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Molecular Detection of Certain Virulence Genes of Some Food Poisoning Bacteria Contaminating Raw Milk

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A B S T R A C T

The current study was planned to detect some pathogenic microorganisms that may contaminate raw milk beside application of molecular methods in a trail to detect virulence genes in the isolated bacteria. A grand total of 100 samples of raw milk were randomly collected from small breeders and supermarkets then transferred directly to the laboratory with a minimum of delay, where they were examined bacteriologically for detection of some food poisoning bacteria including; Escherichia coli, Staphylococcus aureus and Salmonella. The obtained results clarified that the prevalence of E. coli, Staph. aureus and Salmonella in raw milk samples was 64, 35 and 3%, respectively. Serological identification of E. coli isolates revealed that only 4 isolates of E. coli (6.25%) were belonged to Enteropathogenic E. coli where serological identification revealed the presence of O86, O119: H6, O26: H11 and O125: H21 serotypes. Moreover, multiplex PCR was employed for detection of stx1, stx2 and eaeA virulence genes specific for Enteropathogenic E. coli. Also, it was found that the prevalence of Coagulase positive Staph. aureus based on coagulase test was 60% (21 out of 35 isolates) while it was 54.3% based on presence of clfA gene. Finally, serological identification of Salmonella isolates revealed the presence of S. Infantis, S. Typhimurium and S. Enteritidis. The recorded results in the current work highlighted the role of raw milk in transmitting food poisoning bacteria to consumers so strict hygienic measures must be considered during production and distribution of raw milk.

Keywords: Milk, Food Poisoning Bacteria, Isolation, PCR

1. Introduction

Billions of people consume milk and dairy products every day. Besides its beneficial effects on nutrition, it represents an ideal nutritive environment for numerous pathogens so milk easily deteriorates to become unsuitable for processing and human consumption. Bacterial contamination of milk can originate from different sources: air, milking equipment, feed, soil, faeces and grass. Rinsing water for milking machine and milking equipment washing also involve some of the reasons for the presence of a higher number of microorganisms including pathogens in raw milk (Anil and Sangeeta, 2015).

Bacterial food poisoning can be divided into three principal types; food infection (invasive infection or ingestion of invasive organisms) representative as Escherichia coli and Salmonella; food intoxication (ingestion of preformed toxin) representative as Staphylococcus aureus and food toxico-infection (non-invasive infection) representative as some serotypes of E. coli.

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Escherichia coli is the most common contaminant of raw and processed milk. It is a reliable indicator of fecal contamination of water and food such as milk and dairy products. Presence in food may be of public health concern due to the possible presence of entero-pathogenic and/or toxigenic strains. Udder with sub-clinical mastitis and wet environment has led to contamination of milk and hence raw milk reaches the consumers with elevated coliforms count (Abd El-Samae, 2016).

There are six categories of pathogenic E. coli that affect the intestines of humans: Shiga toxin-producing Escherichia coli (STEC) recognized as toxin producing group of E. coli and one of the most significant foodborne pathogens worldwide (Farrokh et al., 2013). STEC is also called vero-cytotoxin-producing E. coli or (VTEC), STECs are associated disease symptoms. STECs are so named because they produce one or more cytotoxins, called Shiga toxin 1 (stx1) and Shiga toxin 2 (stx2), of which entero-haemorrhagic E. coli (EHEC) are a pathogenic sub-group; enteropathogenic E. coli (EPEC); entero-toxigenic E. coli (ETEC); entero-aggregative E. coli (EAEC); entero-invasive E. coli (EIEC); and diffusely adherent E. coli (DAEC) (Mhone et al., 2011). In addition, it causes wide range of human clinical symptoms comprising diarrhea, haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) have been attributed to both non-O157 STEC and O157 isolates (Wang et al., 2013).

