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Effects of Incubation Time and Temperature on Microbiologic Sampling Procedures for Hemodialysis Fluids

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To prevent pyrogenic reactions and bacteremia in hemodialysis patients, the Association for the Advancement of Medical Instrumentation and the Centers for Disease Control recommend culturing of hemodialysis fluids (water and dialysate) at least once a month. The recommendations for total microbial counts are (i) ≤200 CFU/ml in water used to prepare dialysate or reprocess hemodialyzers and (ii) ≤2,000 CFU/ml for the dialysate. In accordance with the Association for the Advancement of Medical Instrumentation recommendations all cultures should be incubated at 37°C for 48 h on suitable culture media, such as Trypticase soy agar, standard methods agar, or one of several commercially available assay systems. There have been suggestions that lower temperatures and longer incubation might improve the recovery of bacteria from water and dialysate. In this study bacterial recovery from various dialysis fluids (water, bicarbonate dialysate, and bicarbonate concentrate) at 30 and 37°C was compared. Duplicate sets of samples were membrane filtered (pore size, 0.45 μm); one set was incubated at 30°C and the other was incubated at 37°C for 72 h. The number of visible colonies was counted every 24 h by using a dissecting microscope. No significant difference was observed in specimens incubated at 37°C for 48 h compared with those incubated at 30°C for 72 h. Also, bacterial recovery was significantly better when samples of bicarbonate dialysate or bicarbonate concentrate were plated on Trypticase soy agar as opposed to standard methods agar.

To prevent pyrogenic reactions and bacteremia in hemodialysis patients, the Association for the Advancement of Medical Instrumentation (AAMI) and the Centers for Disease Control recommend that bacteriologic assays of dialysate and water used to prepare dialysate be performed at least monthly (4, 21). Bacteriologic and/or endotoxin assay is recommended for water used to reprocess hemodialyzers in dialysis centers with a hemodialyzer reuse program (5). A variety of bacterial culture methods are currently used, including the standard spread plate, the pour plate, or commercial assay systems (3, 5).

It is recommended that hemodialysis fluids be cultured on standard methods agar (SMA) or Trypticase soy agar (TSA) and incubated at 37°C for 48 h before bacterial growth is quantified. Colony counts in water used to prepare dialysate should be ≤200 CFU/ml; dialysate should contain ≤2,000 CFU/ml (4). These guidelines were made on the basis of the results of epidemiologic investigations of pyrogenic reactions and bacteremia in patients undergoing hemodialysis (9).

Several studies have demonstrated increased recovery of heterotrophic bacteria from water sources through the use of nutrient-poor media and longer incubation times (16, 20). It has been suggested that these assay techniques should be used in the culturing of hemodialysis fluids in an attempt to improve bacterial recovery (13, 17). However, it has been shown that media containing salt (which is not present in low-nutrient media such as R_2A and SMA) are necessary for the recovery of bacteria from bicarbonate dialysate and concentrate (7). These findings have led to increased discussion concerning the current AAMI guidelines pertaining to bacteriologic examination of hemodialysis fluids. The purpose of this study was to compare bacterial recovery from samples of hemodialysis fluids, collected from three dialysis centers, that were incubated at 30 and 37°C for 48 or 72 h and cultured on SMA, R_2A, TSA, and salt-supplemented media.

MATERIALS AND METHODS

Dialysis fluids were collected weekly from three dialysis clinics in the Atlanta metropolitan area for 5 weeks. Specimens included reverse osmosis (RO) water, liquid bicarbonate concentrate, and bicarbonate dialysate. In addition to these specimens, liquid bicarbonate concentrate was prepared in the laboratory. Specimens were serially diluted 10^{-1} to 10^{-4} in either sterile physiologic saline or sterile buffered distilled water (pH 7.4). RO water samples were diluted in sterile buffered distilled water, whereas all others were diluted with sterile physiologic saline. From each sample dilution, 1 ml was aseptically withdrawn and membrane filtered through a sterile, 47-mm-diameter, cellulose-nitrate filter (pore size, 0.45 μm) (Micro Filtration Systems, Dublin, Calif.). These membrane filters were rinsed with sterile physiologic saline or buffered distilled water, depending on the sample diluent. Membranes were then placed on appropriate media for incubation.

