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Detection of Listeria Group by Conventional and PCR Techniques from River Nile Water

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Abstract: The main objective of this study is detection of Listeria group in River Nile water and comparison between conventional (membrane filtration (MF), multiple tube fermentation (MTF) techniques and polymerase chain reaction (PCR)). To achieve these aims, 60 samples were collected from River Nile water at Greater Cairo, during a period extended from December, 2005 to November, 2006 and 14 water samples were collected from River Nile (Rossita Branch) during two months. The results of Listeria group showed that, 54 out of 60 samples (90.0%) were positive using PCR technique, 58 out of 60 samples (96.6%) were positive using MF technique. On the other hand, 52 out of 60 samples (86.6%) were positive using MTF technique. In case of water samples which collected from Rossita Branch, 13 out of 14 samples (78.57%) were positive using PCR, MF and MTF techniques.

Key words: Listeria group · PCR · MF technique · MTF technique · River Nile water

INTRODUCTION

The name of the Nile originates from the Greek word "Nelios", meaning River Valley. The River Nile is the longest river in the world (4176 Miles) and has been the source of civilization for more than 5000 years, flowing from the south to the north of Egypt. As today, about 160 million people depend on the River Nile for their livehood, quality of the River Nile water as a source of drinking water is a critical issue for the Nile Basins countries, where chemical run-off from industry and agriculture creates pollution and higher concentrations of salt affect irrigated soils, increase waterborne diseases and lead to loss of fisheries and biodiversity [1]. With the increases of population on the Nile banks, a remarkable increase in industrial, agricultural, human activities and recreational activities have also occurred. The effluents of such activities are discharged directly into the Nile or through some agricultural drains which finally discharged their wastes into the Nile [2]. The genus *Listeria* comprises six characterized species: *Listeria monocytogenes, L. innocua, L. seeligeri, L. welshimeri, L. ivanovii* and *L. grayi* [3]. Among these gram positive, nonsporulating and motile species, only *L. monocytogenes* is a human and animal pathogen, capable of causing severe infections like septicemia, encephalitis and meningitis, especially in immunocompramized individuals, newborns and pregnant women [4].

L. monocytogenes is ubiquitously distributed throughout the environment. Forest soil, cultivated and uncultivated fields, mud, feed, feeding grounds, wildlife faeces and birds [5] have been found to be extensively contaminated with *listeria*. In addition, a large proportion of fecal samples collected from healthy animals with no clinical symptoms of listeriosis may contain *L. monocytogenes* [6]. Moreover, the bacterium has also been detected in different kinds of fish, squid and crustaceans [7, 8, 9]. Hence it is no surprise that contamination with *Listeria* spp. has been found in water environments such as coastal sea water and rivers containing organic load [10]. In addition, even springs and groundwater wells have been discovered to harbour the bacterium [11, 12]. Watkins and Sleath [13] stated that attention should be drawn to the presence of *L. monocytogenes* within the water cycle in order to assess its epidemiological significance. One of the routes to discharge the effluents of sewage treatment plants is by dispensal in surface water. Thus, the aim of this study is detection of Listeria group in River Nile water in

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addition to compare between the two conventional techniques (MF and MTF) and PCR technique for detection of Listeria group as pathogenic bacteria in River Nile water.

MATERIALS AND METHODS

Sampling Sites and Collection: River Nile water samples were collected monthly intervals from five sites in Greater Cairo as follow Kafr El-Elw, El-Maasara, Giza, Embaba and El-Galatma during one year (Dec. 2005 - Nov. 2006). Also 14 samples were collected from Rossita Branch (seven sites allocated along 60 Km of Rossita Branch which are a point source of pollution named as followed in the front of El-Rahawy drain, Abo El-Khawey (next to El-Rahawy with 15 Km), El-Nigela (next to El-Rahawy with 30 Km), Kafr El-Ziat (in the front of Menof drain), salt and soda production company (next to El-Rahawy drain with 45 Km), next to El-Rahawy drain with 50 Km and next to El-Rahawy drain with 60 Km during two months (July- Aug. 2006). Samples were collected from the middle of the river (30 cm deep from the surface) in a wide mouth sterile glass bottles. The samples were preserved in an ice box and examined within 4-6 h.

