

OCCURRENCE OF *Proteus mirabilis* ASSOCIATED WITH TWO SPECIES OF VENEZUELAN OYSTERS

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SUMMARY

The fecal contamination of raw seafood by indicators and opportunistic pathogenic microorganisms represents a public health concern. The objective of this study was to investigate the presence of enteric bacteria colonizing oysters collected from a Venezuelan touristic area. Oyster samples were collected at the northwestern coast of Venezuela and local salinity, pH, temperature, and dissolved oxygen of seawater were recorded. Total and fecal coliforms were measured for the assessment of the microbiological quality of water and oysters, using the Multiple Tube Fermentation technique. Analyses were made using cultures and 16S rRNA gene sequencing. Diverse enrichment and selective culture methods were used to isolate enteric bacteria. We obtained pure cultures of Gram-negative straight rods with fimbriae from *Isognomon alatus* and *Crassostrea rhizophorae*. Our results show that *P. mirabilis* was predominant under our culture conditions. We confirmed the identity of the cultures by biochemical tests, 16S rRNA gene sequencing, and data analysis. Other enterobacteria such as *Escherichia coli*, *Morganella morganii* and *Klebsiella pneumoniae* were also isolated from seawater and oysters. The presence of pathogenic bacteria in oysters could have serious epidemiological implications and a potential human health risk associated with consumption of raw seafood.

KEYWORDS: *Proteus mirabilis* in bivalves molluscan; *Isognomon alatus*; *Crassostrea rhizophorae*; Venezuelan coastal area; Public health and environmental impact; Antibiotic resistance.

INTRODUCTION

After *Escherichia coli*, *Proteus mirabilis*¹⁰ is one of the most frequent etiological agents associated with urinary tract infections (UTIs), particularly in catheterized patients or individuals with structural abnormalities of the urinary tract¹⁷. *P. mirabilis* is a member of the normal microbiota of the mammalian intestinal tract and has been isolated from humans, dogs, monkeys, pigs, sheep, cattle, raccoons, cats, rats, and other mammals⁷. Additionally, *P. mirabilis* is widely distributed in the environment, occurring in polluted water, manure, and soil, where it plays an important role in decomposing organic matter of animal origin¹⁶. Several potential *P. mirabilis* virulence factors related to UTI have been described, including fimbrial-mediated adherence to the uroepithelium, swarming motility mediated by flagella, outer-membrane protein (OMP) expression, cell invasiveness, urease production, hemolysin production, and iron acquisition^{16,17}. Besides UTI, this pathogen has been described as an opportunistic etiological agent in infections of the respiratory tract and of wounds, burns, skin, eyes, ears, nose, and throat, as well as in gastroenteritis resulting from the consumption of contaminated food^{10,11,16}.

Proteus is part of predominant microflora in fresh seafood including finfish and shellfish⁴. Also, it has been isolated from tissues and fluid of oysters from a coastal marine environment of Nigeria¹. Oysters, as

filter-feeding organisms, magnify public health problems associated with environmental contamination, because they accumulate microbial pathogens manifold over the densities found in overlying waters¹⁴. The fecal contamination of seafood by microorganisms like *P. mirabilis* is one of many major problems of coastal environments indicating a potential human health risk associated with consumption of raw oysters. The objective of this study was to investigate enteric bacteria in oysters collected from a marine coastal area, in view of the popularity of these mollusks as a source of raw food, and to verify sensitivity to antibiotics.

MATERIALS AND METHODS

Sampling site and sample collection: The first species of oyster (*Isognomon alatus*, Gmelin, 1791²⁰) was collected from a sunken ship close to Chichiriviche, a rural town in northwestern Venezuela (10°18'17" N and 68°05'01" W), during August, October and December 2004. *Isognomon alatus* was harvested at a depth of half a meter under the water surface over a sunken ship, a place where people dive and collect oysters for raw consumption. Live specimens of the other oyster species (*Crassostrea rhizophorae* Guilding, 1828 or mangrove oyster⁸) were purchased from local fishermen at the nearby town of Chichiriviche (10°55'30" N and 68°5'1" W). Two samples of approximately 60 individuals were collected or purchased from each oyster species, immediately washed with sterile distilled water and

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maintained in separate containers. Samples were transported under refrigeration to the laboratory, where they were processed in fewer than eight hours from capture. Duplicate seawater samples were taken 10 cm below the surface at the first site and the local values of pH (pHep1, Hanna Instruments), salinity (RHS-10ATC refractometer, Westover), temperature, and dissolved oxygen (OXDP-02 oxygen meter, VWR) were measured.

