Determination of pathogenic bacteria in wastewater using conventional and PCR techniques

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ABSTRACT

The objectives of this investigation were to detect and determine some pathogenic bacteria in raw and treated wastewater and to compare and evaluate different techniques. To achieve this aim, pathogenic bacteria (salmonellae group, total vibrios and Listeria group) were determined in 30 samples collected from inlet and outlet of the treatment plants using PCR, MPN and surface plate techniques. The results of salmonellae group showed that all 30 samples (100%) were positive using PCR technique, 28 samples (about 93%) were positive using direct surface plate technique, and 27 samples (90%) were positive using MPN technique. In case of total vibrios 27 samples (90%) were positive using direct surface plate technique while 28 samples (93%) were positive using MPN technique. When using multiplex PCR technique, detection of three species of *Vibrio* (*V. vulnificus, V. parahaemolyticus, V. cholerae* and Classical or El-Tor) were negative. Listeria group was detected in 29 samples (97%) using PCR technique, in 28 samples (93%) using surface plate technique, and in 27 samples (90%) using MPN technique. The removal percentages showed that oxidation pond of El-Sadat City is more efficient in removal of these pathogenic bacteria than the activated sludge system. Statistical analysis (Student t-test) showed that there were significant differences between MPN and surface plate techniques in revealing the three selected pathogenic groups.

INTRODUCTION

Selection of wastewater treatment process depends on the wastewater composition, such as biological oxygen demand, pH, suspended solids, presence of toxic compounds, etc. (Negm et al. 1995). There are numerous conventional wastewater treatment plants in operation in Egypt. The effluent from these plants is mostly discharged to irrigation canals (Abdel-Wahaab 1995).

Wastewater may contain million of bacteria per milliliter including coliform, *Streptococci*, *Staphylococci*, anaerobic sporeforming bacilli, the proteus group, and many other types organisms. Wastewater is also potential source of many human pathogenic forms including bacteria, viruses, and protozoa. In addition, certain bacterial viruses (bacteriophages) are readily isolated from wastewater (Pelczar et al. 1986; Younis et al. 2003). The presence of microbial pathogens in polluted, untreated and treated waters poses a considerable health risk to the general public. Despite large advances in water and wastewater treatment, waterborne diseases still pose a major world-wide threat to public health.

Waterborne pathogens infect around 250 million people each year resulting in 10-20 million deaths (Anon. 1996). Many of these infections occur in developing nations which suffer from lower levels of sanitation, problems associated with low socioeconomic conditions, and less public health awareness than in more developed nations. The risk from microbial pathogens in water necessitates monitoring water and wastewaters for various types of microbial pathogens. For an appropriate risk assessment to be made, the type of microbial pathogen present and its relative numbers need to be determined. This is particularly important regarding wastewater treatment and the reuse of wastewater.

Different microbial pathogens have different infectious doses (i.e., the number of infectious units required to cause an infection). For example, most enteric viruses and protozoa usually require only ten or less infectious particles or cysts to cause infection. Bacteria, however, do not usually cause infection unless more than 10^3 infectious cells are ingested (Lee and Kim 2002; Molleda et al. 2008; USEPA 1992). Thus, determination of the numbers of different microbial pathogens in a water or wastewater sample is imperative. Also, the efficient enumeration of microbial pathogens in a wastewater sample pre- and posttreatment can allow an effective

assessment of the treatment process. The ideal detection method would be rapid, sensitive, highly accurate, easy to perform, able to be run in high numbers and inexpensive.

Detection, isolation and identification of different types of microbial pathogens contaminating water and wastewater would be time consuming and expensive if done in a conventional way. Instead, indicator organisms, commonly fecal coliforms, are used to estimate a risk of the presence of pathogenic microorganisms in a sample. Indicator organisms have a disadvantage, however, as they often show survival different to that of a variety of pathogens (Hellard et al. 1997).

Conventional methods of pathogen detection in wastewater, including the total and fecal coliform assays, have significant limitations (Rousselon et al. 2004). These detection methods utilize indicator microorganisms such as *E. coli* and *Clostridium perfringens* to indirectly estimate the number of pathogens in water and wastewater samples (Ulrich et al. 2005). Therefore, this quantity may not reflect the actual number of pathogens in the environment, nor does it take into consideration the variation in infectious doses between different microorganisms. Additionally, the cultivation and analysis of indicator microorganisms is labour intensive, time consuming and difficult (Lemarchand et al. 2005). Also, the ability of microorganisms to persist in a viable but nonculturable (VBNC) state is common (Alexandrino et al. 2004). Consequently, rapid, accurate and culture-independent alternatives are being investigated to enable monitoring of pathogens in water and wastewater (Straub and Chandler 2003).

Traditionally, detection and enumeration of pathogenic bacteria have been largely based on the use of selective culture and standard biochemical methods. Although these methods are simple, inexpensive and do not require well-trained personnel (De Boer and Beumer 1999; Klein and Juneja 1997), but such methods suffer from a number of drawbacks.

