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A SELECTIVE PROCEDURE FOR THE FIELD ISOLATION OF PATHOGENIC *STREPTOCOCCUS* SPP. OF RAINBOW TROUT *(SALMO GAIRDNERI)*

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ABSTRACT

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A procedure established for the selective isolation of the species of *Streptococcus* responsible for rainbow trout streptococcosis in South Africa, consisted of the inoculation of samples into nutrient broth which had been supplemented with 100 µg/mℓ of nalidixic acid, 160 µg/mℓ of oxolinic acid or 200 µg/mℓ of sodium azide. After incubation, the sample was plated onto tetrazolium agar on which the rainbow trout pathogenic *Streptococcus* species grew as a red colony. The colonies were isolated from the tetrazolium agar and identified as rainbow trout pathogenic isolates by biochemical and serological tests.

In the laboratory the selective procedure is capable of detecting about 2 bacteria per mℓ. This procedure was used in the field and biochemically identical *Streptococcus* species were found in the mud and a freshwater crab from the water source of a site with a history of streptococcosis.

INTRODUCTION

During the last 10 to 15 years, *Streptococcus* spp. have been isolated from fish and are now recognized as serious pathogens of a number of cultured fish species. Serious economic losses, attributed to streptococcosis, have been reported in yellowtail (Kusuda, Kawai, Toyoshima & Komatsu, 1976), Japanese eels (Kusuda, Komatsu & Kawai, 1978), tilapia (Kitao, Aoki & Sakoh, 1981), bullfrogs (Chung & Kou, 1985) and rainbow trout (Boomker, Imes, Cameron, Naudé & Schoonbee, 1979; Bragg & Broere, 1986).

Various aspects, including the characteristics of streptococcosis, the isolation of the pathogen and characterization of the isolates, were recently reviewed (Austin & Austin, 1987). It was found that substantial biochemical diversity existed between *Streptococcus* species isolated from different species of fish, as well as from the same species of fish from different geographical distributions. It is apparent from this review (Austin & Austin, 1987) that little is known about streptococcosis.

Streptococcosis of rainbow trout appears to be limited to South Africa (Boomker *et al.*, 1979; Bragg & Broere, 1986), Australia (Humphrey, Lancaster, Gudkovs & Copland, 1987) and Japan (Kitao, Iwata & Ohta, 1987). One of the characteristics of the South African isolates is that they are biochemically and serologically identical, irrespective of date of isolation or geographical distribution. Streptococcosis of trout in South Africa was recently reviewed by Bragg, Todd & Lordan (1989).

One approach to controlling streptococcosis is to identify reservoirs, vectors or carrier-fish and attempt to eradicate them by preventing their entry onto the site or reducing the bacterial load of the carriers, if present. From previous experience (Bragg *et al.*, 1989) it was found that the pathogenic *Streptococcus* sp. could only be isolated from diseased fish or ponds housing severely diseased fish. This inability to isolate the bacterium from the environment indicated that the bacterium, if present, was present in low numbers which made detection by conventional methods difficult.

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Other *Streptococcus* spp. and contaminants in the environment presented problems when isolating the pathogenic species. Selective media for the genus *Streptococcus* have been developed (Lichstein & Snyder, 1941; Pike, 1945; Barnes, 1959), but these methods were not effective for the selective isolation of the rainbow trout pathogenic *Streptococcus* sp. (Bragg & Todd, 1987, unpublished data). There was thus a need for a selective medium for the isolation of the *Streptococcus* sp. responsible for streptococcosis in rainbow trout.

MATERIALS AND METHODS

Bacterial strains

Streptococcus spp. isolates 7845, 104/87, 14/86, 67, 7877 and 7958 were all isolated from clinical cases in the laboratory of the authors. Bacterial strains such as *Aeromonas hydrophila, A. salmonicida, Yersinia ruckeri, Pseudomonas aeruginosa, Escherichia coli, Alcaligenes* sp., *Chromobacter* sp., *Citrobacter* sp., *Enterobacter* sp., *Serratia* sp., *Vibrio* sp. and various unidentified yeast species were also isolated in the laboratory of the authors and were chosen to represent contaminating organisms frequently encountered in water samples or from fish samples. Samples of *Streptococcus lactis, S. faecium, S. equi, S. zooepidemicus* and *S. faecalis* were obtained from the Bacteriology Section of the Veterinary Research Institute.

