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ORIGINAL ARTICLE

Pseudomonas aeruginosa isolated from otitis externa associated with recreational waters in some public swimming pools in Tehran

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ABSTRACT

Background: *Pseudomonas aeruginosa* is the most significant bacteria capable of multiplying in water especially in recreational waters. This bacterium is also the most commonly bacterial pathogen in ear infections. Although the bacterium is infrequently found in the normal ear, but the users of swimming pools may be at risk of acquiring disease.
Patients and methods: The bacteriological quality and health risk of the water of eleven public outdoor and indoor swimming pools in East and North-East of Tehran was assayed. Useful information was recorded at the time of sampling. Samples tested for aerobic colony count, coliforms, *Escherichia coli* and *P. aeruginosa* according to the standard method. Ear swabs were collected from 179 users with a history of ear problems during the previous two weeks. An adequate control group was chosen randomly from those who never used the investigated pools.
Results: *P. aeruginosa* was isolated from 9 (81.8%) of the pools. *P. aeruginosa* was the only bacterium grew in 7 (63.6%) swimming pools, while in the other 2 (18.2%) swimming pools high rates of total bacterial count, total coliforms and fecal coliform counts were also found besides *P. aeruginosa*. Meanwhile, *P. aeruginosa* was isolated from the ear swabs of 142 (79.3%) swimming pool users, as compared to 4% of the controls.
Conclusion: Results revealed that otitis externa was strongly associated with swimming in pools, due to *P. aeruginosa*. Furthermore, contamination of the swimming pools with *P. aeruginosa* and often the chlorination process could not remove the pollution, especially when high numbers of people led to overuse of the pools, hence, more strict bathing water standards should be met in the public swimming pools in the city.

Keywords: *Pseudomonas aeruginosa, Otitis externa, Recreational waters.*
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INTRODUCTION

Microbiological analysis of recreational water is aimed largely at detecting markers of the coliforms group and generally fecal coliforms pollution especially as colony plate counts (1,2). As routine indicators, coliforms are employed to assess the microbiological quality of swimming pools water (3,4). The coliforms group consists of several genera of bacteria belonging to the family of *Enterobacteriaceae* (5,6). Definition of this group is based on the method used for detection (lactose fermentation). When fermentation technique is used, this group is defined as all facultative anaerobic, gram-negative, non-spore forming, rod-shaped bacteria that ferment lactose with gas and acid formation within 48 h at 35^{o}C (7). In this method, fecal coliforms are those thermotolerant coliforms bacteria that ferment lactose and produce gas within 24±2 hours in EC

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or A-1 broth after incubation at 44.5±0.2°C (7,8). *E. coli* and fecal coliforms are subsets of the total coliform group and flourish in the intestinal tracts of mammals, including humans (5,6). These bacteria are not necessarily harmful but indicate the possible presence of parasites and pathogens that could be harmful to livestock and humans (9,10). Most species classified as total coliforms are wide-spread in the environment. Characteristics and behaviour of these microbial indicators in the environment is relatively well known (11). Most fecal coliforms are present in large numbers among the intestinal flora of human and other warm-blooded animals, and are thus commonly found in fecal material. As a result, the presence of these non-pathogenic microorganisms in water indicates the potential presence of pathogenic microorganisms of fecal origin and consequently a health hazard (12,13). It is estimated that most of communicable diseases in the world are waterborne (13,14). Analyzing for total coliform bacteria and *E. coli* is the most common method used to test the hygienic quality of swimming pools water (15,16).

