Identification of bacteria in scuba divers’ rinse tanks

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ABSTRACT

Scuba divers typically rinse equipment in communal tanks. Studies show these tanks are contaminated with bacteria, but the types of bacteria have not been studied. We sought to identify bacteria in rinse tanks at a dive facility at San Pedro, Belize, to determine the origin of the bacteria and determine whether the bacteria represented potential threats to human health. The identity of bacteria was investigated using reverse line blot (RLB) assays based on 28 different rDNA probes designed to detect known pathogens of sepsis, as well as by sequencing 23S rDNA from isolates and performing VITEK identification of several isolates. Based on the identities of bacteria in divers’ rinse tanks, many likely originate from the ocean, and others likely originate from the divers themselves. None of the bacteria identified would be considered overt human pathogens. However, some of the bacteria found in the tanks are known to be associated with unsanitary conditions and can cause opportunistic infections, which may pose health problems to some individuals. Rinsing scuba equipment in communal tanks has the potential to transmit disease among some divers. Equipment, especially regulators and masks, should be rinsed/cleaned individually and not be placed in communal tanks.

INTRODUCTION

Recreational scuba divers typically rinse equipment in “communal” freshwater rinse tanks after diving. The equipment includes wetsuits, booties, buoyancy control devices (BCDs), fins, masks and regulators with mouthpieces. On a number of occasions, divers have complained of becoming sick during or after dive trips and speculated that illness may be spread among divers via communal rinse tanks.

The first study to investigate microbial contamination of scuba diver rinse tanks reported that water samples taken from two communal rinse tanks at a dive facility in Roatan, Honduras, indeed harbored many bacteria (1). Based on morphologies and swimming patterns, many different types of bacteria appeared to inhabit the rinse tanks. However, it was not possible to determine whether bacteria were introduced into tanks by rinsing equipment or by the water used to fill the rinse tanks, or if any of the bacteria were human pathogens.

Recently a report documented an outbreak of conjunctivitis among recreational scuba divers at Vitu Levu Island, Fiji (2). Twenty-nine individuals had been scuba diving from two boats. Of the 29 divers, 14 were clinically diagnosed with conjunctivitis during the six-day dive trip or upon return to the United States. The pattern of conjunctivitis transmission from diver to diver was documented and appeared to originate with a single person, the dive-master. The divemaster reported having an eye infection prior to the outbreak on the dive boats, and had placed his dive mask in a communal mask container with other divers’ masks (2).

This report documented an outbreak of illness among scuba divers, apparently spread via containers in which communal equipment had been placed. Recently we reported (3) that at a dive facility on Bonaire, Netherlands Antilles, bacteria were introduced into communal equipment-, regulator- and mask rinse tanks by rinsing of equipment, but not by
the water source used to fill these rinse containers. We also documented that cleaning an equipment rinse tank daily in the morning with bleach did not reduce the subsequent bacterial load. However, no studies to date have attempted to identify the bacteria in scuba divers’ rinse tanks.

The purpose of this study was to identify bacteria in water samples taken from equipment- and mask rinse tanks, as well as from ocean water. A PCR-based reverse line blot hybridization assay (RLB) targeting 23S rDNA was initially used to identify pathogenic bacteria in water samples. Subsequently, 23S rDNA was sequenced to identify other bacteria not detected using the pathogen-specific RLB, and VITEK 2 system was used to identify several of the isolates.

METHODS
Water samples
Samples were collected during a dive trip based at a facility on San Pedro, Ambergris Caye, Belize, in June 2008. During a five-day period, water samples were collected in sterile tubes from the following sources:

- a hose used to fill a communal equipment rinse tank,
- the communal equipment rinse tank,
- buckets on boats in which masks were rinsed or stored during transit,
- several dive sites in the ocean at various depths, and
- ocean water at the dive facility.

Thirty samples in total were collected. As soon as possible after collection, samples were placed in a refrigerator until the day of departure. During transit to the United States (~8 hours) samples were stored at ambient temperature and then transferred to a refrigerator upon arrival. The next day 15 µl aliquots were plated on terrific broth/agar plates, which were incubated at 33° C overnight and photographed.

Individual colonies from plates were subcultured in terrific broth overnight. Where bacteria on a plate were too dense to obtain individual colonies, samples were streaked onto other plates from which individual colonies were obtained and subcultured. Twenty-one isolates from different water samples and with various morphologies and/or swimming patterns were inoculated into terrific broth “slants” for RLB analysis and rDNA amplicon sequencing.