Bacteriological quality of water and oyster tissues: The total and fecal coliforms in the samples of seawater and oyster tissue homogenates (10 g in 90 mL PBS buffer (0.32 M) at pH 7.2) were estimated as the Most Probable Number (MPN) in aliquots of 0.1, 1 and 10 mL. The initial dilutions for this analysis were 1:10 and 1:100, using the Multiple Tube Fermentation (MTF) technique². For the determinations of total coliforms Lauryl Sulfate (Merck) and Bright Green Bile (BRILA, Merck) were used for incubations at 35 °C for 24-48 h, and the presence of fecal coliforms was confirmed in EC medium (*E. coli*, Merck) at 44.5 °C for 24-48 h. From the dilutions which were positive for total and fecal coliforms, 50 µL aliquots were inoculated in MacConkey (HIMEDIA) and ENDO (Merck) media and incubated at 35 °C for 18-24 h. Isolates from these enteric bacteria were Gram-stained and observed under an optical microscope to aid colony selection and later identification by means of conventional biochemical assays and the API ATB/Plus system (BioMérieux, Lyon, France).

Bacterial isolation from oyster tissue: The oyster shells were first washed thoroughly with sterile water and then opened and the tissues removed; 10 g of the latter were homogenized 1:10 in PBS buffer (0.32 M) at pH 7.2. Aliquots of 300 µL were then inoculated into MacConkey (Difco) and Nutrient Broth (Difco) media, and into the selective Agar Chocolate medium (Blood agar base plates, Difco), supplemented with 10% sheep blood, and a mixture of antibiotics and fungicides consisting of (final concentrations, per mL) vancomycin (10 µg), polymyxin B (2.5 IU), trimethoprim (5 µg) and amphotericin B (2.5 µg) (Sigma Chem. Co.). These cultures were grown at 37 °C for 24-48 h.

Biochemical tests and antibiotic resistance: Routine microbiological tests, including those for Gram-stain reaction, oxidase, catalase, urease, arginine dihydrolase, and API ID 32E test (BioMérieux) were performed according to standard methods on all strains². The biochemical characteristics of our isolates were determined with the API ID 32E and API rapid ID 32E systems. A dendrogram based on the API ID 32E biochemical reactions of our isolates was produced with the computer package MINITAB 14.2 (www.minitab.com) by using the single linkage of cluster analysis applied to similarities based on the Manhattan distance. Growth of presumptive *Proteus* isolates at various salt concentrations and antibiotic resistance patterns were tested in Nutrient Broth (Difco) and Möeller-Hinton Agar (Becton Dickinson), respectively. The *Proteus* isolates MA (No. 1740) and O (No. 1749) were deposited at the Centro Venezolano de Colecciones de Microorganismos (CVC, Venezuela).

DNA extraction, PCR and 16S rRNA gene sequencing: Aliquots of the isolates (700 µL of nutrient broth culture) were placed in sterile Eppendorf tubes and centrifuged at 14,000 g for one min. The supernatants were discarded and pellets were then stored at -20 °C

until DNA extraction could be performed. Total genomic DNA was extracted with a MO BIO Laboratories kit (Cat. No. 12224250) used according to the manufacturer recommendations. The 16S rDNA from the isolates was amplified by PCR with puReTaq Ready-To-Go beads (Cat. No. 27-9557-01, Amersham Biosciences, NJ) and two universal primers, 8Fpl (5'-AGA GTT TGA TCC TGG CTC AG -3') and 1525R (5'-AAG GAG GTG ATC CAG CC -3'), derived from highly conserved regions of the 16S rRNA genes. Amplification was performed using a thermal cycler model Gen AMP 9700 (Applied Biosystems, CA), as follows: one cycle at 94 °C for six min, 30 cycles at 94 °C for 45 s, 55 °C for 45 s and 72 °C for one min, with a final 10 min extension step. The PCR products were visualized by running the reaction mixture in a TBE agarose gel (2.0%), staining it with ethidium bromide, and visualizing it under an UV transilluminator. The amplicon was purified for sequencing using the CONCERT™ Rapid PCR Purification System kit (GibcoBRL-Invitrogen, CA.) according to the manufacturer recommendations. Both strands of the purified DNAs were sequenced at the CeSAAN facility (IVIC, Venezuela) with an ABI PRISM™ 377 sequencer (Perkin-Elmer, USA). The primers used for sequencing were those mentioned above, plus the internal primers 515F (5'-GTG CCA GCM GCC GCG GTA A -3'), 519R (5'-GWA TTA CCG CGG CKG CTG -3'), 926F (5'-GGG CCC GCA CAA GCG GT -3') and 926R (5'-ACC GCT TGT GCG GGC CC -3'). Sequences were compared to the compilation of 16S rRNA genes available in the GenBank nucleotide library by BLAST searching through the NCBI site. Alignments of 16S rRNA genes were done manually using EDITVIEW version 1 and bl2seq through the NCBI site. The 16S rRNA gene sequence (~1.4 kb) of isolates MA and O was deposited in GenBank under the accession numbers DQ449630 and DQ449631, respectively.

