

Ex situ study of *Enterococcus faecalis* survival in the recreational waters of the southern coast of the Caspian Sea

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ABSTRACT

Background and Objectives: The US Environmental Protection Agency has suggested faecal enterococci as the primary bacterial indicators. Of more importance is their direct correlation with swimmer-associated gastroenteritis in recreation water quality monitoring. In contrast to other seawater bodies with 3.5% salinity, the recreational waters in the southern coast of the Caspian Sea possess its own salinity (about 1% w/v) and thus require further investigations to determine the capacity of *Enterococcus faecalis* as the sole primary microbial index in this unique aquatic environment.

Materials and Methods: The survey of the presence and survival of *E. faecalis* as a microbial index in the recreational waters of the southern Caspian Sea was carried out using a microcosm as an experimental model. The concentration of *E. faecalis* cells in samples of seawater were estimated by a standard membrane filtration method using m-Enterococcus agar as the selective culture medium. As the current standard culture-based methods are not reliable enough for the detection of non-growing, damaged and under-tension bacteria, PCR was used to identify the possible VBNC form of the bacterium after disappearance of the culturable cells.

Results and Conclusion: A continuous decline in the number of culturable *E. faecalis* cells resulted in apparent elimination of the bacteria from seawater in a defined period. Detection of intact DNA was possible in the following 60 days. The salinity of about 1% and the self-purification properties of the Caspian Sea make the conditions feasible for the use of this microorganism as a measure of water quality throughout the region. The results confirmed the presence of damaged bacterial cells, namely VBNC forms, indicating the necessity of examining of the sea water samples by using molecular approaches or repair procedures.

Keywords: Caspian Sea, Microbial indices, Molecular methods, *Enterococcus faecalis*

INTRODUCTION

Fecal enterococci are normal inhabitants of the

gastrointestinal tract of warm-blooded animals (1-2). Their densities correlate better with the incidence of gastrointestinal illnesses among recreational bathers than fecal coliform densities. Compared to other enterococci, *Enterococcus faecalis* survives longer in the aquatic environments (5), and therefore has been suggested as the primary bacterial indicator to monitor the quality of recreational seawaters (3-4). Furthermore, this Gram-positive, non-speculating bacterium is known as an opportunistic pathogen that causes urinary tract infection and is responsible for the majority of cases of sub-acute bacterial

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endocarditis (6).

In 1999, the U.S. Environmental Protection Agency (USEPA) set forth an Action Plan for Beaches and Recreational Waters, as Americans faced the risk of illness associated with swimming in surface waters contaminated with disease-causing microorganisms. Previous epidemiological studies performed by the USEPA had demonstrated a direct relationship between the density of *E. coli* and enterococci in surface waters and an increase in swimmer-associated gastroenteritis (7). Limits were established as guidelines for recreational water quality based on this information. For freshwater, the present single-sample advisory limits are 235 CFU/100 ml for *E. coli* and 61 CFU/100 ml for enterococci. The 5-day geometric mean should not exceed 33 CFU/100 ml for Enterococci (8). Previous studies have demonstrated that when released into the environment, *E. faecalis* can activate the VBNC state in response to unfavorable conditions (e.g., low temperatures and oligotrophic conditions) (9-10).

Standard microbiological methods used to detect bacteria in the aquatic environment are based on colony-forming unit counts, thus allowing detection of only those bacteria capable of dividing. However, over the past few decades, studies have shown that non-culturable bacteria, including human bacterial pathogens, make up part of the microbial population in the aquatic environment. These bacteria are usually unable to divide in oligotrophic environments or culture media subject to stressful conditions (11). Since the VBNC cells could constitute a potential hazard to human health, it became mandatory for the right monitoring of the microbiological quality of waters, to develop and apply methods, which are also capable of detecting non-culturable bacterial forms.

Among the different molecular methods, PCR has been proven to be very useful in detecting low amounts of a specific DNA against a large background of prokaryotic and eukaryotic cells and organic material present in environmental samples (10-12).

In this study, the presence and survival of culturable and non-culturable forms of *E. faecalis* were tracked in a microcosm, designed in our laboratories, using standard selective culture media and PCR. The results from such *ex situ* studies could have implications in the prediction and management of *E. faecalis* pollution in the Caspian Sea.