RLB analysis
Slant cultures were streaked to BD trypticase soy agar plates, and single colonies were used to inoculate TSB liquid media; these cultures were grown with aeration until turbid (one-two days). Total DNA was extracted from 1 ml of pelleted cells using bead beating in SDS/NaCl; DNA was purified using Qiagen mini DNA kits. Two µl of each DNA preparation was PCR-amplified with broad-range, digoxigenin-labeled primers targeting the 23S rDNA region (4); 10 µl of each PCR reaction was run on an agarose gel and stained with ethidium bromide to verify amplification. Aliquots (0.1 µl) of the digoxigenin-labeled amplicons were then tested by reverse line blotting, using a set of 28 probes targeting 23S rDNA sequences of a series of common bacterial agents of sepsis. Probes were derived from published studies (4) or designed by Immunetics, Inc. Hybridization, washing and detection with alkaline phosphatase-labeled anti-digoxigenin antibodies were done using a semi-automated flow-through hybridization instrument (CodaXcel™) developed by Immunetics. Three samples were later analyzed using an automated CodaXcel™ prototype, modified to provide finer control of hybridization conditions through more precise temperature control. Reference bacteria strains (Citrobacter freundii, Enterobacter cloacae, Klebsiella pneumoniae, Staphylococcus aureus and Staphylococcus warneri) for probe and phenotypic tests were obtained from the American Type Culture Collection.

DNA sequencing
Many of the bacterial isolates were not identified based on the pathogen-based RLB probe set alone. All 21 bacterial DNA samples were re-amplified using unlabeled 23S rDNA primers, and PCR amplicons were purified using Qiagen minElute columns. DNA was then sent to Cogenics for sequencing using an unlabeled version of the 23S rDNA reverse primer from the original amplification pair (4). Sequence chromatograms were manually edited in Sequencher to correct obvious miscalls and to

http://archive.rubicon-foundation.org
exclude sequences on the ends that were of poor quality. The edited sequences were used to search for homologies in the NCBI nucleotide collection (nr/nt) using the blastn algorithm. Species identifications were based on BLAST matches. Sequence homologies for best matches were usually >98%, but in some cases the species identity was ambiguous. Supplemental data showing the DNA sequences that were used, and the top BLAST matches are available upon request.

Additional microbial identity tests
To confirm identities of some species via an independent method, simple microbial tests were performed, including assessing motility with motility agar tubes (PML) and determination of carbohydrate fermentation, gas production and H₂S formation with triple sugar iron agar tubes (PML). Two isolates were sent to Nelson Laboratories for identification via VITEK (bio-Merieux Industry, Hazelwood, Missouri).

RESULTS
Plates
Figure 1 (above) shows the results of plating water samples on terrific broth/agar plates. No bacteria were found in water samples from a hose used to fill the equipment rinse tank (not shown). The actual number of bacteria in 15 µl aliquots of plated samples was not determined; however, Figures 1A and 1B

FIGURE 1 — Bacterial growth from water samples

A: Equipment rinse tank

B: Mask rinse tank

C: Ocean water at resort

D: Ocean water

FIGURE 1: Bacterial growth from indicated water samples (15 ul); dates of sampling are indicated below plates (samples with same date were taken at different times), depth of ocean water (1D) is indicated in feet, below plates.
show that all water samples from the equipment and mask rinse tanks contained significant levels of bacteria.

Mask rinse containers were only present on dive boats on three different days. The extent to which bacteria were present in ocean water samples by the resort dock where people swam and snorkeled (Figure IC) as well as ocean water sampled during dives on different days and different depths (Figure ID), varied greatly from low levels to high levels, with no apparent correlation with day or depth of sampling. From these plates, individual colonies were isolated and examined by dark field microscopy. To increase the prob-
ability of detecting different types of bacteria, isolates from various water samples were selected for further analysis based upon apparent different morphologies and/or swimming patterns.

Reverse line blot studies
A total of 21 isolates were selected for further analysis; DNA from all 21 bacterial isolates were successfully PCR-amplified using the digoxigenin-labeled rDNA primers (not shown). Aliquots (0.1 µl) of the 21 labeled amplicons were hybridized on blots with 28 different 23S rDNA probes designed to detect common bacterial agents of sepsis, and positive hybridization was detected, as described in Methods.

Figure 2A (facing page) shows: 13 of the labeled amplicons hybridized with probe GN2, designed to recognize most common gram-negative bacteria, with the exception of Acinetobacter baumannii; one amplicon reacted with the gram-positive probe GP3, and seven amplicons reacted with neither of these probes.