RESULTS

Environmental parameters and bacteriological assessment: At the collecting sites of oysters and during the sampling period the physicochemical parameters of the water were pH 8.9, temperature 29.9 °C, salinity 11‰ and dissolved oxygen 4.8 mg O₂ L⁻¹. These values differed from those in dry season at this same location, as salinity and temperature were lowered⁶, possibly due to run-off of rainwater at sampling time. The initial estimates for total and fecal coliforms were 13 and 4 MPN/100 mL in the water and more than 160,000 MPN/g of tissue in both oyster species evaluated for both indicators, confirming the capability of these bivalves to concentrate organic matter and microorganisms.

Characterization of isolates, biochemical tests, and antibiotic resistance: Eleven *Enterobacteriaceae* were isolated from seawater (Col11), *I. alatus* (Col13, MA, M4 and M) and *C. rhizophorae* (Col 15, Col18, Col21, Col22, Col 27 and O) tissue. Results of the biochemical tests and the API ID 32E identification procedure of the *P. mirabilis* isolates obtained from the two species of oysters are in Table 1. Several species were isolated, such as *Escherichia coli*, *Morganella morganii* and *Klebsiella pneumoniae*, as well as *P. mirabilis* which prevailed in both oyster species. All the isolates from *P. mirabilis* showed biochemical features common to the species (oxidase negative, catalase and urease positive, H₂S and lack of indole production and motility). It should be pointed out that the M4 and Col22 isolates grew well at high NaCl concentrations (6 and 8%) in less than 48 h (Table 1).

Table 1
Results of biochemical tests of *P. mirabilis* isolates from oyster tissue collected from a sunken ship close to Chichiriviche during December 2004

Isolates	Source	Kliger ^a	SIM ^b	Citrate ^c (Simmons)	NaCl growth ^{c,d}				ID 32E Identification
					0%	3%	6%	8%	
MA	<i>I. alatus</i>	K/A H ₂ S	+ - +	+	+	+	-	-	<i>Proteus mirabilis</i> (99.9%)
M4	<i>I. alatus</i>	K/A H ₂ S	+ - +	+	+	+	+	+	<i>Proteus mirabilis</i> (99.9%) ^e
M	<i>I. alatus</i>	K/A H ₂ S	+ - +	+	+	+	-	-	<i>Proteus mirabilis</i> (99.9%)
O	<i>C. rhizophorae</i>	K/A H ₂ S	+ - +	+	+	+	-	-	<i>Proteus mirabilis</i> (99.9%)
Col22	<i>C. rhizophorae</i>	A/A H ₂ S	+ - +	+	+	+	+	-	<i>Proteus mirabilis</i> (99.9%)

^a K/A, alkaline/acid; A/A acid/acid; ^b SIM: H₂S and Indole production, and Motility; ^c +, positive; -, negative; ^d Positive results for the NaCl test were seen at 24 h for 0 and 3%, and at 48 h for 6 and 8%, except in strain Col22 which grew in 6% NaCl in 24 h; ^e The API rapid ID 32E gallery was used to identify strain M4.

The dendrogram (Fig. 1) shows the biochemical relationships between the isolates and reference microorganisms used at the ID 32 E System strains. The isolates Col22, O, M and MA formed a phenon with *P. mirabilis* (88% similitude), Col11 with *M. morgani* (100%), Col15, Col18 and Col21 with *E. coli* (76%), and the isolates Col27 and Col13 with *K. pneumoniae* (92%). For comparative purposes, *Vibrio cholerae* was included as a reference strain from outside the *Enterobacteriaceae* family.