Although general routine markers of fecal contamination of swimming pools water is based on detecting of total coliforms and fecal coliform (17,18), some other primary studies reveal that coliforms may not be suitable indicators of some other opportunistic pathogens such as *Pseudomonas aeruginosa* which may consistently associated with recreational waters (19-23). *P. aeruginosa* has been described as an environmental bacterium which has a hardy resistance to mechanical cleansing and flushing as well as to disinfectants and antibiotics (24-26). *P. aeruginosa* can inhabit the nasopharynx and lower digestive tract, but is only occasionally associated with disease, primarily as an opportunistic pathogen in immunocompromised hosts (27). In humans, *P. aeruginosa* is the second most frequent gram-negative nosocomial pathogen in hospitals and has the highest case-fatality rate of all hospital-acquired bacteremias (28-31). *P. aeruginosa* is the predominant bacterial pathogen in some cases of external otitis including "swimmer's ear". The bacterium is infrequently found in the normal ear (32). In swimming pools, the primary health effect is otitis externa or swimmer's ear. Otitis externa is characterized by injury, maceration, inflammation, swelling, redness, itching, fluid leakage and pain in the external auditory canal (33). Risk factors that increase the occurrence of otitis externa in ambient waters include amount of time spent in the water prior to the infection reported before (33). During the years 1991-1992, *Pseudomonas* spp. were the most commonly identified etiologic agents causing dermatitis, conjunctivitis, or otitis in humans following recreational water exposures (34).

Studies on *P. aeruginosa*, reveal that this bacterium is becoming increasingly resistant to many disinfectants and antibiotics (35,36). Over the past few years, a notable increase in antibiotic resistance *P. aeruginosa* has been reported, especially for critically ill patients (37). Infections caused by multidrug resistant *P. aeruginosa* have been associated with increased morbidity, mortality and costs (38,39). Infection caused by *P. aeruginosa* after swimming in surface and recreational waters, is particularly challenging because of its resistance to most antimicrobial agents (40,41). Since this bacterium is widely distributed in the environment and may also be present in or on bather's bodies, thus swimming pools water may be contaminated with this very virulent opportunistic pathogen. Also as *P. aeruginosa* is the most commonly isolated bacteria from ear infections associated with recreational waters, in this study, we attempted to investigate more about the situation in some public swimming pools in east and north-east districts of Tehran.

PATIENTS and METHODS

Over a period of about one year (2008-2009), eleven public swimming pools in east and north-east of Tehran in addition to the bathers of the

pools were monitored for bacteriological examinations. For this purpose, the study was performed in two general phases. The first phase was the bacteriological examinations of the pools water samples and the second phase was the cultivation of ear swabs samples prepared from bathers of the swimming pools.

Sampling procedures was performed as directed in APHA section 9060A (42), 480-ml sterile bottles were used to collect samples. Before sampling, 0.4ml of a 10% solution of sodium thiosulfate ($Na_2S_2O_3$) was added to the bottles for dechlorination and then the bottles with the caps were sterilized in an autoclave at 121°C for 15 min. (42). Samples were collected by carefully removing cap of the sterile bottles and holding bottles near the base at an angle of about 45 degree (42). The bottles were filled in one slow sweep down through the water, with the mouth of the bottles always ahead of the hand so that any contamination of the samples was avoided. Cap of the bottles were replaced and the collected samples were then transferred to laboratory for further examinations (42). Additional samples of the surface microlayer from the area in 1-m-deep water were collected from each pool (42). Another microlayer sample were collected by plunging a sterile glass plate (approximately 20 cm by 20 cm) vertically though the water surface and withdrawing it upward at a rate of approximately 6cm/s (42). The surface film and water layer adhering to both sides of the plate was then removed with a sterile silicone rubber scraper and collected in another sterile glass bottle. This procedure was repeated to obtain desired volume of the water samples (42). To minimize microbial contamination, sterile rubber or plastic gloves was worn during all sampling procedures (42). Thus, according to the mentioned sampling procedures, three samples were collected from each of the selected pools during periods of maximum bather load in different days and work shifts. Useful information such as date and time of sampling, bather load in the pools, total number of users per day, total water capacity of the pools, average flow rate of filtration of water in the pools and other important tests like water clarity, temperature, pH, and alkalinity of the water, were recorded at the time of sampling. Microbiological analysis of the samples was achieved as soon as possible after collection. Free and combined residual chlorine levels were determined by DPD colorimetric method using a spectrophotometer set at wavelength of 515nm and providing a light path of 1cm as described in APHA section 4500-Cl G (42).