First, pathogenic bacteria which normally occur in low numbers tend to incur large errors in sampling and enumeration (Fleisher 1990). Second, detection of pathogenic bacteria by bacteriological methods is time consuming and usually requires 5–11 days (Ferretti et al. 2001; Humbert et al. 1997). Tedious, laborious, invariably monospecific (i.e. detecting only one type of pathogen), and low throughput. Third, many pathogenic organisms in the environment, although viable, are either difficult to culture or nonculturable (VBNC) (Roszak et al. 1987), but can still cause illnesses (Rahman et al. 1996).

Increased public awareness related to health and economic impact of waterborne contamination and illnesses has resulted in greater efforts to develop more sensitive methods of pathogenic detection and identification. Therefore, efforts have been made by many workers to reduce time required and to increase the sensitivity of the methods to detect pathogenic bacteria (Carli et al. 2001; Notermans et al. 1997).

The introduction of nucleic acid-based methods, such as the polymerase chain reaction (PCR) for pathogen detection research, has resolved some of the problems encountered using conventional methods. PCR has high specificity, speed and sensitivity in pathogen detection, with a detection limit of <10 copies of a specific gene present in a mixed sample (Call et al. 2001). Thus the main objective of this study was detection and determination of some pathogenic bacteria in raw and treated wastewater using different techniques; another objective was to compare and evaluate these different techniques.

MATERIAL AND METHODS

Sampling sites and collection

Fifteen runs were collected, one run per month (eighteen and twelve samples were collected from inlet and outlet of oxidation pond in El-Sadat City (Menoufia Governorate, Egypt) and activated sludge from Zenin wastewater treatment plant (Giza Governorate, Egypt), respectively, during years 2006 and 2007. Samples were collected in a wide mouth, sterile glass bottles one liter volume, and immediately transferred to the laboratory at NRC within one hour and analyzed for detection and enumeration of salmonellae group, total vibrios and Listeria group. The techniques used for detection were surface plate technique, most probable number technique (MPN) and polymerase chain reaction technique (PCR).

Microbiological examination

Salmonellae group was detected and enumerated by MPN technique on Rappaport-Vassiliadis (RV) broth then streaked on bismuth sulfite agar and by direct detection on bismuth sulfite agar according to ISO (2000) and APHA (2005). Identification of salmonellae group was carried out according to APHA (2005). Total Vibrios were detected and enumerated by MPN technique on alkaline peptone broth (pH 8.4) then streaked on thiosulfate citrate bile sucrose (TCBS) agar and by direct detection on TCBS agar according to Koch (1994) and APHA (2005). Identification of *V. cholerae* was carried out according to Robert and Noel (1981) and APHA (2005). Detection of Listeria group was carried out by MPN and by direct detection on *Listeria* selective broth and agar according to Fenlon (1985). Beside biochemical identification of isolates, the isolates were also confirmed by PCR.

Preparation of wastewater samples for PCR and DNA extractions

The samples were prepared as follows: 100-200ml was filtered with nitrocellulose membrane (0.45μm pore size and 47mm in diameter (Whattman Co.)). The membrane filters were transferred to 10ml tryptic soy broth (TSB) (DIFCO Co.) (with 10% glycerol), plates were incubated overnight at room temperature with gentle shaking. DNA extractions were carried out according to Kapperud et al. (1993) and Waage et al. (1999), from each overnight culture 100μl was transferred into Eppendorf tubes and centrifuged at 14900g for 10min in a microcentrifuge (Labfuge, 460). The resulting pellets were resuspended in 50μl PCR buffer with 1μl of Proteinase K ($0.2 mg \cdot ml^{-1}$). After incubation at 37°C for 1h, the bacteria were lysed by boiling for 10min. The samples were stored at 20°C overnight prior to PCR. After thawing at room temperature and centrifugation at 14900g for 5min, 5μl of supernatant was used for PCR reaction.

Identification of isolates by PCR

Loopful of isolates was taken from stored slants to 5ml TSB tubes, incubated at 37°C for 18-24h, then streaked on nutrient agar and incubated at 37°C for 18-24h. Single colony was picked into 50ml TSB incubated at 37°C for 18-24h then centrifuged at 3700g for 15min in cooling centrifuge (HERMLE - LABORTECHNIK), the supernatant discarded and twice resuspended the pellets in PBS (pH 7.2) then centrifuged at 3700g for 15min, the supernatant discarded and the pellets resuspended in 1000μl PBS, finally stored at -20°C until used for DNA extraction.