MIC determination procedure for trout Streptococcus *sp. and other bacterial species*

Minimum inhibitory concentrations (MIC) of nalidixic acid[1], oxolinic acid[1] and sodium azide[2] against *Streptococcus* isolates 7845, 104/87, 14/86, 67, 7877 and 7958, *Yersinia ruckeri, Aeromonas hydrophila, A. salmonicida, Pseudomonas aeruginosa* and *Escherichia coli* were determined. This was done in 96-well plates by first filling all the wells with 100 µℓ of nutrient broth[3]. The chemicals were made up to contain 256 µg/mℓ (m/v) in nutrient broth. One hundred µℓ of the antimicrobial containing broth was added to each well in the second vertical

[1] Sigma, Organochem, P.O. Box 1355, Kempton Park 1355
[2] SAARCHEM, P.O. Box 144, Muldersdrift 1747
[3] Biolab, E. Merck, P.O. Box 1998, Halfway House 1685

row on the 96-well plate. Twofold serial dilutions of the antimicrobial broth were made. Fifty µℓ of a 1/2 000 dilution of an 18 h old culture of each bacteria to be tested was added to the wells in 2 horizontal rows. Two horizontal rows of wells were inoculated with 50 µℓ nutrient broth as negative controls. The plates were incubated for 24 h at room temperature and turbidity read on a Biotek EIA[4] reader Model EL 307 at 610 nm.

Solid medium

An agar medium containing 1,4 % m/v agar, 1 % m/v peptone, 1 % m/v lablemco[5] 0,5 % m/v NaCl and 1 % glucose was prepared. A solution containing 0,01 % m/v tetrazolium salt[6], which had been filter sterilized was added to the agar medium before the plates were poured.

These plates were inoculated with isolate 7845, *Streptococcus lactis, S. faecium, S. equi, S. zooepidemicus, S. faecalis, A. hydrophila, A. salmonicida, Y. ruckeri, P. aeruginosa* and *E. coli*. The plates were incubated at room temperature for 24 h and growth and colour reactions recorded.

Determination of the most effective concentrations of antimicrobial compounds to use in the selective procedure

Samples of nutrient broth containing either 25 µg/mℓ, 50 µg/mℓ, 100 µg/mℓ, 160 µg/mℓ, 200 µg/mℓ or 250 µg/mℓ of nalidixic acid, oxolinic acid or sodium azide were made up in 10 mℓ tubes. Equal quantities of 18 h old cultures of *Streptococcus* isolate 7845, *A. hydrophila, A. salmonicida, Y. ruckeri, P. aeruginosa* and *E. coli* were mixed, and 100 mℓ was used to inoculate each tube. All the tubes were incubated at room temperature for 24 h and growth, as seen by an increase in turbidity, was recorded. Gram stains were made of each tube which showed an increase in turbidity and samples from each of these tubes were plated onto tetrazolium agar plates. Any red colonies were isolated and subjected to Gram staining.

Sensitivity of selective procedure

Streptococcus isolate 7845 was plated onto blood tryptose agar (BTA) plates and incubated at room temperature overnight. The colonies were removed from the agar with a sterile swab and suspended in sterile phosphate-buffered saline (PBS) at a pH of 7,2. Tenfold serial dilutions of the bacterial suspension were made and 0,1 mℓ from dilutions 10^{-6}–10^{-10} were plated onto BTA plates in such a way that an even distribution of colonies was obtained. Five plates per dilution were prepared. These plates were incubated at room temperature and the colonies counted after 24 h incubation. Tubes containing nutrient broth plus 100 µg/mℓ nalidixic acid were inoculated with either 0,1 mℓ, 0,5 mℓ or 1 mℓ from all of the dilutions of the suspension of isolate 7845. The tubes were incubated and growth was recorded. The results were used to calculate the lowest number of bacteria per mℓ which could be detected by the method.

