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Evaluation of Media for Monitoring Fecal Streptococci in Seawater

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The selectivity of KF streptococcus agar (KF) for monitoring fecal streptococci (FS) in seawater was examined in 234 samples of Mediterranean water and compared with the selectivity of M-Enterococcus agar (M-Ent) for 124 samples and with bile-esculin-azide agar (BEA) for 17 samples. KF was found to be unsuitable for marine water because *Vibrio alginolyticus* and other gram-negative bacilli indigenous to this environment grew well on it and produced red colonies identical to those of FS. In 26% of samples, some with high counts of red colonies on the membrane filters (MF), there were no streptococci, only gram-negative bacilli and staphylococci, and in an additional 23.1% the streptococci constituted less than 50% of the "typical" red colonies on the MF. *V. alginolyticus* also produced FS-like colonies on MF incubated on BEA but was not isolated from MF incubated on M-Ent. Although staphylococci grew and produced FS-like colonies on all three media, M-Ent was the most selective since no gram-negative bacilli were isolated from MF incubated on it.

Fecal streptococci (FS) are considered good indicators of fecal pollution; therefore, they are of great sanitary as well as clinical significance. Attempts to find a good selective medium for the isolation of all FS or just enterococci date back to 1918 (18), and the fact that it is no easy task is demonstrated by the 68 media, based on various inhibitory substances, that were proposed for this purpose till 1972, when Pavlova et al. (18) reviewed the subject. These media were designated for the examination of water, sewage, feces, and other clinical material, and therefore their efficacy, specificity, and selectivity were tested against these environments or the bacterial species usually present in them, such as members of the family *Enterobacteriaceae*, and the media were found to be satisfactory (14, 15, 20). Comparative studies between some of the media or modified versions of them (3, 7, 9, 18, 19) were also done on feces, sewage, food, and water from rivers, lakes, or springs, but very few samples of seawater were studied (7).

KF streptococcus agar (KF), introduced by Kenner et al. in 1961 (15), is considered one of the best media for the enumeration of FS in water on membrane filters (MF), and it is recommended in *Standard Methods for the Examination of Water and Wastewater* (1) for recreational as well as drinking water. It was also adopted tentatively for the examination of seawater by the World Health Organization Programme of Pollution Monitoring and Research in the Mediterranean (MED-POL). However, experience showed that in some samples of seawater the number of FS calculated on the basis of red and pink colonies enumerated on MF incubated on KF exceeded that of the fecal coliforms by 2 to 3 orders of magnitude, a discrepancy which could not be attributed merely to the longer survival of FS in seawater. This led to the isolation and Gram staining of some typical colonies, which revealed that many of them were gram-negative bacilli or gram-positive cocci in clusters. The catalase test showed that they released O_2 from 3% H_2O_2, and hence they were not streptococci. Since the nonstreptococcal bacteria were the dominant flora in a considerable percentage of the samples examined, and their number exceeded several hundred per 100 ml of water, it was felt that the rate of false-positive results on KF should be evaluated in a large number of samples in order to reassess the suitability of this medium for marine water. It was also important to examine other commercially available media, such as M-Enterococcus agar (M-Ent) and bile-esculin-azide agar (BEA), which is equivalent to PSE (10), to see whether one of them was more suitable for the purpose. This comparative study was therefore undertaken, and the results are presented here.

MATERIALS AND METHODS

Media and incubation. KF, M-Ent, BEA, brain heart infusion broth (BHI), and tryptic soy broth (TSB) were all from Difco Laboratories. Nutrient agar with 0.5% NaCl (NA) was from the Institut Pasteur. Modified KF was prepared from the ingredients, omitting NaCl (KF^1) and also sodium glycerophosphate (KF^2). Incubation was done at 35 ± 0.5°C and 42 ± 0.5°C for 48 h for KF and M-Ent and for 24 h for BEA. The membrane filters (MF) used were Gelman GN-6, with grids.