Staphylococcus aureus usually acts as a commensal bacterium, asymptomatically colonizing about 30% of the human population; it can sometimes cause disease and it has been implicated in cases of severe diarrhea as well as it may be one of the main causes of food poisoning gastroenteritis among consumers (Tong et al., 2015). In addition, Staph. aureus is commonly associated with intoxications due to its ability to produce a variety of potent enterotoxin. Identical Staph. aureus strains have occasionally been isolated from dairy cows and hands of milking persons, but strains originating from bovine mastitis in general represent a genetically different cluster than the human strains, suggesting host specificity (El-Leboudy et al., 2014).

Salmonella is a member of the Enterobacteriaceace, Gram negative, motile, with peritrichous flagella and non-spore forming rods. Also, Salmonella is a facultative anaerobic (can grow with or without oxygen) catalase positive and oxidase negative bacteria. However, Salmonella is not included in the group of organisms referred to as coliforms (Lawley et al., 2008). More than 2,500 different types of Salmonella exist, some of which cause illness in both animals and people. Some types cause illness in animals but not in people. Some serotypes are only present in certain parts of the world (Brands, 2006).

Severe cases of salmonellosis can result in systemic infections and even death. Fecal wastes from infected animals, storage material and ways of handlings are important sources of Salmonella contamination of the raw milk. Previous studies indicated that milk and milk products are important sources of Salmonella particularly among those raw milk consumers (Teshome and anbessa, 2012). Salmonella has long been responsible for the largest number of food poisoning outbreaks worldwide and it is considered as one of the major causes of human gastroenteritis worldwide (CDC, 2007).

So, this study was planned to detect some pathogenic microorganisms that may contaminate raw milk beside application of molecular methods in a trail to detect virulence genes in the isolated bacteria.

2. Material and methods

1. Sampling:

A grand total of 100 samples of raw milk were randomly collected from small breeders and supermarkets. Milk samples were collected in sterile jars (250 ml. capacity), then transferred to the laboratory in an insulated ice box with a minimum of delay, where directly examined bacteriologically for detection of some food poisoning bacteria.

2. Isolation and identification of some pathogenic bacteria:

2.1. Preparation of milk samples was carried out according to APHA (2013).

2.2. Detection of Enteropathogenic Escherichia coli according to FDA (2002) and serological identification according to Kok et al., (1996).

2.3. Detection of Staphylococcus aureus were carried out according to Bailey and Scott, (1998).

2.4. Detection of Salmonellae was performed according to ISO 6579, (2017).

2.5. Detection of some virulence genes in bacterial isolates using PCR:

2.5.1. Primer sequences:

Bacteria	Gene	Oligonucleotide sequence (5′ → 3′)	Product size	References
Shiga toxin-producing *E. coli*	stx1 (F)	5′ ACACTGGATGATCTCAGTGG ′3	614 bp	Dhanashree and Mallya, (2008)
	Stx 1 (R)	5′ CTGAATCCCCCTCCATTATG ′3		
	Stx 2 (F)	5′ CCATGACAACGGACAGCAGTT ′3	779 bp	
	Stx 2 (R)	5′ CCTGTCAACTGAGCAGCACTTTG ′3		
	eae A (F)	5′ GTGGCGAATACTGGCGAGACT ′3	425 bp	Mazaheri et al., (2014)
	eae A (R)	5′ CCCCATTCTTTTTCACCGTCG ′3		
Coagulase +ve *Staph. aureus*	*clf*A	5′ GCAAAATCCAGCACAACAGGAA ACGA ′3	638 bp	Mason et al., (2001)
		5′ CTTGATCTCCAGCCATAATTGGT G ′3		
Salmonella	inv A	5′GTGAAATTATCGCCACGTTCG GGCA ′3	284 bp	Rahn et al., (1992)
		5′ TCATCGCACCGTCAAAGGAACC ′3		

2.5.2. DNA Extraction using QIA amp kit according to Shah et al., (2009).

2.5.3. Amplification reaction:

For E. coli, it was performed according to Fagan et al., (1999), For Staph. aureus, it was performed according to Mason et al., (2001) and finally for Salmonella, it was performed according to Singer et al., (2006).