Selection and synthesis of primers and DNA amplification

Detection of *Salmonella* by nested PCR was carried out according to Waage et al. (1999). Two pairs of PCR primers were selected from published DNA sequences of a randomly cloned fragment of the *Salmonella* Typhimurium chromosome (Aabo et al. 1993). Two outer primers for the first PCR step were SAL-1F and SAL-2R, and the other two inner primers for second PCR step were SAL-3F and SAL-4R. Primers SAL-1F and SAL-2R (Table 1) were derived from the conserved sequences ST15 and ST11, respectively, while Primers SAL-3F and SAL-4R were derived from the conserved sequences ST14 and ST11, respectively. The first PCR step amplifies a fragment with size 438 bp, while the size of the final PCR product is 312 bp. The primers were synthesized by Bio-Basic Inc., Canada. The PCR reaction mixture was 1X buffer (50mM KCl, 10mM Tris-HCl (pH 8.3), 1.5mM $MgCl_2$, 0.001% (w/v) gelatin, 200μM (each) dNTPs (Fermentas), 0.1μM (each) primer and 1U *Taq* polymerase (Fermentas)) per 50μl reaction volume. The first PCR was done in a total volume of 50μl. The second PCR (nested) was carried out in a total volume of 50μl. A 1.0μl aliquot of the first PCR product was used as template. The first PCR was carried out under the following conditions: 95°C for 3min followed by 40 cycles (95°C for 30s, 60°C for 1min and 72°C for 1.5min), and then 72°C for 10min. The second PCR was carried out under the following conditions: 95°C for 3min followed by 20 cycles (95°C for 30s, 67°C for 1min and 72°C for 1.5min), and then 72°C for 10min. DNA from the bacterial strain *Salmonella* Typhimurium ATCC 14028 was used as positive control.

Table 1. Primer sets and target genes for *Salmonella* spp., *Listeria* spp. and three *Vibrio* spp.

Pathogen	Target gene	Primer sequences	Amplicon size (bp)	Reference
V. vulnificus	*vvh*	F 5' TTCCAACTTCAAACCGAACTATGAC 3' R 5' ATTCCAGTCGATGCGAATACGTTG 3'	205	Brasher et al. 1998
	viuB	F 5' GGTTGGGCACTAAAGGCAGATATA 3' R 5' CGGCAGTGGACTAATACGCAGC 3'	504	Panicker et al. 2004
V. parahaemolyticus	*tlh*	F 5' AAAGCGGATTATGCAGAAGCACTG 3' R 5' GCTACTTTCTAGCATTTTCTCTGC 3'	450	Bej et al. 1999
	tdh	F 5' GTAAAGGTCTCTGACTTTTGGAC 3' R 5' TGGAATAGAACCTTCATCTTCACC 3'	269	Bej et al. 1999
	trh	F 5' TTGGCTTCGATATTTTCAGTATCT 3' R 5' CATAACAAACATATGCCCATTTCCG 3'	500	Bej et al. 1999
	ORF8	F 5' AGGACGCAGTTACGCTTGATG 3' R 5' CTAACGCATTGTCCCTTTGTAG 3'	369	Myers et al. 2003
V. cholerae	*ompU*	F 5' ACGCTGACGGAATCAACCAAAG 3' R 5' GCGGAAGTTTGGCTTGAAGTAG 3'	869	Rivera et al. 2001
	toxR	F 5' CCTTCGATCCCCTAAGCAATAC 3' R 5' AGGGTTAGCAACGATGCGTAAG 3'	779	Rivera et al. 2001
	tcpI	F 5' TAGCCTTAGTTCTCAGCAGGCA 3' R 5' GGCAATAGTGTCGAGCTCGTTA 3'	862	Rivera et al. 2001
Classical or El Tor	*hlyA*	F 5' GGCAAACAGCGAAACAAATACC 3' R 5' GGCAAACAGCGAAACAAATACC 3'	738 or 727	Rivera et al. 2001
***Salmonella* spp.**	SAL-1F SAL-2R	F 5' GTA GAA ATT CCC AGC GGG TAC TG 3' R 5' GTA TCC ATC TAG CCA ACC ATT GC 3'	438	Waage et al. 1999
	SAL-3F SAL-4R	F 5' TTT GCG ACT ATC AGG TTA CCG TGG 3' R 5' AGC CAA CCA TTG CTA AAT TGG CGC A 3'	312	
***Listeria* spp.**	S1F S1R	F 5' AGT CGG ATA GTA TCC TTA C 3' R 5' GGC TCT AAC TAC TTG TAG GC 3'	460	Paillard et al. 2003
	S2F S2R	F 5' GGC TCT AAC TAC TTG TAG GC 3' R 5' ACT GGT ACA GGA ATC TCT AC 3'	890	

F = Forward, R = Reverse.