Procedure. Samples of seawater from beaches of the central part of Israel that were brought to the laboratory for routine monitoring of fecal coliforms (FC) once a week in the summer and once a month in the winter were also monitored for FS by the MF method. Portions (10 and 50 ml) of water were filtered, and the MF were incubated on KF. Later, parallel samples were also filtered and incubated on M-Ent and BEA. On KF and M-Ent, all pink and red colonies were counted with a magnifying glass, and on BEA light to dark brown colonies with brown haloes were counted. Every group of colonies with the same morphological appearance was counted separately, and two to three colonies from each group were subcultured on NA for observation of colonial morphology on this medium and performance of the catalase test. Well-isolated colonies from NA were subcultured in BHI or TSB, Gram stained, and examined microscopically. The catalase test was performed on microscopic slides with 3% H_2O_2.

According to the colonial and microscopic morphology and the catalase reaction, the bacteria that grew on the MF were designated streptococci, staphylococci, and gram-negative or gram-positive bacilli. All gram-positive cocci that did not shown any chain formation and were catalase positive were considered staphylococci. The number of each group of bacteria per 100 ml of water was calculated. When the representative colonies from each morphological group on the MF showed two or three different types, the count of that group was divided equally among them (28 of 1,332

TABLE 1. Distribution of bacterial flora on MF incubated on KF at 35°C for 48 h

Bacteria[a]	No. of samples with indicated count (CFU/100 ml of seawater)			
	≤100	101–1,000	>1,000	Total (%)
Streptococci only	42	18	5	65 (27.8)
Streptococci > NS	37	11	6	54 (23.1)
NS > Streptococci	13	27	14	54 (23.1)
NS only	22	30	9	61 (26.0)
Total (%)	114 (48.7)	86 (36.8)	34 (14.5)	234 (100)

[a] NS, Nonstreptococci; includes staphylococci and gram-negative bacilli.

TABLE 3. Selectivity of KF and M-Ent

Total count (CFU/100 ml of seawater)	No. (%) of samples with the following dominant flora (>50%)					
	KF			M-Ent		
	Strepto-cocci	Staphy-lococci	Gnb[a]	Strepto-cocci	Staphy-lococci	Gnb
≤100	20	8	12	62	22	0
101–1,000	18	14	31	25	13	0
>1,000	5	7	9	2	0	0
Total	43 (35)	29 (23)	52 (42)	89 (72)	35 (28)	0 (0)

[a] Gnb, Gram-negative bacilli.

groups of colonies counted). Selected colonies from each group were identified by the API 20 system (La Balme les Grottes, 38390 Montalieu-Vercieu, France).

RESULTS

During a period of 2 years, 234 samples of seawater from 32 beaches were monitored on KF; 124 were also monitored on M-Ent and 17 were monitored on BEA as well. From the first eight samples on KF, an additional set of MF were incubated at 44.5 ± 0.2°C, but growth at this temperature was poor, so it was replaced by 42 ± 0.5°C. Comparison of incubation temperatures (35 versus 42°C) was done for 33 samples on KF and for 17 of them on M-Ent and BEA as well. From the various MF, 2,670 representative typical colonies were subcultured, gram stained, and examined for catalase activity. Identification by the API 20 system was performed on 266 randomly selected colonies, and 253 of them were identified to the species level.

The distribution of bacterial groups that grew on KF according to gram stain and catalase activity is summarized in Table 1. Streptococci alone were found in only 27.8% of samples. In 23.1% of samples, streptococci were more than 50% of the flora, and in an additional 23.1% they were a minority and in 26% of the samples there were no streptococci among the "typical" colonies counted as FS. In 23 of the 34 samples with more than 1,000 CFU per 100 ml of water (10% of all samples), streptococci were absent or constituted only a small fraction of the flora, erroneously indicating high pollution in water that was actually clean, with low FC counts, as illustrated by the results of several samples presented in Table 2.

In Table 3, the selectivity of KF is compared with that of M-Ent on the basis of the dominant flora obtained on MF incubated on each medium. The percentage of samples in which staphylococci were the dominant flora was similar on both media (23 and 28%). However, none of the gram-negative and few gram-positive bacilli that were isolated from KF were found on M-Ent. This made the latter much more selective, with streptococci as the dominant flora in 72% of samples, versus 35% on KF.