Specificity of selective medium

The specificity of the selective medium was tested by inoculating 39 samples of environmental isolates or *Streptococcus* isolates into nutrient broth supplemented with 100 µg/mℓ nalidixic acid, 160 µg/mℓ oxolinic acid or 200 µg/mℓ sodium azide. Tubes were incubated at room temperature. The pH of other batches of medium supplemented with the antimicrobials was adjusted to 9,6 (using 1 N NaOH) and tubes, inoculated with the 39 isolates, were incubated at room temperature. Growth was recorded and Gram stains carried out on all tubes showing positive growth.

Use of selective medium in the field

Nutrient broth, supplemented with 100 µg/mℓ of nalidixic acid was prepared and bottled in 10 mℓ McCartney bottles. The bottles were taken on various field trips to 'Maloney's eye', a trout farm near Johannesburg, 'Waterval', a trout farm near Lydenburg, Transvaal, and various sites in Natal. Samples of water from rivers, production dams and settlement dams were collected. Swabs were taken from rocks in rivers, dam walls of production ponds and mud from rivers, production ponds or settlement dams. Swabs were also taken from the internal organs of fish and on 1 site a dead freshwater crab was also sampled.

All bottles were incubated at room temperature for 3 days and any bottle showing growth was removed and the contents of the bottle plated onto tetrazolium agar plates. All small red colonies were isolated by plating onto BTA plates. Gram stains were carried out on the isolated bacteria and, if gram-positive cocci were seen, the isolates were biochemically identified. Slide agglutination tests and indirect fluorescent antibody techniques (Bragg, 1988) were also performed on the isolates to confirm the isolate's relatedness to the rainbow trout pathogenic isolate of *Streptococcus*.

Colonies that did not grow with a red colour on tetrazolium agar were subjected to Gram staining. All gram-positive cocci were isolated onto BTA, and the catalase, oxidase and oxidation/fermentation tests were carried out. If the isolates were catalase-negative, oxidase-negative and fermentative, they were plated onto BTA slants and stored for future analysis.

Control of yeast contaminants in cultures

Besides the increased pH described above, amphotericin B[7] was added to the liquid broths at levels of 128 µg/mℓ in an attempt to control the growth of yeasts.

RESULTS

MIC determination for trout Streptococcus *sp. and other bacterial species*

The MIC's of nalidixic acid, oxolinic acid and sodium azide for various *Streptococcus* isolates, *Y. ruckeri, A. salmonicida, A. hydrophila, P. aeruginosa* and *E. coli* were determined and the results are presented in Table 1.

It can be seen that all isolates of *Streptococcus* sp. are resistant to all three antimicrobials (Table 1). The gram-negative isolates tested were all sensitive in varying degrees to all three antimicrobials.

Solid medium

Isolate 7845 was plated onto tetrazolium agar plates and this isolate was found to grow as a small red colony (Fig. 1).

[4] Sterilab Services, P.O. Box 2021, Kempton Park 1620

[5] Oxoid, C. A. Milsch (Pty) Ltd, P.O. Box 943, Krugersdorp 1740

[6] BDM, E. Merck, P.O. Box 1998, Halfway House 1685

[7] Fungizone, Squibb, Electron Ave, Isando 1600

TABLE 1 Minimum inhibitory concentrations of nalidixic acid, oxolinic acid and sodium azide for trout pathogenic *Streptococcus* sp. and various gram-negative organisms

Isolate	MIC in µg/mℓ		
	Nalidixic acid	Oxolinic acid	Sodium azide
7845	R	R	R
104/87	R	R	R
14/86	R	R	R
67	R	R	R
7877	R	R	R
7958	R	R	R
Yersinia ruckeri	0,065	0,065	0,25
Aeromonas hydrophila	0,065	0,25	0,5
A. salmonicida	0,065	0,125	0,5
Pseudomonas aeruginosa	8	16	8
Escherichia coli	0,125	0,125	0,25