Detection of *Vibrio* species were carried out according to Panicker et al. (2004) by multiplex PCR for detection *V. vulnificus, V. parahaemolyticus, V. cholerae* and Classical or El-Tor. The oligonucleotide primers selected for PCR amplification of the three *Vibrio* spp. were specific for their respective targeted gene segments (Table 1), and selected according to Bej et al. (1999); Myers et al. (2003); Panicker et al. (2004); Rivera et al. (2001, 2003). Each multiplex-PCR amplification was performed in a 45μl reaction volume consisting of 10ng of purified genomic DNA, 200μM dNTPs (Fermentas), 3U *Taq* polymerase (Fermentas) and 1X buffer (60mM Tris-HCl (pH 9.0), 15mM $(NH_4)_2SO_4$, 2mM $MgCl_2$) or 1X buffer (50mM Tris-Cl (pH 8.9), 50mM KCl, 2.5mM $MgCl_2$). Amplification of the targeted genes for *V. cholerae* and *V. parahaemolyticus* was carried out with 1μM each oligonucleotide primer (Table 1). However, for the detection of *V. vulnificus*, 1μM each of oligonucleotide primers F-*vvh* and R-*vvh* and 2μM each of oligonucleotide primers F-*viu*B and R-*viu*B were used in order to achieve optimum multiplex PCR amplification of *vvh* and *viu*B, respectively (Table 1). All PCR amplifications were achieved on the thermocycler (BIOER; model TC- 25/H & BIORAD; model PTC 196) under the following conditions: 94°C for 3min; 30 cycles (94°C for 1min, 55°C for 1min) for *V. parahaemolyticus*; 65°C for *V. vulnificus*; or 60°C for *V. cholerae*, and 72°C for 1min, then 72°C for 5min. DNA from the bacterial strain *Vibrio cholerae* (Reference strain from VACSERA Co., Egypt) was used as positive control.

Listeria spp. were identified by amplification of 23S rRNA gene using two sets of primers. The PCR reaction mixture was 50mM KCl, 10mM Tris-HCl (pH 8.3), 1.5mM $MgCl_2$, 0.2mM (each) dNTPs (Fermentas), 1.5U of *Taq* polymerase (Fermentas), 5μl of sample, and 0.5μM (each) designed primer for every reaction. PCR experiments were performed under the following conditions: 94°C for 5min, followed by 35cycles (94°C for 1min, 50°C for 1min and 72°C for 1min), then 72°C for 7min. DNA from the bacterial strain *Listeria monocytogenes* ATCC 25152 was used as positive control. Amplified products were analyzed by agarose gel electrophoresis. Gels were stained with ethidium bromide (0.005%, w/v) (Sambrook et al. 1989) with BenchTop ΦX174 DNA/HaeIII Marker (Promega Co.).

Statistical analysis

In order to study the relationship between MPN and surface plate techniques for detection of pathogenic bacteria, linear correlation analyses were used. The quantitative analysis for two techniques and pathogenic bacteria were carried out with 30 samples as a total for each wastewater treatment plant. All the data were transformed in decimal logarithms and processed by SPSS 10.0 (Student t-test).

RESULTS

Pathogenic bacteria were determined from the inlet and outlet of two wastewater treatment plants during this study using three techniques (MPN, surface plate and PCR) and the results are in Table 2 and Table 3. It was found that salmonellae group, total vibrios and listeria group were detected in all samples of the inlet of treatment systems.

Table 2. Comparison between the conventional technique (MPN (MPN-index·100ml^{-1}) and surface plate technique (cfu·100ml^{-1})) and PCR technique and removal percentages in samples collected from inlet and outlet of oxidation ponds of El-Sadat City wastewater treatment plant.

