MULTIPLEX PCR AND LATEX AGGLUTINATION FOR IDENTIFICATION AND CHARACTERIZATION OF TOXIGENIC CLOSTRIDIUM DIFFICILE FROM RAW MILK

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ABSTRACT

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Community-acquired *C.Diff.* infections have recently been increasing in incidence and severity. Several studies have isolated *C. Diff.* spores from livestock and food suggesting that food may play a role in transmission. The overall goals of this research were to: 1) determine prevalence of *C. Diff.* from raw milk using selective (cycloserine-cefoxitin fructose agar [CCFA]; 2) determine toxigenic profiles of *C.Diff.* isolated using PCR targeting *tpi and tcdB* genes. A total of 50 random samples of raw marketable milk were collected from different localities in Assuit city, Egypt. The obtained results indicated that about 11 (22%) out of 50 examined raw milk samples were positive for *C.Diff.* using conventional method. While, 2 strains of *C.Diff.* (18.2%) out of the positive 11 strains could be detected using the latex agglutination test. The prevelance rate of *C.Diff* using multiplex PCR was one (50 %) out of the two tested strains containing the DNA of *C.Diff.*, which was negative for toxin B production. The public health concern of *C.Diff* and the suggestive measures were disscused. The prescence of toxigenic *C.Diff.* in raw milk is of great concern and suggests its transmission through milk, which needs proper milking and handling to reduce risk to the public health.

Keywords: *Clostridium Difficile, Raw milk, latex agglutination, toxigenic, PCR.*

INTRODUCTION

Milk is one of the few food stuffs consumed in its natural state. It is the only article in the diet, with exception of honey, whose sole function in nature is to serve as a food. It presents a favourable physical environment for the multiplication of contaminating microorganisms. One of these microorganisms, *Clostridium difficile*.

C. Diff. is a Gram positive spore forming obligatory anaerobic bacillus. Spores can persist in a wide range of aerobic environments (2, 3) was first isolated in 1935 from the stool of healthy neonates by Hall and O'Toole (2). It was only in 1978, over 40 years later, that *C.Diff.* was found to cause human disease in the form of diarrhea and in severe cases pseudomembranous colitis [PMC] (4, 5). *C.Diff.* has been associated with clinical disease in dairy calves (6,7). It has toxigenic characteristics that toxin producing strains of *C.Diff.* Produce two toxins – toxin A, an enterotoxin, and toxin B, a cytotoxin (8). There are four broad categories listed as sources of acquiring *C. Diff.* infection (CDI): environment-to-person, person-to person, animal-to-person and consumption (9).

Mortality rates associated with *C.Diff.*-related disease in the U.S. increased nearly three-fold from 1999 to 2002. A study, which included *C. Diff.*-related cases where *C.Diff.* infection was present but not listed as the underlying cause of death, demonstrated an increase in deaths from 5.7 per million population in 1999 to 23.7 per million in 2004. It is possible that the increased rates were due to the emergence of a highly virulent strains (10).

Owing to the incrimination of *C.Diff.* in PMC and littile information about it's occurrence in milk, this study aims to determine the prevelance of *C.Diff.* from raw milk samples by comparing cultural method by using selective medium (*C.Diff.* medium), latex agglutination and multiplex PCR. In addition to determination of toxigenic profile of *C.Diff.* isolated from raw milk using multiplex PCR.

MATERIALS and METHODS

1- Collection of samples:

A total of 50 random samples of raw marketable milk were collected from different localities in Assuit city, Egypt. Samples were transferred to the laboratory in clean, dry and sterile containers in an ice box.