MATERIALS AND METHODS

Study site and water sampling. The beach examined in this study is located in the Chaloos area (Radio Darya beach: N36° 40.731; E051° 26.347) on the southern coast of the Caspian Sea, Mazandaran Province, Iran. This area is a major travel destination for many swimmers and tourists especially in the hot summer months. Furthermore, it is contaminated by sewage from human inhabitants and agricultural wastewater and pollutants from industries, which are carried by the rivers into the coastal seawater. Water samples were taken from April to January 2011 covering the four months of April, August, October and January. Samples were collected in sterile polypropylene containers from zero point at water surface level to 30 cm below the surface, where the water depth reached 1m and was thus immediately tested in main station field laboratory using Membrane Filtration (MF) method as described below.

Measuring physicochemical parameters.

Physicochemical parameters of the recreational water in the Radio Darya beach (Chaloos station) were studied in four months including April, August, October and January. Temperature, pH value, dissolved oxygen, turbidity and electrical conductivity, as the indicator of salinity were measured daily. All samples were taken daily between 8 am to 4 pm and immediately examined in place using portable Hach instruments. All instruments were calibrated and used according to the manufacturer's instructions. Since rainy and stormy weather adversely affect the different measurements, all the data obtained in such circumstances, were omitted and not used.

Microcosm model. An 800-l glass walled container with dimensions of 50 cm (w)× 200 cm (L)× 80 cm (h) and working volume capacity of 600 l was constructed as a model of the microcosm (Fig. 1). The model was equipped with a permanently working air pump 80 l min⁻¹, two alternately working water circulators with 10 s intervals (collectively 600 l min⁻¹), heating and cooling devices and an electromechanical wave-simulating apparatus which was built in our laboratory with 200 W electromotor and a 30× 40 cm stainless steel paddle reciprocally moving within a 40° angle, 40 times per hour. All parts of the system electronically integrated and controlled to



Fig. 1. The seawater containing tank equipped with instruments to simulate environmental conditions of recreational water in the southern coast of the Caspian Sea and used as a model microcosm.

work in a manner to simulate the conditions very close to the actual conditions in coastal recreational water under natural daylight using an indirect sunlight through the window. Temperature and dissolved oxygen were measured off-line using Hach portable instruments. Water loss was compensated with deionized water, each time when it was necessary.

All parts of instruments were steam sterilized or sanitized by sinking in 70% ethyl alcohol for 30 min and then washed thoroughly with the same sea water before immersing within the aquarium.

The sea water was taken in 20 l container from the coastal Caspian Sea water at the Chaloos station at 5am and transferred under cold and dark condition to the container in our laboratory in Tehran at 10am on the same day (in October, 2011). All the surrounding physicochemical parameters of the microcosm were measured as mentioned before.

Microbial analyses. Selective count of enterococci was performed by Membrane Filtration method (7). The procedure provides a direct count of bacteria based on the development of colonies on the surface of a membrane filter. Volumes of water samples, including 10 ml, 1 ml, and volumes of 0.1% dilution in sterile normal saline, including 10 ml and 100 ml, were filtered through a 0.45 μ m pore size filter of 47mm diameter, gridded, sterile, nitrocellulose membrane filter (Millipore Co., Bedford, MA, USA) and rinsed with 20ml phosphate-buffered saline (PBS) according to the standard membrane filtration protocol (7). Filters were then placed with the grid side up, on m-Enterococcus agar (Merck) as a standard selective culture medium. Colonies that produced a

pink to red hue on m-Enterococcus agar were designated as enterococci. When the colony count came close to 0, 100ml aliquots of the raw, undiluted water sample was filtered and bacteria were counted until it reached <0.1 CFU/ 100 ml.

Verification Tests. Verification tests on the randomly selected typical colonies for Enterococci were carried out by streaking these colonies on Brain-Heart Infusion agar (Merck) (24h at 35 °C) followed by sub-culturing of 5-10 colonies in Brain-Heart Infusion broth (Merck) (48h at 45 °C) and Bile Aesculin Azide agar (Merck)(48h at 35 °C). For 6.5% Sodium Chloride growth test, colonies were cultured on the base medium, Brain-Heart Infusion broth, at 35 °C. To distinguish *E. faecalis* from other Enterococci, biochemical tests were carried out to determine fermentation of mannitol, ribose, and arabinose in Phenol Red Broth Base (Himedia) and deamination of arginine amino acid were carried out in Moller Decarboxylase Broth Base (Merck). Additionally, Gram staining and the catalase test along with other differentiation tests were conducted (18).