Aerobic colony count: For aerobic colony count (ACC) and measuring the number of live heterotrophic bacteria or heterotrophic plate count (HPC) in the swimming pools water samples, pour plate method was used as described in APHA section 9215 B (42). Peptone water was used for dilution of the samples. Peptone water was prepared as directed in APHA section 9015 C (42). Final diluted sample volumes or actual volume of the samples in culture dishes was 1, 0.1, 0.01 and 0.001. After cultivation of the diluted samples, plates between 30 and 300 colonies were selected for counting and measuring. If needed, a colony magnifying device was used for counting. The medium used in this procedure, was tryptone glucose yeast agar and the incubation temperature and time were 35°C and 48 hours, respectively (42). The term colony-forming unit (CFU) was descriptive of the method used; therefore all counts were reported as colony-forming units per milliliter (CFU/mL) and computed by the following equation (42): CFU/ml=colonies counted/actual volume of sample in dish (ml).

Total coliforms: For measuring the density of total coliforms in the samples, the standard total coliform multiple-tube fermentation technique was carried out using method 9221 A of APHA (42). Results of the examination were reported in the terms of the Most Probable Number (MPN) of organisms present in the samples (42). The media used in this procedure, were lauryl tryptose broth in

the presumptive phase, brilliant green lactose bile broth in the confirmed phase, MacConkey agar, single-strength lauryl tryptose broth, and nutrient agar in the completed phase as described in APHA 9221 B (42). In the presumptive phase, five tubes test were used. Each tube in a set of five was inoculated with diluted sample volumes of 10, 1.0, and 0.1 ml. Inoculated tubes were incubated at 35±0.5°C. After 24±2 h, each tube swirled gently and examined for growth, gas, and acidic reaction (shades of yellow color) and, if no gas or acidic reaction was evident, the tubes were reincubated and reexamined at the end of 48±3 h. Results were recorded as presence or absence of growth, gas, and acid production. The absence of acidic reaction or gas formation at the end of 48±3 h of incubation constituted a negative test and all positive presumptive tubes were submitted to confirmed phase (42). In the confirmed test, inoculated brilliant green lactose bile broth tubes were incubated at 35±0.5°C. Formation of gas in any amount in the inverted vial of the brilliant green lactose bile broth within 48±3 h constituted a positive confirmed phase. The MPN value was calculated from the number of positive brilliant green lactose bile broth tubes as described in section 9221 C. To provide quality control data, at least 10% of positive confirmed tubes were submitted to completed test (42). In the completed phase, cultured MacConkey agar inverted plates were incubated at 35±0.5°C for 24±2 h. Typical colonies were transferred to single-strength lauryl tryptose broth fermentation tube and onto a nutrient agar slant. The secondary broth tubes were incubated at 35±0.5°C for 24±2 h. If gas was not produced within 24±2 h, the tubes were reincubated and reexamined again at the end of 48±3 h. Colonies from the cultured nutrient agar slants after 24 h incubation at 35±0.5°C, were microscopically examined by gram-staining preparations. Formation of gas in the secondary tube of lauryl tryptose broth within 48±3 h and demonstration of gram-negative, nonspore-forming, rod-shaped bacteria from the nutrient agar slant culture under microscopically observation, were constituted a positive result for completed test (42).

Fecal coliforms: To establish the presence of fecal coliforms in the samples, simultaneous with inoculation into brilliant green lactose bile broth for total coliforms (in the confirmed test) inoculation into EC broth for fecal coliforms and inoculation into EC-MUG broth for *E.coli* was carried out as described in APHA sections 9221 E and 9221 F (42). Positive EC and EC-MUG broth at elevated temperature (44.5°C), considered the presence of fecal coliforms and *E. coli* in the samples, respectively. Parallel positive brilliant green lactose bile broth culture with negative EC or EC-MUG broth culture was indicated the presence of non-fecal coliforms in the samples (42). Therefore, to indicate the presence of fecal coliform in the samples, all positive presumptive fermentation tubes were inoculated into EC broth and incubated in a water bath at 44.5±2°C for 24±2 h. All EC tubes were placed in water bath within 30 min after inoculation. Gas production with growth in the EC broth culture within 24±2 h was considered a positive fecal coliform reaction. Failure to produce gas, constituted a negative reaction. The MPN value was calculated from the number of positive EC broth tubes as described in section 9221 C (42).