Run	Salmonellae								*Vibrio* spp.								Listeria group							
No.	PCR		MPN			Surface			PCR		MPN			Surface			PCR		MPN			Surface		
	In	Out	In	Out	%	In	Out	%	In	Out	In	Out	%	In	Out	%	In	Out	In	Out	%	In	Out	%
1	+	+	$9.0{\cdot}10^4$	$3.5{\cdot}10^3$	96.1	$8.0{\cdot}10^4$	$1.2{\cdot}10^2$	99.8	-	-	$1.6{\cdot}10^4$	$1.7{\cdot}10^3$	89.3	$2.3{\cdot}10^4$	$5.8{\cdot}10^3$	74.7	+	+	$2.5{\cdot}10^3$	32	98.7	$3.6{\cdot}10^3$	72	98.0
2	+	+	$1.6{\cdot}10^4$	$3.0{\cdot}10^2$	98.1	$4.5{\cdot}10^3$	$1.6{\cdot}10^2$	96.4	-	-	$9.0{\cdot}10^4$	$1.3{\cdot}10^3$	85.3	$2.8{\cdot}10^4$	$1.2{\cdot}10^2$	99.5	+	+	$6.4{\cdot}10^4$	$2.1{\cdot}10^2$	99.6	$2.5{\cdot}10^4$	32	99.8
3	+	+	$3.0{\cdot}10^4$	$1.1{\cdot}10^2$	99.6	19	ND	100	-	-	$9.0{\cdot}10^4$	$5.0{\cdot}10^3$	94.4	$6.2{\cdot}10^3$	$5.5{\cdot}10^2$	91.1	+	+	$1.6{\cdot}10^3$	$1.7{\cdot}10^2$	89.3	$6.3{\cdot}10^3$	$9.0{\cdot}10^2$	85.7
4	+	+	$2.8{\cdot}10^3$	ND	100	2.0·10	ND	100	-	-	$3.0{\cdot}10^4$	$5.0{\cdot}10^3$	83.3	$1.9{\cdot}10^4$	$1.6{\cdot}10^3$	91.5	+	+	$1.6{\cdot}10^3$	20	98.7	$6.0{\cdot}10^3$	21	99.6
5	+	+	$2.4{\cdot}10^3$	80	99.6	$5.0{\cdot}10^2$	20	96	-	-	$2.4{\cdot}10^5$	$3.0{\cdot}10^3$	98.7	$8.8{\cdot}10^5$	$4.2{\cdot}10^3$	99.5	+	+	$9.0{\cdot}10^3$	ND	100	$6.6{\cdot}10^3$	10	99.8
6	+	+	$1.4{\cdot}10^3$	$2.3{\cdot}10^2$	83.5	$4.5{\cdot}10^3$	$2.0{\cdot}10^2$	95.5	-	-	$2.2{\cdot}10^5$	$3.3{\cdot}10^3$	98.5	$7.2{\cdot}10^5$	$2.6{\cdot}10^3$	99.6	+	+	$1.7{\cdot}10^3$	ND	100	$3.2{\cdot}10^3$	26	99.1
7	+	+	$3.0{\cdot}10^3$	ND	100	$5.4{\cdot}10^5$	50	99.9	-	-	$3.1{\cdot}10^4$	$1.1{\cdot}10^2$	99.6	$2.4{\cdot}10^5$	$1.0{\cdot}10^2$	99.9	+	+	$1.3{\cdot}10^2$	70	46.1	$5.6{\cdot}10^3$	ND	100
8	+	+	$1.6{\cdot}10^3$	20	98.7	$1.8{\cdot}10^4$	$3.0{\cdot}10^2$	98.3	-	-	$1.6{\cdot}10^4$	$1.1{\cdot}10^3$	93.1	$1.5{\cdot}10^5$	$1.6{\cdot}10^3$	98.9	+	+	$1.6{\cdot}10^3$	ND	100	$4.3{\cdot}10^3$	40	99.0
9	+	+	$9.0{\cdot}10^4$	$3.0{\cdot}10^2$	99.6	$2.2{\cdot}10^5$	$9.2{\cdot}10^2$	99.5	-	-	$3.0{\cdot}10^5$	$3.0{\cdot}10^2$	99.9	$2.3{\cdot}10^5$	$1.1{\cdot}10^3$	99.5	+	-	$5.0{\cdot}10^3$	20	99.6	$6.5{\cdot}10^3$	ND	100
Min.			$1.4{\cdot}10^3$	20	98.5	19	20	89.4			$1.6{\cdot}10^4$	$1.1{\cdot}10^2$	99.3	$1.9{\cdot}10^4$	$1.0{\cdot}10^2$	99.4			$1.3{\cdot}10^2$	20	84.6	$3.2{\cdot}10^3$	10	99.6
Max.			$9.0{\cdot}10^4$	$3.5{\cdot}10^3$	96.1	$5.4{\cdot}10^5$	$9.2{\cdot}10^2$	99.8			$3.0{\cdot}10^5$	$5.0{\cdot}10^3$	98.3	$8.8{\cdot}10^5$	$5.8{\cdot}10^3$	99.3			$6.4{\cdot}10^4$	$2.1{\cdot}10^2$	99.6	$2.5{\cdot}10^4$	$9.0{\cdot}10^2$	96.4
Ave.			$2.8{\cdot}10^4$	$5.0{\cdot}10^2$	98.2	$9.6{\cdot}10^4$	$1.9{\cdot}10^2$	99.8			$1.1{\cdot}10^5$	$2.3{\cdot}10^3$	97.9	$2.6{\cdot}10^5$	$1.9{\cdot}10^3$	99.2			$9.6{\cdot}10^3$	5.0·10	99.4	$7.4{\cdot}10^3$	$1.2{\cdot}10^2$	98.3

In = Inlet, Out = Outlet, % = Removal Percentage, ND = Not Detected.

The average MPN values of pathogenic bacteria in the inlet of El-Sadat wastewater treatment plant were $2.8 \cdot 10^4$, $1.1 \cdot 10^5$ and $9.6 \cdot 10^3$ MPN-index·100ml^{-1} for salmonellae group, total vibrios and Listeria group, respectively. While the average values of surface plate technique of the same groups were $9.6 \cdot 10^4$, $2.6 \cdot 10^5$ and $7.4 \cdot 10^3$ cfu·100ml^{-1}, respectively (Table 2).