2- Isolation of *Clostridium difficile*: (11)

a- Enrichment of samples:

C. Diff. broth (CDB) which is composed of proteose peptone (40.0g), disodium hydrogen phosphate (5.0g), potassium dihydrogen phosphate (1.0 g), magnesium sulphate (0.1g), sodium chloride (2.0 g), fructose (6.0g), sodium taurocholate (0.1) was used for enrichment with addition of 2 vials of *Cl. difficile* supplement (Oxoid, SR96), 7% defibrinated horse blood (Oxoid SR50) and 2ml tween 80 (12). 3 gram of each sample was added to 10ml CDB with thorough mixing. Then incubated in an anaerobic cabinet (10% CO_2 and 37°C) for 7 days (13).

b- Plating: using direct streaking method:

By streaking a 3 mm loopful to at least 3 quadrants of the plate which contains *C.Diff.* agar media (Oxoid CM 0601) added to it 1gm sodium taurocholate and 2ml tween 80 that improve the recovery of *C. Diff.* (12) and supplemented with 2 vials *C.Diff.* selective supplement (Oxoid SR96) and 7% defibrinated horse blood (Oxoid SR50). Supplement and horse blood added to the media at 50˚c not higher, which provides extra enrichment allowing for better growth (14). Plates were incubated anaerobically in 37º C for 2 days.

3- Identification of *C.Diff.*:

a- Characteristic appearance of colonies:

Typical colonies of *C. Diff.* appear as greyish, non hemolytic, swarming colonies (12). With typical odor of horse manure (15). Suspected colonies were subcultured on brain heart infusion agar slopes (BHI) for further identification and toxin detection.

b- Biochemical tests:

Colonies were identified by biochemical tests that they were tested for indole production and sugar fermentation which includes glucose, lactose and sucrose (16). Acid was produced with gas from glucose. Sucrose was fermented with production of acid only. lactose was not fermented by *C.Diff.* The pathogen didn't produce indole.

c- Latex Agglutination test:

The isolated strains of *C.Diff.* were tested for its antigen. This is an antigen antibody reaction using Oxoid agglutination kits (Oxoid DR1107A, Basingstoke, UK).

d- PCR ribotyping: (17)

Multiplex PCR for *C.Diff.* identification and toxigenic type characterization.

1-Primers used:

a-tpi-F(5`-AAAGAAGCTACTAAGGGTACAAA-3`)
tpi-R(5`-CATAATATTGGGTCTATTCCTAC-3`)

b- tcdB-F(5`-GGAAAAGAGAATGGTTTTATTAA-3`)
tcdB-R(5`-ATCTTTAGTTATAACTTTGACATCTTT-3`)

2- Procedure:

a- extraction of DNA by boiling and then centrifugation for 2 min. to settle bacterial debris.
b- 10 micron litre of supernatant containing DNA used for subsequent PCR amplification.
c- Thermal cycler conditions.

			Additional comments
Initial PCR activation step:	30 sec	95°C	Hot Star Taq DNA Polymerase is activated by this heating step. Omniscript and Sensiscript Reverse Transcriptases are inactivated and the cDNA template is denatured.
3-step cycling			
Denaturation:	0.5–1 min	94°C	
Annealing:	30 sec	55 -65°C	Approximately 5°C below Tm of primers. Down touch procedure was implanted, annealing for 30 sec at decreasing temperature during first 11 cycles.
Extension:	30 sec	72°C	For RT-PCR products of 1–2 kb, increase the extension time by 30–60 s. For RT-PCR products over 2 kb.
Number of cycles:	40		The cycle number is dependent on the amount of template RNA and the abundance of the target transcript.
Final extension:	10 min	72°C	

d- Agarose gel electrophoresis of PCR product:

Amplified reverse transcriptase–polymerase chain reaction (RT-PCR) products were analyzed by electrophoresis on 1% agarose gels and stained with ethidium bromide.

RESULTS

Table 1: Incidence of *C.Diff.* in the examined raw milk samples using conventional methods.

No. of examined samples	Positive samples	%
50	11	22

Table 2: Frequency % of *C.Diff.* isolated from raw milk samples using Latex agglutination.

No. of examined strains	Positive strains	%
11	2	18.2

Table 3: Frequency % of *C.Diff.* isolated by using Latex agglutination from raw milk samples using Multiplex PCR**.**

No. of examined strains	positive strains	%
2	1	50

Table 4: Frequency % of *C.Diff.* toxigenic type (B) isolated by using Latex agglutination from raw milk samples using Multiplex PCR.