Escherichia coli: For *E. coli* test, all positive presumptive fermentation tubes were inoculated into EC-MUG broth and incubated in a water bath at 44.5±2°C for 24±2 h. All EC-MUG tubes were placed in water bath within 30 min after inoculation. All tubes exhibited growth was examined for fluorescence using a long-wavelength UV lamp. The presence of bright blue fluorescence was considered a positive response for *E. coli*. A positive control consisting of a known *E. coli* culture, a negative control consisting of a thermotolerant *Klebsiella pneumonia* culture, and an uninoculated medium control were used

simultaneously (42). The MPN value was calculated from the number of positive EC-MUG broth tubes as described in section 9221 C (42).

Pseudomonas aeruginosa: For isolating and identifying of *P. aeruginosa* in the samples, multiple-tube technique was carried out according to 9213 F of APHA (42). The culture media used in this procedure, were asparagine broth for presumptive test and acetamide agar slants for confirmed test (42). In the presumptive test, a five-tube multiple-tube test was performed, then, 10ml single-strength asparagine broth was used for inoculation of 1ml of the samples and 10ml double-strength asparagine broth was used for inoculation of 10ml of the samples (42). The inoculated tubes were incubated at 35 to 37°C. After 24 h and again after 48 h of incubation, tubes were examined under long-wave ultraviolet light (black light) in a darkened room. Production of a green fluorescent pigment constitutes a positive presumptive test (42). In the confirmed test, positive tubes from presumptive test were selected and 0.1ml of the culture was inoculated onto the surface of acetamide agar slants. Development of purple color (alkaline pH) within 24 to 36 h of incubation at 35 to 37°C was considered a positive confirmed test for *P. aeruginosa* (42). Results were computed and recorded according to APHA Table 9221: V and section 9221 D (42).

Ear swabs preparation and cultivation: Two ear swabs samples from each right and left ears of bathers of the swimming pools and control groups were prepared separately (four swabs for each individual). One of the ear swabs samples were cultivated onto a blood agar plates and the other swab was placed into a selective Koser's citrate broth (Difco). The cultivated blood agar plates were incubated at 37˚C for 18 to 24 h as previously recommended (32). Growth of beta hemolytic colonies with a metallic sheen and blue or green pigment on blood agar plates was considered as the possibility presence of *P. aeruginosa*. Gram staining was performed for typical colonies. Gram negative rods with no particular arrangement were considered as positive presumptive test of *P. aeruginosa* as microscopically examination. The inoculated samples into Koser's citrate broth were incubated at 35°C for 24 and 48 h. Displayed turbidity was indicated a positive citrate utilization and was considered as the possibility presence of *P. aeruginosa* in the samples. For confirmation, 0.1ml of the positive cultures was inoculated onto the surface of acetamide agar slants. Development of purple color (alkaline pH) within 24 to 36 h of incubation at 35 to 37°C was considered a positive confirmed test for *P. aeruginosa* (42).

Questionnaire preparation: At the same time with swab sampling, users of the swimming pools were asked to complete a questionnaire. Totally, 179 bathers with a history of ear problem during two weeks before the exam took part in the survey for whom ear swabs were prepared. Eventually, 149 subjects returned the completed questionnaires. Additionally, 97 subjects (about 65% of the cases) belonging to different age and sex groups were chosen randomly as control group, none of whom attended the proposed pools. Ear swab sampling, cultivation, and questionnaire were prepared for the control group accordingly. The following initial information were gathered: age, sex, time spent in the pools, frequency of swimming pool attendance (per week), attending other swimming pools at the same time, history of ear problems (if yes; when, how many times, and how long did it take), presence of ear-associated signs and symptoms such as earache, redness, itching, edema, discharge, and hearing loss, use of drugs including immunosuppressive drugs, family history of ear problems, and finally other problems such as dermatitis, conjunctivitis, etc.

RESULTS

Samples of eleven public swimming pools water were examined for total bacterial colony counts, coliforms, fecal coliforms and *P.*

aeruginosa as described in materials and methods. Results showed that 9 (81.8%) outdoor and indoor pools were contaminated while the other 2 (18.2%) pools, both of which were indoor, revealed bacteria-free.

