The distribution of fecal streptococci and their application to the interpretation of pollution problems is a matter of considerable current interest. The study reported here investigated the streptococci present in the feces of human beings and of a few domestic animals.

FECAL STREPTOCOCCI. II. QUANTIFICATION OF STREPTOCOCCI IN FECES

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THE STREPTOCOCCAL densities of 23 surface water and two sewage samples were measured by Kenner, Clark, and Kabler using four different streptococcal media. Two of the media were used with the multiple tube test, one medium for a membrane filter method, and the fourth, experimental KF Streptococcus Medium, by both procedures. There were significant differences between the three MPN procedures and between the two membrane filter methods, with the KF Streptococcus Medium yielding higher densities by both MPN tests and membrane filter counts. A detailed study was made of 698 colonies growing on the membranes which had KF Streptococcus Medium as a nutrient solution. They were able to confirm streptococci in all 698 colonies and failed to observe any nonstreptococcal bacteria in this series of samples. These data suggested that the differences on KF Medium by both MPN test and MF technic could be due to the number of species of streptococci which were able to grow on the more productive medium.

This study investigated the streptococci present in the feces of human beings and a few domestic animals, such as the cow, pig, sheep, and fowl, and classified them into broad groups by a minimum number of selected biochemical reactions. The study should yield information which would have value in the interpretation of the significance of the streptococci as indicators of the various types of animal pollution. The investigation of the groups of streptococci in feces may indicate whether there are other streptococci, in addition to the enterococcus group, which have some importance as indicators of fecal contamination in polluted waters or in tracing sources of pollution.

The enterococcus group[1] is defined for the purpose of this study as streptococci which grow at 45° C and 10° C, grow in 6.5 per cent sodium chloride in broth or at pH 9.6, and are tolerant to 0.1 per cent methylene blue in milk, consisting of the species Streptococcus fecalis and its varieties of liquefaciens and zymogenes and Streptococcus durans. Fecal streptococci in this study are defined as the group composed of species consistently present in significant numbers in fresh fecal excreta, which includes all of the enterococcus group

in addition to other groups of streptococci.

Methods

One gram of fresh feces was added to 99 ml of sterile phosphate buffered distilled water containing glass beads. This was shaken until a uniform suspension of the fecal material was produced. Appropriate quantities of the suspension were filtered through membrane filters and cultivated on KF Streptococcus Medium[2] for a colony count of the streptococci and the identification of a representative number of colonies from each animal feces tested. The streptococcal densities were reported as colony counts per gm (wet weight) of feces for each group of animals.

Between 20 and 50 colonies from each fecal sample were cultivated in pure culture in brain heart infusion broth tubes by incubation at 35° C for 24 hours. A gram stain was made and the following media inoculated for each strain:

1. Growth in brain heart infusion broth at 45° C in two days;
2. Reactions in litmus milk as observed at intervals of one, two, three, four, and seven days;
3. Reaction in sterile milk containing 0.1 per cent methylene blue at intervals of one, two, three, and seven days;
4. Growth in 6.5 per cent sodium chloride in brain heart infusion broth after three days;
5. Reduction of potassium tellurite (1:2500) in sterile milk within seven days;
6. Growth in brain heart infusion broth adjusted to pH 9.6 in three days;
7. Growth in 40 per cent bile contained in brain heart infusion broth in three days; and for some strains;
8. Fermentation of 1 per cent sorbitol in cystine trypticase agar.

The incubation temperature was 35° C for the above reactions unless otherwise noted. Composition and preparation of the media is described in the Appendix of this report.

Results

Streptococcal counts by the membrane filter procedure were calculated in millions per gm (wet weight) of feces. The median density for human feces was 3 million, for cows 1.3 million, for sheep 38 million, for pigs 84 million, and for fowl 3.4 million per gm. These values represent fecal samples from 18 adult human beings, six cows, five sheep, five pigs, and ten fowl.