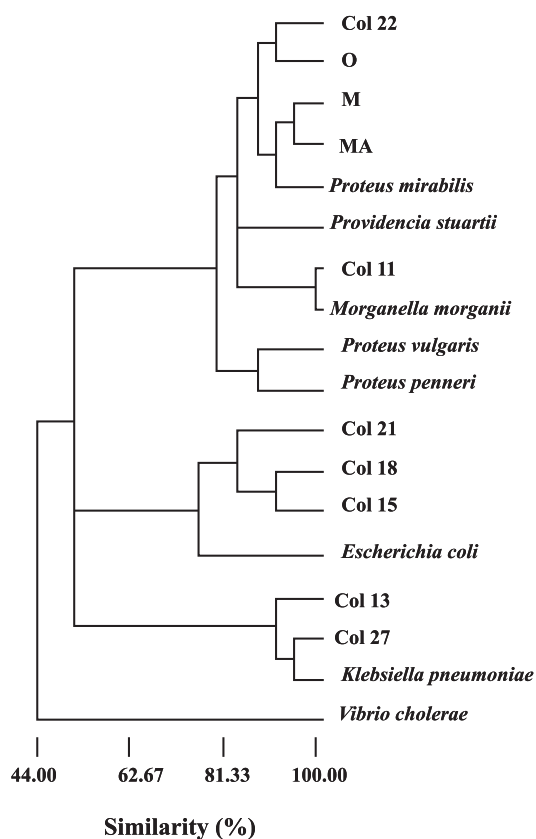


Fig. 1 - Dendrogram based on API ID 32E biochemical reactions showing the levels of similarity between all the isolates (Col 22, O, M, MA, Col 11, Col 21, Col 18, Col15, Col 13, Col 27) and reference microorganisms from the ID 32 E System of *Enterobacteriaceae*. The graph shows similarity percentages by single-linkage method with Manhattan distances. Strain M4 was not included in this analysis because it had been identified with the API rapid ID 32E gallery.

The responses of the *P. mirabilis* isolates to diverse therapeutic antibiotics are recorded (Table 2). The Col22 and MA isolates were resistant to seven of the 13 antibiotics evaluated, whereas O was resistant to only five of them. All isolates were resistant to penicillin G and to cefoxitin, while being sensitive to quinolones (norfloxacin and lomefloxacin).

Table 2

Antibiotic resistance of *P. mirabilis* isolates from oyster tissue collected from a sunken ship close to Chichiriviche during December 2004. Concentrations are given per mL

Isolates	MA	M4	M	O	Col22
Tetracycline (30 µg)	R*	R	R	S	R
Cefoxitin (30 µg)	R	R	R	R	R
Ceftriaxone (30 mg)	S	S	S	R	S
Cefixime (5 µg)	S	S	S	R	S
Cephadrine (30 µg)	I	I	I	S	R
Cefazolin (30 µg)	R	I	R	S	R
Ampicillin (10 µg)	S	R	R	S	R
Penicillin G (2 U)	R	R	R	R	R
Ampicillin/sulbactam (10 µg)	R	R	R	S	R
Netilmicin (30 µg)	R	S	S	R	S
Sulfametoxazol-trimethoprim (10 µg)	R	R	S	S	S
Norfloxacin (10 µg)	S	S	S	S	S
Lomefloxacin (10 µg)	S	S	S	S	S

* R, resistant (< 5 mm); I, Intermediate (5-12 mm); S, sensitive (> 12 mm).

Sequencing analyses of *P. mirabilis* isolates: Full 16S rRNA gene sequences were obtained from two bacterial isolates cultured from oyster tissue, MA and O strains. The 16S rRNA sequences of strains MA and O were highly similar (99%) to *P. mirabilis* (GeneBank accession number AF008582). The sequences differed between the isolates by twenty bases (2%) but they were phenotypically identical, and thus appear to represent the same species.

DISCUSSION

Our finding reveals the presence of *P. mirabilis* and other bacteria in the tissues of two species, *I. alatus* and *C. rhizophorae*. To date, there are rather few reports in the literature concerning the obtainment

of *P. mirabilis* from samples of marine invertebrates, especially bivalves⁵; the great majority of isolates come from clinical samples related to urinary tract infections or from feces or intestines of other animals¹³. The presence of *P. mirabilis* in aquatic environments may be attributed to the run-off of contaminated water bodies which increases during rainy periods, and which modifies the physicochemical and perhaps the biological conditions of coastal areas.