Media. Several types of media were used in this study and included the following: (i) commercially prepared TSA (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.), (ii) TSA supplemented with 5% NaCl (TSA-NaCl; 40 g of TSA [Becton Dickinson], 45 g of NaCl [Sigma, St. Louis, Mo.], 1,000 ml of distilled water), (iii) SMA (Becton Dickinson) (23 g of SMA, 1,000 ml of distilled water), (iv) SMA^+ (23.5 g of SMA, 11.8 g of NaCl, 33.7 g of $NaHCO_3$, 1,000 ml of distilled water) (7), (v) R_2A agar (Difco Laboratories, Detroit, Mich.) (2, 20), and (vi) R_2A supplemented with 5% NaCl (R_2A-NaCl; 18.5 g of R_2A [Difco], 50 g of NaCl, 1,000 ml of distilled water).

Culture. In the first part of the study the following media

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TABLE 1. Mean bacterial recovery from samples of liquid bicarbonate concentrate on media with and without salt supplement incubated at 30°C for 72 h

Sample no.	Log_{10} CFU/ml						
	TSA	TSA-NaCl	SMA	SMA^+	SMA-NaCl	R_2A	R_2A-NaCl
1	3.897	4.230	0	3.785	3.362	0	4.146
2	3.924	4.176	0	3.322	3.740	0	4.114
3	2.968	4.079	0	3.544	4.041	2.342	4.041
4	2.921	2.964	2.301	2.886	2.914	2.724	3.079
5	2.863	2.556	2.756	2.591	2.612	2.806	2.707
6	2.491	2.505	0.778	2.176	3.518	0	2.415
7	2.813	2.286	0	2.785	2.813	0	2.832
8	2.556	2.929	0	3.230	2.939		
9	2.447	2.778	1.447	3.279	2.914	2.114	2.707
10	2.792	2.763	1.398	2.886	2.204	0.903	3.079
11	3.079	3.041	0.903	2.968	2.255	0	2.534
12	3.146	3.176	1.462	3.398	2.431	1.431	2.477
Mean	2.992	3.124	0.920	3.017	2.979	1.12	3.103

were analyzed for the recovery of microorganisms from liquid bicarbonate concentrate: SMA, SMA^+, TSA, TSA-NaCl, R_2A, and R_2A-NaCl. Each dilution was assayed six times by membrane filtration, and the filters were placed aseptically on one of the six media being compared. Culture plates were incubated at 30°C for 72 h. Colonies were counted and recorded at 24, 48, and 72 h.

In the second half of the experiment specimens of water, bicarbonate dialysate, and liquid bicarbonate concentrate were serially diluted as described above. Each sample dilution was assayed six times, and the membrane filters were placed aseptically onto two R_2A plates, two SMA plates, and two TSA plates. One set of culture plates (R_2A, SMA, and TSA) was incubated at 30°C for 72 h, and the other set was incubated at 37°C for 48 h. After incubation, culture plates were removed from the incubators and viable colonies were counted and recorded. All plates were counted by using a ×7 to ×30 variable-power dissecting microscope (Bausch & Lomb, Rochester, N.Y.).

Statistical analysis. Statistical analysis of data was accomplished by using analysis of variance.

RESULTS

Bacterial recovery from liquid bicarbonate concentrate was significantly greater from media containing salt (Table 1). Low-nutrient media containing no salt (Na^+), such as R_2A and SMA, had less potential for recovering organisms ($P \leq 10^{-5}$) from liquid bicarbonate concentrate than any of the media containing salt. There was no significant difference (analysis of variance, $P = 0.936$) between the recovery of bacteria on TSA, SMA^+, or any of the three types of media supplemented with 5% NaCl. Also, no significant difference ($P = 0.311$) was noted between the use of TSA and SMA^+ for bacterial recovery from liquid bicarbonate concentrate when culture plates were incubated at 37°C for 48 h (Fig. 1).