Raising the incubation temperature from 35 to 42°C did not improve the selectivity of the media (Table 4). The total number of colonies on KF was higher at 35°C in 10 samples, at 42°C in 13 samples, and equal at both temperatures in 10 samples. The selectivity of BEA was not better than that of KF and inferior to that of M-Ent, since some of the gram-negative bacilli also produced brownish colonies on it (Table 4).

Since the most frequently isolated gram-negative bacilli on KF were marine halophilic bacteria that required NaCl for

TABLE 2. Examples of discrepancies[a]

Location	Sampling point	Date (day/mo/yr)	FC (CFU/100 ml)	"FS"[b]	
				Count (CFU/100 ml)	Confirmed results
Nathania	M23	27/5/85	16	2,300	100% *Staphylococcus xylosus*
	M24	27/5/85	16	1,200	67% *S. xylosus*, 33% gpb
	M29	29/5/85	40	2,000	73% gnb (*V. alginolyticus*)
	M31	7/8/85	12	500	90% gnb (*V. vulnificus*) + staphylococci
	M32	7/8/85	<2	360	100% gnb (*V. alginolyticus*)
	M34	7/8/85	<2	320	99% gnb (*V. alginolyticus* + *Pasteurella pneumotropica*)
Tel-Aviv	M48	31/7/85	22	2,700	75% gnb (*V. parahaemolyticus*)
Bat-Yam	M49	17/6/85	46	3,200	90% gnb (*V. alginolyticus*)
	M50	7/8/85	<2	1,000	99% gnb (delicate, oxidase negative, not identified)
Rishon-Lezion	M52	6/8/85	4	1,800	99% *S. xylosus*
	M52	17/6/85	<2	1,000	99% Nfs (*Gemella haemolysans*)
	M52	1/7/85	2	2,800	100% gnb (*V. parahaemolyticus* + *Plesiomonas shigelloides*)
	M53	1/7/85	<2	200	100% gnb (*Pasteurella* sp.)
	M56	17/6/85	2	1,100	100% gnb (*V. alginolyticus* + *P. multocida*)

[a] Discrepancies between actual contamination rate and false-positive results obtained by counting typical red colonies on MF incubated on KF.
[b] "FS," Colonies that would have been considered fecal streptococci without confirmation. gpb, Gram-positive bacilli; gnb, gram-negative bacilli; Nfs, nonfecal streptococci.

TABLE 4. Effect of temperature on selectivity of media

Bacterial flora[a]	No. of samples with stated flora					
	KF (n = 33)		M-Ent (n = 17)		BEA (n = 17)	
	35°C	42°C	35°C	42°C	35°C	42°C
Streptococci only	12	11	6	5	2	4
Streptococci + staphylococci	10	12	6	8	8	5
Streptococci + Gnb	2	5	0	0	2	4
Staphylococci only	3	2	3	3	3	3
Streptococci + Gnb	0	1	0	0	2	0
Gnb only	5	2	0	0	0	1
No growth	1	0	2	1	0	0

[a] Gnb, Gram-negative bacilli.

growth, it was attempted to improve the KF medium by omitting the NaCl in it (KF^1). When this had no effect and *Vibrio alginolyticus* was isolated from this medium as well, the sodium glycerophosphate was also omitted (KF^2). The recovery rate of gram-negative bacilli was lower, but *V. alginolyticus* was isolated on several occasions.

The identification of randomly selected strains of gram-negative bacilli isolated from MF on KF and BEA is given in Table 5. The dominant species were marine vibrios, mainly *V. alginolyticus* (13) and several *Pasteurella* species. Some strains of oxidase-negative vibrios that grew best with 3% NaCl could not be identified, even by the Centre de Formation API in Montalieu-Vercieu, France, where several strains were sent for identification. There were also a few strains of gram-positive bacilli, probably *Bacillus* spp., which I did not attempt to identify. Some of them also grew better with 3% NaCl.

It was realized that colonial and microscopic morphology, even combined with the catalase test, was not always sufficient for distinguishing certain strains of *Enterococcus faecalis* from staphylococci. On rich media, colonies of most group D streptococci are larger than usual and may be confused with staphylococcus and micrococcus colonies (8). Rarely, there is also catalase activity in some strains (8). It was therefore deemed necessary to identify some of the gram-positive cocci in clusters and pairs to the species level, which proved that they were indeed staphylococci (Table 6). They were isolated from all three media.