R = resistant up to 128 µg/mℓ

TABLE 2 Growth of isolates on tetrazolium agar plates

Isolate	Growth	Colour
7845	+	red
S. lactis	+	white
S. faecium	+	pink
S. equi	+	white/cream
S. zooepidemicus	+	white/cream
S. faecalis	+	pink
A. hydrophila	+	white
A. salmonicida	+	white
Y. ruckeri	+	red
P. aeruginosa	+	red
E. coli	+	red

Some of the other organisms such as *Y. ruckeri* also produced a red colony on tetrazolium agar plates but none of the other *Streptococcus* organisms tested grew as red colonies (Table 2).

Determination of the most effective concentration of antimicrobial compounds to be used in minimum concentrations of antimicrobials

The minimum concentrations of antimicrobial needed to inhibit the growth of *A. hydrophila, A. salmonicida, Y. ruckeri, P. aeruginosa* and *E. coli* in a mixture of these organisms and isolate 7845 were calculated and found to be 100 µg/mℓ nalidixic acid, 160 µg/mℓ oxolinic acid and 200 µg/mℓ sodium azide (Table 3).

Sensitivity of selective procedure

The lowest number of bacteria/mℓ which resulted in an increase in turbidity was calculated from the counts of the bacterial suspensions used, the volume of inoculum and dilution of inoculum. A mean result of 2,1 bacteria/mℓ was found.

Specificity of selective medium

The specificity of the selective medium was tested by inoculating nutrient broth containing the different antimicrobials with samples of organisms isolated from the aquatic environment in South Africa or other *Streptococcus* isolates. It was found that most of the *Streptococcus* isolates and yeasts grow in the selective medium (Table 4). The percentage growth of the various organisms is presented in Table 5.

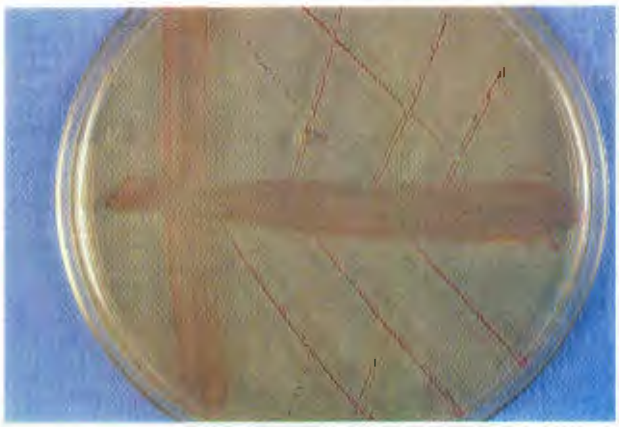

FIG. 1 Small red colonies produced by fish pathogenic *Streptococcus* species on tetrazolium agar

Use of the selective medium in the field

A total of 765 samples from various rivers, production dams, mud, fish and a crab was taken. A *Streptococcus* species, biochemically and antigenically identical with the rainbow trout pathogenic strain, was isolated from a crab and mud from a water source at 'Maloney's eye'. Although numerous *Streptococcus* species were isolated from other sites, these were not biochemically or antigenically related to the rainbow trout pathogenic strain. Numerous yeast strains were also isolated from various sites. No gram-negative organisms were isolated.

DISCUSSION

From the determination of MIC for different antimicrobials it was found that *Streptococcus* isolate 7845 was resistant to nalidixic acid, oxolinic acid and sodium azide. *A. hydrophila,* a ubiquitous environmental bacterium was sensitive to these antimicrobials at levels of 0,065 µg/mℓ of nalidixic acid, 0,25 µg/mℓ of oxolinic acid and 0,5 µg/mℓ of sodium azide (Table 1). From the MIC's obtained (Table 1), nalidixic acid appeared to be the most suitable antimicrobial to inhibit the gram-negative bacteria from samples, although any of the 3 tested could be used.