Colonies were selected at random from the membranes for each of the fecal samples. A total of 1,092 colonies were composed of gram positive cocci in chains of two or more organisms with occasional pleomorphic cocci. None of the colonies picked from the membranes in this study belonged to non-streptococcal species. All of these strains were cultivated in pure culture in brain heart infusion broth for subsequent biochemical tests. Reactions by eight biochemical tests were used to classify the streptococci in groups or species according to reactions described in "Bergey's Manual of Determinative Bacteriology"[1] or those described in Sherman's review.[3] The results are summarized in Table 1.

There were 562 strains included in the enterococcus group, 77 strains in the S. mitis-salivarius group, 186 cultures in the S. bovis group, and 58 representatives of the S. equinus group (S. equinus and S. acidominimus) to account for 884 strains of streptococci. The remaining 208 strains representing 19.1 per cent had one or more biochemical reactions which disagreed with the typical characteristics of the above groups. These were classified as enterococcus group biotypes.

In addition to the reactions described above, the ability of each strain to grow in ethyl violet azide (EVA) broth was determined. There were 548 strains representing 97.5 per cent of the enterococcus group and 62 cultures or 80.5

per cent of the S. salivarius group showing growth in EVA broth. The S. bovis and S. equinus groups contained many strains inhibited by EVA, with approximately 20 per cent of each group producing growth. The biotype group gave growth with 119 cultures or 57 per cent. The above strains are summarized in Table 2 according to number and percentage in each group and the source of fecal samples.

Reference to Table 2 indicates that human feces contain enterococcus and S. salivarius groups with the addition of a few strains (6.8 per cent) of enterococcus biotypes. Feces from quadrupeds contain large numbers of the S. bovis and S. equinus groups, relatively few enterococci (10-31 per cent), and none of the S. salivarius group. There are increasing numbers of biotype strains in the feces of the cow, sheep, and pig. Streptococci in the feces of fowl have a different distribution of species with approximately three-fifths members of the enterococcus group and the remainder being enterococcus biotypes. There is an absence of the other characteristic groups found in the human beings and the quadrupeds. EVA broth showed positive reactions with 94 per cent of human, 85 per cent of fowl, 41 per cent each of cow and pig, and 26 per cent of sheep strains of streptococci.

Table 1—Biochemical Reactions of 1,092 Cultures Isolated from Fresh Feces

Biochemical Reaction	Enterococcus Group	S. mitis-salivarius Group	S. bovis Group	S. equinus-acidominimus Group	Enterococcus Biotype Group
Growth at 45° C	+	(±)	+	+	+
Growth in 6.5 per cent sodium chloride	+	−	−	−	∓
Growth at pH 9.6	+	−	−	−	±
Growth in 40 per cent bile	+	−	+	±	+
Litmus Milk:					
Acid	+	+	+	−	+
Coagulation	+	+	+		+
Reduction	(+)	Partial	+		+
Peptonization	±				±
Reduction 0.1 per cent methylene blue in milk	+	−	−	−	∓
Reduction of potassium tellurite (1:2500)	+	+	− (tr. in 7 days)	(∓)	±
Fermentation sorbitol	±	−	Not used	Not used	±
Final pH in glucose broth	3.9-4.6	4.0-5.8	4.0-4.5	Not used	4.0-5.8
Growth in ethyl violet azide broth:					
Number of strains	548	62	37	11	119
Percentage	97.51	80.51	19.89	19.00	57.21
Total number of strains isolated	562	77	186	58	208

Table 2—Occurrence of Streptococcal Groups in Feces of Man, Cow, Sheep, Pig, and Fowl

		Source of the Fecal Samples				
		Human	Cow	Sheep	Pig	Fowl
Enterococcus group	Number	360	21	31	10	139
	Percentage	76.3	12.3	24.8	10.0	61.8
S. salivarius group	Number	77	None	None	None	None
	Percentage	16.3				
S. bovis group	Number	None	104	50	32	1
	Percentage		61.2	40.0	32.0	0.4
S. equinus group	Number	3	24	8	24	None
	Percentage	0.6	14.1	6.4	24.0	
Enterococcus biotypes group	Number	32	21	36	34	85
	Percentage	6.8	12.4	28.8	34.0	37.8
Growth in EVA broth at 35° C	Number	444	69	32	41	191
	Percentage	94.1	40.6	25.6	41.0	84.9
Total streptococcal cultures	Number	472	170	125	100	225

