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Research article

Gene expression study of pathogenic hemolysin producing *E. coli* isolated from cattle by using reverse transcription real-time PCR

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Abstract

The main goal of current study is an investigation of detection and expression of hemolysin toxin in E.coli isolates in clinical veterinary infection cases included Mastitis milk, Abortion samples and diarrhea samples from cattle in Al-Diwaniyah city by using real time-polymerase chain reaction technique. Total samples that used in this study are (24) isolates, included (9) isolates from mastitis cases, (5) isolates from abortion cases, and (10) isolates have taken from Diarrhea cases in cattle. The total prevalence of hemolysin in E.coli isolates is 18/24 (75%), where the percentage of occurrence of hemolysin production E.coli isolates by using real-time PCR was 7/9 (77%), 5/5 (100%), and 6/10 (60%) in Mastitis milk, Abortion and Diarrhea cases in cattle respectively. In concluding, production of hemolysin toxin by E.coli isolates have contrast activity depend on the type of sample; the study found the abortion isolates have high gene expression followed by diarrhea isolates, then milk isolates.

Keywords: Gene expression, Hemolysin, *E.coli*, Cattle, Real-Time PCR.

Introduction

There are a lot of the diseases which cause great loss economic in dairy cattle in animal's production section Such as abortion, mastitis, and diarrhea. Where these diseases are causing loss of milk production, loss of new birth, and loss of body fluids following by death respectively. The abortion in cattle is a loss of the fetus during pregnancy period (1)(2). Mastitis is inflammation of tissue of the mammary gland, it is causing a great problem and great economic loss in the dairy industry (3), either the diarrhea is a loss of fluids from the bowl due to disorder in the intestine (4). Many of etiological agents cause theses veterinary diseases. *Escherichia coli* is most prevalent. *E. coli* is a gram-negative bacteria and normal inhabitant of many of organs like gut, uterine, and another site in human and animal. It is worldwide spread and causes a hazard to public health (5) and has many of the virulence factors like intimin, Shiga toxins and enterohemolysin (*hlyA*) (6). The hlyA gene encodes hemolysin toxin, and it can lyse of erythrocytes and therefore provide iron to the bacterium (7). *HlyA* is a common virulence factor in *E. coli* (8) (9). *HlyA* has attacked granulocytes and erythrocytes (10, 11), The Monocytes (12), endothelial cells (13), and primates, renal epithelial Cells of mice and ruminants (14) (15). *E. coli HlyA* causes changes in permeability of host cells by form pores in host cells leading to their destruction then causing lysis of erythrocytes that provide iron for bacteria (16). Some of the *E.coli* isolates carry the *hlyA* gene. The *hlyA* gene encoding hemolysin toxin and secret outside the bacteria cell. The hemolysin *E.coli* isolates secret the toxin at different amounts and activities, that will cause different

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degrees of affecting, also acts of the gene (sometimes called gene expression) are the contrast depending type of isolates and another factor (17-18), we will discuss it in details later. The main purpose of this paper is to report the investigation expression gene of hemolysin toxin that secreted by *E.coli* strains isolated from many of Veterinary cases included mastitis, abortion and diarrhoea in cattle**.**

Materials and Methods

Ethical approval

The Animal Ethical Committee of Veterinary Medicine College, University of Al-Qadisiyah, Iraq, has approved the present study under permission No: 432

Bacterial isolates:

Twenty-four *Escherichia coli* isolates were isolated from mastitis milk cases, uterine fluids of abortion cases, and cases of diarrhoea infection of dairy cattle from different fields in Al-Diwaniyah city.

Total RNA extraction:

Extracted RNA of *Escherichia coli* isolates was done by use (Accuzol® reagent kit. Bioneer. Korea) depend on company instructions. as follow 200µl of 4 h incubation *Escherichia coli* isolates on LB broth was placed in sterile 1.5ml Eppendorf tube, and 1 ml of Accuzol reagent was added and mixed well by using micropestle then the tubes were shaken vigorously for one minute. After that, add chloroform (200) µl to all tube and shake strongly (15) seconds. The mixture compound was a dip in ice (5) min. And centrifuged at (12000) rpm, then cooled at (4) C° (15) minutes. Transmitted supernatant layer for new Eppendorf tube, and add (500) µl of isopropanol to the mixture. The mixture should be inverting the tube about (4-5) times and cooling at (4) C° (10) min. Follow at (12000) rpm, (4C°) (10) min by use centrifuge tube. Discarded supernatant layer and add (1) ml 80% Ethanol and use vortex for mixing. Next step, centrifuge at (12000) rpm, (4C°) for (5) min. The supernatant is discarded, and the RNA pellet was left to air to dry. Finally, 50µl DEPC water was added for all samples to dissolve RNA pellet, and then the extracted RNA sample was kept at (-20) the extracted RNA was measured by Nanodrop apparatus (THERMO. USA).