In order to see whether the streptococci isolated were all FS, 86 strains, most of them from KF, were classified. The results (Table 7) show that 26 of the 72 strains (36%) taken from KF were not FS.

TABLE 5. Gram-negative bacilli that produced "typical" red colonies on KF or brown colonies on BEA, identified by the API 20NE system

Genus	Species	No. of strains identified[a]	
		KF	BEA
Vibrio	*V. alginolyticus*	75	5
	V. parahaemolyticus	7	
	V. vulnificus	3	
	V. damsela[b]	1	
Pasteurella	*P. multocida*	6	
	P. pneumotropica	5	
	P. haemolytica	1	
	P. aerogenes	1	
	Pasteurella spp.	1	
Aeromonas	*A. hydrophila*	2	1
Plesiomonas	*P. shigelloides*	2	
Moraxella	*M. phenylpyruvica*	1	
Agrobacterium	*A. radiobacter*	2	
Pseudomonas	*P. vesicularis*	1	
	P. paucimobilis	1	
CDC group IIB		2	
CDC group VE		1	
Not identifiable[c]		13	

[a] Totals: 125 and 6 strains on KF and BEA, respectively.
[b] Identified in Centre de Formation API, France.
[c] Four of them were designated by the Centre as oxidase-negative *Vibrio* sp., Na^+ required for growth, and one as probably *Serratia liquefaciens*.

TABLE 6. Staphylococci that produced "typical" red colonies on KF and M-Ent or brownish colonies on BEA, identified by API 20 staph system

Species	No. of strains identified[a]		
	KF	M-Ent	BEA
S. aureus	8	5	1
S. xylosus 2	13	3	
S. hominis 1	3	4	3
S. saprophyticus	2	2	1
S. epidermidis		1	1
S. warneri	2	2	
S. sciuri	2	1	
S. lentus		1	

[a] Totals: 30, 19, and 6 strains by KF, M-Ent, and BEA, respectively.

DISCUSSION

Epidemiological studies indicate that enterococci are the indicator of choice for assessing the quality of marine water (6). It is possible that this group of bacteria may eventually replace FC, or at least be of equal sanitary significance in monitoring seawater. It must therefore be ascertained that the medium employed for enumerating enterococci in seawater is selective against marine bacteria, so that accurate results may be obtained without much additional confirmation.

This study shows that KF, which has proven to be a good medium for monitoring FS in other environments, is definitely unsuitable for marine water, in which the background flora contains many bacteria that grow on it and produce red

TABLE 7. Classification of streptococci isolated from seawater on MF incubated on KF, M-Ent, and BEA identified by the API 20 strept system

Genus	Species	No. of strains identified[a]		
		KF	M-Ent	BEA
Enterococcus	*E. faecalis* 1	3		
	E. faecalis 3	2		
	E. faecium 1	6	2	1
	E. faecium 2	15	5	2
	E. faecium 3	10		
	E. durans 2	6	2	
	E. avium	3		1
	E. gallinarum	1		
Aerococcus	*A. viridans* 1	2		
	A. viridans 2	10		
	A. viridans 3	9		
Streptococcus	*S. sanguis* I/1	2		
Gemella	*G. haemolysans*	3		

[a] Totals: 72, 9, and 5 strains by KF, M-Ent, and BEA, respectively.

colonies like those of FS, leading to grossly erroneous results (about 50% in this case). In 26% of the 234 samples, some of them with very high counts, FS were absent, and in an additional 23.1% they constituted less than 50% of the bacterial flora. The organisms isolated most frequently were marine vibrios, mainly *V. alginolyticus*, which is very common in the marine environment (13). In 1964, Slanetz and Bartley (21) compared KF and M-Ent for recovery of FS from feces, sewage, and water from various sources, including shellfish water and oysters. They reached the conclusion that KF did not appear suitable for selective detection of FS in seawater and seafood. They isolated and identified selected red and pink colonies and found that from salt water and oysters, only 28 and 42% of colonies from KF, respectively, were FS. The contaminating organisms were mainly *Pseudomonas* spp., filamentous gram-negative rods, and micrococci. Their findings are in good agreement with those of the present work. The filamentous rods are probably identical to the vibrios isolated here, among which many filamentous forms were observed. The rate of streptococcal isolation is also similar; however, they claim that 98.5 and 100% of colonies on M-Ent were confirmed as FS, while I isolated quite a few strains of staphylococci from M-Ent as well as from KF (Tables 3 and 6). Modification of KF medium by omitting the NaCl and sodium glycerophosphate to inhibit the growth of halophilic marine bacteria improved its selectivity only partially. It seems that some strains of *V. alginolyticus* can grow without the addition of Na^+ ions, and complete selectivity of the medium may require the inclusion of inhibitory substances.

