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## **Polymerase Chain Reaction for Detection of Toxigenic Strains of *Corynebacterium diphtheriae* in Milk and Some Milk Products**

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### **ABSTRACT**

Three hundred random samples were collected from different localities in Assiut city including raw milk (150) and some dairy products as Kareish cheese, Domiati cheese, Ras cheese, small scale producers ice cream and street vendors ice cream (30 samples for each). These samples were examined for the incidence of *C. diphtheria* using two selective media: Mueller-Hinton medium with Rabbit serum and Medium D2. The incidence of *C. diphtheriae* was (0.9%) and (3.5%) on Mueller-Hinton medium with Rabbit serum and Medium D2, respectively. Comparison between two selective media used for isolation of *C. diphtheria* proved the superiority of Medium D2 in the isolation of *C. diphtheria* from milk and some milk products. Polymerase Chain Reaction (PCR) was done to 8 *C. diphtheria* strains previously identified by biochemical tests and negative results were obtained. These strains may be non toxogenic or may be genetically varied. So we suggest making genetic sequencing for the isolated *C. diphtheria* strains and creating specific primers for locally isolated strains. More efforts are needed to enhance and promote farms and sale points of milk.

**Key words:** Polymerase chain reaction, *Corynebacttrium diphtheriae*, milk products, toxogenic strains

### **INTRODUCTION**

*C. diphtheriae* causes diphtheria which was a highly infectious disease with fatality rates between 5 and 10%. In children under 5 years and adults over 40 years, the fatality rate may be as much as 20%. Outbreaks, although very rare, still occur worldwide, even in developed nations (Todar, 2004). Hull (1963) reported that in the United States during the years 1919 to 1948 there were only 11 milk-borne outbreaks of diphtheria recorded. CDC (2010) recorded that Egypt was the third country that endemic with diphtheria.

Diphtheria affects the upper respiratory tract with symptoms including sore throat, low-grade fever, headache and the formation of a pseudo membrane on the tonsil(s), pharynx, and/or nose. Absence of treatment may lead to complications like kidney damage, heart failure and paralysis due to the toxins produced by bacteria (Santo-pietro, 2007). Cardiac enlargement due to myocarditis is common and Central Nervous System (CNS) may develop signs of hemorrhage, meningitis and encephalitis. Death is mainly due to respiratory obstruction by the membrane or toxic effects in the heart or nervous system (Frassetto, 2010). The exotoxin of *C. diphtheriae* is one of the well-studied bacterial toxins and acts to inhibit protein synthesis particularly in the heart muscle and neural cells (Baron *et al.*, 1994). On the other hand, the classical respiratory diphtheria is rare in the UK

and between 1986 and 2007, only eight cases of classical respiratory diphtheria caused by toxigenic *C. diphtheriae* were reported, all of them had a history of travel to endemic countries (Perkins *et al.*, 2010).

The advances in the biotechnology over the past decade have resulted in the development of many methods for the detection of pathogenic microorganisms, such as *C. diphtheriae* in food. These tests are less time consuming than conventional microbiological methods. PCR is an *in vitro* amplification technology that has been widely adapted for rapid assay development (Nakao and Popovic, 1997). Several Polymerase Chain Reaction (PCR) systems for the detection of *C. diphtheriae* have been described, mainly targeting the virulence genes, here, a PCR detection system based on the diphtheria toxin gene and its use has allowed for the rapid differentiation between toxigenic and non toxigenic strains.

## **MATERIALS AND METHODS**

### **Isolation and identification of *C. diphtheriae* from milk and some milk products samples:**

**Collection of samples:** A total of 300 random samples of raw milk and some milk products (cheeses and ice cream) were collected from different sources and localities in Assiut Governorate.

**Raw milk samples:** A total of 150 raw milk samples including bovine milk (90 samples) from dairy farms, dairy shops and street vendors (30 samples of each) and 60 samples of sheep and goat milk (30 samples of each).

### **Milk product samples**

**Cheese samples:** Ninety samples of Kareish, Domiati and Ras cheese (30 samples of each) were collected randomly from famous markets, supermarkets and dairy shops.

**Ice cream samples:** Sixty Ice cream samples including small scale producers and street vendors (30 samples of each) were purchased from dairy shops and street vendors.

**Preparation of samples:** The samples were prepared according to the technique recommended by APHA (1992).

### **Isolation of *C. diphtheriae***

**Enrichment procedure:** One milliliter of well homogenized milk or milk products samples was aseptically inoculated into sterile cotton plugged test tube, containing 10 mL of Dubos broth with Horse serum and incubated at 37°C for 24-48 h (Atlas and Parks, 1993).