The minimum concentrations of antimicrobials needed to inhibit gram-negative organisms in a mixture of gram-negative organisms and *Streptococcus* isolate 7845 were found to be 100 µg/mℓ for nalidixic acid, 160 µg/mℓ for oxolinic acid and 200 µg/mℓ for sodium azide (Table 3).

TABLE 3 Minimum concentrations of nalidixic acid, oxolinic acid and sodium azide which will prevent the growth of gram-negative organisms in a mixture of gram-negative and gram-positive organisms

Concentration of antimicrobial	Gram stain		
	Nalidixic acid	Oxolinic acid	Sodium azide
25 µg/mℓ	Gram + and Gram −	Gram + and Gram −	Gram + and Gram −
50 µg/mℓ	Gram + and Gram −	Gram + and Gram −	Gram + and Gram −
100 µg/mℓ	Gram +	Gram + and Gram −	Gram + and Gram −
160 µg/mℓ	Gram +	Gram +	Gram + and Gram −
200 µg/mℓ	Gram +	Gram +	Gram +
250 µg/mℓ	Gram +	Gram +	Gram +

TABLE 4 Specificity of selective medium

Isolate	% growth greater than 0																	
	100 µg/mℓ nalidixic acid						160 µg/mℓ oxolinic acid						200 µg/mℓ sodium azide					
	pH = 7,2			pH = 9,6			pH = 7,2			pH = 9,6			pH = 7,2			pH = 9,6		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Acinetobacter sp	66	100	100	33	50	83	50	50	50	—[3]	—	66	—	—	33	—	—	—
Aeromonas sp.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	33	33	100
A. hydrophila	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	66	89	89
Alcaligenes sp.	—	—	8	—	—	—	—	—	—	—	—	—	—	—	—	—	33	66
Chromobacter sp.	—	—	—	—	—	33	—	—	—	—	—	—	—	—	—	—	33	66
Citrobacter sp.	—	—	—	33	33	33	—	—	—	—	—	—	—	—	—	—	66	66
Enterobacter sp.	50	50	50	16	33	83	25	25	25	—	—	—	—	—	—	33	66	66
Pseudomonas sp.	—	—	33	—	—	—	—	—	—	—	—	—	—	—	—	—	—	66
Serratia sp.	—	—	33	—	—	16	25	25	25	—	—	—	—	33	33	—	33	60
Streptococcus ractis	83	83	100	100	100	100	75	100	100	100	100	100	66	100	100	100	100	100
S. faecalis	66	100	100	83	100	100	25	25	75	100	100	100	66	100	100	100	100	100
Steptococcus Isolate:																		
7845	83	83	83	83	100	100	75	75	75	66	100	100	33	33	100	33	66	100
103/87	83	83	83	83	100	100	75	75	75	66	100	100	33	66	100	33	66	100
7877	100	100	100	66	83	100	75	75	75	66	100	100	33	66	100	33	66	100
26/86	100	100	100	83	83	83	75	75	75	66	100	100	66	100	100	100	100	100
KA 46[1]	100	100	100	100	100	100	75	75	75	66	100	100	33	100	100	100	100	100
Vibrio sp.	—	—	12	—	—	—	—	6	6	—	—	—	—	—	8	8	25	33
Yeast 1[2]	50	66	83	66	83	100	50	75	75	33	33	66	—	—	—	—	33	66
Yeast 2[2]	60	100	100	50	100	100	50	50	75	33	33	100	—	—	33	—	33	66
Yeast 3[2]	33	83	83	33	50	83	25	75	75	—	33	33	—	—	33	—	66	100
Yersinia ruckeri	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	33	100	100