DNase I Treatment:

Pure Extracted RNA was treated with DNase I enzyme to discard genomic DNA by use (DNase I enzyme kit), and it was done depend on PRomega company, USA instructions as the following Table (1):

Table (1): DNase I Treatment master mix preparation

The Mixture	The Volume
Total RNA 1µg	10µl
DNase I enzyme	2 µl
DEPC water	4 µl
10X buffer	4 µl
Total	20 µl

Use It (37C°) for (30) min. For incubation. Then, added 1µl stop solution then incubated the samples at (65C°) for (10) min. For the decreased activity of the enzyme of DNase.

cDNA synthesis:

By using AccuPower® RocktScript RT PreMix kit with DNase-I treatment, RNA samples used in cDNA synthesis step that provided from Bioneer company, Korea and done according to company instructions as the following table:

Table (2): RT master mix for cDNA synthesis

RT master mix	The Volume
The Random Hexamer primer (10pmol)	(1) µl
The Total RNA 100ng/ul	(10) µl
The DEPC water	(9) µl
The Total	(20) µl

Then the mixture put in Accu Power Rocket Script RT PreMix specific tubes. Converting the RNA to cDNA was done by a thermocycler apparatus as the table below :

Table (3): Thermocycler conditions for cDNA synthesis

The Step	The Temperature	The Time
cDNA synthesis	(50 °C)	(1) h
Heat inactivation	(95 °C)	(5) min.

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Quantitative Real-Time PCR (qPCR):

qPCR was performed for detection and quantification of relative gene expression of hemolysin mRNA transcript levels from *E. coli* that isolated from clinical infection cases in cattle whereas, it was carried out by using ($2^{-\Delta\Delta CT}$ Livak method) (Livak and Schmittgen, 2001)**(1)**. The qPCR reaction was done on a Real-Time PCR system (BioRad. the USA) by using SYBER Green dye qPCR master mix that used in detection and amplification of target genes and rssA housekeeping gene for normalization of gene expression. The Primers were designed using the primer3 and (Primers sequences are a list in Table (4).

Table (4): RT-qPCR primers with their sequence.

Primer		Sequence	Amplicon size	GenBank
hlA	F	AATGGGAAAAGGAGCATGGC	143bp	NC_007365.1
	R	GCAAAACCAAGTTGGGTGTG		
rssA	F	TTGCCCGCAAATATCTACGC	102bp	NC_000913.3
	R	ACCAACGGCAAAAGTTCGTC		

qPCR master mix was prepared for the *hlyA* target gene and rssA housekeeping gene according to (AccuPower^{TM}2XGreen Star qPCR master mix kit. Bioneer .Korea) instructions as following Table (5):

Table (5): qPCR master mix preparation

qC master mx	The volume
2X green star master mx	25 µl
Forward primer (10pmol)	2 µl
Reverse primer(10 pmol)	2 µl
cDNA template (10ng)	5 µl
DEPC water	16 µl
Total	50 µl

The qPCR master mix reaction component placed in qPCR white tube strips and mixed by Exispin vortex centrifuge for three minutes, then the strips placed in Miniopticon Real-Time PCR system BioRad. The USA as following thermocycler conditions Table (6):

Table (6): qPCR thermocycler conditions.

qPCR step	The Temperature	The Time	The Repeat cycle
Initial Denaturation	50°C	1h	1
Denaturation	95°C	20 sec	40
Annealing/Extension Detection (scan)	60°C	30 sec	
The Melting	60-95°C	0.5 sec	1

Results

1-Real-time PCR detection results

The results appeared that only (18) *Escherichia coli* have hemolysin production as the following table:

Table (7): Number and percentage of hemolysin production isolates.

Escherichia coli isolate the source	No of tested isolate	Positive isolates and percept %
Mastitis milk	9	7/9 (77%)
Abortion	5	5/5 (100%)
Diarrhea	10	6/10 (60%)

2-Relative gene expression analysis results:

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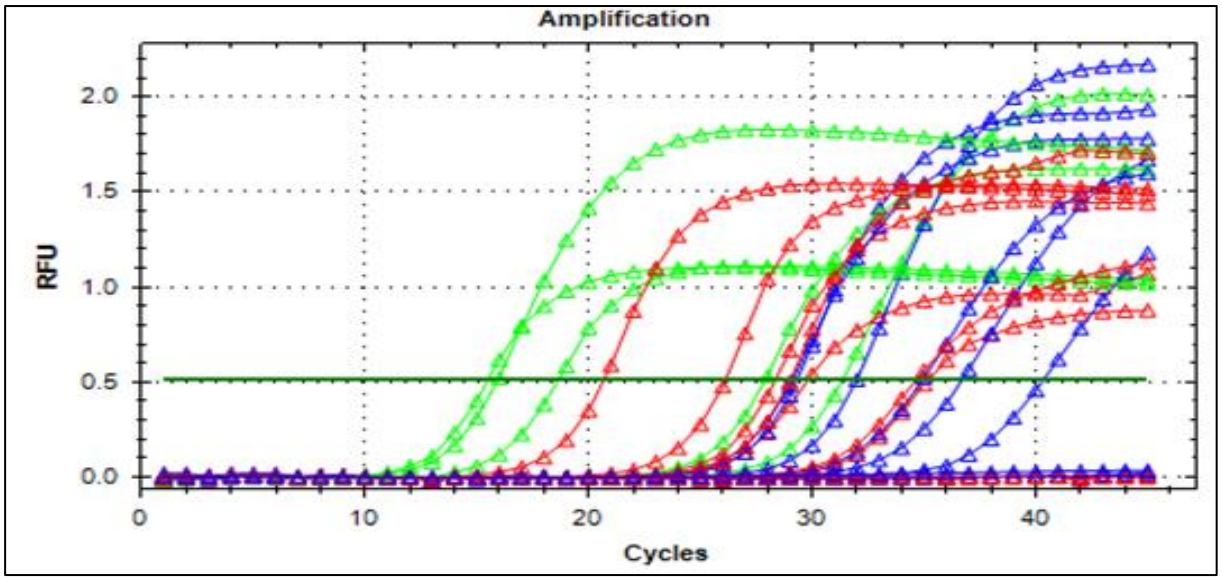

Figure (1): Real-Time PCR amplification plots based SYBER green for detection hemolysin gene producing *Escherichia coli* isolate, Where the red plot (diarrhea), blue plot (Mastitis milk), and green plot (abortion).

Table (8): The relative gene expression analysis of hemolysin gene producing *Escherichia coli* isolate reference Livak method.

Isolate source	CT (*hlyA*)	CT (*rssA*)	ΔCT	Fold change expression	Mean ± St. error
Milk	33.634	33.723	0.089	1.064	
Milk	32.354	33.123	0.769	1.704	
Milk	33.743	33.873	0.130	1.094	
Milk	32.152	34.332	2.180	4.532	1.689
Milk	33.136	33.341	0.205	1.153	
Milk	32.346	32.952	0.606	1.522	
Milk	33.142	32.734	-0.408	0.754	
Abortion	30.352	32.922	2.570	5.938	
Abortion	30.225	33.216	2.991	7.950	
Abortion	29.762	33.143	3.381	10.418	7.728
Abortion	30.763	33.531	2.768	6.812	
Abortion	30.723	33.634	2.911	7.521	
Diarrhea	32.972	33.582	0.610	1.526	
Diarrhea	31.631	33.634	2.003	4.008	
Diarrhea	31.531	33.723	2.192	4.569	4.316
Diarrhea	31.345	33.182	1.837	3.573	
Diarrhea	31.341	34.345	3.004	8.022	
Diarrhea	31.462	33.532	2.070	4.199	

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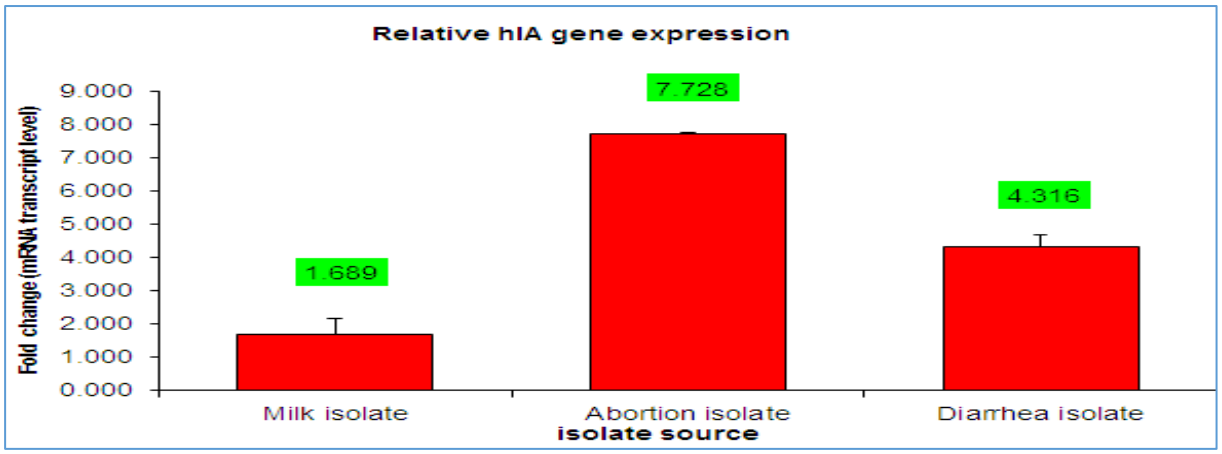

Figure (2): Gene expression levels of *hlyA* mRNA. The values are normalized for rssAmRNA expression. Bars display the mean and the standard Error (ST.E) of three independent experiments, significant at **p<0.05.