**Selective plating:** A loopfull from the incubated broth culture was streaked on to plates of Mueller-Hinton medium with Rabbit serum and Medium D2. Streaked plates were incubated at 37°C for 24-48 h (Atlas and Parks, 1993). These organisms usually appear as opaque, white or gray colonies (Bailey *et al.*, 1986).

**Identification of isolates:** Microscopic examination using Gram Stain was by using different tests (Baron *et al.*, 1994), catalase test (Land *et al.*, 1991) and motility test (APHA, 1992).

Confirmation of *C. diphtheriae* was done according to Mahon and Manuselis (1995) applying the following tests:

Urease test (Koneman *et al.*, 1992), starch hydrolysis (Peter *et al.*, 1986), gelatin hydrolysis (Shawar *et al.*, 1990) and hemolysis on blood agar ( $\beta$ -Hemolysis) (Cruickshank *et al.*, 1975).

**Detection of toxigenic *C. diphtheriae* by PCR:** This part has been done in the Molecular Biology and Genetic Engineering Research Centre in Assiut University.

**Isolation of the genomic DNA from cultured cells and preparation of the cell suspension:** This protocol is designed for rapid isolation of up to 25  $\mu$ g genomic DNA from cultured cells.

**PCR assay:** PCR amplification was performed using Master Mix. The reaction mixture contained a total reaction volume of 25  $\mu$ L in 0.5 mL tubes included: 12.5  $\mu$ L of Master Mix., 2.0  $\mu$ L of primer F, 2.0  $\mu$ L of primer R, 2.0  $\mu$ L of free water and 6.5  $\mu$ L of Template DNA.

**Cycling conditions (thermal profile):** In the present study two primers were used (Tox 1, 2 and Dipht 6F, Dipht 6R (Nakao *et al.*, 1997). The two primers used for amplification of *C. diphtheriae* DNA were purchased from The Midland Certified Reagent Company Inc. of Midland, Texas.

- Tox1: 5'-ATCCACTTTTGTGCGAGAACCTTCGTCA-3'
- Tox2: 5'-GAAAACCTTTTCTTCGTACCACGGGACTAA-3'
- DIPHT 6F: 5'-ATACTTCCTGGTATCGGTAGC-3'
- DIPHT 6R: 5'-CGAATCTTCAACAGTGTTCCA-3'

The mixture was initially denatured at 95°C for 2 min, followed by 35 amplification cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 1 min. ending with a final 10 min extension at 72°C according to Nakao and Popovic (1997).

**Agarose gel electrophoresis:** The commercial DNA standard markers and the samples were loaded one in the wells. Gels were run for 60 min. at 90 V in Tris-acetate buffer (Kaufman *et al.*, 1995).

**Detection of PCR products:** The gel was stained with ethidium bromide and amplicons were visualized on a UV Tran illuminator.

## RESULTS

Results illustrated in Table 1 revealed that by using conventional methods, 2 (0.9%) and 6 (3.5%) of the isolates were identified as *C. diphtheriae* on Mueller-Hinton medium with Rabbit serum and Medium D2, respectively. Two isolates obtained from dairy farm raw milk and goat milk samples cultured on Mueller-Hinton medium with Rabbit serum. Two isolates recovered from Kareish and Domiati cheese each; one isolate from Ras cheese and one isolate from small scale producers ice cream samples cultured on Medium D2.

Table 1: Incidence of *C. diphtheriae* in the examined samples of milk and some milk products (according to the biochemical tests)

Milk and milk product samples	<i>C. diphtheriae</i> strains isolated on			
	Mueller-hinton medium with Rabbit serum		Medium D2	
	No./30	%	No./30	%
Bovine milk samples:				
Dairy farm	1	3.3	0	0
Dairy shops	0	0	0	0
Street vendors	0	0	0	0
Sheep milk	0	0	0	0
Goat milk	0	0	1	3.3
Cheese samples:				
Kareish cheese	0	0	2	6.7
Domiaty cheese	0	0	2	6.7
Ras cheese	0	0	1	3.3
Ice cream samples:				
Small scale producers	1	3.3	0	0
Street vendors	0	0	0	0
Total	2	0.9	6	3.5

