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Detection of Enteroviruses in Raw Milk by Nested RT-PCR

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Abstract: This study aimed to evaluate the incidence of enteroviruses (EV) in milk samples. Raw milk samples (n=150) were collected from different dairy farms, bulk tanks and farmer house (50 sample each) in Kafr-El-Sheikh and Gharbia governorates, Egyptthroughout one year and were analyzed for EVby Nested RT-PCR.Viral RNA was extracted using BIOZOL total RNA extraction method. The primers were selected from the 5' non-translated region, which is the most conserved region in an enterovirus genome. Eleven raw milksamples (7.33%); 4 dairy farm milk samples (8%), 2 bulk tank milk samples (4%) and 5 farmer house milk samples (10%) were positive for EV RNA.EV were found to be more prevalent in milk fat (4.66%) than the partially skimmed milk (2.66%). This is the first report on the detection of EV RNA incow'sand buffalo'smilk samples in Egypt. This study highlights the variations in the incidence of EV in dairy farms, bulk tanks and farmer house raw milk samplesaccording tothe various sources of contamination,the public health significance of EV as well as suggested control and prevention measure to safeguard the consumer were discussed.

Key words: Foodborne viruses · Enteroviruses · Cow's Milk · Buffalo's milk · Nested RT-PCR

INTRODUCTION

Milk is a highly nutritious food and it is a source of vital macro- and micronutrients for the growth, development and maintenance of human health. Though, it may also be a source of natural food contaminants that may cause diseases and constitute potential infectious hazard [1].Milk has been well documented as a vehicle for transmission of assortment of bacterial diseases [2]. However, limited information is available on the transmission of viral diseases through milkwhich may attributed to the limitations of detection methods and culturing techniques for foodborne viruses, research has focused on bacterial pathogens. It has only been in the past few years that rapid and reliable methods have become available for virus detection. The development of molecular methods has increased the diagnoses of illnesses linked to foodborne viruses[3].

Enteroviruses (EV), genus*Enterovirus*, family *Picornaviridae*, are among the most common foodborne viruses infecting humans worldwide. EV are small (approximately 30 nm), single-stranded RNA, nonenveloped viruses with an icosahedral capsid composed of 60 subunits consisting of four structural proteins with approximately 7.5 kb long RNA. EV are sub grouped into *polioviruses*, *coxsackievirus* (groups A and B), *echovirus* and *enterovirus* 68 to 71[4-6]. Human EV infect millions of people worldwide and cause clinical manifestations such as aseptic meningitis, myocarditis, acute haemorrhagic conjunctivitis and other acute and chronic illnesses [7, 8]. Many EV are transmitted by the fecal-oral route and are excreted in stool but do not generally cause gastroenteritis however the first recorded outbreak associated with foodborne viruses was an outbreak of poliomyelitis linked to consumption of raw milk in 1914. Many

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outbreaks associated with raw milk consumption were reported after [9,10].

The objective of this studyis toinvestigate the presence of EV inrawcow's and buffalo's milk samples collected from different sources; different dairy Farms (Representing machine milking), bulk tanks (Representing market samples) and farmer house (Representing hand milking)in Kafr-El-Sheikh and Gharbia governorates, Egypt and to evaluate the probability of presence of EV in milk fat and partially skimmed milk.

MATERIALS AND METHODS

Raw Milk Samples: A total of 150 raw cow's and buffalo's milk samples were collected from; different dairy farms (Representing machine milking), bulk tanks (Representing market samples) and farmer house (Representing hand milking) (50 samples each) in Kafr-El-Sheikh and Gharbia governorates, Egyptthroughout one year.

Centrifugationof Raw Milk Samples: A Volume of 100 ml of raw milk samples were centrifuged at low speed centrifugation; 1000 xg (R.F.C) for 10 minutes. The milk sample is separated into two layers; upper fat and lower partially skimmed milk.

Viral Nucleic Acid Extraction: RNA was extracted from all milk fat and partially skimmed milk using BIOZOL total RNA extraction reagent (BIOFLUX, Japan) according to the manufacturer's instructions.