[1] Isolate supplied by Allen Chung, Inst. Fish Science. Nat. Taiwan University

[2] Unidentified yeast species isolated by this laboratory

[3] No growth

TABLE 5 Percentage growth in specificity test

Mean % positive for:	Nalidixic acid		Oxolinic acid		Sodium azide	
	pH 7,2	pH 9,6	pH 7,2	pH 9,6	pH 7,2	pH 9,6
Fish *Streptococcus* sp. after 24 h	88,6	77,3	75	66	33	33
Fish *Streptococcus* sp. after 48 h	88,6	94,3	75	100	55	66
Fish *Streptococcus* sp. after 72 h	88,6	100	75	100	100	100
Fish *Streptococcus* sp ×	88,6	90,53	75	88,6	62,6	66,3
Streptococcus spp. after 24 h	87,8	85,4	67,9	75,7	47,1	71,28
Streptococcus spp. after 48 h	92,7	95,1	71,4	100	80;7	85,42
Streptococcus spp. after 72 h	95,1	97,6	78,6	100	100	100
Streptococcus sp. ×	91,86	92,7	72,63	91,9	75,93	85,56
Yeasts after 24 h	49,6	49,6	41,7	22	0	0
Yeasts after 48 h	83	77,6	66,7	33	0	44
Yeasts after 72 h	88,6	94,3	75	66,3	22	88,67
Yeasts ×	73,73	73,83	61,13	40,43	7,3	44,22
Gram – after 24 h	6,8	4,8	5,9	0	0	19,5
Gram – after 48 h	8,8	6,8	7,4	0	1,9	44,8
Gram – after 72 h	16,47	9,7	7,4	3,88	5,8	62,35
Gram – ×	10,69	7,10	6,9	1,29	2,57	42,21

The fish pathogenic *Streptococcus* bacterium grew as a small red colony on tetrazolium agar. Some of the gram-negative organisms tested, such as *P. aeruginosa* and *Y. ruckeri,* also produced red colonies on tetrazolium agar plates. These organisms would, however, be inhibited if samples were first incubated in nutrient broth with the appropriate concentration of antimicrobials. None of the other *Streptococcus* species tested produced a red colony.

It was decided to use a two-phase selective procedure, consisting of a selective enrichment phase in liquid medium followed by an isolation phase on tetrazolium agar medium. This approach was followed because lower numbers of bacteria per mℓ in samples could be detected. If the organisms occurred in the environment at levels of 1–2 organisms per mℓ, they could multiply in the selective liquid medium and result in pure or almost pure cultures on the agar medium and enhance the possibility of detection. If the same sample, containing 2 organisms per mℓ was plated onto tetrazolium agar which had been supplemented with the appropriate concentrations of antimicrobials, only 2 colonies would grow. The antimicrobials would inhibit the gram-negative organisms, but other gram-positive organisms and yeasts in the sample would grow and could possibly overgrow the small *Streptococcus* colonies.

The selective procedure for the fish pathogenic *Streptococcus* sp. was thus established and consisted of inoculation of samples into 10 mℓ nutrient broth supplemented with 100 µg/mℓ of nalidixic acid, incubation, and plating out onto tetrazolium agar plates. All small red colonies were isolated and biochemically and serologically identified.

It was found that the selective phase was capable of detecting the fish pathogenic *Streptococcus* sp. at levels of 2,1 bacteria per mℓ. The sensitivity of this stage could be enhanced either by using larger volumes of medium and thus permit the collection of larger volumes of water samples, or by filtering the water samples through a 0,45 µm filter and placing the filter in the selective medium. Both of these methods would, however, be impractical in the field, particularly if large numbers of samples were collected.

From the specificity studies (Table 4 & 5) it can be seen that a medium containing either 100 µg/mℓ nalidixic acid or 160 µg/mℓ oxolinic acid, with the pH adjusted to 9,6 gave the best results. In both cases, 100 % of the fish pathogenic *Streptococcus* sp. tested grew after 72 h incubation. In the case of oxolinic acid, 100 % growth of fish pathogenic *Streptococcus* sp. was achieved after 48 h. Some of the environmental gram-negative organisms tested were found to grow in both media, but the lowest average number was found in the medium containing oxolinic acid at a pH of 9,6. The only gram-negative organism to grow in this medium was *Acinetobacter* sp. which grew after 72 h incubation. *A. hydrophila* did not grow in any of the media tested except in nutrient broth with a pH of 9,6 supplemented with sodium azide. This is the single most important organism which must be inhibited in the selective process.