Microbiological Examination: Detection of Listeria group was carried out by MTF according to Fenlon [14], MF techniques on *Listeria* selective agar supplemented with 0.01% esclalin and 0.05% ferric citrate according to Shaban and El-Taweel [15] and PCR technique was carried out according to Paillard *et al.* [16].

Preparation of Water Samples for PCR: The samples were prepared as follows 1000-2000 ml was filtered with nitro-cellulose membrane (0.45 μm pore size and 47 mm in diameter (Whattman Co.). The membrane filters were transferred to 10 ml tryptic soya broth (TSB) (DIFCO Co.) with 10% glycerol. Plates were incubated overnight at room temperature with gently shaking. DNA extractions were carried out according to Kapperud *et al.* [17] and Waage *et al.* [18], from each overnight culture 100 μl was transferred into Eppendorf tube and centrifuged at 14900 g for 10 min in a microcentrifuge (Labfuge, 460). The resulting pellets were resuspended in 50 μl PCR buffer with 1 μl of Proteinase K (0.2 mg ml^{-1}). After incubation at 37°C for 1 h, the bacteria were lysed by boiling for 10 min. The samples were stored at -20°C overnight prior to PCR. After thawing at room temperature and centrifugation at 14900 g for 5 min, 5 μl of supernatant will be used for PCR reaction.

Identification of *Listeria* Isolates by PCR: Loopful of isolates were taken from stored slants to 5 ml TSB tubes, incubated at 37°C for 18- 24 h, then streaked on nutrient agar and incubated at 37°C for 18- 24 h. Single colony was picked into 50 ml TSB incubated at 37°C for 18- 24h then centrifuged at 3700 g for 15 min in cooling centrifuge (HERMLE- LABORTECHNIK, 79564, NR: 43960039 and Labofuge 460), the supernatant discarded and twice resuspended the pellets in phosphate buffered saline (PBS) (pH 7.2) then centrifuged at 3700g for 15min, the supernatant discarded and the pellets resuspended in 1000 μl PBS, finally stored at -20°C till used for DNA extraction.

Selection and Synthesis of Primers DNA Amplification: Detection of *Listeria* species were carried out according to Paillard *et al.* [16]. Listeria group was identified by amplification of 23S rRNA gene using two sets of primers, the first is S1F 5' AGT CGG ATA GTA TCC TTA C 3' and S1R 5' GGC TCT AAC TAC TTG TAG GC 3', which amplify fragment size 460 bp (S1) and the second set is S2F 5' GCC TAC AAG TAG TTA GAG CC 3' and S2R 5' ACT GGT ACA GGA ATC TCT AC 3', which amplify fragment size 890 bp (S2) of the 23S rRNA gene. The primers were synthesized by Bio-Basic Inc., Canada. The PCR reaction mixture was 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM (each) dNTPs (Fermentas), 1.5U of *Taq* polymerase (Fermentas), 5 μl of sample and 0.5 μM (each) designed primer for every reaction. PCR experiments were performed under the following conditions: 94°C for 5 min, followed by 35 cycles (94°C for 1 min, 50°C for 1 min and 72°C for 1 min), then 72°C for 7 min. Amplified products were analyzed by agarose gel electrophoresis. Gels were stained with ethidium bromide (0.005%, w/v) [19] with BenchTop ÖX174 DNA/HaeIII Marker (Promega Co.).

Sequencing of Amplified Products for Typing: PCR products of selected positive samples of Listeria group were sequenced. Fifty to one hundred μl of the PCR products were purified using a high pure PCR products purification Kit (Qiagen) following the manufacturer's instructions. Cycle sequencing was performed on 1 to 7 ml of the purified products with an ABI Prism Big dye termination cycle sequencing ready reaction (Applied biosystem) using the same primers as in the PCR and following the manufacture instructions. The DNA was sequenced with an ABI Prism (model: 310) automated DNA sequencer. Sequence data from both strands of the PCR products and extensive sequence information from the GenBank database were aligned and compared by using the clustal X and blast programs [20].