The five isolates that were identified as *P. mirabilis* by API ID 32E in our study exhibited differences regarding their tolerance to NaCl. The strains M4 and Col22 withstood high (6%) NaCl levels and in particular strain M4 grew well in up to 8% NaCl for 24 to 48 h. Hence, M4 may be defined as an halophilic strain and Col22 as a halotolerant strain²¹. Strains MA, M and O grew in NaCl concentrations from 0 to 3%, in agreement with other reported results showing that *P. mirabilis* strain AF008582 (DSM 4479) grows at levels of about 4% NaCl¹⁵.

By comparing the 16S rRNA gene sequences of our isolates O and MA, a similitude of 99% with the *P. mirabilis* strain AF008582 was found, with differences between 5 to 14 bp from a total of 1451 and 1444 bp, respectively. The morphology and the biochemical tests were sufficiently discriminating to confirm the identity of genus and species of the isolates. The API ID 32E has been widely used for the biochemical characterization of enterobacteria and it revealed a considerable variability between the examined isolates (Fig. 1). In particular, the phenon of *P. mirabilis* showed a variation of up to 12% between isolates and these remained separated according to the source of the samples (*I. alatus* or *C. rhizophorae*). Further, the discrimination between the group comprising *Proteus-Providencia-Morganella* and that of *Escherichia-Klebsiella* could be clearly seen.

Analyses of resistance to antibiotics in the five isolates from oysters used for the present study showed resistance to tetracycline, penicillins (penicillin G, ampicillin/sulbactam and ampicillin), and cephalosporins (cefotaxime and cefazolin) (Table 2). Comparisons of these results with other reports based on clinical isolates clearly show their similar resistance to tetracyclines. This may be used as an identification marker for this microorganism¹². However, contrasting with reported susceptibility to penicillins and cephalosporins^{3,12} our isolates were fairly resistant to them. Such differences may be ascribed to strain source, either clinical or environmental, and to serological group⁹. Additionally, halophilic bacteria often show natural resistance to antibiotics. The mechanism behind the resistance remains to be elucidated¹⁹. We isolated several *P. mirabilis* strains that showed elevated resistance to a wide variety of antibiotics and NaCl. AJAYI & AKONAI¹ reported multiple antibiotic resistances on environmental *Proteus* isolates from a coastal lagoon and their presence indicated recent contamination with sewage which is of significant public health concern. If such microorganisms are consumed by marine organisms, they could spread within the food chain. Similar health and environmental risk could also be occurring in our study area.

To conclude, while *P. mirabilis* is of common occurrence in the human urinary tract, from which it may become an opportunistic pathogen under certain conditions¹⁰ and spread to diverse environments^{4,13,18}, our findings suggest that oysters can accumulate this bacterium. This, in turn, may pose a potential threat to the health of those using these water bodies for recreational purposes and who

choose to eat those bivalves raw. Monitoring the microbiological quality of consumable seafood species and commercially important shellfish fisheries should be a priority issue for any public health program.

RESUMO

Ocorrência de *Proteus mirabilis* associado a duas espécies de ostras venezuelanas

A contaminação fecal de frutos do mar crus por microrganismos oportunistas patogênicos representa problema de saúde pública. O objetivo deste estudo é investigar a presença de bactérias entéricas que colonizam ostras coletadas em área turística da Venezuela. Amostras de ostras foram coletadas na costa noroeste da Venezuela e foram registrados a salinidade local, pH, temperatura e o oxigênio dissolvido na água do mar. O total de coliformes fecais foi medido para a avaliação da qualidade microbiológica da água e das ostras, usando a técnica de fermentação em tubos múltiplos. Análises foram feitas usando culturas e seqüência do gene 16S rRNA. Enriquecimento diversificado e métodos de cultura seletivos foram usados para isolar a bactéria entérica. Obtivemos culturas puras de bastões retos Gram negativos com fímbrias de *Isognomon alatus* e *Crassostrea rhizophorae*. Nossos resultados mostram que *P. mirabilis* foi predominante nas nossas condições de cultura. Confirmamos a identidade das culturas por testes bioquímicos, seqüência do gene 16rRNA e a análise de dados. Outras enterobactérias como *Escherichia coli*, *Morganella morganii* e *Klebsiella pneumoniae* foram também isoladas da água do mar e ostras. A presença de bactérias patogênicas em ostras podem ter implicações epidemiológicas e potencial risco para a saúde humana quando do consumo de frutos do mar crus.

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