On the other hand, the selected pathogenic groups were determined in the outlet of El-Sadat oxidation ponds and their counts are in Table 2. Total vibrios were detected in all samples using MPN and surface plate techniques and their counts fluctuated from $1.1 \cdot 10^2$ to $5.0 \cdot 10^3$ MPN-index·100ml^{-1} and $1.0 \cdot 10^2$ to $5.8 \cdot 10^3$ cfu·100ml^{-1}, respectively. With regard to salmonellae group and Listeria group the results showed that both were detected in 7 and 6 out of 9 samples (about 78% and 67%) using MPN technique and in 7 out of 9 samples (78%) using surface plate technique, respectively, and the counts of their positive samples fluctuated from 20 to$3.5 \cdot 10^3$ and from 20 to $2.1 \cdot 10^2$ MPN-index·100ml^{-1} using MPN technique, respectively for the two groups, while their counts using surface plate technique fluctuated from 20 to $9.2 \cdot 10^2$ and from 10 to $9.0 \cdot 10^2$ cfu·100ml^{-1}, respectively.

The removal percentage from oxidation pond of El-Sadat City for salmonellae group fluctuated between 83.5-100% and 89.4-100% using MPN technique and surface plate technique, respectively, while *Vibrio* spp. removal percentage ranged between 85.5-99.9% and 74.7-99.9% using MPN technique and surface plate technique, respectively. For Listeria group the efficacy percentage fluctuated from 46.1-99.6% and 85.7-100% using MPN technique and surface plate technique, respectively (Table 2).

In case of Zenin wastewater treatment system the counts of selected pathogenic bacteria by MPN technique fluctuated from 40 to $1.1 \cdot 10^5$, $1.4 \cdot 10^3$ to $2.4 \cdot 10^4$, and 80 to $2.4 \cdot 10^3$ MPN-index·100ml^{-1}, for salmonellae group, total vibrios and Listeria group, respectively. While their counts using surface plate technique fluctuated from $1.4 \cdot 10^2$ to $4.3 \cdot 10^5$, $3.0 \cdot 10^3$ to $5.0 \cdot 10^5$ and $1.5 \cdot 10^2$ to $7.5 \cdot 10^3$ cfu·100ml^{-1}, respectively (Table 3).

Concerning the pathogenic bacteria (Table 3) determined in the outlet of Zenin treatment system our data showed that Listeria group was detected in all samples (100%) using two techniques. Salmonellae group was detected in 5 out of 6 samples (about 83%) using MPN technique, while total vibrios were detected in 4 and 3 out of 6 samples (67 and 50%) using MPN and surface techniques, respectively, and their counts of positive samples ranged from 15 to 28 MPN-index·100ml^{-1} and 5.0 to 81 cfu·100ml^{-1}, respectively.

The density of salmonellae group, total vibrios and Listeria group using MPN technique from inlet of two wastewater treatment plants fluctuated between 40.0 to $1.1 \cdot 10^5$, $1.4 \cdot 10^3$ to $3.0 \cdot 10^5$ and 80 to $6.4 \cdot 10^4$, while the density in the outlet ranged from 20 to $3.5 \cdot 10^3$, 15 to $5.0 \cdot 10^3$, and 20 to $2.4 \cdot 10^2$ MPNindex·100ml^{-1}, respectively. In addition slight variation was found in the density of the same group using surface plate technique (Table 2 and Table 3).

The removal percentage from activated sludge of Zenin wastewater treatment plant for salmonellae group fluctuated between 80-100% and 12.8-99.9% using MPN technique and surface plate technique, respectively, while *Vibrio* spp. removal percentage ranged between 98.5-100% and 99.4-100% using MPN technique and surface plate technique, respectively. For Listeria group it is clear that the efficacy percentage fluctuated from 12.5-93.7% and 20.0-98.6% using MPN technique and surface plate technique, respectively (Table 3).

Finally, the results of removal percentage showed that oxidation pond of El-Sadat City was highly more efficient in removing pathogenic bacteria than was activated sludge system.

Table 3. Comparison between the conventional technique (MPN (MPN-index·100ml^{-1}) and surface plate technique (cfu·100ml^{-1})) and PCR technique and removal percentages in samples collected from inlet and outlet of Zenin wastewater treatment plant.