Other accepted methods for enumerating FS in water were found inadequate for seawater. Buck (4) found that by the most-probable-number method, 45% of the results confirmed in ethyl violet azide broth were false-positive, and the most commonly isolated false-positive organism was a gram-negative nonpigmented short rod, which was presumed to be a species of *Vibrio*. He also isolated gram-positive cocci in clusters and, from one sample, gram-positive bacilli. These findings are similar to mine on KF. Buck later increased the concentration of azide to 0.1% and tried Pfizer PSE broth for the examination of 28 samples of coastal and estuarine water (5). His results indicated that false-positive reactions occurred frequently in AD broth, and increased azide concentrations were inhibitory to streptococci. In PSE broth, three samples showed false-positive tests, in one case rod-shaped organisms and in the other two gram-positive cocci in clusters.

Bile-esculin provides a reliable means for identifying group D streptococci (10, 12). Selective media based on hydrolysis of esculin as a marker for FS were found to be very satisfactory for the examination of clinical specimens, feces, sewage, and water from various sources, including some samples of seawater (7, 14, 17, 18). Nevertheless, it was shown here that for seawater with the indigenous flora of this region, BEA was not selective enough, just like KF. Of the 17 samples examined on this medium, some typical light and dark brown colonies were identified as *V. alginolyticus*, *Aeromonas hydrophila*, and several species of staphylococci (Tables 4 and 5). Farmer et al. (13) state that only 3% of *V. alginolyticus* strains hydrolyze esculin; however, according to the API 20 NE scheme for identification of gram-negative bacilli, 69% of *V. alginolyticus* hydrolyze esculin, as do 95% of *V. vulnificus*, 82% of *A. hydrophila*, and 99 to 100% of *Pseudomonas vesicularis* and *P. paucimobilis*, which were all found in these water samples (Table 4). Staphylococci usually do not hydrolyze esculin (2), yet some of the brownish colonies I isolated on BEA were identified as staphylococci (Table 6). The medium of Levin et al. (17) is also based on esculin hydrolysis as a marker for FS, but it contains actidion and nalidixic acid as inhibitors. The authors examined 2,231 colonies isolated from polluted marine and estuarine waters and found that 90% of the typical colonies and 11.7% of the other colonies were enterococci. It is possible that the marine vibrios so often isolated from the coastal waters of Israel are scarce in the New York bight or that they were inhibited by the antibiotics in the medium.

Staphylococci were isolated from all three media, and they included pathogenic and nonpathogenic species (16). Most of the randomly selected streptococci that were identified came from KF, and only 64% of them were FS (Table 7). Of the 26 nonfecal streptococci, 21 were *Aerococcus viridans*, two were *Streptococcus sanguis*, and three were *Gemella haemolysans* (11), which constituted most of the flora in two samples. The nine strains from M-Ent and the five strains from BEA were all enterococci, but their number was too small (compared with the 72 strains from KF) to determine the rate of specificity of these media for FS. Pavlova et al. (18) identified 721 isolates and found that 28.3% were nonfecal streptococci. Brodsky and Scheimann (3), who examined secondary sewage effluent, obtained on KF a confirmation rate of 83% for FS, with 54% enterococci. On PSE, their confirmation rate was 90% FS, with 86% enterococci.

The data of this study show that none of the three media examined is completely selective for FS, but M-Ent seems to be the best since the gram-negative bacilli that produced false "typical" colonies on the other media did not grow on it. In order to be efficient for monitoring FS in marine water, the medium should inhibit the growth of marine vibrios and other gram-negative bacilli listed in Table 5, as well as staphylococci and other streptococci.

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