In all cases, except for sodium azide, the adjusting of the pH to 9,6 enhanced the selective media. The average number of fish pathogenic *Streptococcus* species as well as the average number of the other *Streptococcus* species which grew were increased while the average number of gram-negative organisms which grew was reduced.

The adjusting of the pH to 9,6 in media containing oxolinic acid also reduced the number of yeasts that grew (Table 5). The lowest average number of yeasts which grew was in media containing sodium azide, but the lowest average number of *Streptococci* spp. also grew in this medium, thus making it unsuitable. The growth of yeasts in the selective medium is not generally a problem as the yeast colonies are easily distinguishable on the tetrazolium agar plates (Fig. 2) as large colonies. These are very easily distinguishable from the small red fish pathogenic streptococci colonies.

From the specificity experiments it can be seen that the medium of choice for the selective phase of the procedure is nutrient broth with a pH of 9,6 supplemented with 160 µg/mℓ of oxolinic acid. Unfortunately, the work done on the specificity of the selective media was carried out only after extensive field work was done using a selective medium consisting of nutrient broth supplemented with 100

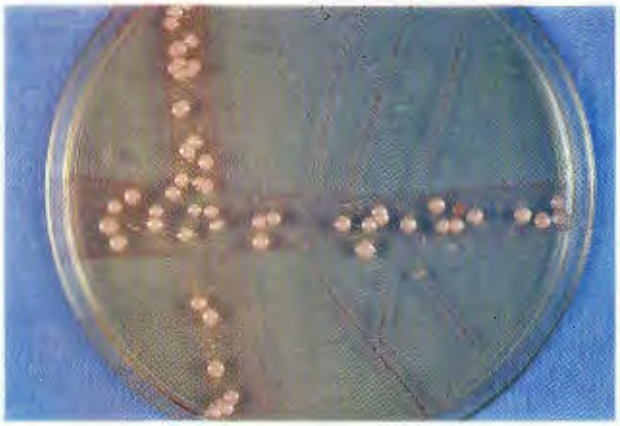

FIG. 2 Tetrazolium agar plate showing small red *Streptococcus* colonies and large yeast colonies. Small white colonies were also a *Streptococcus* sp., not biochemically or serologically related to the fish pathogenic *Streptococcus* sp.

µg/mℓ nalidixic acid at a pH of 7,2. After extensive work with this medium, it was noted that a large member of yeast organisms was isolated. The pH of the media was adjusted to 9,6 in the hope of eliminating the yeasts, which were found to be resistant to amphotericin B at levels of 128 µg/mℓ.

From field work, using a selective medium of nutrient broth containing 100 µg/mℓ nalidixic acid, a *Streptococcus* sp. biochemically and antigenically identical to the fish pathogenic *Streptococcus* sp., was isolated from the mud and from a dead freshwater crab from the water source on a farm with severe streptococcosis problems. The source of water on this site is a spring with a constant water temperature of 18 °C. Unfortunately, trout stocked in this source made the interpretation of results difficult. As streptococcosis is related to water temperature and approximately 18 °C appears to be the critical temperature for initiation of infection, the fish in the source may have subclinical streptococcosis and may be continually shedding the *Streptococcus* organism into the environment. The isolation of the *Steptococcus* sp. from the mud and crab on this site could be significant in that these isolations may indicate that the organism is a natural environmental organism, or that the freshwater crab may be a reservoir of infection. However, the fact that the *Streptococcus* sp. was found only on this site and all the other sites tested were negative, indicates that the isolation of this organism from the mud and freshwater crab was a result of shedding by subclinically infected trout in the water source.

In conclusion, the most effective selective procedure for the isolation of fish pathogenic streptococci involves inoculation of samples into a liquid medium containing 160 µg/mℓ of oxolinic acid at a pH of 9,6, incubation at room temperature for 48 h, followed by inoculation onto tetrazolium agar plates. Any small red colonies are isolated by plating onto BTA plates and the organism identified by Gram stain, biochemical tests, slide agglutination and IFAT.

Further investigation on the epidemiology of trout streptococcosis is needed and the development of this selective prodecure will assist in this investigation.

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