Revitalization experiments. Subsequent to the reduction of the bacterial count on m-Enterococcus agar plates to <0.1 CFU/ 100 ml, efforts were directed at resuscitating the probable VBNC bacteria and hence, enterococcal cells, which had recovered from the VBNC form, were obtained as previously described (13-14). Liquid cultures were used since no recovery was seen on agar plates. Briefly, 10ml aliquots of the seawater in the microcosm composed of cells that had just entered the VBNC state was transferred into 10ml aliquots of Brain-Heart Infusion broth double strength (Merck) and were placed in shaking incubator for two hours at 35 °C. Bacteria were collected by filtering the medium through a 0.45 μ m poresize nitrocellulose membrane filter which was then placed on m-Enterococcus selective agar and incubated at 35 °C for a further three days.

Sample processing for PCR analyses: Bacterial cells collection and DNA extraction. A 100ml volume of the water sample was filtered through 47-mm in diameter, 0.22- μ m pore size acetate cellulose filters (Millipore Co., Bedford, MA, USA) and the sides of the funnels rinsed twice with 20ml of sterile distilled water. Bacteria from seawater were

collected using two different approaches, subsequent to which DNA was extracted from the samples. In the first case, the filters were transferred to a petri dish with the sample side facing up. DNA was recovered from the organisms, retained on the filters by the addition of 1ml of SET buffer (20% Sucrose, 50mM EDTA, 50mM Tris-HCl, pH 7.6) and DNA extracted as previously described (15-16).

In the second approach, the filters were aseptically cut into 2cm² pieces and placed into tubes containing 50ml PBS, followed by a 10min vortex to remove bacteria from the surface of the filter pieces. The filters were subsequently removed and pellets obtained by centrifugation and the concentrated samples were suspended in 1ml SET buffer (20% Sucrose, 50mMEDTA, 50mM Tris-HCl) (5-10).

The efficiency of the DNA extraction and purification protocol was assessed. Briefly, DNA was extracted and suspended in 50µl of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.5). The DNA concentration was determined by co-migration on 0.8% agarose gel with markers of standard concentrations as described (10).

PCR conditions. The target sequence for PCR amplification was a 444-bp fragment located on the *E. faecalis* chromosome within the *pbp5* gene, coding for penicillin binding protein 5 (PBP5) which is species-specific (17). The fragment was amplified by PCR with two primers, the sequences of which were selected from within the gene: primer FWD (5' CATGCGCAATTAATCGG 3') and primer REV (5' CATAGCCTGTGCGAAAAC 3').

The PCR protocol used in this study (with modifications) has been previously described (10): amplification was carried out with 30 cycles consisting of 1.5min denaturation at 94 °C, 1.5 min annealing at 60 °C and 2min extension at 72 °C with a final 5min extension period at 72 °C.

RESULTS

The average readings of conditions and characteristics of seawater as well as the concentration of *E. faecalis* during three seasons (in 2011) at Chaloos Beach (Radio Darya station) is presented in Table 1.

The microcosm built in our lab, properly simulated the abiotic environmental condition of southern recreational waters of the Caspian Sea at Chaloos Station. The system provided the same temperature, about 90% similarity with measured dissolved oxygen.

In the laboratory, efforts were directed at maintaining the physicochemical characteristics of the sampled water in the microcosm close to the natural readings during the fall. The median of these values obtained from microcosm were as follows: DO 6.5±0.3mgO₂l⁻¹, pH 8.2±0.1, temperature 24±0.5 °C, turbidity of 4.57±3.2 NTU and salinity 8.5±0.5 gl⁻¹.

A total of 75 putative enterococci isolates on m-Enterococcus agar were subjected to confirmatory tests for the genus, and 50 isolates exhibited all the characteristics associated with *Enterococcus* spp. (18). The isolates were Gram positive, catalase negative, esculin cleavage positive; they were capable of growing in 6.5% sodium chloride, 0.05% sodium azide, at pH 9.6 and 45 °C. The probable enterococci cells were examined for further differential characteristics as mentioned in the verification tests.