Statistical Analysis: In order to study the relationship between MTF and MF for detection of Listeria group, linear correlation analysis was used. The quantitative analysis for two techniques and Listeria group was carried out within the average counts of the five sites of raw River Nile water sites. All the data were transformed in decimal logarithms and processed by the Microsoft excel (office 2003) under Microsoft windows 2002, computer application.

RESULTS

In this study Listeria group was determined in 5 sites at River Nile samples collected from Greater Cairo during one year (Table 1), in addition to 7 sites along Rossita Branch during two months (Table 2). *Listeria* counts were in the range of range of 2.0- 900 MPN-index/100ml and 3.0- 490 cfu/100ml using MTF and MF techniques, respectively. Results of listeria group at Greater Cairo showed that it were detected in 52 (86.6%) and 58 (96.6%) out of 60 samples from different sites using MTF and MF techniques, respectively. The highest counts were recorded in Embaba followed by Giza and El-Massara, while the lowest count was recorded in Kafr El-Elw (Figure 1).

On the other hand, Listeria group was detected in 13 (92.8%) samples out of 14 using both MTF and MF techniques during two months. The highest densities of each were recorded at sites no. 2 ($7.0x10^3$ MPN-index/100ml and $1.8x10^3$ cfu/100ml) and site no. 6 ($4.3x10^3$ MPN-index/100ml and $4.3x10^3$ cfu/100ml) for MTF technique and MF technique respectively (Figure 2). Listeria group was detected in River Nile water samples collected from Greater Cairo (Kafer El-Elw, El-Maasara, Giza, Embaba and El-Galatma) from Dec. 2005 till Nov. 2006 using PCR technique, 54 (90.0%) out of 60 samples were positive in samples collected from River Nile Greater Cairo (Table 1).On the other hand, Listeria group was detected in all River Nile water (Rossita Branch) collected from seven sites during two months using PCR technique 13 (92.8%) out 14 samples only negative sample at site no. 1 (July) Table (2). Listeria group was detected in two samples using PCR techniques at fragment size (S2) at sites no. 3 and no. 7 (Figure 4). By the way, twenty *Listeria* isolates which determined by MTF and MF techniques were confirmed by PCR technique and were positive.

In order to study the relation between MTF and MF techniques for determination of Listeria group in River Nile water, linear correlation coefficients analyses were used. It was found that highly significant correlations between MF and MTF techniques for detection of listeria group (r= 0.98 and b=2.23) (P=0.05) Figs. 1-4.

Table1: Comparison between different techniques for detection of Listeria group during one year from 5 sites at Greater Cairo

	Kafr El-Elw			El- Maasara			Giza			Embaba			El- Galatma		
Months	PCR	MTF	MF	PCR	MTF	MF	PCR	MTF	MF	PCR	MTF	MF	PCR	MTF	MF
Dec.	+	2.0	28	+	2.0	20	+	4.0	60	+	900	380	+	4.0	53
Jan.	+	ND	10	+	2.0	30	+	4.0	72	+	280	490	+	17	28
Feb.	+	7.0	10	-	2.0	10	-	4.0	20	+	170	360	+	11	32
March	+	4.0	20	+	30	10	+	4.0	43	+	50	72	+	30	37
April	+	50	14	+	4.0	84	+	80	96	+	160	240	+	26	12
May	+	ND	10	+	ND	44	+	2.0	14	+	240	360	+	8.0	12
June	-	ND	4.0	+	30	86	+	23	80	+	80	200	+	8.0	14
July	+	2.0	60	+	4.0	47	+	23	18	+	800	12	+	4.0	80
Aug.	-	ND	ND	+	7.0	96	+	13	60	-	700	310	-	2.0	ND
Sept.	+	20	84	+	20	15	+	90	15	+	70	10	+	ND	80
Oct.	+	50	20	+	17	40	+	4.0	84	+	ND	3.0	+	8.0	22
Nov.	+	ND	22	+	9.0	12	+	8.0	10	+	40	36	+	22	13
Min.		2.0	4.0		2.0	10		2.0	10		40	3.0		2.0	12
Max.		50	84		30	96		90	96		900	490		30	80
Ave.		11	23		10	41		21	47		291	206		12	40