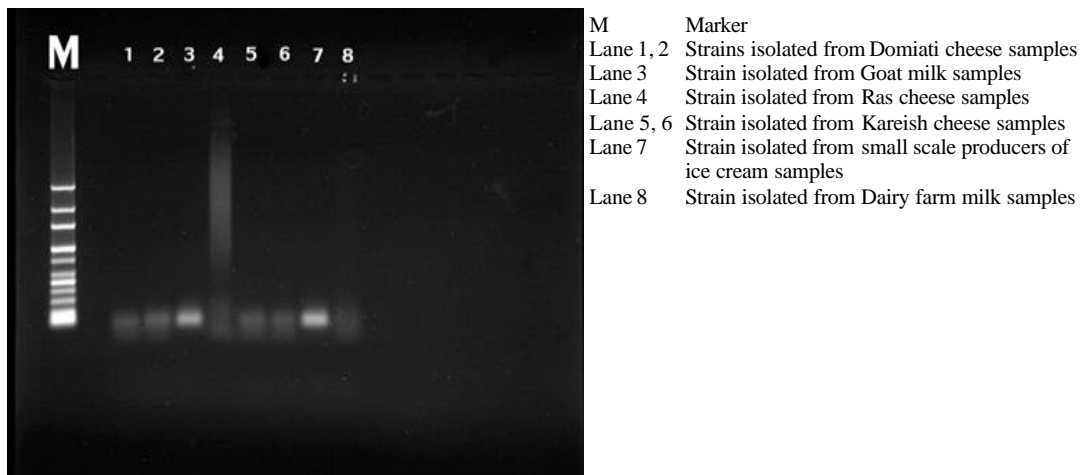


Fig. 1: Results of PCR for detection of toxigenic strains of *C. diphtheriae* using Tox 1- Tox 2 primers

Electrophoresis analysis of 1.2% agarose gel stained by ethidium bromide (from left to right) showed that lanes (1-8) were negative by using both primers Tox 1- Tox 2 ( 248 bp; A subunit) and primers Dipht 6 F- Dipht 6 R (297 bp; B subunit) (Fig. 1, 2).

## DISCUSSION

The risk of infection with zoonotic Corynebacteria appears greatest in those who drink unpasteurized milk (Palmer *et al.*, 1998). The important member of the genus *Corynebacterium*

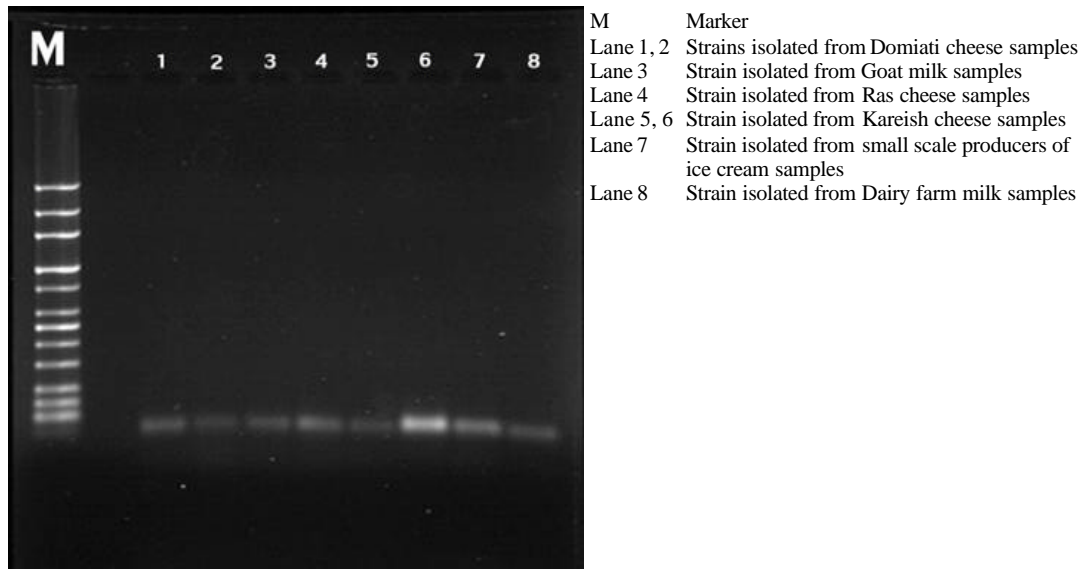


Fig. 2: Results of PCR for detection of toxigenic strains *C. diphtheriae* using Dipht 6 F-Dipht 6 R primers

from human health point of view is *C. diphtheriae*, which produces a powerful exotoxin and is the cause of human diphtheria (Sharma and Adlakha, 1997). Although *C. diphtheriae* is not thought to have animal reservoir, it has occasionally been isolated from the udder and teats of cows (Palmer *et al.*, 1998). In the United States during the years 1919 to 1948 there were 11 milk-borne outbreaks of diphtheria recorded and non thereafter (Hull, 1963), in many years later, *C. diphtheriae* endocarditis included 49 cases (Huber-Schneider *et al.*, 1995).