No. of examined Strains	Positive strains	%
2	0	0

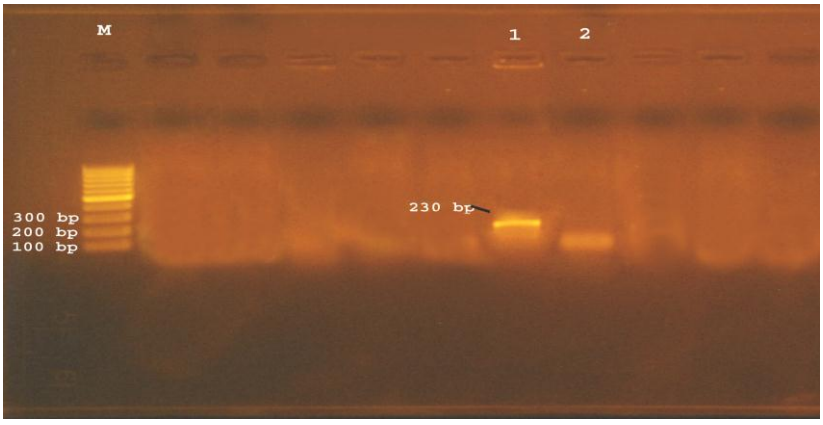

Photo1: Incidence of *C.Diff.* in the examined raw milk samples using multiplex PCR.
Lane M = Ladder marker
Lane 1= non toxigenic (B-) *C.Diff* strain.
Lane 2=negative *C.Diff*.

DISCUSSION

Table 1 showed that 11 (22%) out of 50 examined raw milk samples were positive for *C.Diff.* using conventional method. While, in Table 2, only 2 strains of *C.Diff.* (18.2%) out of the positive 11 samples could be detected using the latex agglutination test. It's clear from the obtained result that latex agglutination test is more accurate for detection of *C.Diff* than the biochemical reactions. Although latex agglutination test is available for the detection of *C.Diff.*, culture of Clostridium remains essential (15).

Most strains of *C.Diff.* produce both toxin A and B which cause gastrointestinal disease with severe

complications such as colonic perforation ,sepsis and death. (18,19).

In the current work, Table 3, cleared that only 1 strain of *C.Diff.* (50 %) out of the tested 2 strains containing the DNA of *C.Diff.* using multiplex PCR, but there was no strain positive to the *C.Diff.* toxigenic type B (Table 4).

The multiplex PCR-toxigenic culture scheme may be proposed as a reliable diagnostic method, since it provides both strain isolation and toxigenic type characterization within 36 to 48 hrs (17).

Previous studies have reported various prevelance rates of toxigenic *C.Diff.*0.2 % in the united states (20).2.5-3% in the European countries (21,22) and 6.7-9.30% in Japan (23, 24).

Higher results were obtained by (25) that analyzed 132 samples of raw bovine milk for toxigenic *C.Diff* in western Italy, the organism could be detected from 5 (3.8%) samples of bovine bulk tank milk.

CONCLUSION

The prescence of toxigenic *C.Diff.* in raw milk is of great concern and suggests its transmission through milk, which needs proper milking and handling to reduce risk to the public health.

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تفاعل البلمرة المتسلسل المتعدد واختبار التجلط للتعرف على وتمييز الكلوستريديام ديفيسيل في اللبن الخام

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تعتبر الكلوستريديام ديفيسيل من الميكروبات اللاهوائية والتي تؤدى إلى الحالات المعوية والتي من أخطرها ثقب القولون وفى هذه الدراسة تم جمع خمسين عينة عشوائية من اللبن الخام المباع في الأسواق ، وأوضحت النتائج أن ١١ عينة (٢٢%) كانت ايجابية للكلوستريديام ديفيسيل وذلك باستخدام الطرق التقليدية. بينما كانت هناك عترتان ايجابيتان للميكروب باستخدام اختبار التجلط ، أما باستخدام اختبار البلمرة المتسلسل المتعدد أثبتت النتائج أن (٥٠%) من العترات المختبرة كانت ايجابية والتي لم تكن بدورها مفرزة للسم B. وبذلك اثبت تفاعل البلمرة المتسلسل المتعدد انه طريقة سريعة وحساسة للكشف عن ميكروب الكلوستريديام ديفيسيل مقارنة بالطرق التقليدية. هذا وقد تم ذكر الشروط الصحية الواجب إتباعها لمنع تلوث الألبان بهذا الميكروب.