Run	Salmonellae								*Vibrio* spp.								Listeria grou							
No.	PCR		MPN			Surface			PCR		MPN			Surface			PCR		MPN			Surface		
	In	Out	In	Out	%	In	Out	%	In	Out	In	Out	%	In	Out	%	In	Out	In	Out	%	In	Out	%
1	+	+	40	ND	100	$1.4 \cdot 10^2$	20	85.7	-	-	$1.4 \cdot 10^3$	20	98.5	$3.0 \cdot 10^3$	ND	100	+	+	80	70	12.5	$1.5 \cdot 10^2$	$1.2 \cdot 10^2$	20.0
2	+	+	$1.1 \cdot 10^4$	$1.5 \cdot 10^2$	98.6	$7.0 \cdot 10^2$	$6.1 \cdot 10^2$	12.8	-	-	$2.0 \cdot 10^3$	23	98.8	$4.9 \cdot 10^3$	ND	100	+	+	$1.1 \cdot 10^3$	$2.4 \cdot 10^2$	78.1	$4.3 \cdot 10^3$	$3.0 \cdot 10^2$	93.0
3	+	+	$1.1 \cdot 10^5$	$1.5 \cdot 10^2$	99.8	$4.3 \cdot 10^5$	3.9·10	99.9	-	-	$1.1 \cdot 10^4$	28	99.7	$5.4 \cdot 10^3$	30	99.4	+	+	$1.1 \cdot 10^3$	$2.0 \cdot 10^2$	81.8	$4.3 \cdot 10^3$	$1.0 \cdot 10^2$	97.6
4	+	+	$1.1 \cdot 10^5$	$2.0 \cdot 10^2$	99.8	$3.6 \cdot 10^5$	$3.8 \cdot 10^2$	99.8	-	-	$2.3 \cdot 10^3$	15	99.3	$3.6 \cdot 10^3$	5.0	99.8	+	+	$1.1 \cdot 10^3$	$2.1 \cdot 10^2$	80.9	$6.6 \cdot 10^3$	$1.4 \cdot 10^2$	97.8
5	+	+	$1.1 \cdot 10^4$	$1.1 \cdot 10^2$	99.0	$1.1 \cdot 10^5$	$9.0 \cdot 10^2$	99.1	-	-	$2.4 \cdot 10^4$	ND	100	$5.0 \cdot 10^4$	81	99.8	+	+	$2.4 \cdot 10^3$	$1.5 \cdot 10^2$	93.7	$7.5 \cdot 10^3$	$2.5 \cdot 10^2$	96.6
6	+	+	$2.0 \cdot 10^2$	40	80.0	$2.1 \cdot 10^4$	32	99.2	-	-	$1.5 \cdot 10^4$	ND	100	$2.0 \cdot 10^4$	ND	100	+	+	$2.0 \cdot 10^2$	90	55.0	$1.2 \cdot 10^3$	16	98.6
Min.			40	40	0.0	$1.4 \cdot 10^2$	20	85.7			$1.4 \cdot 10^3$	15	98.9	$3.0 \cdot 10^3$	5.0	99.8			80	70	12.5	$1.5 \cdot 10^2$	16	89.3
Max.			$1.1 \cdot 10^5$	$2.0 \cdot 10^2$	99.8	$4.3 \cdot 10^5$	$9.0 \cdot 10^2$	99.7			$2.4 \cdot 10^4$	28	99.8	$5.0 \cdot 10^4$	81	99.7			$2.4 \cdot 10^3$	$2.4 \cdot 10^2$	90.0	$7.5 \cdot 10^3$	$3.0 \cdot 10^2$	96.0
Ave.			$4.0 \cdot 10^4$	$1.0 \cdot 10^2$	99.7	$1.5 \cdot 10^5$	$3.3 \cdot 10^2$	99.7			$9.2 \cdot 10^3$	14	99.8	$1.4 \cdot 10^4$	19	99.8			$9.9 \cdot 10^2$	$1.6 \cdot 10^2$	83.8	$4.0 \cdot 10^3$	$1.5 \cdot 10^2$	96.2

In = Inlet, Out = Outlet, % = Removal Percentage, ND = Not Detected.

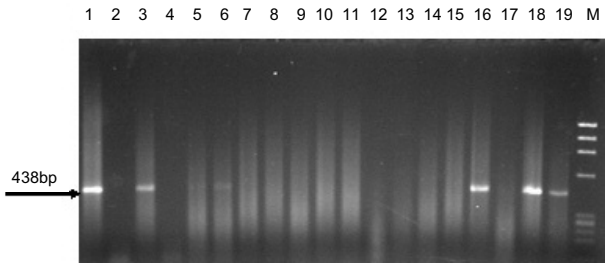

Figure 1. First PCR for detection of salmonellae group; lane 1 - positive control, lanes 2–19 - inlet and outlet of wastewater samples, lane M - marker (ØX 174).

The results of PCR techniques applied in case of two systems of wastewater treatment were that salmonellae group (Figure 1 and Figure 2) and Listeria group were positive in 30 and 29 out of 30 samples (100 and about 97%, respectively). The three species of vibrios, however, were negative in all wastewater samples (Table 2 and Table 3).

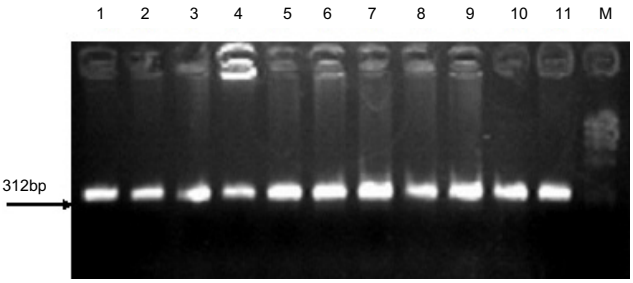

Figure 2. Nested PCR for detection of salmonellae group; lane 1-11 - inlet and outlet of wastewater samples, lane M - marker (ØX 174).