Discussion

Virulence factors enable *E. coli* to colonies lining layer of many of the tissue and the organs such as udder, intestine and uterine.These genes make the bacteria more virulence, and more resistance to immune defence, the capacity of *E. coli* to produce many virulence factors that contribute its pathogenicity (19), hemolysin production produce by *E.coli* isolates (20). According to our result, the percentage of total hemolysin production *E.coli* isolates by using real-time PCR was (75%). That near to results of (21) and (22) which they recorded were (68.45%) and (68.5%) respectively, while (23) recorded (90%) more than our results and (24) found (41.36%) that considered less than our result. The seasons and geographic determinants have a great and important role in the prevalence of virulence genes of *E.coli* isolates. Where it was recorded in summer more than winter and other seasons, there are significant differences in rates of the distributions of virulence genes in *E.coli* isolates that isolated from milk samples taken in different seasons also from the different area, it has different values of the *hlyA* gene (25). Where E.coli isolates carried the percentage of occurrence of HLyA gene by using real-time PCR was 7/9(77%), 5/5(100%), and 6/10(60%) in Mastitis milk, Abortion and Diarrhea cases in cattle respectively. But (26) found (40%) of isolates able to produce hemolysin toxin on blood agar that less than the rate of our results. Also (27), (28) and (29) recorded lower rates. It were (41.36%), (23.7%) and (11.6%) respectively. (35) found the range of prevalence (16.6% – 41%) where it depends on the type of samples that taken from different tissues that less than our results, while (30) recorded high percentage (93.7%) that considered more than our study. In the USA, (31) record prevalence of *hly A* gene in *E.coli* was (63%), and in Spain (32) record the rate was (56%). While (32), (33) and Egypt (34) found the prevalence of *hlyA* gene that isolated from *E.coli* from milk were (23%), (35%) and (50.2%) respectively; all above represent less than the value of our results. Many of studies recorded the prevalence of *hly A* gene in *E.coli* close to our rates like (35) where record (71.3%) in Canada. According to our results, hemolysin production *E.coli* isolates were more expression of toxin production (more activity and more effective) in abortion samples followed by diarrhoea sample then milk samples; We can explain that by a suggestion by aborted uterine fluids contain a large amount of blood because of the destruction of blood vessels. While mastitic milk and diarrheic feces either contain little blood

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amount or empty from blood, amount of blood will stimuli the *E.coli* isolates to increase gene expression of a *hlyA* gene, in another meaning the gene expression depends on the amount of blood in the environment. The bacteria produce hemolysin toxin only when needed, whenever amount of the blood is larger, it will hemolysin secreted more (36), the hemolysin is synthesised intracellular and needs Ca2+ ions in the extracellular medium wherever the activity depends on internal and external factors (37) and (38). There are some other causes play a great role to control the expression of hemolysin gene by *E.coli* isolates where some strains will produce loss hemolysin production in the presence of streptomycin, sodium cyanide, rifampin and nalidixic acid. Also, it shows maximum expression at pH (7-8) (39). Some of the compounds present in the meat extract medium have a great role for stimulation *E.coli* isolates to the production of hemolysin (40). (41) Confirm the calcium is important ion for increased gene expression of hemolysin. Also, the growth time of the colony is considered another important factor for increasing haemolysin production. It was detected bacteria that grow in a specific atmosphere at temperature 37 *"C* for 16 h, that similar to animal body temperature (42) (43), the oxygen, glucose and iron are considered important additive factors to stimuli up-regulation of the *hlyA* gene in *E.coli* isolates (44) (45). Hemolytic *Escherichia coli* becomes more active in the medium that contains a mineral, salt and haemoglobin. The producing and releasing hemolysin toxin was stopped in the presence of energy metabolism inhibitors such as sodium azide, potassium cyanide and dinitrophenol. Also, a similar effect that observed in the presence of neuroactive drugs and procaine which decrease protein production (46). Furthermore, there are some drugs have an effect on gene expression of *E.coli* isolates in the presence of nalidixic acid, rifampin, streptomycin, and chloramphenicol (46). All these factors play an important role in determined gene expression of hemolysin toxin in *E.coli* isolates. All these factors explain the contrast in the degree of expression among the samples. At finally; secretion hemolysin toxin by *E.coli* depend on several internal and external factors that present in around the environment; these factors will determine gene expression.

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