E. faecalis was the most frequently identified *Enterococcus* species (76% of confirmed enterococci), and nearly all isolates were identified with 90% or greater assurance by the biochemical test system. Members of the *E. faecalis* species were found in all seawater samples.

The culturable form of *E. faecalis* in the water samples was detected during a 16-days period. In the first 12 days, the test was performed by direct growth of colonies on m-Enterococcus medium after incu-

Table 1. Values of physiochemical parameters and concentration of indicator bacteria at Chaloos station (Radio Darya beach).

Date	<i>Enterococci</i> CFU/100 ml	Dissolved oxygen (mgO ₂ l ⁻¹)	pH	Turbidity (NTU)	Temperature (°C)	Salinity gl ⁻¹
April 2011	0.3×104±7.0×103	9.47±0.6	8.45±0.1	37.76±23.7	16.25±2.8	8.15±0.3
August 2011	4.2×104±1.6×103	7.45±0.5	8.53±0.1	8.24±0.2	30.65±1.7	8.2±1.2
October 2011	1.5×104±5.9×103	6.85±0.3	8.31±0.1	4.86±6.5	23.2±5.2	8.7±0.5
January 2011	3.9×104±0.1×103	10.1±0.2	8.76±0.1	114±2.0	12.1±3.4	8.3±0.4

bation and during the final 4 days, the revitalization procedure as mentioned in the revitalization experiment's section, was required to ensure the growth of colonies on m-Enterococcus plates. The latter provided sufficient reason to prove the presence of VBNCs. Growth and survival of this bacterium showed a continuous decline in concentration of enterococci from 1.2×10^4 to <0.1 CFU/100 ml during the 16-days period, with an average of a 10-fold reduction in the number of CFU every four days during the period (Fig. 2).

Intact *E. faecalis* cells containing the whole genome were tracked during the following few days. PCR, performed with DNA extracted from the VBNC form of the bacteria, obtained by direct membrane filtration of seawater, yielded amplicons which formed sharper bands on agarose gel than those obtained by the indirect method (centrifugation), indicating that perhaps a greater number of VBNC forms were detectable by the former method. We examined four different series of water samples collected from the model microcosm over a three-month period (Fig. 3). To detect intact *E. faecalis* cells (or probable VBNC forms of *E. faecalis*), after the culturable number of bacteria reached <0.1 CFU/100 ml⁻¹, water samples were filtered immediately (Fig. 2: lane A) or were obtained by centrifugation (lane B). The second series (lane C) was collected one month later and the third series (lane D) two months later all which showed positive results of the presence of the *E. faecalis* *pbp5* gene as indicated by PCR. No amplicons were detected after a three-month period (lane E).

DISCUSSION

Fecal *streptococci/enterococci* are standard indicators of fecal contamination in aquatic environments.

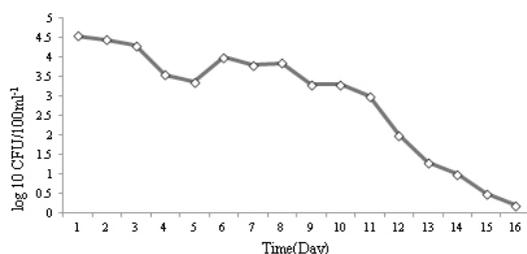


Fig. 2. Decline of enterococcus faecalis survival in the Caspian Seawater microcosm. Colony counts on m-Enterococcus agar plates. Each point is the mean of triplicate samples.

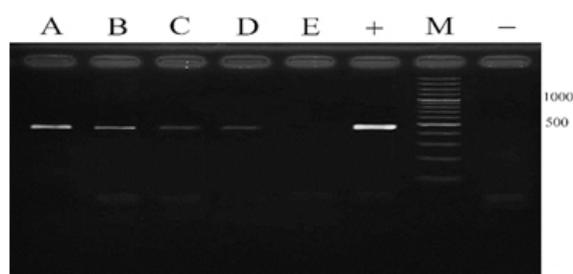


Fig. 3. Ethidium bromide-stained gel with products from amplification by PCR of *pbp5* (A and B), after the beginning of the viable but non-culturable (VBNC) state; A: direct filtration method and B: indirect filtration method, (C) one month, (D) two months and (E) three months after bacteria was not detected any longer on culture media. The PCR-positive (+) and -negative (-) controls contained *Enterococcus faecalis* PTCC: 1393 DNA and sterile water, respectively. Lane M contained markers. The sizes of the amplification products were 444 bp for *Enterococcus faecalis* PTCC: 1393.