Statistical analysis

Results of statistical analysis (Table 4) showed that raw wastewater samples using both MPN and surface plate techniques counts showed no significant correlation with total vibrios and Listeria group whereas salmonellae group showed highly significant correlations with two techniques in case of raw wastewater. In treated wastewater samples there were no significant correlations between MPN and surface plate techniques with three selected pathogenic groups.

DISCUSSION

El-Taweel (1994) found that salmonellae group and *V. cholerae* were detected in the majority of raw wastewater samples at oxidation pond of Mit-Mzah treatment plant (Dakahlia governorate) in numbers ranging from 10^2 to 10^5 and from 10^4 to 10^5 cfu·100ml^{-1} using surface plate technique. In the outlet of the treatment plant, salmonellae group was recorded four times out from 12 samples (about 33%), while *V. cholerae* was absent during four months only (67%). In addition, their counts in positive samples ranged from 4.0 to $1.0 \cdot 10^2$ and from $8.0 \cdot 10^2$ to $9.0 \cdot 10^3$ cfu·100ml^{-1} for salmonellae group and *V. cholerae*, respectively. El-Hawarry et al. (1997) examined 106 samples collected from 13 different station intakes covering 8 governorates for microbiological characterization of raw wastewater in Egypt. They have found that the density of salmonellae group, total vibrios and Listeria group were in ranges between 10^2 to 10^5, 10^4 to 10^7 and 10^5 to 10^7 cfu·100ml^{-1}, respectively. El-Taweel et al. (2000) followed up this work and found that the density of salmonellae group, total vibrios and Listeria group from the outlet of the same 13 stations fluctuated from 10 to 10^4, from 10^2 to 10^5 and from 10^3 to 10^6 cfu·100ml^{-1}, respectively, using surface plate technique. Additionally salmonellae group was isolated from 8 out of the 13 treatment plants, total vibrios were not detected in most of the tested samples and Listeria group was not detected in four treatment plants.

Shaban and El-Taweel (1999) examined the presences of Listeria group from different aquatic environments using MPN and surface plate counts with the selective media. They have found that counts for Listeria group as well as those for *L. monocytogenes* in wastewater varied from few cells to about 10^5 cfu·100ml^{-1}, the surface plate technique gave an order of magnitude higher count than the selective enrichment MPN technique. Variations in counts by both methods were rather small in case of chlorinated sewage effluent. Surface plate technique gave higher counts, sometimes reaching 1-2 log cycles over the corresponding MPN value. Thus, surface plate technique appears to be the most practical method for the enumeration of Listeria group in wastewater samples. In addition Shaban and El-Taweel (2002) in a study on the plant working by activated sludge system have found that the density of salmonellae group, total vibrios and Listeria group in raw wastewater ranged from $5.0 \cdot 10^4$-$2.6 \cdot 10^7$, $8.8 \cdot 10^4$ to $2.0 \cdot 10^7$ and $2.6 \cdot 10^5$ to $2.6 \cdot 10^7$ cfu·100ml^{-1} using surface plate techniques. While in the

Table 4. Student's t-test (significance, *P*-value) between MPN and surface plate technique in inlet and outlet of two wastewater treatment plants.

Sample type	n	Salmonellae group	Total vibrios	Listeria group
Inlet	15	0.001*	0.124***	0.202***
Outlet	15	0.402***	0.696***	0.202***

* P≤0.001), *** P≥0.05.

treated effluents salmonellae group was isolated 8 times out from 12 samples and their counts in positive samples ranged from $2.0 \cdot 10^3$ to $1.0 \cdot 10^4$ cfu·100ml^{-1}. Total vibrios and Listeria group were detected in all tested samples, and the counts ranged from $2.0 \cdot 10^3$ to $4.4 \cdot 10^5$ and from $2.0 \cdot 10^5$ to $2.5 \cdot 10^6$ cfu·100ml^{-1}, respectively.

The variation in sensitivity between the first and nested PCR was clear in our results. We observed higher sensitivity of nested PCR in comparison to the PCR of *Salmonella*. In one field study some samples assigned as negative by conventional techniques were positive using nested PCR technique, especially in detection of *Salmonella* spp. In some samples of *Listeria* spp. and the three species of *Vibrio*, negative results using PCR technique was observed. The reason could be the lower sensitivity of multiplex PCR in detection of *Vibrio* in comparison with nested PCR, or it may due to the detection of only three species of *Vibrio* (*V. vulnificus, V. parahaemolyticus, V. cholerae* and Classical or El-Tor) by multiplex PCR when compared with detection of total vibrios by conventional technique. Absence of nested PCR in the detection of *Listeria* may be the reason of having some negative samples compared with conventional techniques. In this study, according to statistical analysis, there were no differences between using MPN and surface plate techniques for determination of pathogenic bacteria from wastewater; also nested PCR was more sensitive than first PCR and conventional techniques for detection of *Salmonella*.

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