Table 2: Comparison between different techniques for detection of Listeria group during two months from 7 sites at Rossita Branch

	site no. 1			Site no. 2			site no. 3			site no. 4			site no. 5			site no. 6			site no. 7		
Month	PCR	MTF	MF	PCR	MTF	MF	PCR	MTF	MF	PCR	MTF	MF	PCR	MTF	MF	PCR	MTF	MF	PCR	MTF	MF
July	-	ND	ND	+	7000	1800	+	160	180	+	280	570	+	900	390	+	17	45	+	17	50
Aug.	+	160	210	+	420	920	+	140	180	+	170	320	+	160	250	+	4300	4300	+	210	120
Ave.		80	105		3700	1300		150	180		220	440		530	320		2100	2100		110	85

ND: Not detected

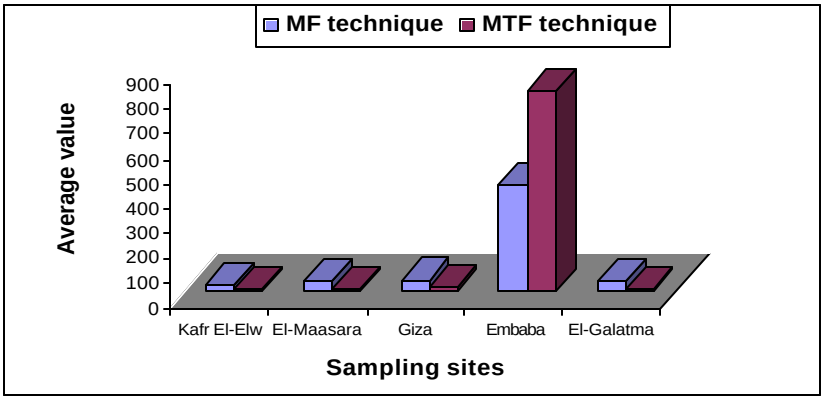

Fig. 1: Comparison between average counts of MF technique and MTF technique for detection of Listeria group during one year from 5 sites at Greater Cairo

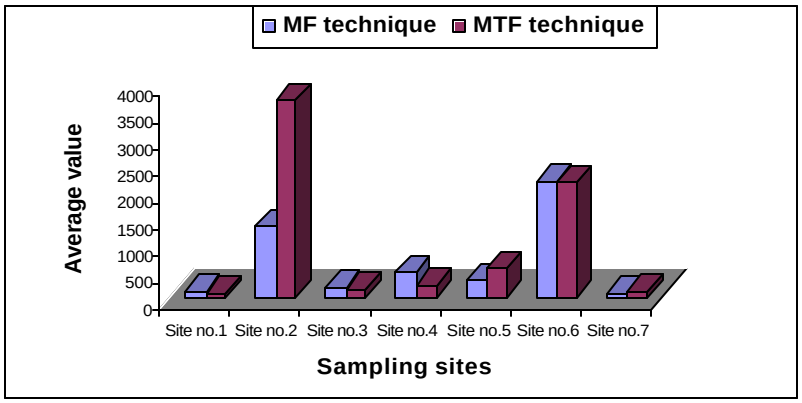

Fig. 2: Comparison between average counts of MF technique and MTF technique for detection of Listeria group during two months from 7 sites at Rossita Branch

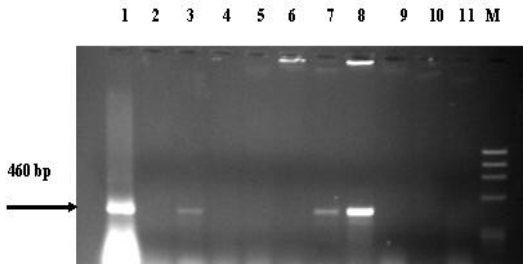

Fig. 3: Results of PCR of Listeria group, lane1: positive control, lane 2: negative control, lane 3 till lane11: River Nile water samples lane12: Marker (ØX 174)

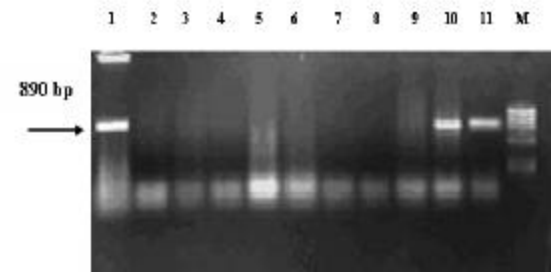

Fig. 4: Results of PCR of Listeria group fragment (S2), lane 1: positive control lane 2: till lane 11: River Nile water samples (Rossita Branch) lane 8: Marker (ØX 174)