2.5.4. Agarose gel electrophoreses was carried out according to Sambrook et al., (1989).

3. Results:

Table (1): Prevalence of some food poisoning bacteria in raw milk samples (n=100)

Bacterial isolates	Positive samples	
	No.	%
E.coli	64	64.0
Staph. aureus	35	35.0
Salmonella	3	3.00

The recorded data in Table (1) clarified that the prevalence of E. coli, Staph. aureus and Salmonella was 64, 35 and 3%, respectively.

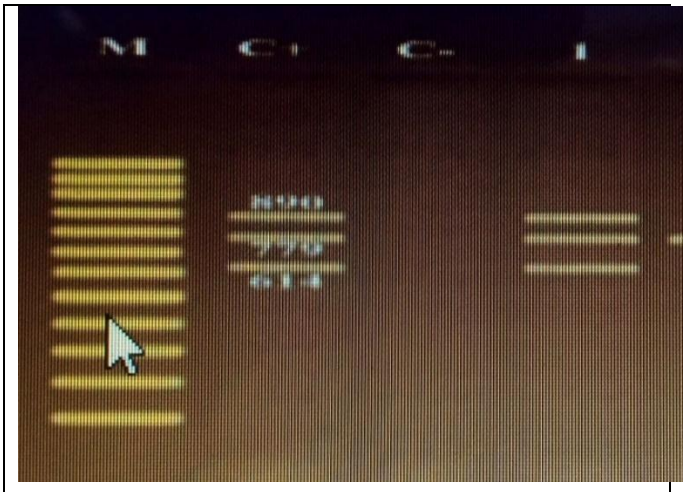
Table (2): Occurrence of virulence genes of Shiga toxin-producing E. coli isolated from the examined raw milk samples.

	Positive samples			*E. coli* Serovars	Character	Shiga-toxin 1 gene stx1	Shiga-toxin 2 gene stx2	intimin gene eaeA
	No.	%*1	%*2					
Shiga toxin-producing *E. coli*	6	4.0	6.25	O_{86}	EHEC	-	+	-
				O_{119} : H_6	EPEC	-	+	-
				O_{26} : H_{11}	EHEC	+	+	+
				O_{125} : H_{21}	ETEC	+	+	-

*1 referred to percentage in relation to total examined.

*2 referred to percentage in relation to total positive.

The recorded data in Table (2) clarified that only 4 isolates of E. coli (6.25%) were belonged to Enteropathogenic E. coli where serological identification revealed the presence of O86, O119 : H6, O26 : H11 and O125 : H21 serotypes



Photograph (1): Agarose gel electrophoresis of multiplex PCR of stx1 (614 bp), stx2 (779 bp) and eaeA (890 bp) genes for characterization of Enteropathogenic *E. coli*.

Lane M: 100 bp ladder as molecular size DNA marker.

Lane C+: Control positive E. coli for stx1, stx2 and eaeA genes.

Lane C-: Control negative.

Lane 1 (O26): Positive E. coli strain for stx1, stx2 and eaeA genes.

Lanes 2 (O86) and 3 (O119): Positive E. coli strains for stx2 gene.

Lane 4 (O125): Positive E. coli strain for stx1 and stx2 genes

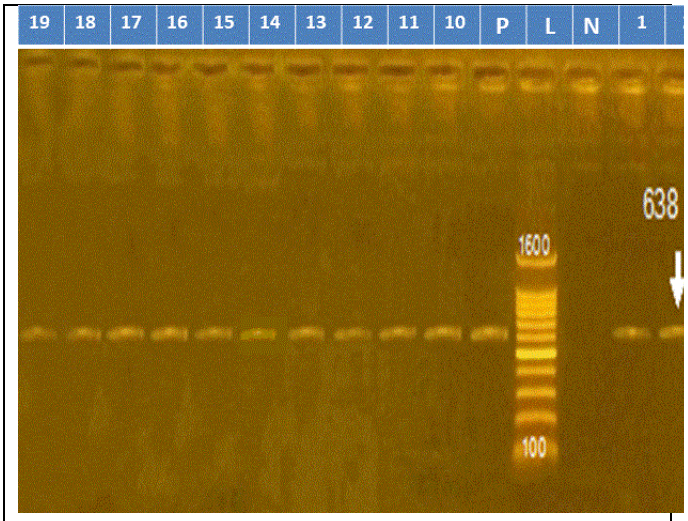
Table (3): Prevalence of coagulase positive Staph. aureus in raw milk samples

Examined samples	Staph. aureus positive culture		Coagulase positive Staph. aureus based on coagulase test			Coagulase positive S. aureus based on presence of clfA		
	No.	%	No.	%*1	%*2	No.	%*1	%*2
Milk	35	35.0	21	21.0	60.0	19	19.0	54.3%

*1 referred to percentage in relation to total examined.