Samples #10, #11, #19 and #20 hybridized with an Acinetobacter probe, Ab.1; samples #12 and #14 hybridized with subsets of the Enterobacteriaceae probes (Ec.2, Pm.1, ENT.2, ENT.3.); sample #15 hybridized with probes STPH.1, STPH.2 and Sw.1.

Based on the pattern of amplicon hybridization in Figure 2A, sample #12 resembled C. freundii, #14 resembled K. pneumoniae and #15 resembled S. warneri. These three samples were retested in the automated CodaXcel™ prototype, modified for finer temperature control, along with labeled 23S rDNA amplicons prepared from K. pneumoniae, C. freundii and S. warneri strains from ATCC.

Figure 2B shows that samples #12, #14 and #15 appeared to closely resemble C. freundii, K. pneumoniae and S. warneri, respectively.

rDNA sequence and BLAST Studies
To identify bacteria not detected by RLB, and to confirm the identity of isolates that did hybridize with 23S rDNA probes (Figure 2), bacterial DNA from all 21 water sample isolates was PCR-amplified using the reverse 23S rDNA primer. DNA sequences were obtained and edited as described and used to BLAST the NCBI nr database. These results are summarized in Table 1, which shows the water sample # as designated in Figure 2, the site of sampling, the likely organism, the percentage of the query DNA covered in the BLAST analysis (BLAST Coverage %) and the % Maximum Identity. The criteria for good level of confidence in the organism assignment was chosen to be Coverage % >98 and % Maximum Identity >98%.

The BLAST analysis usually allowed assignment of genus with good confidence, but not always species. For example, BLAST analysis of samples #1 and #7 could not differentiate between three species of Aeromonas. Regarding sample #15, only a partial 23S rDNA sequence was available at NCBI for S. warneri, resulting in nearly half of the query sequence missing from the data bank; the portion of the query that was available showed 99% identity to S. warneri.

Additional microbial tests
The BLAST results for samples #12 and #14 did not allow clear species identification. Number 12 had best matches of 97% homologous to Klebsiella pneumoniae and Citrobacter freundii, and #14 had 97% matches to Klebsiella pneumoniae, Citrobacter koseri, and Enterobacter aerogenes. To confirm the identities of sample #12 and #14 by independent methods, some simple microbial tests (motility and triple sugar iron) were performed. Results from these tests (not shown) were consistent for #12 as C. freundii, as predicted by RBL, but were not consistent with #14 as K. pneumoniae. Therefore, those two isolates were sent for biochemical identification by VITEK (bioMerieux); results (data not shown) confirmed sample #12 was C. freundii (97% probable), but identified #14 as Enterobacter cloacae (99% probable).

DISCUSSION
Most of the isolates from the water samples were not identified by RLB using the 28 different bacterial 23S rDNA probe set, which is designed to detect pathogens involved in human bloodstream infections. RLB did identify Acinetobacter in several equipment-mask rinse tanks (samples #10, #11, #19, #20).
Acinetobacter is commonly found in the environment, and some Acinetobacter species, especially A. baumannii, have increasingly been recognized as opportunistic human pathogens (5). Based on rDNA sequence data (Table 1, facing page), Acinetobacter [baumannii/calcoaceticus] was identified in equipment- and mask rinse tank samples (samples #10, #19). Three samples (#12, #14 and #15) were identified as organisms that may have originated from the divers. S. warneri (sample #15) is a coagulase negative Staphylococcus found on human skin, and sometimes causes illness in hospitalized patients (6). The RLB results indicated that samples #12 and #14 are in the Enterobacteriaceae family (Figure 2A and Figure 2B). Sample #12 was further identified by both RLB and VITEK as C. freundii.

Although sample #14 was similar to K. pneumoniae in the RLB test, #14 was identified by VITEK as E. cloacae; the identification of #14 as E. cloacae is likely correct. E. cloacae is an unusually diverse “nomen-species” that consists of at least five groups, based on DNA hybridization studies (7, 8), and cannot be assembled into a single cluster in rDNA comparisons (9).

In tests with ATCC strains, the difference in the CodaXcel™ RLB for K. pneumoniae vs. E. cloacae is the presence of Ec.2 probe hybridization. However, based on the available sequences at NCBI, some E. cloacae isolates are expected to cross-hybridize with the Ec.2 probe. Additional probes in the RLB may make it possible to unambiguously distinguish E. cloacae from K. pneumoniae.