More specifically, *E. faecalis* is currently considered to be the indicator of choice for coastal recreational waters regardless of *Escherichia coli* as the other well-known microbial index of water contamination into human feces (2-27). This is because of the salinity (35g salt l⁻¹) of the coastal waters all over the world. However, the Caspian Sea possesses a unique salinity of about 10g salt l⁻¹ in its south coastal waters, which would require further investigations for the identification of microbial indices and limits of microbial concentrations. Few studies have been published regarding the survival of enterococci in the southern Caspian Sea waters and all have been conducted by evaluating the concentration of enterococcal cells present in the water samples through their growth as colonies on nutrient media (19). In the aquatic ecosystem, the survival of microbial indices is affected by complex environmental stresses. Stress factors, potentially killing agents that trigger damage to cell and then induces cellular responses, are still poorly understood (20, 28- 29).

This study aimed at devising an experimental model to monitor the pollution of the Caspian Sea by surveying the survival of the indicator bacterium, *E. faecalis*. Different laboratory microcosms representing several environmental conditions have been used. Absence of growth on a solid culture medium following inoculation of 10ml microcosm sample is an indication of

bacterial population entry into the VBNC state (19) which is the criteria being used in this study. It is now well known that bacteria can activate survival strategies, which allow them to persist even in adverse environmental conditions when cell division is restricted. In addition, factors including low nutrient concentrations, low or high temperatures, extreme salinity and pH induce the activation of the VBNC state in *E. faecalis*. In such conditions, the cultivability of this bacterium is maintained for about three weeks (21-29).

Since a remarkable concentration of *E. faecalis* is found in the Caspian Sea water, it would be interesting to determine whether this bacterial species could also be present in the viable but non-culturable form within this type of natural environment. Signoretto et al. (2004) reported that *E. faecalis* could enter a VBNC state; other studies reported the application of the RT-PCR method in detection of mRNA in VBNC of *E. faecalis* (8). In view of these findings, it seems that traditional methods used to monitor the microbiological quality of the Caspian Sea water may have become obsolete as they are only capable of detecting culturable cells.

Previous studies have demonstrated that VBNC cells retain viability and infectivity; if culture-based methods had been used, it is unlikely that indicator bacteria such as *E. coli* cells would have been detected in the drinking water and river water systems that were investigated (22-27). Our data support the demand for the use of molecular methods to accurately monitor microbiological quality of the environment. As this study report, both biochemical and PCR techniques were required to detect culturable and non-culturable *E. faecalis* in a seawater model.

The likelihood of amplifying free *E. faecalis* DNA seems remote, since in a complex environment, such as the Caspian Sea, free DNA degrades very rapidly, i.e. within hours (11-23). On the other hand, it is well known that injured bacteria can recover in nonselective media but do not grow on selective media (24) and non-culturable bacteria form colonies on neither selective nor non-selective media (25), so we considered the possibility of injured bacteria being present in our samples. This possibility was ruled out by examining selective and non-selective media.

Water quality monitoring studies that employ enterococci as indicator organisms could benefit from the knowledge of the percentage of enterococci isolated that are *E. faecalis*, particularly as this species occurs

with high frequency in human feces and sewage (26-28).

Lio and colleagues demonstrated that non-culturable *E. faecalis* cells are capable of expressing the *pbp5* gene for at least three months, indicating that they remain viable in a low-nutrient-concentration microcosm for this period of time (21).

Tracking the presence of *E. faecalis* cells in the Caspian Sea model with the standard culture method and specific PCR protocol indicates a surveillance period of about two and a half months. Despite the apparent self-purification properties of the southern coastal waters of the Caspian Sea, it is persistently prone to pollution from animal and human sources and hence stricter monitoring procedures should be prioritized to ensure the safety of these recreational waters.

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