Discussion of Results

The median streptococcal density per gm of feces yields no information on the total number of streptococci discharged per day from the gut of the typical animal of the group. There is considerable variation in densities between animals of the same species, and these data come from a relatively short series. Even with these limitations, the following calculations should have considerable value in estimating the quantity of pollution from groups of animals located on a watershed or from a surface drainage area. These estimates are summarized in Table 3.

Based on the average percentage of the enterococcus group (from Table 2) the respective number of the enterococcus density for various animals per day would be approximately 372 million for the human being, 384 million for the chicken, 3,670 million for the cow, 10,735 million for sheep, and 22,680 million for the pig. In the routine examination of a surface water, the enterococcus group density alone would give no indication as to the source of the pollution, such as human, quadruped, or fowl.

Eight or, in some instances, nine biochemical tests were used in the study of the 1,092 strains isolated from feces of animals, human beings, and fowl. Considering the fecal source of the samples, the group classification by the biochemical reactions as presented in Table 1 appears to be a reasonable and proper procedure. In addition to these biochemical reactions, tests for growth at 10° C; hydrolysis of starch; tolerance to 0.01 per cent methylene blue, 2 per cent and 4 per cent sodium chloride; and thermal resistance at 60° C for 30 minutes should be included where a careful and detailed classification of the streptococci is required. The streptococci in domestic wastes from human origin should yield between 70 per cent and 80 per cent of the strains showing growth in both 6.5 per cent sodium chloride and in medium at pH 9.6. A markedly lower percentage in positive tests by the salt tolerance and pH 9.6 growth would

be suggestive of quadrupeds. Such a procedure would give no significant differences in streptococci from human and those of fowl origin.

Pollution of human origin would be indicated by the presence of a high percentage of the enterococcus group, qualitative demonstration of the S. salivarius group, and very few strains of enterococcus biotypes. Finding the S. bovis group or S. equinus group indicates pollution of animal sources and with a reasonable certainty that, as the percentage of these two groups increases, the quantity of animal fecal pollution is increasing. Fowl fecal pollution would be indicated by a large percentage of the group classified as biotypes and the enterococcus group. In mixtures of all five types of fecal pollution the problem is more complicated, but it is possible to make qualitative interpretation as to the possible source of the pollution. The absence of the S. salivarius group does not exclude the presence of human fecal pollution, since it is a relatively sensitive organism with a rapid death rate in polluted surface waters.

The classification of the enterococcus group biotypes requires additional investigation. They cannot properly be considered to belong to the enterococcus group since they vary in one or more of the commonly accepted critical characteristics used in the classification of the enterococcus. However, due to their close resemblance in reactions to the enterococcus group and greater variations from the other fecal streptococcal groups, they have been tentatively described as biotypes. This heterogenous group of biotypes tends to show some characteristic variations according to the host species from which it was derived.

The relation of species of streptococci with animal species has been suggested by other investigators. Barnes, Ingram, and Ingram[4] believed S. faecium to be the dominant species on the skin of pigs. Cooper and Ramadan[5] have pointed out that S. bovis definitely is of animal origin. Fewins, Newland, and Briggs[6] found the streptococci density much higher than the coliform densities in pig feces. Results of this investigation are in agreement with all of these investigators.

Table 3—Estimated per Capita Discharge of Fecal Streptococci and Enterococci from Human, Chicken, Cow, Sheep and Pig

	Human	Chicken	Cow	Sheep	Pig
Average weight of 24-hour fecal discharge (wet weight in grams)	150*	182†	23,600‡	1,130‡	2,700‡
Streptococci per gram in millions	3.0	3.4	1.3	38.0	84.0
Total number of streptococci discharged per day in millions	450.	619.	30,680	42,900	226,800
Enterococcus group—percentage	76.3	61.8	12.3	24.8	10.0
Total enterococci discharged per day in millions	372.	384.	3,670	10,740	22,680

* Best, C. H., and Taylor, N. B. The Physiological Basis of Medical Practice, Baltimore, Md.: Williams and Wilkins, 1955, pp. 846-848.