AGTCGGATAGTATCCTTACGCGTGAGGAAGCAGACCCAGGGA
ACTGAAACATCTAGTACCTGGAGGA AGAGAAGAAAATCGATT
TCCTGAGTAGCGGCGAGCGAAACGGAAAGAGCCCAAACCAAG
AAGCTTGCTTCTTGGGGTTGTAGGACACTCTATACGGAGTTACA
AAAGAAAGTTATAAATGAAGCGGTCTGGAAAGGCCCGCCAAAG
ACGGTAACAGCCCGGTAGTTGAAATAGCTTTCCCTCCAGAGTGG
ATCCTGAGTACGGCGGAACACGTGAAATTCCGTCGGAATCCGGG
AGGACCATCTCCAAGGCTATATACTCCCTAGTGACCGATAGTGA
ACCAGTACCGTGAGGGAAAGGTGAAAAGCACCCCGGAAGGGGA
GTGAAACAGTTCCTGAAACCGTGTGCCTACAAGTAGTTAGAGCC

Fig. 5: Sequence analyses of 460 bp DNA fragment of River Nile water samples using S1F and S1R primers. Sequence analyses of the positive *Listeria* found that 98% homology with *Listeria welshimeri* strain C15 23S ribosomal RNA gene. (Accession no. EF690671.1)

AGTCGGATAGTATCCTTACGCGTGGGAGCAGACCCAGCACTGAC
ATCTAGTACCTGGAGGAGAGAAGAAAATCGATTTCCTGAGTAGC
GGCGAGCGAAACGGAAAGAGCCCAAACCAAGAAGCTTGCTTCTT
GGGGTTGTAGGACACTCTATACGGAGTTACAAAAGAAAGTTATA
AATGAAGCGGTCTGGAAAGGCCCGCCAAAGACGGTAACAGCCCG
GTAGTTGAAATGGCTTTCCCTCCAGAGTGGATCCTGAGTACGGCG
GAACACGTGAAATTCCGTCGGAATCCGGGAGGACCATCTCCAAG
GCTATATACTCCCTAGTGACCGATAGTGAACCAGTACCGTGAGGG
AAAGGTGAAAAGCACCCCGGAAGGGGAGTGAAACAGTTCCTGAA
ACCGTGTGCCTACAAGTAGTTAGAGCC

Fig. 6. Sequence analyses of 460 bp DNA fragment of River Nile water samples using S1F and S1R primers. Sequence analyses of the positive *Listeria* found that 96% homology with *Listeria innocua* strain AB2497 23S ribosomal RNA gene. (Accession no. EF690672.1)

AGTCGGATAGTATCCTTACGCGTGGGAGCAGACCCAGCACTGAC
ATCTAGTACCTGGAGGAGAGAAGAAAATCGATTTCCTGAGTAGC
GGCGAGCGAAACGGAAAGAGCCCAAACCAAGAAGCTTGCTTCTT
GGGGTTGTAGGACACTCTATACGGAGTTACAAAAGAAGGATGTA
GATGAAGCGGTCTGGAAAGGCCCGCCAGAGACGGTAAAAGCCCG
GTAGTCGAAACGTCTTTCCCTCCAGAGTGGATCCTGAGTACGGCG
GAACACGTGAAATTCCGTCGGAATCCGGGAGGACCATCTCCAAG
GCTATATACTCCCTAGTGACCGATAGTGAACCAGTACCGTGAGGG
AAAGGTGAAAAGCACCCCGGAAGGGGAGTGAAACAGTTCCTGAA
ACCGTGTGCCTACAAGTAGTTAGAGCC

Fig. 7: Sequence analyses of 460 bp DNA fragment of River Nile water samples using S1F and S1R primers. Sequence analyses of the positive Listeria found that 96% homology with *Listeria grayi* strain Li07 23S ribosomal RNA gene. (Accession no. EF690667.1)

Sequences Analysis Results: Sequence analysis of PCR positive Listeria group samples showed that the most frequent strains were *Listeria welshimeri* sorovar 6b strains (SLCC5334) with 98% homology (Figure 5), *Listeria innocua* strain AB2497 with 96% homology (Figure 6) and *Listeria grayi* (Figure 7) strain 96% homology Figs. 5-7.