Table 1 compares positive tubes for the outdoor and indoor public swimming pools. Of a total of 33 samples obtained from 7 outdoor and 4 indoor pools, 11 (33.3%) and 2 (6%) were contaminated with *P. aeruginosa*, respectively (table 1). In addition, coliforms and fecal coliforms were isolated from 5 (15.1%) and 2 (6%) samples of outdoor pools, respectively (table 1). Meanwhile, *P. aeruginosa* grew in 7 (63.6%) swimming pools' water samples. In the other 2 (18.2%) pools, in addition to *P. aeruginosa*, high rates of total bacterial count, total coliforms and fecal coliform counts were also found (table 1).

Table 1. Comparison of positive tubes in the major tests done for the outdoor and indoor public swimming pools

Pools	Total count			Coliform			Fecal Coliform			*E. coli*			*P. aeruginosa*		
Samples	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Outdoor 1	+	+	-	+	+	-	-	+	-	-	+	-	+	+	-
Outdoor 2	+	-	-	-	-	-	-	-	-	-	-	-	+	-	+
Outdoor 3	+	+	+	+	-	-	+	-	-	+	-	-	+	+	-
Outdoor 4	+	-	+	-	-	-	-	-	-	-	-	-	+	-	+
Outdoor 5	+	+	-	+	-	-	-	-	-	-	-	-	-	+	-
Outdoor 6	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+
Outdoor 7	+	-	-	+	-	-	-	-	-	-	-	-	+	-	-
Indoor 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indoor 2	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Indoor 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indoor 4	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Total positives	15			5			2			2			13		
% of positive outdoor pools	39.3			15.1			6.0			6.0			33.3		
% of positive indoor pools	6.0			0			0			0			6.0		
% of positive pools/total pools	81.8			18.2			18.2			18.2			81.8		

Results of microbiological analysis were then matched with other useful information recorded at the time of sampling such as the amount of residual chlorine, number of bathers in the pools, total number of bathers per day, area of the pools, total water capacity of the pools, etc. Results revealed that the mean total number of bather per day in the positive outdoor and indoor pools was 1.7 and 1.1 people per one square meter of the pools, respectively. Meanwhile, the mean amounts of water per each individual bather, in the outdoor and indoor pools were 81.7, and 66.6 liter, respectively. The mean free residual chlorine was 1.3 and 1.7mg/l for the positive outdoors and indoors pools, respectively. The mean temperature of the positive outdoors and indoors water pools were 26.1 and 26.7˚C, respectively. The mean pH of the positive outdoors and indoors water pools were 6.8 and 6.7, respectively. Finally the mean MPN/100 ml for *P. aeruginosa* was 18 and 13 in the positive outdoors and indoors pools, respectively.

Results of the ear swabs samples showed that among 179 bathers who had ear problem during the past two weeks before the survey, 142 (79.3%) were positive for *P. aeruginosa*, however, among controls *P. aeruginosa* was isolated from 4 (4.1%). Table 2 summarizes age, sex, and time spent in the pools in both subjects and controls.

Table 2. Comparison of age, sex and time spent (per week) in pools between cases and controls

	Age		Sex		Duration/week		
	≤ 20	**≥ 20**	**Male**	**Female**	**≤6 h**	**6-12 h**	**≥ 12 h**
Number of cases	96	53	101	48	32	81	36
Percentage of cases (n=149)	64	36	36	32	21	54	24
Number of control	42	55	56	41	-	-	-
Percentage of controls (n=97)	43	57	58	42	-	-	-

Table 3. Comparison of four major otitis externa symptoms results between cases and controls

Cases	Positive for *P. aeruginosa*	Otitis externa symptoms			
		Earache	Edema	Discharge	Hearing Loss
No. of cases	142 *	123	95	53	8
Percentage of cases (n=149)	79.3 *	82	64	36	5
No. of controls	4	4	2	-	-
Percentage of controls (n=97)	4.1	4	2	-	-

* (n=179)

All of positive cases of *P. aeruginosa* had at least one symptom of otitis externa while among controls only 2 (50%) had both earache and edema.