When dialysate was cultured onto R_2A, SMA, and TSA at 37°C for 48 h, the greatest recovery was found on TSA with a 3-log-greater recovery over both R_2A and SMA ($P \leq 10^{-6}$) (Fig. 1). When bacterial recovery from RO water was compared, there was no statistically significant difference between the use of SMA, R_2A, and TSA (analysis of variance, $P = 0.265$), although bacterial recovery was greatest on R_2A (Fig. 1). Samples of RO water, bicarbonate concentrate, and dialysate, when cultured onto TSA and incubated at both 37°C for 48 h and 30°C for 72 h, showed no significant difference in bacterial recovery, although the mean bacterial counts were slightly higher when the samples were incubated at the lower temperature for 72 h (Fig. 2).

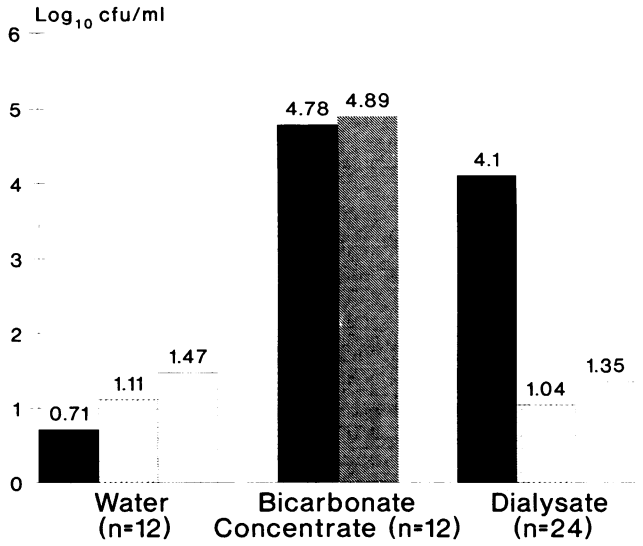

FIG. 1. Effects of culture medium on mean bacterial recovery from hemodialysis fluids incubated at 37°C for 48 h. ■, TSA; ▨, SMA; □, R_2A; ▧, SMA^+.

DISCUSSION

Gram-negative bacteria can grow in all types of waters, even those with small amounts of organic matter (e.g., RO, distilled, or deionized water) (8). These bacteria present a potential hazard to hemodialysis patients in two ways. Under the right conditions, viable cells have been found to

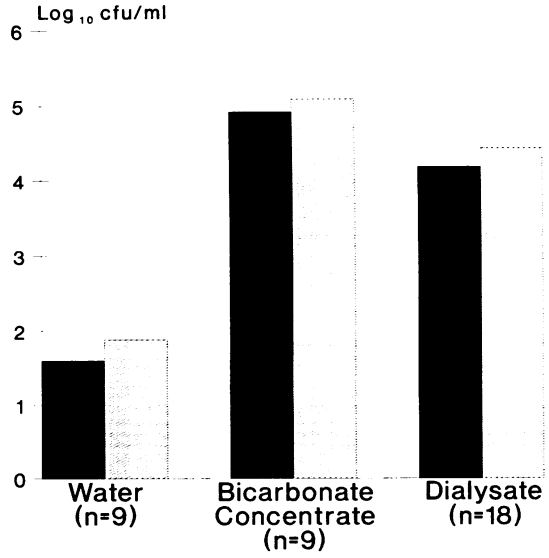

FIG. 2. Effects of incubation time and temperature on the mean bacterial recovery from hemodialysis fluids cultured on TSA. ■, 37°C for 48 h; ▨, 30°C for 72 h.

produce episodes of bacteremia and septic shock in these patients (10–12, 14). Also, the cell wall components of these organisms, which can exist as either cell-associated or free moieties in fluids, contain lipopolysaccharide (or endotoxin) and can produce pyrogenic reactions (15, 18, 19). Epidemiologic studies (10) suggest that if bacterial contamination of dialysate exceeds 3 log CFU/ml, the risk of pyrogenic reactions increases.