DISCUSSION

Water pollution is considered to be one of the most dangerous hazards affecting Egypt. Pollution in the Nile River System (main stream Nile, drains and canals) has increased in the past few decades because of increases in population, several new irrigated agriculture projects and other activities along the Nile. Cairo with 13 million people is the largest city in the Middle East region. As typical of giant cities, it has continuous rapid population growth and spatial expansion. Since the city is an open environmental system, Cairo's surrounding regions are burdened with heavy wastewater discharges and increasing water demand. Also the city's water resources are affected by discharges from other regions [21]. River Nile is considered as the longest river in the world. Its length is approximately 6740 Km. It follows from south at Ethiobia plateau to Egypt. In the north of Cairo about 25 Km at Barrage River Nile bifuciate into two brunches, namely, Damietta and Rossita. Rossita Branch represents, the area of investigation, its length is about 60 Km. The width of the branch varies from 150- 200 m and the average depth from 2- 2.5 m. The estimated flow of industrial wastewater discharge to the Rossita Branch is about 0.05 milion m^3/day [22]. Abdo [23] pointed that, industries were identified as being the major source of water pollution in this area. The metal industry represents almost 50% of the total wastewater discharges. Besides, about 88 of drains are located on both sides along the River Nile from Aswan to the Mediterranean sea. These drains often also receive manucipal and industrial wastes which are then discharged into River Nile [24].

Thus in this study water samples of River Nile at Greater Cairo were collected to evaluate Listeria group by different techniques for these regions. Monitoring of different pathogens in water could be used as a tool to assess the health status of the community. Thus, Listeria group was determined in this investigation as pathogenic bacteria. The results showed that, the average values of Listeria group by MTF technique ranged between 2.0-9.0x10^2 MPN-index/100ml. While the average values of previous mention group by MF technique ranged between 2.3x10- 4.3x10^2 cfu/100ml from River Nile at Greater Cairo during the study. On the other hand, the average values by MTF technique ranged between 80-3700 MPN-index/100ml and 85-2100 cfu/100ml by MF technique from samples of River Nile at Rossita Branch.

The results of Listeria group showed that, 54 out of 60 samples (90.0%) were positive using PCR technique, 58 out of 60 samples (96.6%) were positive using MF technique. On the other hand, 52 out of 60 samples (86.6%) were positive using MTF technique. In case of water samples which collected from Rossita Branch, 13 out of 14 samples (78.57%) were positive using PCR, MF and MTF techniques. The results indicated that, PCR technique was more sensitive than the conventional techniques in our study although, some samples of *Listeria* negative results using PCR technique was observed. It may return to absence of nested PCR compared with conventional techniques. In addition to that, *Listeria grayi*, *Listeria welshimeri* and *Listeria innocua* strains which were observed in the sequenced samples may be frequent in River Nile water (Figures 5, 6, 7).

Shaban and El-Taweel [15] found that, Listeria group was detected in 83-94% of surface fresh water samples taken from the Nile and streams. Numbers of *Listeria* spp. ranged from a few hundreds to about 10^4/100ml. Ali *et al.* [2] observed that the counts of Listeria group at El-Gizera site were higher during the summer and winter than in other seasons. Biochemically, *Listeria monocytogenes* represent 58% of Listeria group during the period of study. El-Taweel and Shaban [25] observed that Listeria group was detected in 88 out of 96 samples with mean values ranging between 9.8x10^2 and 4.4 x10^4 cfu/100ml. *L. monocytogenes* represents 32- 61% of the *Listeria* spp. at different sites from the Greater Cairo according to the biochemical reaction. Bernagozzi *et al.* [26] found that the main concentration of Listeria group around 2 MPN/100ml in fresh water. While, Colburn *et al.* [10] found that, *L. monocytogenes* appears to be predominant in fresh water when cows, horses and farms of other animals are located in the surrounding area.

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