Nested RT-PCR forEV: Nested Reverse Transcription PCR (Nested RT-PCR) was performed to the RNA extracted from all milk fat and partially skimmed milk using specific primers for EV detection selected from 5' nontranslated region (5' NTR) of the EV genome according to Puig, Jofre [11] with minor modifications. The primer sequences used reported in Table 1, the procedures were done as follows:

cDNA Synthesis for EV RNA: RNA extracted samples (5 µl) were heated to 99°C for 5 min and immediately placed on ice. Five-µl of the heat shocked RNA were mixed with 5 µl of the reaction mixture containing; 1x RT-buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTP's, 100 U of Reverse Transcriptase and 2.5 µM of Reverse primer Ent2. The samples were incubated for 60 min. at 42°C for the RT reaction.

First round PCR forEV: The first round PCR amplification was performed in 25 µl end volume reaction mixture containing; (Five µl of the RT product was mixed with 20 µl of the reaction mixture containing PCR-buffer (Biobasic, Canada), 1.5 mM $MgCl_2$, 2.5 U of DNA polymerase(Biobasic, Canada), 0.1 mM dNTP's (Biobasic, Canada)and 0.5 µm of each primer (Ent1 and Ent2)). The amplification conditions were performed as follow; after a denaturation step at 94°C for 4 min, 40 cycles of amplification at 92°C for 1.5 min, 55°C for 1.5 min and 72°C for 2 min. were performed with a final extension of 72°C for 10 min.

Nested PCR for EV: A Nested PCR was performed, to amplify 138 bp fragment of the 5' NTR of enterovirus,in 25 µl end volume reaction mixture containing; (2 µl of first-round PCR product were mixed with 23 µl of the reaction mixture containing PCR-buffer,1.5 mM $MgCl_2$, 2.5 U of DNA polymerase, 0.1 mM dNTP's and 0.2 µm of each primer (Ent3 and Ent4)). Amplification conditions were as described for the first PCR amplification.The PCR products were analyzed on 3% agarose gel stained with ethidium bromide and examined with Gel Documentation System. The nested PCR amplification for EV positive samples yielded amplicon of 138-bp size.

Table 1: Primers used in this study

Primer	Primer sequences (5'-3')	Size of amplified product (bp)	Reference
Ent1	CGGTACCTTTGTACGCCT GT	534 bp	[11]
Ent2	ATTGTCACCATAAGCAGCCA		
Ent3	TCCGGCCCCTGAATGCGGCTA	138 bp	
Ent4	GAAACACGGACACCCAAAGTA		

RESULTS

Partially skimmed milk and Milk fat samples were investigated for the presence of EV by Nested RT-PCR. Four samples (2.66%) of partially skimmed milk; two dairy farm milk samples and two farmer house milk sampleswere positive for EV RNA.while, seven samples (4.66%) of milk fat; two dairy farm milksamples, two bulk tank milk samples and three farmer house milk samples contained EV RNATable2. Overall, out of the examined one hundred fifty raw milk samples 11 (7.33%)with an amplicon fragment size of 138 bp in Nested RT-PCR Figure 1; 4dairy farm milk samples,2 bulk tank milk samples and 5 farmer house milksamples were proved to be contaminated with human enteroviruses Table2.

Table 2: Incidence of EV in partially skimmed milk,milk fat and raw milk samples

Type of sample	Number of examined samples	Positive partially skimmed milk samples		Positive milk fat samples		Positive both partially skimmed milk and fat layer samples		Total positive raw milk samples	
		No.	%	No.	%	No.	%	No.	%
Dairy farm milk samples (Machine Milking)	50	2	4 %	2	4%	0	0%	4	8 %
Bulk tank milk samples (Market samples)	50	0	0%	2	4%	0	0%	2	4 %
Farmer house milk samples (Hand milking)	50	2	4 %	3	6%	0	0%	5	10 %
Total	150	4	2.66%	7	4.66%	0	0%	11	7.33%