These studies, upon which the AAMI microbial standards for dialysate were based, were conducted by using assay techniques involving incubation times and temperatures of 48 h and 37°C, respectively. The microbial standard for water was a best-guess extrapolation from the dialysate microbial standard and was not based on any epidemiologic data. A 1-log reduction in the water standard from the dialysate standard was selected because of the potential for bacterial amplification as the water flows through the distribution system and the dialysis machine.

AAMI recommends that bacteriologic assays of hemodialysis fluids be performed at least once a month (5, 6, 9). Cultures of dialysate and RO water are collected for quantitative heterotrophic culture on SMA or TSA by either the spread plate or the membrane filtration technique (2, 4). These cultures are then incubated at 37°C for 48 h, and a heterotrophic plate count is obtained (4). Identification to the species level is not required, because naturally occurring gram-negative water bacteria, none of which appears epidemiologically more important than another, will be isolated (10, 11).

Some researchers and clinicians (13, 17) suggest that the AAMI recommendations for assaying RO water are not adequate for the recovery of bacteria, because the bacterial floras of water have become adapted to a carbon- and nutrient-poor environment. Indeed, their studies as well as several previous studies have concluded that R_2A had a strong selectivity for waterborne bacteria compared with TSA or plate count agar (or SMA) and also suggested incubating these cultures at much lower temperatures (down to 20°C) for longer periods of time (3 to 4 weeks). However, our studies show no significant difference between water cultures grown at a lower temperature (30°C) for 72 h and those grown at 37°C under the AAMI and Centers for Disease Control guidelines. The mean colony counts on R_2A were higher than those on TSA, but not at a statistically significant level.

Earlier studies by Bland and others (7) demonstrated that a salt-supplemented, low-nutrient medium (SMA^+) achieved significantly greater bacterial recovery from both liquid bicarbonate concentrate and bicarbonate dialysate than SMA. They concluded that the medium used to culture bicarbonate dialysate (now used in more than 63% of the chronic hemodialysis centers in the United States [1]) or bicarbonate concentrate should include salt. Media containing salt (i.e., TSA, TSA-NaCl, SMA^+, SMA-NaCl, and R_2A-NaCl) were all found to have significantly better recovery than nutrient-poor R_2A or SMA from bicarbonate concentrate (Table 1). There was no significant difference ($P = 0.936$) between the commercially available TSA and the other media supplemented with salt. Bacterial recovery from bicarbonate dialysate was found to agree with earlier work by Bland and others (7), with TSA having significantly better bacterial recovery. No significant difference was observed when lower temperature and longer incubation times were compared with those conditions recommended by AAMI.

Changes in incubation times or temperatures, made to improve bacterial recovery, would invalidate the basis for the AAMI standards. The primary purpose of the assays is to determine the degree of magnitude of microbial contamination and not to precisely quantify or identify bacteria in these fluids. Although R_2A did provide higher counts (Fig. 1), statistically significant differences were not observed in this study between the use of low-nutrient media (such as R_2A and SMA) and TSA when water was cultured. Neither incubation temperature nor increased incubation time causes a significant increase in recovery. Probably the highest bacterial recovery from environmental water samples is obtained when culture plates are incubated at 20°C for 20 to 30 days; however, this procedure is not practical and would not serve the purpose of quality control in a dialysis setting. In addition, gram-negative organisms responsible for producing significant amounts of endotoxin will grow to be visible on a culture plate within 24 to 48 h. The ready availability of TSA in clinical and commercial laboratories makes this the medium of choice in the culturing of dialysis fluids.

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