The relatively high results obtained from cheeses samples were not surprising since *C. diphtheria* could be growing up to 9% NaCl (Smith, 1969). This may be attributed to the neglected sanitary control adopted during manufacturing, handling and distribution of cheese. Therefore, it is advisable to obligate strict hygienic measures during preparation and handling of such product to improve its quality, as well as to safe guard consumers against infection.

*C. diphtheriae* is a Gram positive, fermentative, pleomorphic rod, Catalase positive, Urease negative and Nitrate positive (Efstratiou and George, 1999), hemolytic on blood agar and produce acid from glucose, maltose, sucrose and xylose (Songer and Post, 2005). Comparison between the two selective media (Mueller-Hinton medium with Rabbit serum and Medium D2) used for isolation of *C. diphtheria* revealed that 6 *C. diphtheriae* strains could be isolated on Medium D2 that emphasized the superiority of Medium D2 in the isolation of *C. diphtheria*.

Although the PCR test completely correlated with the standard biochemical and commercial identification for all *C. diphtheriae* strains tested (Pimenta *et al.*, 2008), the results in this study concluded that all strains isolated and identified biochemically as *C. diphtheriae* were negative by using PCR. These strains may not harbour the toxin gene so, it could not be expressed. Efstratiou and George (1999) described that the presence of tox gen does not always indicate toxin production and isolates should be tested phenotypically for toxin production by the Elek test or Vero cell cytotoxicity assay.

The non toxogenic *C. diphtheria* strains were detected by other investigations. Demikhovskaia *et al.* (2001) found that 1 out of 5 non toxigenic strains had tox gene. Melnikov *et al.* (2004) found that among the 828 *C. diphtheria* isolates, 114 were non toxigenic cultures (13.8%) had the gene of diphtheria toxin (gene tox) and were thus called non toxigenic tox-carrying (NTTC) strains. Moreover, 26 non toxogenic strains were isolated by De Zoysa *et al.* (2005). Dewinter *et al.* (2005) recorded that only 14 from 89 isolates of *C. diphtheriae* produced diphtheria toxin and harbored the diphtheria toxin gene. Pimenta *et al.* (2008) isolated 91 *C. diphtheriae* and found that 54 strains were non-toxigenic.

Although universal vaccination has resulted in a very low incidence of diphtheria in Canada, human clinical isolates which harbour and produce the diphtheria toxin remain in circulation. Between 1999 and 2003, 16% (14 of 89) of referred *C. diphtheriae* isolates produced the diphtheria toxin or harboured the diphtheria toxin gene without expressing it (Dewinter *et al.*, 2005). Furthermore, non toxigenic strains of *C. diphtheriae* represent a potential reservoir for the emergence of toxigenic *C. diphtheriae* strains if they possessed functional diphtheria toxin repressor (dtxR) genes (De Zoysa *et al.*, 2005).

The type of samples may affect on the result of PCR test, in the present study all *C. diphtheriae* strains were isolated from raw milk, cheese and ice cream samples it may be transmitted from milkers, workers during handling the milk or milk products through skin contact and coughing. These strains were mainly non toxogenic (Gruner *et al.*, 1994). Moreover, Tiley *et al.* (1993) postulated that non toxogenic *C. diphtheriae* strains occur more often in people who have been previously immunized. In spite of all *C. diphtheriae* strains were non toxogenic the danger may be due to that these non toxigenic strains could become toxigenic by acquiring the tox gene, assuming that the chromosomal diphtheria toxin repressor gene (dtxR) is functional (Cianciotto and Groman 1997). De Zoysa *et al.* (2005) stated that non toxigenic types of *C. diphtheriae* have been isolated from various sporadic or epidemic cases of cutaneous diphtheria. Finally, genetic variation between strains isolated from local samples and the others all over the world affect the results. So, we suggest making a genetic sequencing for the isolated microorganism and creating specific primers for the locally isolated strains.

## CONCLUSION

The aforementioned data proved that great attention must be paid to the problems of these pathogens in our food. Consequently, more restriction and preventive measures should be taken to improve the quality of raw milk to protect consumers from being infected by this and other organisms.

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