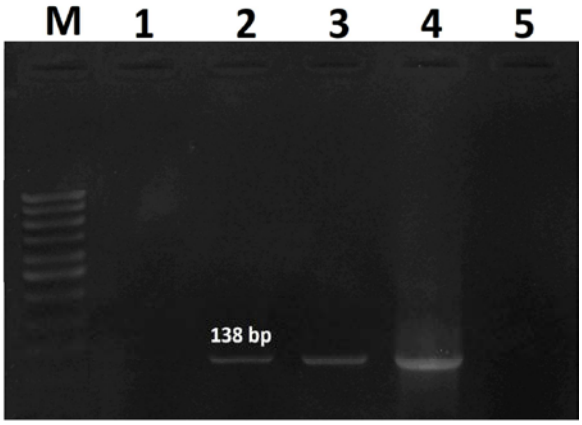

Fig. 1: Agarose gel electrophoresis 3 % showing PCR products amplified from Enteroviruses RNA extracted from milk samples at (138 bp)
Lane M=100bp ladder, lanes 1=negative sample that did not demonstrate the expected 138bp fragment. Lane 2,3=Positive samplesthat demonstrated the expected 138 bp fragment. Lane 4=a positive control. Lane 5= No-template control.

DISCUSSIONS

Enteroviruses were the first viruses shown to befoodborne after transmission by contaminated water and unpasteurizedmilk [12, 13]. Many outbreaks were reported due to consumption of milkcontaminated with EV[9, 10]

The study revealed that, out of 150 raw cow's and buffalo's milk samplesanalyzedby Nested RT-PCR, 11samples (7.33%) were found to be contaminated with EV Table 2. These results were in coincidence with that reported byTerzi, Albayrak [14]. Variations in the incidence level of EV in the examined samples according to the source was detected. The Highest incidence (10%) was found in the samples collected from farmers house followed by (8%) in case of the dairy farm samples while lower incidence (4%) was detected in case of bulk tank samples Table 2.

The lowest incidence of market samples may be attributed to; collection of milk samples from different sources so, these samples are mix between contaminated and free samples which could result in dilution of contaminationwith EV.

On the other hand, the highest incidence was detected in farmer house milk samples may be due to contamination of the milker's hand with EV from stool and the nature of the EVtransmitted by fecal contamination which may occur due to bad hygienic measures during the hand milking[15].

It is suggested that water used for cleaning and washing or asymptomatic infected persons at dairy facilities might be the main source of milk contamination withenteric viruses[3, 10, 14].In this study,the dairyfarm samples were proved to be contaminated with EV which may be due to inefficient sanitization of teat cups of milking machine and the carelessness of worker with their hygiene and the dairy farm hygiene. The four positive dairy farm milk samples were found to be from only onedairy farmwithin one month with two weeks interval which could be resulted from the contamination of milking machine in these farms and inefficient sanitizationfurthermore, the enteric viruses are generally resistant to environmental stressors, including heat and acid and they are stable in the presence of lipid solvents which increase the chances of contamination and impair decontamination [16].

Enteroviral infection is most common in summer and early autumn[10]. In this study EV were found to be more prevalent in samples taken during summer.

The study declared that the incidence of EV were found to be more in milk fat (4.66%) than the partially skimmed milk (2.66%) Table 2. This may be due to smaller size of the virus particles so, it could be separated with fat layer while the presence in the partially skimmed milk is may be due to the partial skimming of the milk was performed under low speed centrifugation and this layer is also containing fat.EV weren't detected in both partially skimmed milk and fat layer of the same sample Table 2.

CONCLUSIONS

In this study, EV were detected in the raw milk samples tested and variations in the occurrence level of EVin dairy farms, bulk tanks and farmer house raw milk samples according to the various sources of contamination was noted. The results also, reflect that the EV were found to be more prevalent in milk fat than the partially skimmed milk. This studyshed light on the importance of sanitation, hygienic practices at dairy facilities, the public health significance of EV as well as suggested control and prevention measure to safeguard the consumer were discussed.

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