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Summary

The median density of streptococci in millions per gram of moist feces was 1.3 for cow, 3.0 for human beings, 3.4 for fowl, 38.0 for sheep, and 84.0 for the pig. Enterococci densities were 0.16, 2.29, 2.10, 9.42, and 8.40 millions per gram, respectively. The average number of streptococci discharged in a 24-hour period increased in the order of human being, fowl, cow, sheep, and pig. The enterococcus and S. salivarius groups predominated in human feces to account for 92.6 per cent of the streptococcal population; enterococcus and streptococcal biotypes represented all the streptococci in feces of fowl; and quadrupeds showed appreciable quantities of the S. bovis and S. equinus groups, which were approximately 75 per cent for cow, 46 per cent for sheep, and 56 per cent for the pig. The enterococcus group on a percentage basis is low in animal feces, but it cannot be used as a differential procedure from human pollution since, on a daily basis, more enterococci are discharged per animal than by the average person. Human being and fowl strains grow well in ethyl violet azide broth, with 94 per cent and 85 per cent showing positive reactions. Growth was reduced to between 41 per cent and 26 per cent for strains from quadrupeds.

A careful classification of the streptococcal groups permits a qualitative interpretation of the possible sources of pollution. The enterococcus group used as an indicator of pollution yields from 77 per cent of the total streptococci from human pollution to a low of 10 per cent from pig feces. The fecal streptococci, based on results of this investigation, consist of the enterococcus group, S. mitis-salivarius group, S. bovis group, S. equinus group, and an atypical group closely resembling the enterococcus group, but with one or more differences in critical biochemical tests. Sufficient data have been accumulated to justify further investigations in the distribution of the fecal streptococci for application to the interpretation of pollution problems. The description as fecal streptococci rather than enterococcus is suggested.

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APPENDIX

Streptococcal Media

1. The 45° C Growth Test—Incubate streptococci in brain heart infusion broth at 45° for two days and examine for growth.
2. Litmus Milk Reaction—Sterilize the litmus milk at 115.2° C (10 lb for 10 minutes). Read reactions of streptococci at one, two, three, four, and seven days.
3. Methylene Blue Test—Add sufficient 1 per cent sterile solution of methylene blue to sterile skim milk to have a final concentration of 0.1 per cent methylene blue. Observe reactions at one, two, three, and seven days.
4. Sodium Chloride (6.5 Per cent) Tests—Inoculate brain heart infusion broth containing 6.5 per cent sodium chloride and observe for growth of streptococci within three days.
5. Potassium Tellurite Reduction—Add sufficient sterile potassium tellurite solution to sterile skim milk to have a final concentration of 1:2500 potassium tellurite. Observe for reduction at three and seven days.
6. Growth at pH 9.6—Add sterile 38 per cent sodium phosphate solution ($Na_3PO_4 \cdot 12H_2O$) to sterile brain heart infusion broth to give a pH 9.6 reaction. This

usually requires approximately 5 ml of phosphate solution per 100 ml of medium. Observe for growth of streptococci within three days.

7. Tolerance Test in 40 Per cent Bile—Add 40 ml of sterile 10 per cent Oxgall solution to 60 ml of sterile brain heart infusion broth. The 10 per cent Oxgall solution is equivalent in concentration to fresh bile originally used in this test. Positive cultures show growth within three days.
8. Sorbitol Fermentation—Sorbitol, 1 per cent, added to phenol red agar base medium or cystine trypticase agar. Incubate three days for fermentation tests.

The above media were prepared by reconstitution of dehydrated media and sterilized according to the recommendation of the manufacturer, except where other time and temperature are indicated in the media descriptions. All tests were incubated at 35° C except where other incubation temperature is specified for the test.

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