*2 referred to percentage in relation to total positive.

The recorded data in Table (3) showed that the prevalence of Coagulase positive Staph. aureus based on coagulase test was 60% (21 out of 35 isolates) while it was 54.3% based on presence of clfA gene.

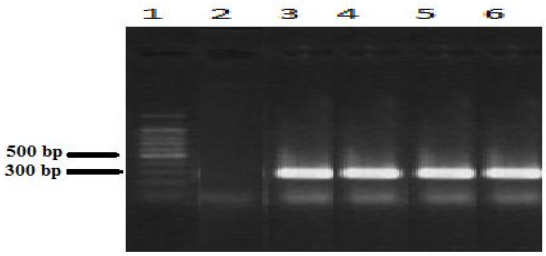


Photograph (2): Agarose gel electrophoresis of PCR products of *Staph. aureus* isolates from milk samples for presence of *clf*A coding gene (638 bp).
Lane L: 100 bp ladder,
Lane N: Negative control,
Lane P: Positive control,
Lanes 1-19: Positive samples for *clf*A coding gene

Table (4): Serodiagnosis of Salmonella isolated from examined raw milk samples

Identified Salmonella strains (n=3)	Group	Antigenic structure	
		O	H
S. Infantis	C1	6,7	r : 1,5
S. Typhimurium	B	1,4,5,12	i : 1,2
S. Enteritidis	D1	1,9,12	g,m : -

The serological identification of Salmonella isolates was recorded in Table (4) and revealed the presence of S. Infantis, S. Typhimurium and S. Enteritidis.



Photograph (3): Agarose gel electrophoresis of PCR products of *Salonella* isolates from milk samples for presence of *invA gene* (284 bp).
Lane 1: 100 bp ladder,
Lane 2: Negative control,
Lane 3: Positive control,
Lanes 4-6: Positive samples for *invA* gene

4. Discussion

Milk is an excellent high quality food providing major nutritional requirement to man at any age. At the same time, it contains abundance of all nutrients required to the growth and multiplication of most microorganisms (Gillespie et al., 2003).

Escherichia coli are a commensal microorganism whose niche is the mucous layer of the mammalian colon. This bacterium is the most abundant facultative anaerobe of the human intestinal microflora. Furthermore, E. coli is widely distributed in the intestinal tracts of warm blooded animals (Ishii and Sadowsky, 2008). E. coli frequently contaminate milk and it is a good indicator of fecal pollution. Its presence in milk indicates the presence of other enteropathogenic microorganisms that constitutes a public health hazard.

As shown in Table (1), the prevalence of E. coli using plating technique on EMB was 64%. This result was higher than that recorded by Bali et al., (2013) (32.5%), Meshref, (2013) (52.6%), Sana et al., (2005) (14.2%), El-Monir et al., (2018) (13.2%) and Ranjbar et al., (2018) (30.16%). Growth of E.coli in raw milk occurred in the absence of cooling system especially in tropical countries and it may reach higher number.

It is evident from the results reported in Table (2) that the prevalence of Shiga toxin-producing E. coli isolated from the examined 100 raw milk samples was 6.25% (4 out of 64 isolates) and they could be identified into four different serotypes including O86, O119: H6, O26: H11 and O125: H21 serotypes. In addition, by using multiplex PCR, it was found that O26:H11 had stx1 gene, stx2 gene and intimin gene; while O86 and O119:H6 serotypes had only stx1 gene. O125:H21 serotype had both stx1 and stx2 genes (Photo, 1).