The frequency of earache, edema, discharge and hearing loss was 82%, 64%, 36% and 5% (table 3).

DISCUSSION

Results revealed that bathers of the public swimming pools are at substantial risk of otitis externa due to *P. aeruginosa*. Monitoring of swimming pools for coliforms, fecal coliforms, *P. aeruginosa* as well as other chemical and physical characteristics showed that bather's health may be at risk. Furthermore, our results showed that water quality of swimming pools were depended on the efficacy of disinfection, sanitary conditions, number of bathers and the total number of bathers per day. While adequate disinfectant residuals and routine maintenance are the key elements to controlling *P. aeruginosa* in swimming pools, the main question is why they could not maintain adequate disinfectant residuals in the pools.

Although maintaining residual chlorine in pools is relatively easy, even under normal operating conditions, recommended residuals can be quickly dissipated. As the efficacy of free and combined available chlorine in swimming pools water is affected by 1) the length of exposure/contact, with longer exposure times producing greater effect, 2) temperature of water at the point of contact/interaction, with higher temperatures of the pool water resulting in more free chlorine. The mean water temperature of all positive pools in our setting was above 26˚C. Thus, they had to use more chlorine and maintain residuals at the level of at least 2mg/l, however, it was much less. 3) pH of water can affect disinfection efficiency. Particularly all of our studied pools were disinfected with chlorine for which pH factor is much more critical. More acidic water is associate with more HOCL, rapid loss of chlorine and less effective chlorination (43). In our setting, pH of all positive pools was low, therefore more HOCL, rapid loss of chlorine and less effective chlorination could be assumed. 4) When chlorine introduces to water and nitrogenous compounds, hypochlorous acid (HOCl), hypochlorite ion (OCl), and chloramines are formed. Although both free and combined chlorine are disinfectant in pools, combined chlorine is not as powerful as free chlorine. Therefore, in our setting there should be more combined chlorine rather than free chlorine in pools. 5) Both hypochlorous acid (HOCl) and hypochlorite ion (OCl-) are free chlorine residual, however, hypochlorite ion (OCl-) is about 100 times less effective as a disinfectant than hypochlorous acid (HOCl). Although all of the positive pools had acidic condition in which more HOCL was produced, their efficacy is lost by the following factors: i) fast forming combined chlorine because of overloading; ii) at lower pH, more trichloramine is produced. This compound is particularly noticeable because it is volatile and is readily aerated out of water by agitation and so much more chlorine is dissipated soon; iii) finally, hypochlorous acid (HOCI) is decomposed under the influence of ultraviolet radiation or sunlight (44). This situation was prepared for all of the positive outdoors pools, too.

In conclusion, investigation of the contaminated swimming pools revealed that chlorination was often inadequate especially when high population of people had to use pools. It is essential that these public swimming pools be adequately disinfected. Overloading of the pools should also be avoided because high pollution entering the pools and the excessive load usually is built up beyond the capacity of the water treatment system. The operators of these public swimming pools should know that the organic load derived from the bathers together with the impinging sunlight can quickly lower the amount of free chlorine available in the pool water. Splashing in the pool also serves to increase the loss of chlorine. For those public swimming pools that the bather load is usually high, it is particularly recommended that the water should be monitored closely and the pH and disinfectant levels of the water should be adjusted

and maintained at the standard levels. To achieve this, it is essential that the water of these pools should be monitored frequently and continuously especially when in use. Routine, thorough cleaning of surrounding surfaces that could harbor pathogens will also help to reduce the spread of *P. aeruginosa*. In addition, public swimming pools operators should oblige users to shower before entering the water, and where possible, control the number of bathers and their duration of exposure.

Although results of this research showed that otitis externa was strongly associated with attending swimming pools due to *P. aeruginosa*, an extensive follow-up study is needed to determine the other possible health risk associated with public swimming pools. Also findings of this study imply that, more strict bathing water standards should be met in the public swimming pools. The risk of otitis externa due to *P. aeruginosa* and other possible illnesses caused by other pathogens should be minimized and studies must decide if standards can be developed.

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