Based on RLB results, VITEK testing and rDNA sequence similarities, 17 of the 21 isolates from various water samples (Table 1) may be identified with good confidence. Samples of ocean water, both near the resort and away from shore (50 and 70 feet deep), were found to contain the marine bacteria Vibrio [alginolyticus/parahaemolyticus], which was not unexpected. Water samples obtained from containers used to rinse masks on boats contained members of the Acinetobacter family (A. [baumannii/ calcoaceticus/twofii/sp.]), widely distributed bacteria which may be part of the ocean environment or, in some cases, human flora. Of all the sites sampled, the equipment rinse tank contained the most diverse types of bacteria, including members of the Acinetobacter, Aeromonas, Citrobacter, Enterobacter, Pseudomonas and Vibrio families.

Some of these bacteria, including Vibrio, were likely introduced by ocean water remaining on divers’ equipment. However, some of the other species may well have originated from the divers themselves. Although none of the bacteria identified in any of the water samples would be considered overt human pathogens, C. freundii and E. cloacae are both coliform bacteria found in the gastrointestinal tract of humans, and whose presence is associated with water contamination (10, 11), and can cause opportunistic infections that could pose a health problem to some individuals. Neither of these species was found in samples from open water. Vibrio species in sea water have been reported to infect wounds (10), and some members of the Acinetobacter family can enter healthy individuals through open wounds and are naturally multidrug resistant (5).

Our previous (1, 3) and current results demonstrate significant bacterial loads in scuba divers’ communal rinse tanks and indicate that some bacteria in these tanks likely originate from the divers themselves. Therefore, it is possible that disease could be spread via these communal rinse tanks; the report of a conjunctivitis outbreak on a dive trip (2), apparently spread by a communal mask container, supports this contention.

Our studies examined only contamination of communal rinse tanks by bacteria but not by viruses or other pathogens that may be introduced into rinse tanks and may cause illness. During diving, masks and regulators are in contact with nasal and oral discharges, respectively; such discharges are known to spread some diseases, including influenza.

To minimize the risk of contracting disease, we recommend divers rinse at least masks and regulators in clean water rather than in communal tanks. Ideally, no equipment should be rinsed in communal rinse tanks, and it might be advisable to spray/wipe masks and mouthpieces with a disinfectant, such as 70% ethanol and allow time to dry, before diving.
### TABLE 1 – Identification of bacteria in water samples based on BLAST or VITEK results

<table>
<thead>
<tr>
<th>Water sample</th>
<th>Identity</th>
<th>BLAST coverage(%)</th>
<th>Maximum identity(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EQUIPMENT RINSE TANK</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td># 1</td>
<td>Aeromonas [sp./hydrophilia/punctata]</td>
<td>100/100/100</td>
<td>100/100/99</td>
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<tr>
<td># 3</td>
<td>Vibrio alginolyticus</td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td># 6</td>
<td>Vibrio [alginolyticus/parahaemolyticus]</td>
<td>100/100</td>
<td>99/98</td>
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<tr>
<td># 7</td>
<td>Aeromonas [sp./hydrophilia/punctata]</td>
<td>100/100/100</td>
<td>100/100/99</td>
</tr>
<tr>
<td># 9</td>
<td>Vibrio [alginolyticus/parahaemolyticus]</td>
<td>100/100</td>
<td>99/98</td>
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<tr>
<td>#12</td>
<td>Citrobacter freundii</td>
<td>VITEK</td>
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</tr>
<tr>
<td>#14</td>
<td>Enterobacter cloacae</td>
<td>VITEK</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[lwoffii/baumannii/caloaceticus]</td>
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<td></td>
</tr>
<tr>
<td>#16</td>
<td>Acinetobacter</td>
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<td>90/87/87</td>
</tr>
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<td>Pseudomonas putida</td>
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<td>99</td>
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<td>Acinetobacter [baumannii/caloaceticus]</td>
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<td>99/99</td>
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<td>#20</td>
<td>Acinetobacter lwoffii</td>
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<td><strong>MASK RINSE TANK</strong></td>
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<td>Acinetobacter [baumannii/caloaceticus]</td>
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<td>99/98</td>
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<tr>
<td>#11</td>
<td>Acinetobacter lwoffii</td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td>#17</td>
<td>Acinetobacter lwoffii</td>
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<td>96</td>
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<td>#21</td>
<td>Acinetobacter baylyi (ADP1)</td>
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<td><strong>OCEAN at RESORT</strong></td>
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REFERENCES