Detection of STECs strains is considered a risk factor because it may cause various human gastrointestinal tract diseases, including watery or bloody diarrhea, and might develop a life-threatening disease, such as Haemorrhagic colitis (HC), thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS) which can occur in 5–10% of patients and is characterized by thrombocytopenia, micro-angiopathic hemolytic anemia and acute renal failure (Pennington, 2010).

Staph. aureus is a common commensal of the skin and mucosal membranes of humans (Argudín et al., 2010). It causes a wide range of diseases including food poisoning due to the production of enterotoxins. Milk and dairy products are often implicated in staphylococcal outbreaks (Pilar et al., 2010). In addition, Staph. aureus is ubiquitous bacteria in mammalian species associated with different types of infections in humans, companion animals and livestock. Moreover, they are the most commonly isolated bacteria from bovine mastitis (Oliveira et al., 2016).

Data summarized in Table (3) illustrated that the prevalence of Staph. aureus was 35%. This prevalence was nearly similar to Abdel-All et al., (2010) (34.1%) and Buyukcangaz, et al., (2013) (33%). At the same time, it was lower than that recorded by Belayneh et al., (2013) (48%), Oliveira et al., (2016) (49%), Haggag et al., (2019) (65.33 %) while it was higher than that recorded by Makovec and Ruegg (2003) (23%), Donkor et al., (2007) (14.6%) and Ibrahim, (2010) (16.47%).

The difference between the recorded result in the current study and other studies may be attributed to different examined area, animal management practices and hygiene conditions during milking.

Also, it was found that the prevalence of Coagulase positive Staph. aureus (CPS) based on coagulase test was 60% (21 out of 35 isolates) while it was 54.3% based on presence of clfA gene. Nearly similar isolation rates of CPS from milk samples were previously recorded by André et al., (2008) (66.7%) while it was lower than that recorded by Kamal et al., (2013) (94.3%) and finally it was higher that obtained by Sumathi et al., (2008) (24%), Korpysa-Dzirba and Osek (2011) (32.5%), Makita, (2012) (43.5%) and Keane et al., (2013) (23%).

These studies concluded that milk may be contaminated by hands of milking men or from the udders of animals harboring microorganisms. Dirty teats with dung and mud are the dirt source of bacteria in milk. Also, contaminated utensils used for the milk are also considered a source of contamination.

Food handlers carry enterotoxin-producing Staph. aureus in their noses or on their hands are regarded as the main source of food contamination, via manual contact or through respiratory secretions. However, Air, dust, and food contact surfaces can also serve as vehicles in the transfer of S. aureus to foods (Argudín et al., 2010). Symptoms of Staph. aureus food poisoning have a rapid onset (2–8 h), and include nausea, violent vomiting, and abdominal cramping, with or without diarrhea. The disease is usually self-limiting and typically resolves within 24–48 h after onset (Pinchuk et al., 2010).

Salmonella is a Gram negative rods genus belonging to Enterobacteriaceae family. It is a ubiquitous and hard bacterium that can survive several weeks in a dry environment and several months in water (WHO, 2016). It is well known that most Salmonella serovars have animal reservoirs and are potentially zoonotic (Rabinowitz and Conti, 2010).

As shown in Table (3), the prevalence of Salmonella in milk samples was 3% and the serological identification of isolates revealed the presence of S. Infantis, S. Typhimurium and S. Enteritidis. This finding was higher than obtained by Bianchi et al. (2013) (0.3 %) while it was lower that obtained by Jayarao et al. (2006) (6%).

S. Typhimurium and S. Enteritidis are the most frequently isolated serovars from food borne out breaks throughout the world (Herikstad et al., 2002).

5. Conclusion:

It could be concluded that raw milk poses a limited risk to public health and the quality of raw milk is still low, and efforts the entire productive

chain are required to attain consumer safety through application of HACCP and GMP programs in all milk chain production. It could be concluded that PCR is considered as sensitive and accurate method for identification of virulence genes of some food poisoning microorganisms
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Conflict of interest statement
No conflicts of interest.

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