and supernatant layer were discarded and streak the sediment on blood agar ,maconcky agar and EMBagar . The plates were incubated aerobically at 37C0 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 Gimesa 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^{O}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.

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

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 ofE.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

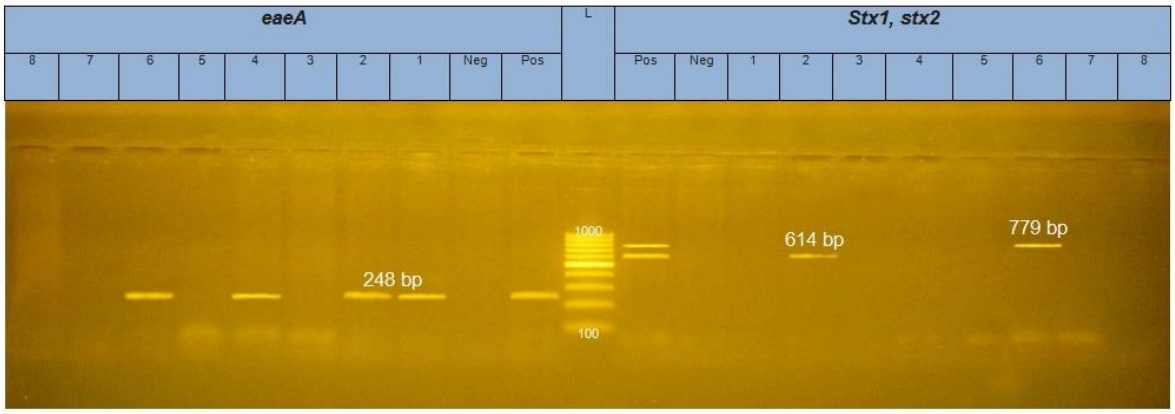

Table 1 explain Primers sequences, target genes, amplicon sizes and cycling conditions which used in preparation of DNA **,**Our results in **Table 2and 3** showed that Characterization of E.coli isolated from mastatic milk by chemical tests which diffrantiation it from other cause of mastitis and other 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.

The statistics was applied by means of SPSS soft ware (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 et[20] and Bradley 2002)[21]. 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 increase in inflamatory parameters IL6,TNF andCRP factors, In our opinion, this elevation may be created as a result of proinflamatory response to infection with E.coli and stmulation 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 al2003[26], Bannerman et al., 2004[27],Eckersall 2001[28] and Hiss et al., 2004)[29.] 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)[4]. The study advanced our standing of the mastatic effect of E.coli on cows . E.coli virulance genes That 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)[31] 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)[30]. 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 .

Aknolgment

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